IDENTIFICATION AND CHARACTERIZATION OF NOVEL EVOLUTIONARILY CONSERVED PROTEIN METHYLTRANSFERASES

Thesis for the degree of *Philosophiae Doctor*

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2015

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 1631

ISSN 1501-7710

Cover: Hanne Baadsgaard Utigard.
Printed in Norway: AIT Oslo AS.

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ACKNOWLEDGEMENTS

The work presented in this thesis was carried out at the Department of Biosciences, University of Oslo. Funding for the PhD position was provided by the Norwegian Cancer Society.

First and foremost I wish to thank my primary supervisor, Professor Pål Falnes, for the opportunity to work in his lab and his expert guidance, constant encouragement and trust in me. I also want to thank my co-supervisors, Angela Ho and Stefan Kernstock, for all they've taught me and for setting me on the right track. In addition, I am grateful to all the co-authors and collaborators for their contributions to the research presented in this thesis, especially to Stefan who spearheaded and put such great effort into the VCP-KMT study.

A big thanks to all past and present members of the Falnes group for the fantastic research and social environment in our lab, as well as to my friends working up and down the corridor for positively contributing to the very special ecosystem that is our 2nd floor. It's been great fun both at work and at various social outings, not to mention some excellent conference trips to Norway and more exotic locations, and I am glad to have you all in my life.

Finally, I would like to thank my family for their constant love and support. I am especially grateful to my partner Nicholas for taking care of me all these years, and to my parents who, by a strange coincidence, have also studied several post-translational modifications of eEF2 for their PhD work in the Soviet Union over thirty years ago. You truly inspire me.

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Kernstock S, Davydova E, Jakobsson M, Pettersen S, Mælandsmo GM, Egge-Jacobsen W, and Falnes PØ. **Lysine methylation of VCP by a member of a novel human protein methyltransferase family.** *Nat. Commun.* 2012;3:1038. Doi: 10.1038/ncomms2041. PMID:22948820

PAPER II

Davydova E, Ho AYY, Moen A, Malecki J, Enserink J, Jakobsson M, Loenarz C, and Falnes PØ. **Identification and characterization of a novel evolutionarily conserved lysine-specific methyltransferase targeting eukaryotic translation elongation factor 2 (eEF2).** *J. Biol. Chem.* 2014;

ABBREVIATIONS

7BS	seven beta strand
AAA+	ATPases associated with diverse cellular activities
Arg (R)	arginine
ATP	adenosine triphosphate
C	carbon
DNA	deoxyribonucleic acid
Efm3	elongation factor methyltransferase 3
eEF1A	eukaryotic translation elongation factor 1
eEF2	eukaryotic translation elongation factor 2
eEF2-KMT	eEF2 lysine methyltransferase
FAM86	family with sequence similarity 86
Fe	iron
GTP	guanosine triphosphate
His (H)	histidine
KMT	lysine methyltransferase
KO	knockout
Lys (K)	lysine
mRNA	messenger RNA
MS	mass spectrometry
MTase	methyltransferase
MTF16	methyltransferase family 16
MW	molecular weight
N	nitrogen

O	oxygen
PRMT	protein arginine methyltransferase
PTM	post-translational modification
Rkm	ribosomal lysine methyltransferase
RPL / Rpl	ribosomal protein of the large subunit
RPS / Rps	ribosomal protein of the small subunit
RNA	ribonucleic acid
rRNA	ribosomal RNA
SAH (AdoHcy)	*S*-adenosyl homocysteine
SAM (AdoMet)	*S*-adenosyl methionine
TAP	tandem affinity purification
tRNA	transfer RNA
VCP (p97)	valosin-containing protein
VCP-KMT	VCP lysine methyltransferase

INTRODUCTION

1. Methylation

Methylation is the transfer of methyl groups from donor to acceptor molecules. This essential biochemical process occurs in every living cell and is catalyzed by enzymes called methyltransferases (MTases). Methylation reactions are central to many biological processes including small molecule biosynthesis, detoxification, signal transduction, DNA repair, epigenetics and transcriptional regulation, translation, and even ageing. Several methyl group donors exist in biological systems, but by far the most common one is *S*-adenosyl methionine (SAM) (Schubert et al. 2003).

1.1 SAM

SAM is a sulfonium compound which consists of an adenosine moiety attached to a methionine molecule (**Figure 1**). The chemically active methyl group is bound to the sulfonium ion, allowing for highly favorable thermodynamics of SAM-dependent methyl-transfer reactions. This makes SAM three orders of magnitude more reactive toward polarizable nucleophiles than methylated folates, the second most common methyl donors (Cantoni 1975). Besides methyl group donation, SAM may also be the source of a variety of other chemical groups, such as methylene (in the synthesis of cyclopropyl fatty acids), amino groups (in the synthesis of a biotin precursor), ribosyl groups (in the synthesis of epoxyqueueosine in tRNAs) and aminopropyl groups (in the synthesis of polyamines). In addition, several [4Fe-4S]-containing enzymes use SAM as a source of 5'-deoxyadenosyl radicals, which initiate many pathways by hydrogen-atom abstraction (reviewed in Kozbial and Mushegian 2005). Given the variety of processes that SAM is involved in, it is unsurprising that this molecule is essential and present in every studied life form, and is the second most abundant cofactor in cells, outnumbered only by ATP (Cantoni 1975).

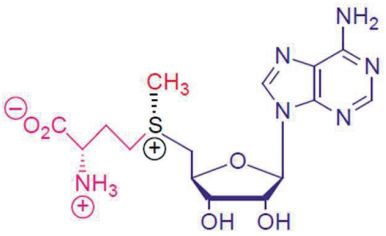

Figure 1. Chemical structure of S-adenosyl methionine (SAM). The central sulfonium ion is shown in black, the deoxyadenosine in blue, the aminocarboxypropyl in magenta, the methyl group in red. Adapted from Fontecave et al. 2004.

The metabolism of SAM is a cycle which is a part of the general metabolism of sulfur-containing amino acid derivatives (**Figure 2**). Its synthesis is catalyzed by SAM synthetase which transfers the adenosyl portion of ATP to a methionine molecule, simultaneously hydrolyzing the remaining triphosphate group of ATP to phosphate and pyrophosphate. Following donation of the methyl group, the demethylated form of SAM is called *S*-adenosyl homocysteine (SAH). This molecule is subsequently hydrolyzed into homocysteine and adenosine by SAH hydrolase. The resulting homocysteine can then either be converted into glutathione, a major cellular antioxidant, or be methylated to generate methionine through the action of the MTase methioinine synthase which uses 5-methyltetrahydrofolate as methyl donor. The regenerated methionine can then be used by SAM synthetase to start the SAM cycle anew. (Mudd and Cantoni 1958, Ludwig and Matthews 1997, Palmer and Abeles 1979, reviewed in Fontecave et al. 2004).

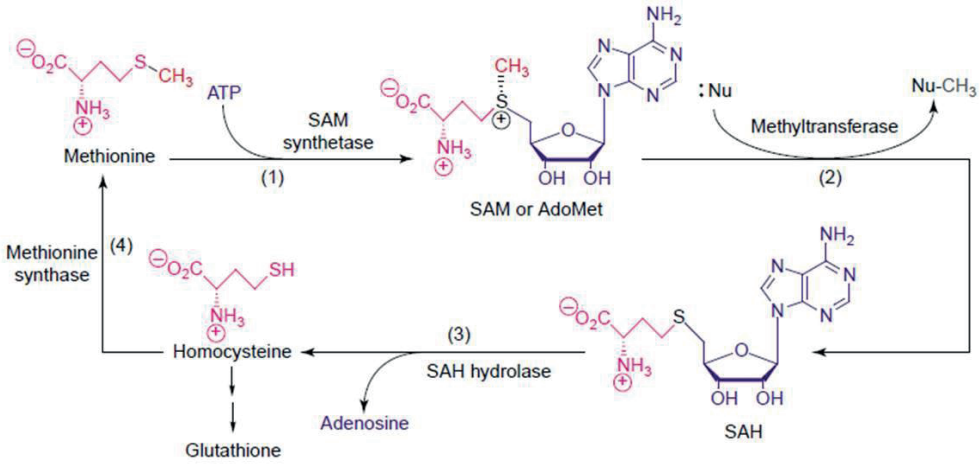

Figure 2. The SAM cycle. Formation of SAM from ATP and methionine by SAM synthetase (1); transfer of the methyl group from SAM to a nucleophile (:Nu) catalyzed by a methyltransferase (2); hydrolysis of SAH into homocysteine and adenosine by SAH hydrolase (3); methylation of homocysteine into methionine catalyzed by methionine synthase (4). Adapted from Fontecave et al. 2004.

1.2 SAM-dependent methyltransferases (MTases)

MTases catalyze methylation reactions by bringing a SAM molecule containing the strongly electrophilic methyl group into close contact with a nucleophilic target (usually S, N, O or C atoms), allowing for nucleophilic attack and transfer of the methyl group in an S_N2 reaction. Collectively, the MTases methylate a very wide range of substrates – from simple molecules such as oxidized arsenic, chloride, or iodine ions to complex molecules such as proteins, lipids or nucleotides, where methylation may play a structural or regulatory role (Thomas et al. 2004, Wuosmaa and Hager 1990, reviewed in Thayer 2002).

Recent bioinformatical studies have shown that in yeast, as much as 1.2% of the protein-coding genome is devoted to MTases. In humans, 208 proteins have been identified as known or putative MTases, corresponding to almost 1% of all human gene products (Petrossian and Clarke 2009, Petrossian and Clarke 2011). About 30% of the human MTases (63 proteins) appear to be associated with disease, most frequently cancer and mental disorders, underlining the importance of such enzymes (Petrossian and Clarke 2011). Interestingly, SAM-dependent methylation reactions may be catalyzed by enzymes that have remarkably distinct structures, making methylation an excellent example of functional evolutionary convergence.

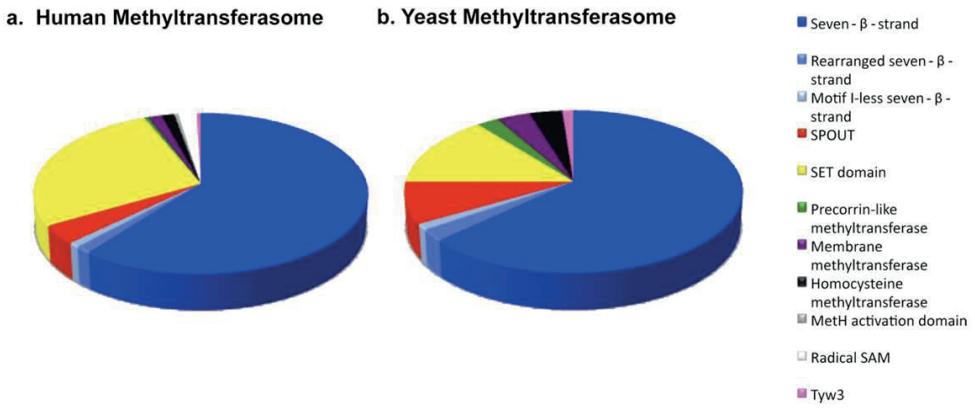

Figure 3. Human and yeast methyltransferases. Fractional composition of the human (a) and yeast (b) methyltransferasomes. Canonical seven-β-strand (7BS) MTases are colored in dark blue, other 7BS MTases in lighter shades of blue. SPOUT family in red, SET domain enzymes in yellow. Adapted from Petrossian and Clarke 2011.

1.2.1 Seven β-strand (7BS) MTases

The first solved MTase structure was that of the HhaI DNA MTase in 1993 (Cheng et al. 1993). It showed the presence of a seven-stranded β-sheet ending with a reversed β-hairpin, and surrounded by α-helices. In the following years, a steady stream of studies reported MTase enzymes with similar structures, and for some time, this structural theme was believed to be common to all SAM-dependent MTases.

While many other MTase structural classes have been subsequently described, the Seven β-strand (7BS) MTases comprise the most abundant family of MTases and constitute over 60% of all human or yeast MTases (**Figure 3**). These enzymes share little sequence identity but contain a characteristic structural core fold consisting of alternating β-strands and α-helices, forming a seven-stranded central β-sheet with three helices on each side, similar to the Rossmann fold of NAD(P)-binding proteins (**Figure 4**). The β-strands are parallel, with the exception of the last one which is antiparallel to the rest. Helices Z, A and B are situated on one side of the β-sheet, and helices C, D, and E are on the other, with helix C not present in many MTases. The N-terminal part of the β-sheet is required for binding to SAM, while the C-terminal part is responsible for substrate binding. In addition, the core fold may be surrounded by N- and C-terminal extensions, which may also have a function in defining substrate specificity (reviewed in Martin and McMillan 2002).

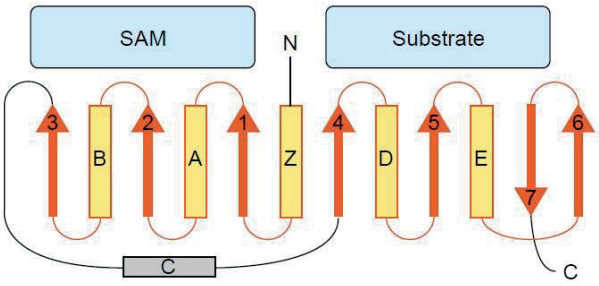

Figure 4. The conserved fold of the Seven β-strand (7BS) MTases. Schematic showing the topology of the core 7BS fold, indicating the SAM- and substrate-binding regions. Helices are shown as rectangles, strands as arrows, the N- and C-termini are labelled. Adapted from Martin and McMillan 2002.

While the primary amino acid sequence varies greatly between MTases, several conserved motifs are present both in the SAM- and substrate-binding regions. The most universal and critical for SAM-binding is the glycine-rich GxGxG motif (also referred to as motif I), located in the loop between β-strand 1 and α-helix A. These residues interact with the methionine portion of SAM. Another conserved motif is an acidic loop between β-strand 2 and α-helix B (motif post-I), which forms hydrogen bonds to the hydroxyl groups of the ribose moiety. The substrate-binding domain of the 7BS MTases also contains the conserved motif II in β-strand 4 and motif III between α-helix D and β-strand 5 (Katz et al. 2003, Martin and McMillan 2002).

However, there are also exceptions to the canonical core fold and motifs. Several human MTases have been identified where the core fold is rearranged, the so-called "rearranged 7BS MTases". For example, in the protein isoaspartyl MTase, the antiparallel β-strands 6 and 7 are reversed, and in the protein arginine-specific PRMT3 these strands appear to be completely missing (Zhang et al. 2000). In many DNA MTases the overall topology of the fold is circularly permutated (Bujnicki 2002). Humans have also several rearranged 7BS MTases whose homologues in yeast have been shown to function as RNA methyltransferases (Clancy et al. 2002, Bujnicki et al. 2002). Most surprisingly, there are even MTases with a canonical 7BS fold but which do not contain the SAM-binding motif I. The human genome appears to encode at least two such enzymes, and a sequence homologue of one of them, the yeast YGR001C, has been recently shown to methylate a lysine residue in eukaryotic translation elongation factor eEF1A, despite previous predictions of DNA *N*6-adenine methylating activity (Dzialo et al. 2014a, Albrecht and Lengauer 2004, Petrossian and Clarke 2011). In addition, several enzymes have been described which contain the canonical 7BS MTase fold, yet catalyze the transfer of other SAM functional groups than the methyl group. For example, spermidine synthase and spermine synthase utilize the aminopropyl group of decarboxylated SAM, and TYW2 catalyzes a SAM-dependent aminobutyryltransferase reaction during wybutosine synthesis. (Wu et al. 2007, Pegg and Michael 2010, Rodriguez et al. 2012).

1.2.2 Other MTase superfamilies

Another abundant family of MTases are the SET-domain enzymes. The SET domain was first recognized as a conserved sequence in several *D. melanogaster* proteins: Suppressor of variegation 3-9, Enhancer of zeste, and Trithorax (Tschiersch et al. 1994, Jones and Gelbart 1993, Stassen et al. 1995). However, proteins containing such domains have since been found in all eukaryotic organisms studied. All MTases of this class characterized so far are lysine-specific, and include all but one of the histone lysine MTases, with the exception being the 7BS enzyme Dot1 (Feng et al. 2002). SET domain proteins make up 27% of all human MTases, but only 14% of the *S. cerevisiae* MTases (**Figure 3**), reflecting the redundancy of many of the histone-specific MTases in humans. Several SET-domain proteins also have non-histone targets, and the most promiscuous of them, SETD7, in addition to methylation of histone H3 at lysine 4, is reported to methylate lysine residues in at least 17 other proteins. SETD7 substrates include such key proteins as p53, the androgen and estrogen receptors, the transcription factor FoxO3 and DNA methyltransferase DNMT1, with lysine methylation affecting their enzymatic activities, interactions or stability (reviewed in Herz et al. 2013).

The SPOUT family is another well-defined and rather large family of MTases, containing 8 members in humans (Petrossian and Clarke 2011). SPOUT MTases generally modify RNA (Tkaczuk et al. 2007). Recently, however, it has been demonstrated that one member of this family, the yeast YOR021c (Sfm1) catalyzes the monomethylation of an arginine residue in the Rps3p protein of the small ribosomal subunit (Young et al. 2012). This finding suggests a wider role for SPOUT methyltransferases than thought previously.

Additionally, there are several small MTase families with only a few human members. For example, the human genome encodes three membrane-bound MTases of unknown function, one methyltetrahydrofolate- and cobalamin-dependent methionine synthase involved in the SAM cycle, one precorrin-MTase-like diphthine synthase (DPH5) involved in diphthamidation of eEF2, and several other putative MTases all of which constitute separate structural families (Petrossian and Clarke 2011).

2. Protein methylation

Post-translational modification (PTM) of proteins is a common method of controlling their structure, localization, stability or activity. PTMs increase the functional diversity of the proteome by the addition of functional groups, proteolytic cleavage of regulatory subunits, or degradation of entire proteins. In addition to acetylation, phosphorylation, ubiquitination, lipidation and glycosylation, the methylation of proteins is one of the most common PTMs. Methylation of proteins usually occurs at the N- or C-termini, or on lysine, arginine, histidine, proline, glutamine, phenylalanine, asparagine, methionine, glutamate and aspartate residues. Most commonly, however, MTases target arginine and lysine residues (reviewed in Karve and Cheema 2011).

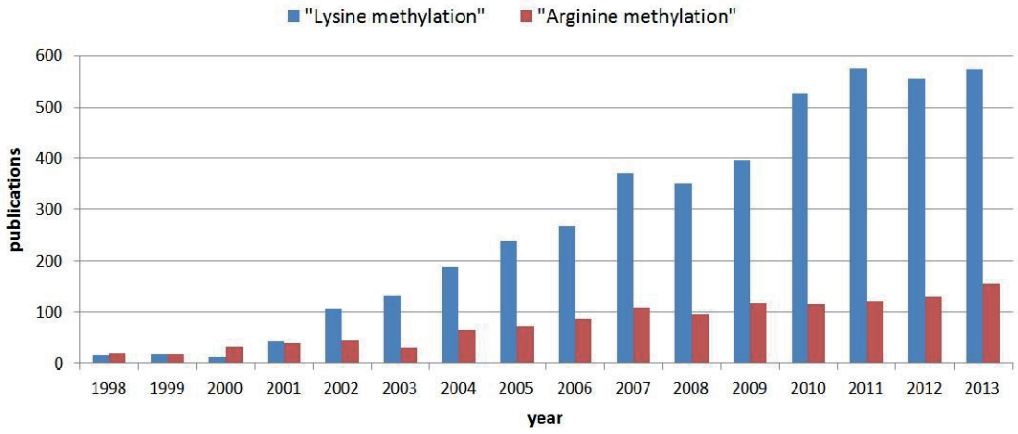

Figure 5. Publications on lysine and arginine methylation from 1998 to 2013. Chart shows the number of publications per year found in PubMed with the search query "lysine methylation" (blue) or "arginine methylation" (red) , from 1998 to 2013. Expanded from Paik et al. 2007.

While protein methylating (and even demethylating) activity had been isolated from cellular fractions many decades ago, and the presence of methylated amino acid residues was found to be widely spread across the evolutionary tree, the methylation of proteins did not receive considerable attention until recently. This is largely due to the discovery of arginine- and lysine-specific MTases that target histone proteins in the early 2000's. Histone methylation was found to play a key role in the "histone code" which regulates chromatin structure and gene expression, and has revitalized the field of epigenetics (Greer and Shi 2012). As can be seen from **Figure 5**, since the late 1990's, the numbers of publications in PubMed on either lysine methylation or arginine methylation have exploded. Interestingly, although methylated arginine residues are apparently more widespread than methylated lysines in human proteins (Bremang et al. 2013) and despite the fact that publications on arginine methylation were proportionally more abundant in the late 1990s, research on lysine methylation currently dominates the field.

2.1 Lysine methylation

Lysine can be modified with either one, two, or three methyl groups on its ε-amino group (**Figure 6**). Methylation does not change the overall positive charge of the residue, however it increases the bulk and hydrophobicity, and full trimethylation makes the positive charge permanent. In addition, methylation blocks for other mutually exclusive modifications of lysine, most commonly acetylation or ubiquitination (Liu et al. 2014).

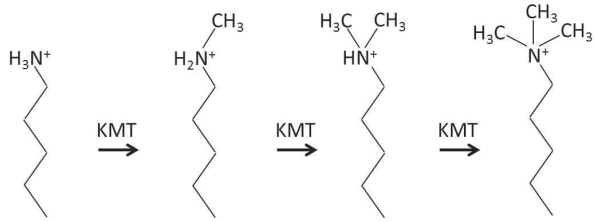

Figure 6. Methylation of lysine. Schematic representation of the chemical structure of the lysine side chain in its unmethylated, monomethylated, dimethylated, and trimethylated states. Methylation catalyzed by a lysine methyltransferase (KMT).

For the first time the presence of methylated lysine residues was observed in the flagellar protein of the bacterium *Salmonella typhimurium*, over 55 years ago (Ambler and Rees 1959). Five years later, the first methyllysine residues were discovered in histones isolated from calf thymus, wheat germ and rabbit organs (Murray 1964). Subsequently, it was demonstrated that the methyllysines present in living organisms could be found in all three possible methylation states, i.e. mono-, di-, or tri-methylated. (Paik and Kim 1967, Hempel et al. 1968). Initially, it was unclear whether such methyllysine residues were a result of post-translational modification, or whether they were pre-formed and incorporated directly into proteins during translation. Finally, in 1970, protein lysine-methylating activity was purified from calf thymus and designated as protein methylase III, showing that the methylation of lysine residues is an enzymatic, post-translational process (Paik and Kim 1970). Interestingly, this enzyme subsequently turned out to be one of the lysine-specific histone methyltransferases, SETD7, which methylates histone H3 on lysine 4 in addition to lysine residues in various non-histone proteins (Martin and Zhang 2005). Over the years, the presence of methyllysine residues was found in a large number of proteins from various organisms, including cytochrome c from plants and fungi, mammalian myosin and citrate synthase, calmodulin (CaM), and the prokaryotic elongation factor Tu as well as various ribosomal proteins (DeLange et al. 1969, Hardy et al. 1970, Bloxham et al. 1981, Watterson et al. 1980, Ames and Niakido 1979, Dognin and Wittmann-Liebold 1980). However, the responsible MTases could not be identified at the time, and besides an indication that lysine-methylation of CaM affects its capacity to stimulate NAD kinase activity (Roberts et al. 1986), few biological functions have been attributed to these modifications.

As mentioned above, a whole family of MTases containing the SET domain was eventually identified, and these enzymes appear to be devoted entirely to the methylation of lysine residues, especially in histone proteins. Additionally, in 2002, a MTase belonging to the 7BS superfamily, DOT1L, was shown to methylate a lysine residue in the core of histone H3, demonstrating that 7BS enzymes may also be lysine-specific (Feng et al. 2002). In 2010, another human lysine-specific 7BS MTase had been identified, the elusive CaM MTase (CaM-KMT) (Magnani et al. 2010). Subsequently, the numbers of reports describing 7BS MTases which methylate lysine residues have steadily increased, and the work in this thesis characterizes the activity of several such novel MTases.

2.2 Protein demethylation

Enzymes catalyzing the removal of methyl marks from protein residues also exist. Evidence for demethylation of lysine residues in cells was provided already in the early 1960s, when an enzyme purified from the rat kidney was shown to liberate lysine from methyllysine in the presence of flavin adenine dinucleotide (FAD) and O_2 (Kim et al. 1964). This enzyme was designated as ε-alkyl-lysinase and is likely to be identical to LSD1, the first protein demethylase cloned and characterized over 40 years later (Shi et al. 2004). The demonstration that LSD1 demethylates mono- or dimethylated lysines 4 and 9 in the histone H3 sparked great interest, as methylation of histones was previously thought to be irreversible, and since then, many more demethylases have been described. Structurally, the human histone lysine demethylases are a diverse group of proteins which can be broadly categorized into two functional enzymatic families. The small FAD-dependent amine oxidase family is composed of the enzymes LSD1 and LSD2, which are able to demethylate only mono- or dimethylated lysines. The Fe(II) and α-ketoglutarate-dependent dioxygenase family of proteins containing the catalytic JMJC domain is much larger, with at least 30 different human members, and these enzymes are able to demethylate also trimethylated lysine residues. The respective demethylation mechanisms of the two families are shown in **Figure 7**. In addition to histones, several demethylases have been shown to act on non-histone substrates such as p53, NF-kB, E2F1, DNMT1, and p65 (Reviewed in Kooistra and Helin 2012).

It has also been suggested that arginine residues can be demethylated. A member of the Fe(II) and α-ketoglutarate-dependent dioxygenase family mentioned above, JMJD6, has been reported by one study to demethylate both symmetrically and asymmetrically dimethylated arginine residues, however demethylating activity of this enzyme has never been subsequently observed by other groups, and instead JMJD6 has been attributed a role in the lysine hydroxylation of RNA splicing factors (Chang et al. 2007, Webby et al. 2009, Hahn et al. 2010). However, monomethylated arginine may be converted by deimination into citrulline, a modification catalyzed by peptidylarginine deiminases (reviewed in Bicker and Thompson 2013). Yet, as no enzyme has ever been found for the conversion of citrulline back to arginine, citrullination does not represent a true demethylation process, although it does prevent re-methylation of the residue, adding a new layer of regulation to the methylation of arginine residues.

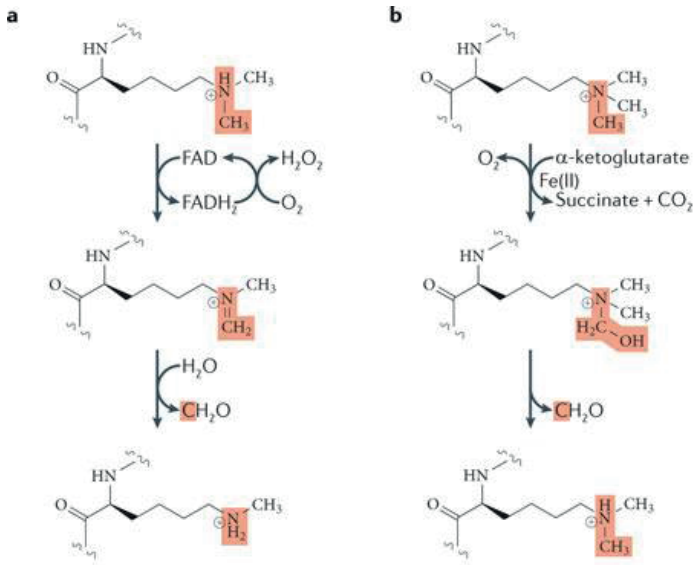

Figure 7. Catalytic mechanisms of lysine demethylation. The two different reaction mechanisms employed by (a) the FAD-dependent amine oxidases LSD1 and LSD2 and (b) JMJC-containing Fe(II) and α-ketoglutarate-dependent dioxygenases. From Kooistra and Helin 2012.

In addition to the arginine- and lysine-specific demethylases, several demethylases acting on other residues have been identified. In *E. coli*, the enzyme CheB removes the carboxylic methyl ester of methylglutamate residues on the methyl-accepting Chemotaxis proteins which control bacterial chemotaxis. The reaction occurs through hydrolysis of the methyl ester to form glutamate and methanol (Park et al. 2006, West et al. 1995). In eukaryotes, protein phosphatase 2A (PP2A) is carboxymethylated at the C-terminal leucine residue. This modification is reversed by a methylesterase, PME-1, regulating the activity of PP2A (Ogris et al. 1999, Tolstykh et al. 2000). Furthermore, one of the enzymes required for the biosynthesis of diphthamide, a modified histidine residue found only in the eukaryotic elongation factor 2 (eEF2), is a demethylase that removes the methyl ether from the methylated diphthine intermediate of the reaction (Lin et al. 2014).

3. Methylation of the translation apparatus

All components of the protein synthesis machinery may be modified by methylation, including ribosomal (rRNA), transfer (tRNA) and messenger (mRNA) RNAs, as well as ribosomal proteins and various translation factors, impacting the translation process in various ways. Strikingly, almost two-thirds of the characterized *S. cerevisiae* MTases are involved in methylating components of the translation apparatus, listed in **Table 1** (as of June 2014). About two-thirds of the translation associated MTases methylate RNA species, while the rest target protein residues.

Table 1. *S. cerevisiae* MTases involved in methylation of translation components. Enzymes are grouped by substrate type. Data correct as of June 2014, modified from Dzialo et al. 2014a.

tRNA	rRNA	mRNA	Translation factors	Ribosomal proteins
Trm1	Spb1	Abd1	Mtq1	Hmt1/Rmt1
Trm2	Nop2		Mtq2	Rmt2
Trm3	Rrp8		Efm1	Ntm1
Trm5	Mrm2		Efm2	Hpm1
Trm7	Dim1		See1	Rkm1
Trm8	Mrm1			Rkm2
Trm9	Emg1			Rkm3
Trm10	Bmt2			Rkm4
Trm11	Rcm1			Rkm5
Trm13	Bmt5			Sfm1
Trm44	Bmt6			
Ppm2	Bud23			
Gcd14				
Tye3				
Trm140				
Ncl1				

3.1 Methylation of RNA

The nucleotides of tRNAs are heavily modified, in fact there are about 25-30 different types of tRNA modifications in an organism, with an average of 13 modifications spread throughout each tRNA in humans (Machnicka et al. 2013). One of the most common is C^5-cytosine methylation, which occurs in various tRNAs and at several positions, and this modification was recently linked to the rate of protein synthesis (Tuorto et al. 2012). Other modifications contribute to increased tRNA stability and translational fidelity (reviewed in Jackman and Alfonzo 2013).

rRNA may be methylated on the 2'-hydroxyl group of the ribose or at several positions in the base, and it is estimated that the human ribosome contains over a hundred such modification sites (Maden 1990). These modifications occur largely in the functionally important areas of the ribosome, such as the peptidyl transferase center, the A, P and E sites, the polypeptide exit tunnel, and at the interface between the large and small ribosomal subunits (Decatur and Fournier 2002). Methylation of rRNA plays a role in ribosome biogenesis and activity, impacts the translation of certain mRNAs and may be important for proper embryonic development (Liang et al. 2007, Sharma et al. 2013, Basu et al. 2011). In bacteria, methylation of rRNA also contributes to their resistance to various antibiotics which target the prokaryotic ribosome (Vester and Long 2009).

Even the mRNA may be methylated, both on the 5' guanosine cap and at internal positions. The most common mRNA modification is N^6-Methyladenosine, and it has been observed since the 1970s. The recent discovery of the responsible MTases and demethylases, as well as "reader"-proteins for such modifications, suggests a possible function for mRNA methylation as a mark for post-transcriptional regulation of gene expression (reviewed in Fu et al. 2014).

3.2 Methylation of ribosomal proteins

At least 15 yeast MTases are involved in the methylation of ribosomal proteins and translation factors (**Table 1**). Of the 10 MTases acting on ribosomal proteins, Rmt1, Rmt2 and Sfm1 methylate arginine residues, Ntm1 methylates N-terminal prolines, Hpm1 methylates a histidine residue, while the rest, Rkm1-5, methylate lysines.

Hpm1 is a fascinating enzyme that belongs to the 7BS superfamily. It catalyzes the trimethylation of a histidine residue in a ribosomal protein of the large subunit, Rpl3. Histidine methylation has been observed in several animal proteins such as actin and myosin but in yeast appears to be present exclusively in Rpl3. This modification was shown to contribute positively to translational fidelity and proper assembly of the 60S subunit (Al-Hadid et al. 2014). It is unknown whether mammalian RPL3 is methylated, but the Hpm1 MTase does have a putative homologue in humans, METTL18, which was found to associate with Rpl3 (Cloutier et al. 2013). Notably, the METTL18 enzyme is part of the MTase Family 16 (MTF16) subfamily of human MTases which is the object of study in this thesis.

The substrates of the five yeast ribosomal lysine (K) MTases, Rkm1-5, are mostly ribosomal proteins of the large subunit, as listed in **Table 2**. Two of the MTases, Rkm1 and Rkm2, each methylate two different lysines within a single protein – Rpl23 or Rpl12, respectively (Porras-Yakushi et al. 2005 and 2006). In addition, Rkm1 also monomethylates a lysine residue in Rps18 of the small subunit (Couttas et al. 2012). The MTases Rkm3 and Rkm4 both monomethylate the same protein, Rpl42, but target different lysine residues (Webb et al. 2008a). Uncommon for a lysine MTase, Rkm5, which monomethylates Rpl1, is a 7BS enzyme rather than a SET-domain enzyme (Webb et al. 2011). These five MTases are responsible for all of the known lysine methylations identified so far in ribosomal proteins of *S. cerevisiae*, with the exception of trimethylation of Rpl11 on lysine 75 (Couttas et al. 2012). Only one of them, Rkm1, has a putative homologue in humans, however neither Rpl23 nor Rps18 appear to be methylated in mammals, so this pattern of ribosomal lysine methylation is likely to be specific to fungi or lower eukaryotes.

Table 2. *S. cerevisiae* ribosomal lysine MTases. me1 - monomethyl, me2 - dimethyl, me3 - trimethyl, * - has a putative human homologue.

MTase	Substrate	Target residue and modification	MTase family
Rkm1*	Rpl23	Lys 106 me2 Lys 110 me2	SET
	Rps18	Lys 48 me1	
Rkm2	Rpl12	Lys 3 me2 Lys 10 me3	SET
Rkm3	Rpl42	Lys 40 me1	SET
Rkm4	Rpl42	Lys 55 me1	SET
Rkm5	Rpl1	Lys 46 me1	7BS

3.3 Methylation of translation factors

As of June 2014, five MTases that are involved in the methylation of translation factors, including release factors and elongation factors, had been identified in *S. cerevisiae* (**Table 1**). Two of these target glutamine residues and the remaining three methylate lysines. The Mtq1 and Mtq2 enzymes are responsible for glutamine methylation of the GGQ motif in two translation release factors, the mitochondrial Mrf1p and the cytoplasmic eRF1, respectively (Polevoda et al. 2006). The GGQ motif is conserved throughout evolution and is critical for the catalytic activity of these release factors, and glutamine methylation of the motif significantly enhances the rate of translation termination (Dincbas-Renqvist et al. 2000). It has also been shown that the Mtq2 MTase has a functional homologue in humans, HEMK2 (Figaro et al. 2008).

The See1 and Efm1 MTases are responsible for lysine methylation of eukaryotic elongation factor 1A (eEF1A) (Lipson et al. 2010). eEF1A is a GTPase that is essential for translation with a key role in delivery of tRNA to the ribosomal A-site, but has various other functions outside of translation (Lamberti et al. 2004). eEF1A is also one of the most highly lysine-methylated proteins (Polevoda and Sherman 2007). Even though yeast eEF1A is highly similar to the mammalian protein (81% identical to the rabbit protein), the pattern of lysine methylation is quite different. eEF1A from animals may contain up to six methylated lysine residues – lysines 36, 55, 79, 165 and 316, and an additional methylation at lysine 290 so far

observed only in chick neural crest cells (Cavallius et al. 1993, Vermillion et al. 2014). In yeast, eEF1A contains four methylated lysine residues, the lysines 30, 79, 316 and 390 (Cavallius et al. 1993, Couttas et al. 2012). Thus, only two methylation sites, lysine 79 and lysine 316, are conserved from yeast to mammals.

Efm1 is a SET-domain MTase, which is responsible for monomethylation of lysine 30 in yeast eEF1A. While the analogous lysine residue in animal eEF1A is present, it appears to be unmodified. There are also no sequence homologues of Efm1 found in higher eukaryotes, indicating that this modification may be specific to yeast. (Lipson et al. 2010, Couttas et al. 2012). The methylation of lysine 316, on the other hand, is conserved from yeast to mammals, though this modification appears to manifest itself as a dimethylation in yeast and as a trimethylation in higher eukaryotes (Cavallius et al. 1993). In yeast this methylation is mediated by the See1 MTase which belongs to the 7BS superfamily. Its human homologue, METTL10, has been identified as a target for phosphorylation during mitosis, suggesting some sort of regulatory mechanism (Dephoure et al. 2008). In addition, the methylation of eEF1A lysine 316 was shown to be affect the replication of several viruses in yeast and plants, providing a glimpse of its functional role (Li et al. 2014).

The 7BS enzyme Efm2 methylates another yeast elongation factor, the eukaryotic elongation factor 2 (eEF2) on lysine 613 (Zhang et al. 2014). eEF2 and its modifications are described in detail below, as the identification of another lysine MTase targeting this protein is the focus of one of the studies presented in this thesis.

4. Eukaryotic translation elongation factor 2 (eEF2)

The process of protein synthesis is conventionally divided into the initiation, elongation and termination phases. Translation elongation, in turn, can be subdivided into three more stages: decoding, transpeptidation and translocation. During decoding, eukaryotic translation elongation factor 1 (eEF1) delivers aminoacyl-tRNA to the ribosome which then selects and binds the tRNA complementary to the mRNA codon in the ribosomal A site. During transpeptidation, the peptidyl group on the P-site tRNA is transferred to the aminoacyl group in the A site through the formation of a peptide bond. Finally, in the translocation step, the A-site and P-site tRNAs, together with the bound mRNA, are moved to the P site and E site respectively, following which the ribosome returns to its pre-translocation state. The process of translation elongation requires the participation of several non-ribosomal proteins, or elongation factors, such as the universally conserved eEF1A and eEF2 (Voet and Voet 2011).

Eukaryotic translation elongation factor 2 (eEF2) is a GTPase that catalyzes the translocation step of translation elongation by binding to the ribosome and hydrolyzing GTP. This enzyme is essential and highly conserved in all eukaryotes, and has functional homologues both in archaea (aEF2) and bacteria (EFG). eEF2 is a highly-abundant monomeric protein, composed of six structural domains (G' and I-V), which fall into three distinct structural blocks that move relative to each other during eEF2 function (**Figure 8**) (reviewed in Jørgensen et al. 2006). eEF2 is subject to several well-studied PTMs which are conserved throughout evolution. One is a reversible phosphorylation event which impacts eEF2 activity, the other is a constitutive formation of a unique diphthamide residue the role of which is still not entirely clear. Several other modifications of eEF2 have also been reported.

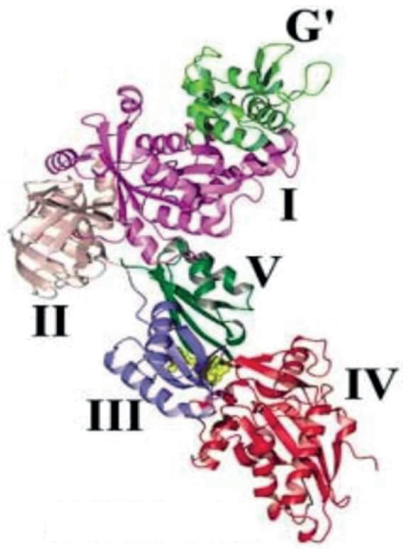

Figure 8. Crystal structure of eEF2 in complex with sordarin. Colored by domain, sordarin shown in yellow in its binding site between domains III, IV and V. Adapted from Jorgensen et al. 2003.

4.1 Threonine phosphorylation of eEF2

During the cell cycle or under various stresses, eukaryotic cells may regulate protein synthesis at the elongation step by modifying eEF2 activity. The major mechanism for regulation of eEF2 is its inactivation through phosphorylation on a threonine residue in its GTP-binding domain by a specific Ca^{2+}/calmodulin-dependent kinase, eEF2K, and subsequent reactivation through dephosphorylation by protein phosphatase 2 (Ryazanov and Davydova 1989, Gschwendt et al. 1989). The eEF2K kinase itself is highly regulated by phosphorylations at several sites, downstream of various signaling pathways impacting translation elongation. It is negatively regulated by signaling through the mammalian target of rapamycin complex 1 (mTORC1) and positively regulated through kinases downstream of cAMP, a catabolic signal which inhibits protein synthesis, as well as the AMP-activated protein kinase, AMPK, a major sensor of low cellular energy status. In addition, eEF2K is dependent on the intracellular levels of Ca^{2+} (reviewed in Kenney et al. 2014).

4.2 Diphthamidation

eEF2 is also post-translationally modified by diphthamidation. Diphthamide is present on archaeal and eukaryotic EF2 and is the only known modification of its kind. The biosynthesis of diphthamide is very complex and involves seven proteins, DPH1-DPH7, one of which is a MTase and one a demethylase. Additionally, this process relies on the co-factor SAM, once as a source of an aminocarboxypropyl group and subsequently as a source of methyl groups (**Figure 9**).

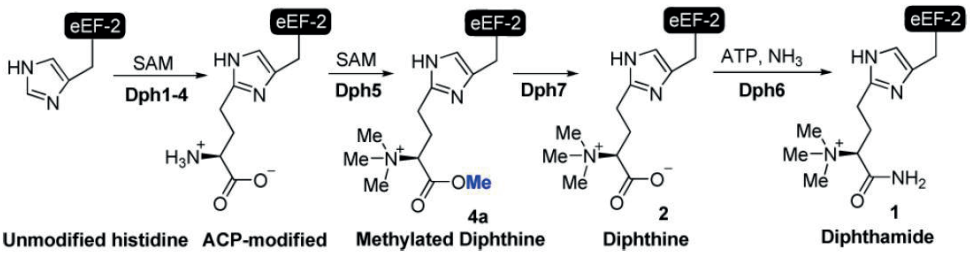

Figure 9. Schematic representation of the biosynthesis of diphthamide. Adapted from Lin et al. 2014.

The first step of diphthamide biosynthesis involves 4 enzymes, DPH1-4, which transfer the aminocarboxypropyl (ACP) group from SAM to the C2 carbon of the imidazole ring. DPH1 and DPH2 are radical SAM enzymes containing a [4Fe-4S]-cluster, which form a complex and use a radical reaction mechanism to transfer the ACP group. DPH3 and DPH4 are thought to chaperone the complex (Zhang et al. 2010). The second step again involves SAM, this time as a source of methyl groups, and the MTase DPH5, also known as diphthine synthase. DPH5 trimethylates the amino group of the ACP-histidine from step one, to form diphthine. Some ambiguity still surrounds this step, however in 2014 it was suggested that DPH5 in fact creates methylated diphthine. That is, DPH5 also catalyzes methylation of the ACP-histidine carboxyl group in addition to the trimethylation of the amino group, apparently having an unusual dual specificity for methylation of both an N and an O atoms in a single residue. The methylated diphthine is then subject to demethylation by the DPH7 demethylase. Lastly, diphthine is turned into the final diphthamide product by the ATP-dependent enzyme, diphthamide synthetase, DPH6 (Lin et al. 2014, reviewed in Schaffrath et al. 2014).

The conservation of the diphthamide modification and the maintenance of such a large number of genes involved in its synthesis throughout all of eukaryotic evolution underlines its fundamental importance. However, its precise biological function is still not well understood (reviewed in Su et al. 2013). Diphthamide lies on the tip of domain IV of eEF2, which comes in close proximity to the tRNA in the P-site of the ribosome, and appears to interact with two universally conserved adenines in 18S rRNA that serve to distinguish cognate from near-cognate codon-anticodon pairs (Spahn et al. 2004, Taylor et al. 2007). These interactions may somehow stabilize the codon-anticodon duplexes during translocation, thereby preventing frameshifts (Jorgensen et al. 2005). The role of diphthamide in maintaining translational fidelity is supported by the observation that the lack of this modification results in increased -1 frameshifting during protein synthesis (Ortiz et al. 2006).

Surprisingly, none of the *Dph* genes in yeast are essential, and their deletion contributes to mostly marginal phenotypes. In mammals, however, the deletion of several *Dph* genes was shown to have detrimental effects on development. In mice, homozygous knockout of *DPH1* or *DPH3* leads to embryonic death and homozygous *DPH5* mutants were retarded in growth and development (Chen and Behringer 2004, Liu et al. 2006, Webb et al. 2008b). It is unclear,

however, whether these effects are due to decreased translational fidelity or rather due to the loss of some other, yet undiscovered function of diphthamide.

Additionally, the diphthamide residue in eEF2 is a specific target for several bacterial toxins, including diphtheria toxin, Pseudomonas exotoxin A and cholix toxin (Jorgensen et al. 2008). Through ADP-ribosylation of diphthamide, these toxins inactivate eEF2, halting protein synthesis and leading to cell death. It has also been suggested that eEF2 may be ADP-ribosylated by endogenous ADP-ribosyltransferases in a wide variety of ekaryotic cell types, serving as a mechanism regulating cellular protein synthesis (Lee and Iglewski 1984, Sitikov et al. 1984). Later, however, this activity was shown to target other eEF2 residues than the diphthamide (Bektas et al. 2006).

4.3 Other eEF2 modifications

In addition to diphthamidation, ADP-ribosylation and EF2K-mediated phosphorylation, eEF2 is subject to several other PTMs. Recently, another phosphorylation event was described for eEF2. The cyclin A-cyclin-dependent kinase 2 (CDK2) phosphorylates eEF2 on a novel site, a serine residue in domain IV, and appears to stimulate eEF2K-mediated threonine phosphorylation, possibly by recruiting eEF2K to eEF2 (Hizli et al. 2013)

As for methylation of eEF2, it has been shown that several arginine residues in eEF2 in mouse fibroblast cells become symmetrically dimethylated upon treatment with the basic fibroblast growth factor. The biological significance of these methylations is unknown, however it was proposed that these methylations are catalyzed by the protein arginine MTase PRMT7, with another protein arginine MTase, PRMT5, coordinating the process. Interestingly, the methylation also appears reversible, suggesting the involvement of an arginine-demethylase (Jung et al. 2011). Additionally, in yeast, eEF2 is the target of a 7BS lysine-specific methyltransferase, Efm2, which methylates lysine 613 and may be involved in maintaining translational accuracy as the knockout of this gene has been reported to increase the incidence of -1 ribosomal frameshifting (Couttas et al. 2012, Dzialo et al. 2014b).

5. Valosin-containing protein (VCP) / p97

Valosin-containing protein (VCP), also known as p97, is a highly abundant essential protein, ubiquitously expressed in all archaeal and eukaryotic cells. It belongs to the AAA+ ATPase superfamily (ATPases associated with diverse cellular activities) and participates in a multitude of cellular processes. Like most other members of the AAA+ superfamily, VCP assembles into oligomeric ring-shaped complexes and uses the energy of ATP hydrolysis to structurally remodel client molecules (Erzberger and Berger 2006). VCP has been linked to diverse cellular processes including endoplasmic reticulum-associated degradation (ERAD), autophagy, post-mitotic membrane fusion, Golgi reassembly, transcription factor regulation, chromatin remodelling in response to DNA damage, and aggregate prevention and disassembly (Meyer and Weihl 2014).

A unifying theme of VCP function is ubiquitination and indeed many of the proteins associated with VCP, as well as VCP itself, can bind ubiquitin, whereas others are involved in further ubiquitination of VCP substrates. VCP segregates the ubiquitinated proteins from their environment i.e. multiprotein complexes, membranes, or chromatin. The specificity of VCP in its various functions is mediated through an array of adaptor proteins which guide the protein to certain complexes or subcellular locations, and different sets of at least 30 cofactors have been shown to be responsible for modulating these processes (Schuberth and Buchberger 2008, Yeung et al. 2008, Meyer et al. 2012).

For example, during ERAD, terminally misfolded membrane and luminal proteins in the ER are ubiquitinated by membrane-anchored ubiquitin ligases and extracted from the ER into the cytosol, where they are subsequently degraded by the proteasome. VCP facilitates this process from the cytosolic side by recognizing the ubiquitinated ER proteins and extracting them out into the cytosol through the formation of different complexes with several cofactors, including Npl4 and Ufd1, erasin and ataxin-3. (Ye et al. 2003, Lim et al. 2009, Zhong and Pittman 2006). By associating with a different cofactor, the ubiquitin regulator-X (UBX) domain-containing protein p47, VCP functions in membrane fusion of the nucleus and of the Golgi cisternae after mitosis (Meyer 2005). Very recently, VCP has also been implicated in the DNA damage response, where it facilitates the specific extraction of ubiquitilated proteins from chromatin through association with the adaptors UBX4 and UBX5 (Dantuma et al. 2014).

5.1 VCP structure

The VCP protein is divided into four domains - two ATPase domains D1 and D2, which in the assembled homohexamer are organized as two stacked rings with a central channel, and the regulatory N- and C-terminal domains which are important for association with various interacting proteins (**Figure 10**). Most of the ATPase activity is attributed to the D2 domain, whereas the D1 domain is proposed to be primarily structural, facilitating homohexamerization of VCP, and exhibits only minor heat-induced ATPase activity (Song et al. 2003). In addition, binding, but not hydrolysis, of ATP in the D1 domain appears to regulate the recruitment of adaptor proteins to the N-terminal domain, possibly linking VCP function to the metabolic status of the cell (Chia et al. 2012). The binding sites in the N- and C-terminal domains facilitate the diversity of VCP functions by coordinating its large collection of cofactors.

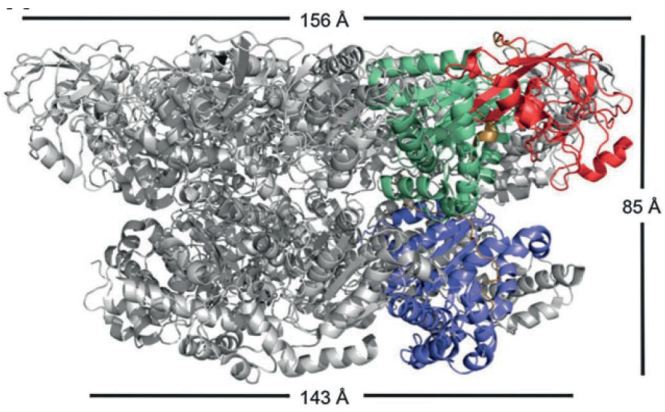

Figure 10. Crystal structure of hexameric mouse VCP in presence of ATPγS. One of the protomers is colored by domain: N-domain is in red, D1 in green, D2 in blue, C-terminal domain not resolved. Size of the hexamer is indicated in Å. From Chapman et al. 2010.

5.2 Post-translational modifications of VCP

VCP is subject to many PTMs. A recent study has revealed the presence of acetylation and phosphorylation marks on at least 60 VCP residues, including the phosphorylation of 18 serines, 15 threonines and 5 tyrosines and the acetylation of 22 lysines. Sixteen of these sites are clustered in the N-terminal region and sixteen more in a 120aa-long region in the D2 domain, while the others are spread throughout the protein. Regrettably, neither the function, nor the enzymes responsible for most of these modifications are yet identified (Mori-Konya et al. 2009).

The phosphorylation of tyrosine 805 in the C-terminus is the most studied VCP PTM and has found several functional roles. This modification has been shown to block the binding of several VCP-interacting proteins containing the PUB or PUL domains *in vitro*, suggesting a regulatory role in cofactor binding (Zhao et al. 2007, Mullally et al. 2006). The most well-characterized PUB-containing VCP adaptor protein is Glycopeptide N-glycosidase (PNGase), which is involved in the deglycosylation of ERAD substrates. VCP normally interacts with this cofactor by inserting tyrosine 805 into a hydrophobic pocket in the PUB domain of PNGase; phosphorylation of this residue disrupts the interaction due to the negative charge of the phosphate group, leading to electrostatic hindrances within the hydrophobic binding pocket (Zhao et al. 2007). It has also been shown that the yeast homologue of VCP, Cdc48, is relocated from the cytosol into the nucleus upon phosphorylation of tyrosine 805, and that in mammalian cells VCP also appears to shuttle between the nucleus and the cytoplasm, possibly depending on tyrosine phosphorylation or some other PTM (Mori-Konya et al. 2009).

The acetylation of VCP has also been ascribed some functional roles. Mutation of one of the acetylation sites, lysine 696, to mimic acetylated and non-acetylated states, was shown to increase and decrease VCP ATPase activity, respectively. Given the large number of acetylated lysines found in VCP, and its direct association with HDAC6, a histone deacetylase, it is plausible that VCP may also be regulated through acetylation (Seigneurin-Berny et al. 2001, Mori-Konya et al. 2009).

AIMS OF STUDY

The aim of the research presented in this thesis was to characterize several novel human seven-β-strand methyltransferases (MTases) belonging to MTase family 16 (MTF16), primarily the enzymes METTL21D (VCP-KMT, **Paper I**) and FAM86A (eEF2-KMT, **Paper II**). In short, our purpose was to identify the substrates targeted by these enzymes, to show direct MTase activity on the substrates *in vitro* and the presence of such an activity *in vivo,* to search for the presence of functionally homologous MTases in other organisms, and to investigate the biological significance of VCP-KMT and eEF2-KMT-mediated methylation by using corresponding gene knockout cells.

SUMMARY OF PAPERS

Paper I

Lysine methylation of VCP by a member of a novel human protein methyltransferase family

In this paper we introduce the 10 members of the human methyltransferase Family 16 (MTF16) and demonstrate protein MTase activity for 3 of them: METTL21A, METTL21C, and METTL21D. We also show that METTL21D trimethylates a conserved lysine residue in the central pore of the molecular chaperone VCP and correspondingly rename the enzyme to VCP-KMT. Additionally, through the use of bioinformatics and *in vitro* activity assays, we identify or infer the lack of functional VCP-KMT homologues in several eukaryotic organisms. Importantly, we show that the ablation of VCP-KMT-mediated methylation via knockout of the *VCP-KMT* gene from several human cell lines reduces their proliferation rate and invasive ability, supporting a role for VCP-KMT in cancer metastasis.

Paper II

Identification and characterization of a novel evolutionarily conserved lysine-specific methyltransferase targeting eukaryotic translation elongation factor 2 (eEF2)

This study unravels the function of another human member of MTF16, the enzyme FAM86A. We show that FAM86A has a functional homologue in *S. cerevisiae*, and that both of these enzymes are able to trimethylate a specific lysine residue in the eukaryotic translation factor eEF2. This eEF2-specific activity leads us to rename the human enzyme eEF2-KMT and the yeast enzyme Efm3 (Elongation factor methyltransferase 3), according to existing nomenclature. We also show that trimethylation of this residue occurs *in vivo* in yeast, rat, rabbit and human cells. Interestingly, in the brain of the rat, eEF2 appears to be hypomethylated, allowing for the existence of a regulatory mechanism. In addition, knockout of the gene encoding Efm3 in yeast increases sensitivity of the cells to the eEF2-specific inhibitor sordarin and leads to increased -1 translational frameshifting, showing that lysine trimethylation of eEF2 has important functional consequences.

DISCUSSION

1. Characterization of VCP-KMT

In **Paper I**, we describe the novel human methyltransferase (MTase) METTL21D (VCP-KMT) which targets a specific lysine residue in valosin-containing protein (VCP). VCP-KMT was initially picked up as a yeast two-hybrid interactant for another enzyme studied in the Falnes group at the time, the putative demethylase ALKBH6. Although the interaction was never confirmed, it sparked interest in VCP-KMT. Bioinformatic analysis showed that VCP-KMT is part of a group of ten closely related human 7BS MTases, annotated as the putative Methyltransferase Family 16 (MTF16) (**Paper I Table 1**, Katz et al. 2003). When we first set out to study the MTF16, none of its ten human members had yet been assigned a function. Soon after, however, another research group reported that one of these enzymes is the MTase responsible for trimethylation of lysine 115 in the Ca^{2+}-sensing protein calmodulin (CaM). CaM methylation has been observed in various organisms since the 1980s and was even shown to have consequences for CaM function, however the responsible MTase was not identified until 2010 (Watterson et al. 1980, Roberts et al. 1986, Magnani et al. 2010). Besides CaM-KMT, no function had been attributed to the remaining nine human MTF16 members until the publication of **Paper I**.

1.1 VCP-KMT *in vitro* activity

To find the function of this novel MTase, we started out with *in vitro* assays investigating the activity of recombinant VCP-KMT on several types of substrates. We observed that VCP-KMT was unable to methylate DNA or RNA, but, at high enzyme concentrations and a higher than physiological pH of 8.5, had some activity on homopolymers of lysine, as well as on several histone proteins, especially histone H2B - the most lysine-rich histone (**Paper I Fig. 1**). Subsequent MS-analysis of the methylated H2B product, however, showed that methyl groups were rather evenly distributed amongst all the lysines in the protein, suggesting that this VCP-KMT activity was not sequence-specific and thus H2B was unlikely to be a *bona fide* substrate of VCP-KMT *in vivo*. Nevertheless, these preliminary results gave a good indication that VCP-KMT is a functional MTase with protein substrates and likely targets lysine residues.

To identify the *bona fide* substrate, we commissioned a yeast two-hybrid screen, with VCP-KMT as bait and a human placental cDNA library as prey. The screen identified VCP as a high-confidence VCP-KMT interactant, and we found that recombinant VCP was efficiently methylated by VCP-KMT *in vitro*. Moreover, a truncated version of VCP lacking the D2 and C-terminal domains (VCPΔD2) was an even better substrate than full-length VCP. MS analysis of the reaction products identified a single trimethylated residue in VCP and VCPΔD2, lysine 315 (**Paper I Fig. 2**). When this residue was mutated, VCP-KMT was no longer able to methylate VCP, confirming that lysine 315 is the only target residue. Additionally, we investigated the mode of substrate recognition for VCP-KMT by monitoring its activity on short peptides containing the VCP target lysine and the surrounding region, and on mutant VCPΔD2 where several residues adjacent the methylation site were mutated to alanines without changing the target lysine. We found that VCP-KMT was unable to methylate the peptides but was just as active on the wild type as on the alanine-mutated VCPΔD2. This suggested that VCP-KMT does not recognize a specific linear sequence but rather requires a larger three-dimensional structure for substrate recognition (**Paper I Fig. 2 and Suppl. Fig. S3**).

Lysine 315 lies in domain D1 of VCP and, in the assembled VCP hexamer, is located in the inner pore, inaccessible for VCP-KMT. Enhanced activity of VCP-KMT on VCPΔD2, which forms a less stable hexamer than the full-length protein, might be explained by more efficient disruption of this hexamer by the MTase (Wang et al. 2003). This is further supported by size exclusion chromatography which showed that VCP-KMT is unable to form a stable complex with full-length hexameric VCP, yet is able to do so with hexameric VCPΔD2 (**Paper I Fig. 2D**). Moreover, VCP-KMT appears to methylate only higher-order aggregates of VCP rather than the biologically functional hexamers, whereas VCPΔD2 is an equally good substrate in both forms (**Paper I Fig. 2E**). The inability to disrupt the VCP hexamer may point to that, *in vivo*, VCP-KMT acts exclusively on VCP protomers prior to their assembly into hexamers, or to the presence of additional factors which are able to disrupt the VCP hexamer and allow access for VCP-KMT to its target residue. The latter hypothesis is supported by a subsequent study which showed that the UBX domain-containing tethering protein ASPSCR1, which interacts with VCP-KMT and has previously been implicated in the conversion of VCP hexamers into monomers, stimulates the methylation of VCP by VCP-KMT (Orme and Bogan 2012, Cloutier et al. 2013). Intriguingly, ASPSCR1 localizes to the ER-to-Golgi intermediate compartment and the ER exit sites, and is implicated in the reassembly of the Golgi apparatus following mitosis (Orme and

Bogan 2012). Among its multitude of functions, VCP is also a key player in Golgi reassembly, and it may be fruitful to investigate whether VCP methylation status affects this process. However, it is not unlikely that other proteins in the horde of VCP adaptors involved in other processes would also be able to disrupt the hexamer and stimulate VCP-KMT-mediated methylation.

1.2 Biological significance of VCP-KMT activity

To elucidate the biological function of VCP methylation, we employed zinc-finger nuclease technology to disrupt the *VCP-KMT* gene in several human cancer cell lines. The zinc-finger nucleases targeted exon 1 of the *VCP-KMT* gene, generating a double strand break which could then be repaired by the process of non-homologous end-joining, which often introduces insertional or deletional mutations. Clones with frameshift mutations in all *VCP-KMT* alleles were obtained for HeLa (epithelial), U87-MG (glioma), and the HEK293-derived, 293 T-REx Flp-In (kidney) cell lines. In all cases, MS analysis showed that whereas VCP was almost exclusively trimethylated on lysine 315 in wild type cells, consistently with our results from various mouse tissues, in the *VCP-KMT* knockout cells this residue was completely unmethylated (**Paper I Fig. 3**). This confirmed disruption of the *VCP-KMT* gene in the cell lines and demonstrated that VCP-KMT is the enzyme responsible for methylation of this residue in human cells.

We then attempted to use the VCP-KMT knockout cell lines to unravel the biological significance of VCP-KMT activity. As VCP is involved in a myriad of different processes, it is difficult to address all possible scenarios where its lysine methylation could be of importance. We started with some of the best studied processes and checked whether there were any differences between the wild type and knockout cells in degradation assays using reporters of the ubiquitin-proteasome system or when monitoring autophagy by LC3B processing. However, no significant differences were detected (**Paper I Suppl. Fig. S6 and S7**). We also examined the amount, complex size and subcellular distribution of VCP in the knockout cells but also found no deviation from the wild type. Furthermore, we investigated whether methylation is important for VCP ATPase activity. To this end, we purified endogenous VCP proteins from wild type or knockout HeLa cells and compared their ATPase activity – yet once again found no appreciable difference (**Paper I Fig. 5**). These results are in contradiction to a later study published by a different group claiming that methylation of lysine 315 in VCP inhibits its ATPase activity

(Cloutier et al. 2013). However, the authors of that study have assayed the ATPase activity of the recombinant VCPΔD2 protein, which lacks the major ATPase domain, D2. The slight difference in residual ATPase acitivity of the D1 domain in our experiments could have been masked by the overwhelming activity of the D2 domain. Moreover, there is a high degree of communication between the ATPase domains of VCP, and it is unclear how informative it is to study the activity of just one domain in isolation (DeLaBarre and Brunger 2003).

Fortunately, during our struggle to attribute biological meaning to VCP methylation, a study was published which demonstrated that expression of *VCP-KMT* was upregulated in several metastatic tumors and was particularly important for migratory behavior and metastatic potential (Thiele et al 2011). This led us to investigate whether our *VCP-KMT* knockout cells showed any abnormalities in the proliferation rate, or migratory and invasive abilities. Indeed, the VCP-KMT-deficient HeLa and 293 T-Rex Flp-In cells showed a significantly lower proliferation rate than the wild type, but the doubling time of U87-MG cells, which grow slowly already in the wild type, was not affected by *VCP-KMT* knockout. U87-MG knockout cells, however, displayed decreased migration and invasion rates as compared to the wild type (**Paper I Fgure 5**). These results demonstrated that the disruption of VCP-KMT expression may lead to slower growth and decreased migratory and invasive capacity of human cancer cells, providing further support to the participation of VCP-KMT in the process of cancer metastasis.

The exact mechanism through which VCP-KMT may promote metastasis is unclear. Given the invariable trimethylation status of VCP lysine 315 we observe in mammalian cells, this is more likely to be a static constitutive modification which subtly optimizes the overall function of VCP, rather than a regulatory mark. It is thus possible that higher levels of VCP-KMT expression in metastatic cells would ensure optimal VCP methylation and function. Indeed, while VCP is one of the most abundant proteins in the cell, VCP-KMT is by far less abundant, and a further elevation of VCP expression has been reported in many fast-growing metastatic cancers (Braun and Zischka 2008). Alternatively, it is plausible that at high intracellular levels VCP-KMT may methylate off-target substrates, for example histone proteins, lysine methylations of which have already been linked tightly with cancer (Varier and Timmers 2011). Another possibility is the existence of other VCP-KMT *bona fide* substrates in addition to VCP, increased methylation of which contributes to metastasis. To test this hypothesis we have subsequently treated protein extracts from HeLa *VCP-KMT* knockout cells with recombinant VCP-KMT and ^{3}H-SAM.

However, analysis of the reaction products by fluorography did not identify any enzyme-specific radiolabelled bands other than a single weak band corresponding in size to VCP, suggesting that VCP may in fact be the only VCP-KMT substrate (V. Aileni unpublished observation). Further experiments are required to elucidate the precise role of VCP-KMT in cancer metastasis.

2. Characterization of eEF2-KMT

In **Paper II**, we describe another human MTF16 member, FAM86A (eEF2-KMT), which targets a lysine residue in eukaryotic translation elongation factor 2 (eEF2). Interestingly, while most mammals have a single *eEF2-KMT* gene, in humans this gene constitutes a whole family of closely-related genomic sequences, *Family with sequence similarity 86* (*FAM86*), and our first goal was to determine the ancestral functional sequence. During the evolution of higher primates, the *eEF2-KMT* gene localized into an unstable genomic region, which resulted in the duplication of the segment containing *eEF2-KMT* to multiple locations in the genomes of humans, gorillas and chimpanzees (Darai-Ramqvist et al. 2008). The actual copy number is highly variable, however in the reference human genome, there are 15 copies of *eEF2-KMT* spread over 7 chromosomes (**Paper II Table 1**). Most of the *eEF2-KMT* copies have been pseudogenized. However, three genes are reported to be transcribed and contain an intact conserved 7BS MTase core fold - *FAM86A*, *FAM86B1* and *FAM86B2*. By analyzing the degree of purifying selection of evolutionarily conserved residues in these sequences, we found that FAM86A is likely to be the enzyme containing the evolutionary conserved activity (**Paper II Fig. 1**). Furthermore, while we were able to PCR-amplify *FAM86A* from HeLa cDNA, we could not amplify any of the *FAM86B* genes. Cloning the *FAM86B1* gene from a commercially-available cDNA clone from a melanoma cell line led to poor bacterial expression of the recombinant protein which, in addition, did not exhibit any detectable MTase activity. All of this allowed us to focus on FAM86A as the evolutionarily conserved MTase in this study.

2.1 Identification of eEF2-KMT activity

Based on the activity of other members of the MTF16 family that had already been characterized, CaM-KMT and VCP-KMT, as well as HSPA-KMT and KIN-KMT which were concurrently studied in our group, we had good indications that eEF2-KMT may also be a protein MTase, possibly targeting lysine residues (Magnani et al. 2010, **Paper I**, Jakobsson et al. 2013, Ho et al unpublished). To find the substrate, we performed tandem affinity purification of eEF2-KMT from a human Flp-In T-Rex HEK293-derived cell line, which identified the eukaryotic translation elongation factor 2 (eEF2) as an interacting protein (**Paper II Fig. 2A**). To investigate whether eEF2 was a substrate for eEF2-KMT-mediated methylation, we initially attempted to express recombinant human eEF2 in *E.coli*. However, we were not successful in purifying recombinant eEF2 from bacterial cells. We then had to turn to a eukaryotic system for

the production of the protein. The yeast *S. cerevisiae* is one of the best-studied eukaryotes, especially in the field of translation, but regrettably we were unable to produce recombinant human eEF2 in this organism either. However, as eEF2 is highly conserved down to the lowest eukaryotes, there existed the possibility that the yeast protein could work as a substrate for eEF2-KMT, much like the cross-species methylation of yeast Cdc48 by the human VCP-KMT which we demonstrated in **Paper I**. In addition, the presence of a putative homologue of the eEF2-KMT enzyme in *S. cerevisiae*, YJR129c (Efm3), and the wide commercial availability of single-gene yeast knockout strains allowed us to study the effects of eEF2-KMT-mediated methylation in a simpler biological setting.

When we performed *in vitro* methylation assays with recombinant eEF2-KMT and Efm3 on protein extracts from wild type and *efm3*Δ yeast cells, we found that eEF2-KMT is in fact an eEF2 MTase, that Efm3 is its functional yeast homologue, and that both enzymes trimethylate lysine 509 of the yeast eEF2. Correspondingly, MS analysis demonstrated that this mark is present in wild type yeast but absent from the *efm3*Δ knockout. Furthermore, the mutation of lysine 509 in eEF2 abolished the activity of both MTases, showing that this residue is the only target for these enzymes (**Paper II Fig. 2**).

2.2 Biological significance of eEF2-KMT activity

When analyzing the phenotype of the *efm3*Δ knockout yeast, we found that neither the proliferation rate nor the rate of protein synthesis of the yeast appeared to be affected by the knockout of Efm3, suggesting that methylation of eEF2 does not drastically alter its function in translation elongation (**Paper II Fig. 4A**). We also tested whether the *efm3*Δ knockout strain displayed altered sensitivity to drugs inhibiting protein translation. And indeed, we found that the knockout strain was more sensitive than the wild type to the eEF2-specific translation inhibitor sordarin (**Paper II Fig. 4B and C**). Sordarin binds to eEF2 in a cleft between domains III, IV and V, and locks it to the ribosome, inhibiting translation. One of the contact points to sordarin is in close proximity to lysine 509 which lies in a highly conserved α-helix in eEF2 domain III. Moreover, a region involved in sensitivity to sordarin has been mapped to an adjacent eEF2 helix (Jorgensen et al. 2003, Shastry et al. 2001). The bulky trimethylation mark on lysine 509 may weaken the binding to sordarin through steric hindrance in the binding cleft or may alter the interaction of eEF2 with the ribosome. Interestingly, presence of the diphthamide residue in eEF2, which is located far away from the sordarin binding region at the tip of domain IV and

reaches into the ribosomal decoding center during translation, has been shown to increase sordarin sensitivity, potentially linking the region around lysine 509 to events occurring in the very center of the ribosome (Botet et al 2008).

As for direct interactions with the ribosome, the trimethylated lysine 509 appears to be in proximity to a ribosomal protein of the small subunit, Rps23. Rps23 has been described as a conserved ribosomal "accuracy center" with various mutations contributing to changes in translational accuracy (Alksne et al. 1993, Synetos et al. 1996). In addition, hydroxylation of a proline residue in Rps23 was shown to either decrease or increase translational accuracy, depending on the stop-codon context (Loenarz et al. 2014). The potential interaction of the methylated eEF2 lysine with Rps23 led us to investigate whether eEF2 methylation affects translational accuracy. Through the use of a dual luciferase assay, we analyzed the frequency of frameshift events in wild type and *efm3*Δ yeast strain during translation of the -1 programmed ribosomal frameshifting sequence of the HIV-1 virus and the +1 programmed ribosomal frameshifting sequence of the yeast retrotransposable element Ty1. We found that in the *efm3*Δ mutant, the frequency of -1 frameshifting was significantly higher than in the wild type (**Paper II Fig. 4D**), suggesting a role for eEF2 lysine 509 methylation in maintaining translational accuracy. A general maintenance role for the modification is supported by the almost invariant trimethylation status we observed at this residue both in yeast and mammalian cells.

Programmed ribosomal frameshifting sequences have been extensively studied in the context of retroviruses, where a frameshift at the overlap of the *gag* and *pol* open-reading frames allows expression of the viral Gag-Pol polyprotein and sets a defined cytoplasmic Gag : Gag-Pol ratio in the cell (Brierley 1995). Recently, however, in yeast and even mammalian cells such endogenous frameshifting sequences have also been shown to have a biological function (Advani et al. 2013, Belew et al. 2014). It is possible that under certain circumstances, eEF2 methylation could be regulated for the translation of some of the endogenous or invading viral frameshifting sequences, while serving to enhance translational accuracy under normal conditions. Further studies must be performed to unravel the precise mechanism and significance of eEF2 methylation, especially in the mammalian systems, yet the apparent conservation of eEF2-KMT activity throughout eukaryotic evolution stresses the importance of this modification.

2.3 Other eEF2 methylation studies

Unexpectedly, eEF2 methylation became a hot research topic in 2014. Two other groups published similar results almost simultaneously with our **Paper II** (Zhang et al. 2014, Dzialo et al. 2014b). Both of these studies, however, focused primarily on the *S. cerevisiae* homologue of eEF2-KMT, Efm3, and not on the human eEF2-KMT. The earliest of the studies, published in Biochemical and Biophysical Research Communications when **Paper II** was in the process of submission, comes from an Australian group headed by Marc R. Wilkins (Zhang et al. 2014). This group has previously extensively studied protein methylation in *S. cerevisiae*, especially in the context of translation, and have recently identified a trimethylated lysine at position 509 and a dimethylated lysine 613 in yeast eEF2. In addition, they had previously demonstrated that methylation of lysine 613 is abolished upon knockout of the *EFM2* gene, but failed to identify Efm3 as the MTase responsible for trimethylation of lysine 509 (Couttas et al. 2012, Hart-Smith et al. 2014). The other study on Efm3, which was published online on the same day and in the same journal as our **Paper II**, comes from the UCLA-based group of Steven Clarke, one of the pioneers of the methylation field (Dzialo et al. 2014b). This group has also long been involved in the study of methylation in *S. cerevisiae* and, amongst a multitude of other discoveries, have characterized Efm1, See1 and Efm5, three yeast lysine MTases acting on the translation elongation factor eEF1A (Lipson et al. 2010, Dzialo et al. 2014a).

Both of these recent Efm3 studies started with screening single gene knockout strains of putative *S. cerevisiae* MTases by immunoblotting whole cell lysates with antibodies against various lysine methylation states. This led them to notice that *efm3*Δ strains showed reduction of methylation signal from the eEF2 region in the blots, and subsequent MS analysis confirmed that these knockout strains lack trimethylation of eEF2 lysine 509 which is present in wild type yeast. The Australia-based (Zhang et al. 2014) study also included experiments with recombinant yeast eEF2 expressed and purified from a knockout strain, demonstrating direct MTase activity of Efm3 *in vitro*. The authors also suggested the human eEF2-KMT to be the functional homologue of Efm3, however their conclusions are based on indirect evidence from literature, and they do not demonstrate any enzymatic activity of eEF2-KMT to support their claims. We, however, clearly show the specific activity of human eEF2-KMT both on the yeast lysine 509 and the analogous lysine 525 in human eEF2, establishing beyond a doubt that these two enzymes are functional homologues (**Paper II Fig. 2 and 3**).

In addition to Efm3, the UCLA-based (Dzialo et al. 2014b) study also investigated the biological significance of the other eEF2 MTase in yeast, Efm2, which targets lysine 613 in the yeast eEF2. The methylation of this residue appears to have a similar effect to Efm3-mediated methylation, as both the *efm2*Δ and *efm3*Δ knockout strains exhibited altered sensitivity to the same translation inhibitor drugs - cycloheximide, verrucarin, and tunicamycin. During our studies of eEF2-KMT, we were also intrigued by the report implicating Efm2 in eEF2 lysine methylation activity (Couttas et al. 2012). Consequently, we have cloned and expressed this enzyme. Treatment of yeast eEF2 with recombinant Efm2 confirmed that this enzyme does in fact methylate eEF2. Surprisingly, Efm2 showed higher activity on arginine and alanine mutants of lysine 509 than on wild type eEF2, which may suggest some cross-talk between the methylations at lysine 613 and lysine 509. We also found that the residue analogous to lysine 613 is conserved in human eEF2, and that recombinant Efm2 is able to methylate endogenous human eEF2 at this residue. A human orthologue of this MTase, however, is unlikely to exist, as MS analysis revealed that this residue is completely unmodified in several human cell lines (Davydova et al. unpublished observation). Since the activity of Efm2 had already been reported and since this MTase appears to be specific to yeast, we chose to solely focus on the characterization of Efm3 instead.

An important difference from our findings is the observation by Dzialo et al. that knockout of Efm3 does not increase translational frameshifting, whereas we do see increased -1 frameshifting in the *efm3*Δ strain, as compared to the wild type. This may be explained by the different -1 programmed ribosomal frameshifting sequences used in the dual luciferase assays, as the other group used the fungal L-A virus sequence, whereas we employed the HIV-1 sequence. Generally, programmed -1 ribosomal frameshifting signals contain several common features, a heptameric "slippery site" followed by a downstream RNA secondary structure which stalls the ribosome over the slippery site as it unwinds the RNA. The slippery site usually has the consensus form of X XXY YYZ, where X can be any three identical nucleotides, Y can be three A or U nucleotides, and Z is either A, U, or C. The downstream RNA secondary structure is typically an RNA pseudoknot located 5-9 nucleotides downstream of the slippery site. The pseudoknot is thought to pause the translating ribosomes with their A- and P-site tRNAs over the slippery site, where they can re-pair with the -1 frame codons, after which translation resumes in the new reading frame (Jacks et al. 1988, Dinman et al. 2002). While the yeast L-A virus contains a typical pseudoknot downstream of the slippery site, the HIV-1 frameshifting signal

appears to contain a different kind of RNA structure, possibly an RNA triplex or two helices (Dinman et al. 1991, Dinman et al. 2002, Dulude et al. 2002). The L-A virus and the HIV-1 signals also differ slightly in the slippery site sequence, and could conceivably induce frameshifting through somewhat different mechanisms which are affected in different ways by the methylation status of lysine 509 in eEF2. In addition, while we did not investigate possible frameshifting effects of Efm2-mediated methylation of eEF2 lysine 613, Dzialo et al. reported that *efm2*Δ yeast displayed increased -1 frameshifting of the L-A virus sequence, as compared to the wild type (Dzialo et al. 2014b). It is thus possible that methylation of eEF2 at two different sites, mediated by either Efm2 or Efm3, may control different aspects of ribosomal frameshifting in yeast. Further studies must be performed to investigate whether the two eEF2 methylations are interconnected in yeast and why Efm2-mediated methylation may not be present in higher eukaryotes.

3. The human Methyltransferase family 16 (MTF16)

The enzymes VCP-KMT and eEF2-KMT constitute a part of the human MTase family MTF16 (**Paper I Table 1**, Katz et al. 2003). All ten members of this family invariably retrieve each other as best hits in BLAST searches and, in addition to the secondary structure and conserved motifs characteristic for most 7BS MTases, contain a specific [D/E]XX[Y/F] motif located C-terminally of motif II in the loop between strand 4 and helix D (**Paper I Fig. 6A**). Based on the activity of VCP-KMT and CaM-KMT, the earliest characterized members of MTF16, this motif initially appeared to be involved in specific interactions with a lysine residue. However, one of the uncharacterized members of the MTF16, METTL18, contains this motif, as does its yeast sequence homologue, Hmt1, which has been shown to monomethylate a histidine residue on the N3 atom in the imidazole ring (Al-Hadid et al. 2014), so it may instead be involved in more general N-methylation. It is also similar to the conserved [D/N/S]PP[Y/F] motif present in the same location of the structural fold in some DNA and protein MTases, which is selective for N conjugated to a planar system such as an amide moiety or a nucleotide (Schubert et al. 2003).

Owing to the work performed in our lab, as well as that of several other groups, most of the MTF16 enzymes are now characterized with respect to their target proteins and residues, which are listed in **Table 3**. Six of the ten enzymes (CaM-KMT, eEF2-KMT, ETFβ-KMT, VCP-KMT and KIN-KMT) have known biological substrates in currently published literature, while ongoing research in our group has in addition revealed the biological substrate of the lysine-specific METTL21B (V. Aileni et al. unpublished). Furthermore, we showed that METTL21C automethylates a lysine residue *in vitro* (**Paper I, Fig. 6B**). Thus all of the MTF16 enzymes, with the possible exception of METTL18, which has a yeast homologue targeting a histidine residue in the ribosomal protein Rpl3, and METTL23, the substrate of which remains elusive, are lysine-specific.

Table 3. Human MTF16 enzymes and their substrates. K - lysine, H - histidine, me1 - monomethyl, me3 - trimethyl, # - activity shown for yeast sequence homologue only, XXX - unpublished data.

Human enzyme	Alternative name	Substrate	Target residue and modification	Reference
C2orf34	CaM-KMT	CaM	K115 me3	Magnani *et al.* 2010
FAM86A	eEF2-KMT	eEF2	K525 me3	**Paper II**, Zhang *et al.* 2014, Dzialo *et al.* 2014
METTL18	--	RPL3 #	H243 me1#	Webb *et al.* 2010, Al-Hadid *et al.* 2014
METTL20	ETFβ-KMT	ETFβ	K200 me3 K203 me3	Malecki *et al.* 2014, Rhein *et al.* 2014
METTL21A	HSPA-KMT	HSPA8 and homologues	K561 me3 and analogous positions	Jakobsson *et al.* 2013, Cloutier *et al.* 2013
METTL21B	XXX-KMT	XXX	KXXX	Aileni *et al.* unpublished
METTL21C	--	METTL21C	K35 me1	**Paper I**
METTL21D	VCP-KMT	--	K315 me3	**Paper I**, Cloutier *et al.* 2013
METTL22	KIN-KMT	--	K135 me3	Ho *et al.* unpublished, Cloutier *et al.* 2013
METTL23	--	--	--	--

Thus far, each MTF16 member appears to be specific to a single substrate protein, with the exception of HSPA-KMT which targets one analogous residue in several highly-similar Hsp70 proteins (Jakobsson et al. 2013, Cloutier et al. 2013). Additionally, ETFβ-KMT methylates two residues in ETFβ, and unpublished research from our group indicates that another MTF16 enzyme is also able to methylate several lysine residues in one target protein (Malecki et al. 2014, Rhein et al. 2014, A. Ho unpublished). Interestingly, most of the enzymes appear to modify their target lysines up to full trimethylation, however intermediate mono- and dimethylation states have been observed for most of them, and they could have distinct functions. The presence of such hypomethylated MTF16 substrates has even been linked to cancer, as is the case for dimethylated HSP70 proteins (Cho et al. 2012).

Most of the identified MTF16 substrates are highly abundant proteins. For example, according to a recent study of the cellular concentration of proteins, VCP, CaM, HSP70 and eEF2 are all in the top 1-2% of the most highly expressed proteins. ETF is also rather abundant and in the top 15%, and only Kin17 is in the bottom third (Beck et al. 2011). Many of the methods employed by us and others for the identification of MTF16 substrates, i.e. fluorography of MTase-treated cellular lysates, immunoblots against methylated lysines, affinity purification from cells, etc., are biased towards the discovery of more abundant proteins. It is thus not unlikely that some of the MTF16 enzymes may have additional, yet undiscovered substrates. In fact, upon prolonged exposure of the fluorography film containing eEF2-KMT activity on hypomethylated protein extracts from human cancer cell lines, the presence of an additional weak substrate band, larger in size than eEF2, becomes apparent. No such band was detected in yeast *efm3*Δ lysates treated with either Efm3 or eEF2-KMT, suggesting that in higher eukaryotes eEF2-KMT may have other substrates than eEF2 (Davydova et al. unpublished observation). Many other protein MTases exhibit a high level of substrate promiscuity. Recently, one MTase, the human fibrillarin, previously implicated in the methylation of rRNA, was shown to additionally methylate a glutamine residue in the histone H2A, though no direct enzymatic activity on RNA has ever been demonstrated for it *in vitro* (Tollervey et al. 1993, Tessarz et al. 2014). Such findings further support the possibility of additional, more obscure and unexpected substrates for some of the MTF16 MTases.

3.1 MTF16 and disease

While the enzymes METTL21C and METTL23 have not yet been attributed a substrate, they have already been implicated in disease. METTL21C was recently suggested to be involved in the bone and muscle diseases osteoporosis and sarcopenia. The study reports that knockdown of METTL21C led to the inhibition of mouse myoblast differentiation and appears to impact the NF-κB signaling pathway (Huang et al. 2014). METTL23 has been shown by two independent studies to be important for human cognition, as the disruption of this gene leads to mild intellectual disability (Reiff et al. 2014, Bernkopf et al. 2014). Interestingly, METTL23 was found to interact with GABPA (GA-binding protein transcription factor, alpha subunit), a component of a transcription factor complex regulating the expression of several genes (Rosmarin et al. 2004). Overexpression or knockdown of METTL23 led to increased or decreased expression of such genes, respectively, suggesting a function of the METTL23 enzyme as a regulator of GABPA. METTL23 and GABPA were reported to interact both in a yeast two-hybrid screen and through co-immunoprecipitation from a mouse cell line (Reiff et al. 2014). However, it is unclear whether GABPA is subject to methylation by METTL23, as there have not yet been reports of any methylated residues in GABPA on Phosphosite (Hornbeck et al. 2012), so the observed effect may be indirect and downstream of some other protein subjected to METTL23-mediated methylation.

Other MTF16 MTases have also been linked to disease. VCP-KMT has been implicated in tumor metastasis and HSPA-KMT-mediated dimethylation of the heat shock protein HSPA1 has been suggested as a biomarker for various cancers (Thiele et al. 2011, Cho et al. 2012). The evolutionary conservation of eEF2-KMT throughout the eukaryotic kingdom also suggests that the alteration of its function is likely to lead to disease. Yeast cells were not greatly affected by the knockout of Efm3, the yeast orthologue of eEF2-KMT, however disruption of genes in the highly-conserved diphthamide biosynthesis pathway similarly leads to mostly minor phenotypes in yeast, whereas in mammalian systems the effects are much more severe, including abnormal embryonic development and even lethality in mice (Su et al 2013). A possible regulatory mechanism of eEF2 methylation in rat brain further necessitates the use of a murine model to study the effects of *eEF2-KMT* knockout as well as a closer inspection of eEF2 methylation in various parts of the mammalian brain.

3.2 MTF16 automethylation

In **Paper I**, we measured the automethylation activity of METTL21C *in vitro* and identified a specific lysine residue in the N-terminus of the enzyme as the target for this activity. This showed that the enzyme is a functional lysine-specific MTase (KMT). We also observed weaker automethylation activity of VCP-KMT, HSPA-KMT (METTL21A) and eEF2-KMT (**Paper I Fig. 6 and Suppl. Fig. 6**, and Davydova unpublished observation). These automethylations could be an *in vitro* artifact of misplaced enzymatic activity in the absence of a substrate, or could potentially have biological functions. In the case of METTL21C which contains a specific automethylation target lysine residue, this activity could have biological function. Indeed, automethylation is biologically relevant for several other MTases. For example, while automethylation of the protein arginine MTase CARM1 does not directly alter its enzymatic capacity, mutation of the automethylation site results in the impairment of several downstream processes, such as CARM1-activated ERα transcription and pre-mRNA splicing (Kuhn et al. 2011). Moreover, automethylation activity of the lysine-specific histone MTase G9a creates a binding site for interacting proteins, whereas automethylation of the arginine-specific PRMT8 affects its enzymatic activity by decreasing its affinity to SAM (Chin et al. 2007, Dillon et al. 2013). It remains to be seen whether automethylation may similarly be a biologically relevant function for some of the MTF16 enzymes.

3.3 Regulatory lysine methylation

In several cases, the methylation of lysines may be reversible and have a regulatory function as part of a complex signaling network. For example, as part of the "histone code", lysine residues in the N-terminal tails of histone proteins are methylated and/or demethylated by MTases and demethylases, leading to distinct methylation states of the residues (Black et al. 2012). The resulting lysine marks recruit specialized "reader domain" proteins which recognize distinct methyllysine states, e.g. proteins containing a Tudor, MBT, PHD, or Chromo domains (Adams-Cioaba and Min 2009, Rothbart et al. 2013), activating different transcriptional responses. Such regulatory lysine methylation mechanisms have also been described outside of the histone code (Esteve et al. 2014, Tong et al. 2015).

While the extent of VCP lysine trimethylation does not appear to vary in any of the mammalian cell cultures and tissues analyzed (**Paper I Fig. 3**), it is unknown whether it may be

regulated in other cell types, under certain conditions or stages of development. We also show that a large fraction of eEF2 from rat brain is hypomethylated, in contrast to almost complete trimethylation present in other tissues, allowing for the possibility of a regulatory mechanism in mammalian brain (**Paper II Fig. 3**). Notably, a recent study reported that, outside of its canonical histone-demethylating function in the nucleus, the cytoplasmic fraction of the lysine-specific demethylase KDM4A interacts with the translation machinery, impacting the distribution of translation initiation factors within polysome fractions (Van Rechem et al. 2015). This leads to the possibility that KDM4A or a related demethylase may also target other translation factors, such as eEF2 or the heavily methylated eEF1A, as part of a dynamic mechanism.

An interesting mechanism involving methylation of lysine residues is the regulation of tumor suppressor p53 during the DNA double strand break damage response (Panier and Boulton 2014). The reader protein involved in this process, the tandem Tudor domain-containing 53BP1, is able to oligomerize and has a strong preference for two dimethylated lysines in the C-terminal domain of p53, as well as dimethylated lysines in several chromatin components such as lysine 36 in histone H3, which is also a modification that correlates with DNA damage (Fnu et al 2011). The mechanism of p53 recruitment to sites of double strand breaks may thus involve formation of 53BP1 homodimers which simultaneously interact with a dimethylated lysine in p53 and dimethylated lysine 36 in histone H3 (Tong et al. 2015). On a larger scale, oligomerization of methyllysine reader domains may lead to the existence of an interaction network between various lysine-methylated proteins throughout the cell.

Conceivably, MTases that bind tightly to their methylated substrates, like some of the MTF16 enzymes (**Paper II Fig. 2A**, Jakobsson et al. 2013, Cloutier et al. 2013), could also act as adaptor proteins or form links between their substrates through oligomerization. One enticing possibility is that eEF2-KMT, which in humans and other higher primates has several highly similar FAM86 sequence homologues some of which contain no apparent eEF2-methylating activity (**Paper II**), could form heterooligomers with the other FAM86 proteins to bridge the interaction of eEF2 and some other cellular component. Such an interaction would be specific to higher primates and, especially in the context of a possible regulatory mechanism in the mammalian brain, could suggest a role for eEF2 methylation in higher brain function. This possibility, however, remains to be investigated.

3.4 Evolutionary conservation of MTF16-mediated methylation

Many of the MTF16 enzymes are conserved throughout large parts of the eukaryotic kingdom. In most cases, the substrate proteins of these enzymes are present in all eukaryotic organisms, however the specific target lysine residues may not be conserved. For example, in species where the target lysine in Kin17 is substituted or deleted, like in *S. cerevisiae*, there also appears to be no KIN-KMT sequence homologue present (Cloutier et al 2014). An eEF2 homologue exists in prokaryotes, yet the lysine analogous to the lysine methylated by eEF2-KMT in eukaryotes is absent, and, correspondingly, there are no prokaryotic sequence homologues of eEF2-KMT. On the other hand, the target lysines in VCP and Hsp70 proteins appear to be invariant and have the potential to be methylated in all eukaryotes, whereas the corresponding MTases have a more patchy distribution. Research performed in our lab showed the existence of a functional VCP-KMT homologue in *C. elegans* through an *in vitro* methylation assay, and allowed us to infer the lack of functional VCP-KMT or HSPA-KMT homologues in *S. cerevisiae* (**Paper I Fig. 4**, Jakobsson et al. 2013 and manuscript in submission). However, it is often difficult to predict functional homology based on sequence similarity. For example, human eEF2-KMT is of rather low sequence similarity to yeast Efm3, yet Efm3 still contains eEF2-methylating activity, whereas VCP-KMT and HSPA-KMT are both highly similar to each other and to the yeast protein YNL024c, yet all three have different functions (**Paper I Table I**, **Paper II**, Jakobsson et al. manuscript in submission). Demonstration of direct MTase activity is thus critical for identification of truly homologous enzymes. Three MTases in *S. cerevisiae* exhibit high sequence similarity to human MTF16 members, YNL024c (Efm6), YJR129c (Efm3), and YIL110W (Hpm1) (**Paper I Table I**). All of these have now been attributed a function, but only Efm3 has so far been directly shown to have a functional human homologue (**Paper II Fig. 2**).

In a flurry of publications during the recent months, all of the MTases responsible for lysine methylation of both universally conserved translation elongation factors, eEF1A and eEF2, have now been described in *S. cerevisiae* (**Table 4**). However, only three of these modifications appear to be conserved in humans, and the human lysine MTases (KMT) targeting sites not present in yeast eEF1A remain elusive. On a global level in yeast, lysine methylation is more common than methylation of any other protein residue (Pang et al. 2010,). This seems to hold especially true in the case of translation factors, and only two other MTases acting on such proteins have been described in yeast, Mtq1 and Mtq2, which target glutamine residues in translational release factors (Polevoda et al. 2006). Interestingly, while methylation of lysine residues was previously considered unusual for 7BS MTases, only one of the six elongation factor KMTs listed in **Table 4** is not part of this class of SAM-dependent MTases. This indicates that KMT activity may be quite common for 7BS enzymes, especially in connection with the translation apparatus.

Table 4. Lysine-specific MTases of the translation factors in *S. cerevisiae*, their targets and human homologues. KMT - lysine-specific MTase. Data correct as of January 2015.

KMT	Alternative name	Target residue and protein	MTase family	Citation	Human homologue
Efm1	YHL039W	Lys 30 eEF1A	SET	Lipson et al 2010	--
Efm2	YBR271W	Lys 613 eEF2 Lys 187 eEF3	7BS	Zhang et al 2014, Dzialo et al 2014a	--
Efm3	YJR129C	Lys 509 eEF2	7BS	**Paper II** Zhang et al 2014, Dzialo et al 2014a	eEF2-KMT
Efm4	YIL064W See1	Lys 316 eEF1A	7BS	Lipson et al 2010	METTL10
Efm5	YGR001C	Lys 79 eEF1A	7BS nonstandard	Dzialo et al 2014b	N6AMT2
Efm6	YNL024C	Lys 390 eEF1A	7BS	Jakobsson et al, manuscript in submission	--

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the work described in this thesis led to the characterization of several MTases belonging to the previously uncharacterized human members of the MTF16 subfamily of human 7BS MTases. We showed that two of them, VCP-KMT and eEF2-KMT, trimethylate specific lysine residues in the chaperone VCP and the translation elongation factor eEF2, respectively. We also indicated that functional homologues for these enzymes are dispersed throughout the eukaryotic kingdom. As for the biological significance of these MTases, the activity of VCP-KMT was implicated to be important in cancer metastasis, whereas eEF2-KMT appears to fine-tune the process of protein synthesis, however future studies must be performed to investigate these mechanisms in detail. Currently, several other members of MTF16 remain uncharacterized, and some are already linked to human disease, and it is imperative to study these enzymes in greater detail. In addition, the human genome still encodes a wealth of other putative MTases, and future investigations must identify their biochemical activities and cellular functions.

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