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Methylation of class I translation termination factors: structural and functional aspects

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ABSTRACT

During protein synthesis, release of polypeptide from the ribosome occurs when an in frame termination codon is encountered. Contrary to sense codons, which are decoded by tRNAs, stop codons present in the A-site are recognized by proteins named class I release factors, leading to the release of newly synthesized proteins. Structures of these factors bound to termination ribosomal complexes have recently been obtained, and lead to a better understanding of stop codon recognition and its coordination with peptidyl-tRNA hydrolysis in bacteria. Release factors contain a universally conserved GGQ motif which interacts with the peptidyl-transferase centre to allow peptide release. The Gln side chain from this motif is methylated, a feature conserved from bacteria to man, suggesting an important biological role. However, methylation is catalysed by completely unrelated enzymes. The function of this motif and its post-translational modification will be discussed in the context of recent structural and functional studies.

149 words.

Highlights

- The recognition of stop codon by release factors (RFs) induces peptide release.
- The universally conserved GGQ motif from RFs is methylated from bacteria to man.
- Trm112 activates several methyltransferases acting on translation components.

Keywords

Translation termination / Post-translational modification / Methyltransferases.

1. Introduction

The termination step of translation allows the release of newly synthesized proteins, in response to the presence of a stop codon in mRNA. In all kingdoms of life, the stop codon is recognized by class I release factors (RFs), which bind to the ribosomal A-site and promote hydrolysis of peptidyl-tRNA in the peptidyl-transferase centre (PTC). These proteins are assisted in their function by class II RFs, but in a different way in prokaryotes and eukaryotes [1].

Despite a similar function, prokaryotic and eukaryotic/archaeal RFs are radically different in primary and tertiary structures, with the exception of an essential and universally conserved tripeptide motif Gly-Gly-Gln (GGQ) located at one tip of the molecule and interacting with the ribosomal peptidyl transferase centre [2-4]. In both bacteria and eukarya, this motif is post-translationally modified by methylation, one of the most common modifications after phosphorylation. The Gln side chain of this motif is converted into N⁵-methyl-glutamine by specific protein methyltransferases (MTases): PrmC in *E. coli* or the Mtq2-Trm112 complex in *S. cerevisiae* [5, 6].

Translation termination requires a perfect coordination between two events on different ribosomal sites, i.e. recognition of the stop codon in the decoding centre, and when this is achieved, hydrolysis of the ester bond of peptidyl-tRNA in the PTC. Different domains from class I RFs are involved in this process, as for tRNAs, leading to the notion of tRNA mimicry [7, 8]. In recent years, many structural data have helped us to understand these mechanisms: structures of RFs alone by X-ray crystallography or in solution by Small-Angle X-ray Scattering (SAXS) [3, 4, 9], followed by cryoelectron microscopy structures of RFs bound to the ribosome [10, 11], and more recently higher resolution crystal structures of these complexes (for reviews, see [2, 12]).

This review summarizes recent findings regarding the mechanism of translation termination and the role and mechanism of RF methylation. In particular, we discuss identification of the enzymes mediating this modification, their physiological roles and structural mechanisms of action.

2. General principles of translation termination mechanism

2.1. Function and structure of class I RFs

2.1.1. Bacterial Release Factors.

In bacteria, two class I RFs detect the three stop codons with overlapping specificity: RF1 recognizes UAG and UAA while RF2 recognizes UAA and UGA stop codons. These proteins share significant sequence identity (for instance, RF1 and RF2 from *E. coli* display 40% sequence identity and 56% homology) and adopt very similar three-dimensional structures [4, 13, 14]. These proteins encompass four domains (from I to IV), among which domains II and IV are associated to form a superdomain (Fig. 1A). Domain II is involved in stop codon recognition in the ribosomal decoding centre (DC) and has long been proposed to contain conserved triplets that act as “anticodon” loops. The recent high resolution structures of ribosomal complexes with the different stop codons and either RF1 or RF2, unravelled the molecular basis of stop codon recognition and revealed that the “anticodon” loop model for stop codon recognition was too simplistic (for more details on stop codon discrimination mechanisms, see recent reviews [2, 15]). Domain III harbours within a loop the universally conserved GGQ motif, which is involved in peptidyl-tRNA hydrolysis in the PTC.

Surprisingly, all the crystal structures of isolated bacterial class I RFs [4, 13, 14] exhibit a “closed” conformation with the two regions predicted to interact simultaneously with the ribosomal DC and PTC distant by only 20-25Å (Fig. 1A), too short to span the corresponding distance in the ribosome (75 Å). Thus, these crystal structures do not reflect the conformation adopted by these proteins once bound to the ribosomal A-site. SAXS studies on isolated release factors [9] together with cryo-EM [10, 11], followed by crystal structures of RF1 and RF2 bound to the ribosome [16-20], confirmed that class I RFs adopt an “open” conformation compatible with their role in both stop codon recognition and catalysis of peptidyl-tRNA hydrolysis (Fig. 1B).

2.1.2. Eukaryotic release factors.

Contrary to prokaryotes, in eukaryotes the three stop codons are recognized by a single protein [21]. This class I RF, eRF1, is composed of three domains, with no structural similarity with those of RF1 and RF2 (Fig. 1C; [3]). The N-terminal and central domains functionally correspond to domains II and III from bacterial RFs, respectively. Several distinct regions from the eRF1 N-terminal domain are involved in stop codon recognition in the DC [22]. The central domain harbours the universally conserved GGQ motif [23]. Finally, the central and C-terminal domains are implicated in the interaction with the class II RF, eRF3, a

GTPase crucial for correct translation termination [24-26]. Since the shape of eRF1 observed in the crystal structure resembles a tRNA, it was supposed to mimic functionally the tRNA: the region centred on the conserved NIKS motif in the eRF1 N-terminal domain corresponds to the tRNA anticodon loop and the GGQ motif from the central domain corresponds to the amino-acyl acceptor stem [7, 8]. In this structure, the distance between the NIKS motif from the N-terminal domain and the GGQ motif is slightly longer than the distance between the DC and the PTC in the eukaryotic ribosome [27], suggesting that further conformational change will be needed for optimal eRF1 function. It was subsequently proposed that eRF1 adopts its functional conformation upon binding to the eukaryotic class II RF, eRF3 [28].

2.2 Class II translation RFs

During translation termination, class I RFs are assisted by class II factors, RF3 in prokaryotes and eRF3 in eukaryotes. These two proteins belong to the translational GTPase family but have different roles. The bacterial RF3 promotes dissociation of RF1/RF2 from the ribosome following peptide release by stabilizing the ribosome in a ratcheted conformation incompatible with the binding of RF1/RF2 to the A-site [29-33]. Its GTPase activity is dependent on the ribosome and is stimulated by RF1/RF2 although structural modelling from crystal and cryo EM structures suggests that the class I and class II proteins do not interact together either in solution or bound to the ribosome [30, 32, 33].

In eukaryotes, eRF1 interacts with the class II RF eRF3, a translational GTPase related to elongation factors EF-Tu and eEF-1A, in the absence of the ribosome [26, 34]. The complex is more stable in the presence of GTP, and eRF3 binds more tightly GTP (but not GDP), in the presence of eRF1 [35-37]. The eRF1 and eRF3 proteins interact mainly through their C-terminal domains as determined both by biochemical and structural data [28, 38, 39]. However, several lines of evidence suggest that the eRF1 central domain harbouring the universally conserved GGQ motif also interacts with the eRF3 GTPase domain. First, the central domain from human eRF1 interacts directly with eRF3 *in vitro* [40]. Second, the central and C-terminal domains from eRF1 are necessary for eRF3 to bind GTP [40]. Third, the recent crystal structure of the Dom34-Hbs1 complex (two proteins related to eRF1 and eRF3, respectively [41, 42]) has revealed that the Dom34 central domain, which is structurally similar to the corresponding domain from eRF1, interacts with the Hbs1 GTPase

domain [43]. Structural modelling of the eRF1-eRF3-GTP complex based on the structure of the Dom34-Hbs1 complex further indicates that the distance between the eRF1 GGQ motif and the region involved in stop codon recognition nicely fits with the distance observed in the crystal structure of the eukaryotic 80S ribosome [27].

The current model regarding eRF1-eRF3 induced peptide release proposes that following binding of the eRF1-eRF3-GTP complex to pre-termination complexes, the eRF3 bound GTP molecule is hydrolysed in a ribosome- and eRF1-dependent manner [44, 45]. This would lead to rearrangement of the eRF1 central domain, which contacts the eRF3 GTPase domain in the GTP-bound form, for perfect stop codon recognition and fit into the ribosomal PTC to induce peptide release, followed by eRF3 release from the ribosome. eRF1 would remain associated with the post-termination complex and interact with the newly identified eukaryotic ribosome recycling factor Rli1/ABCE1, a conserved and essential protein of the ATP-binding cassette family [46]. This latter would then promote eRF1 dissociation from ribosomes as well as subunit dissociation [47, 48]. Release of tRNA and mRNA from recycled 40S subunits is then mediated by eIF3, eIF1 and eIF1A [47].

2.3. Translation termination in archaea

Searches for orthologues of both bacterial and eukaryotic class I RFs within archaeal genomes revealed the presence of a protein related to eukaryotic eRF1 [49]. The recent crystal structure of the archaeal class I RF (aRF1) from *A. pernix* confirmed that aRF1 is the archaeal class I RF [50]. In parallel, the elongation factor aEF-1A has also been shown to act as the archaeal class II RF as well as in RNA quality control [50]. Finally, an ortholog of Rli1/ABCE1, involved in ribosome recycling, is also present in archaea [51]. Altogether, this indicates that translation termination mechanisms are closely similar in archaea and eukaryotes.

3. Post-translational methylation of RFs

3.1. Structural and functional characterization of prokaryotic RF methylation

3.1.1. Bringing to light methylation of prokaryotic RF

RF2 was suspected of being modified because its specific activity and its affinity for ribosomes was lower when overproduced [52, 53]. Dincbas-Renqvist *et al* confirmed this

hypothesis and demonstrated that RF1 was also methylated [54]. In parallel, they showed that *E. coli* K12 strains expressed a peculiar and less efficient RF2 with a Thr instead of an Ala at position 246. Mass spectrometry analysis confirmed the presence of N⁵-methylglutamine on the conserved catalytic GGQ motif of RF1 and RF2.

Although methylation is a frequently observed modification in the translational apparatus (ribosomal proteins, tRNA, rRNA) for example, Gln side chain modification is an unusual one, so far only described for *E. coli* ribosomal protein L3 [55]. After identification of the enzyme methylating L3 protein (YfcB or PrmB), Heurgué-Hamard *et al* showed that a unique candidate, HemK (thereafter renamed PrmC) had a significant sequence similarity to this enzyme [5]. The corresponding gene was located immediately downstream of *prfA*, encoding RF1 at 27 min on the genome. *In vitro* methylation assays performed on RF1/RF2 with purified PrmC enzyme confirmed the role of the protein in this process.

An independent study from another group who previously assigned a different function to HemK (see below) also pointed out its role in RF1/RF2 methylation [56]. Their studies were initiated by the observation that *hemK* was localized in the same operon as *prfA*, and that the HemK sequence was related to a SAM-dependent methyltransferase (MTase) family. *hemK* is a striking example of genome misannotation. A genetic screen for heme biosynthetic enzymes first led to the conclusion that this gene encoded a subunit of protoporphyrinogen oxidase catalyzing one step towards heme biosynthesis (hence its name) [57]. From these data, many homologous proteins in different genomes have been designated as a “protoporphyrinogen oxidase”. When further biochemical data excluded this function and showed the enzyme to be a MTase, the presence of the conserved motif NPPY in HemK homologues, previously thought to be specific to DNA adenine Mtases, then lead to a further misannotation as a nucleic acid MTase. To avoid more confusion, HemK was renamed PrmC (see Table 1 for nomenclature).

prmC is present among the proposed minimal set of genes required for life, suggesting an important role in other organisms [58]. Indeed, methylation of class I RF by a PrmC homolog was also characterized in *Chlamydia trachomatis*, an obligate intracellular bacterial pathogen with a small genome, phylogenetically very distantly related to *E. coli* [59]. In *E. coli*, *prfA* and *prmC* belong to the same operon, and the initiation codon for *prmC* overlaps with the termination codon of *prfA*. Yang *et al* showed that this organization was conserved in

79 out of 126 analysed bacterial genomes [60]. However, at least in two cases, the phylogeny of the PrmC protein was different from that of RF1 and RF2, which is correlated to 16S rRNA, suggesting that horizontal transfer of the *prmC* gene had occurred [60].

3.1.2. Substrate recognition by PrmC methyltransferase

The SAM-dependent PrmC MTase is composed of two domains: an N-terminal α -helical domain connected by a small β -hairpin to the C-terminal class I MTase domain, with a Rossmann-fold consisting of a mixed α/β fold composed of a central seven stranded β -sheet surrounded by 3 α -helices on each side (Fig. 2A; [60, 61]).

The molecular basis for the recognition and methylation of bacterial class I RFs has been clarified by the crystal structure of the complex between the PrmC MTase and RF1 from *E. coli* [62]. In this complex, the RF1 protein adopts the “closed” conformation already observed in the crystal structures of bacterial RF1 and RF2 proteins (Fig. 2B). As expected, the PrmC MTase domain interacts with the RF1 domain III harbouring the GGQ motif while the PrmC N-terminal domain contacts both domains II and III from RF1. This latter interaction is only possible if the RF1 protein adopts the “closed” conformation, suggesting that this RF1/RF2 conformation otherwise observed only in the crystal structures of isolated proteins has biological significance rather than being a crystal artefact. To test this hypothesis, residues from the PrmC N-terminal domain involved in the RF1-PrmC interaction only if RF1/RF2 is in a “closed” form, have been subjected to mutagenesis. These mutants proved to have a significantly decreased *in vitro* RF1/RF2 methylation activity and a reduced affinity for RF1 [62]. Altogether, these results argue that the RF1/RF2 “closed” conformation is the PrmC substrate and hence is biologically relevant.

This first structure of a complex between a MTase and its intact substrate protein has also revealed the molecular basis for RF1/RF2 recognition [62]. In particular, this structure has rationalized the functional importance of the strictly conserved ¹⁸³NPPY¹⁸⁶ motif (*E. coli* PrmC numbers are used for clarity), a hallmark of RF MTases and MTases modifying planar nitrogen atoms [63]. This signature is responsible for the binding and correct orientation of the Gln side chain from the ²³³GGQ²³⁵ motif within the active site (*E. coli* RF1 numbers are used for clarity). The carbonyl groups from the Asn¹⁸³ side chain and from the Pro¹⁸⁴ main chain of this motif are hydrogen bonded to the Gln²³⁵ amide group that will be methylated

(Fig. 2C). The hydroxyl group from the Tyr¹⁸⁶ side chain in the NPPY motif is hydrogen bonded to the main chain amide group of Gln²³⁵ and the Tyr aromatic ring is stacked against the Gln side chain. Another hydrogen bond formed between the PrmC Arg⁹² side chain and the RF1 Gln²³⁵ main chain carbonyl group is also observed. All these interactions occur between conserved residues both from PrmC and RF1/RF2 and participate in a network of interactions that ideally positions the Gln side chain for subsequent methyl transfer from the SAM cofactor bound to PrmC.

3.1.3. Role of the GGQ motif and its methylation in peptide release

As proposed by several studies [23, 64-67], the crystal structures of 70S termination complexes have confirmed that the GGQ motif enters the PTC [16-18, 20, 68]. The two Gly residues from this motif are sandwiched between A2602 and U2585 (Fig. 2D). These residues adopt conformations that would be energetically unfavourable for any of the 19 other natural amino acids, and bring the Gln near the A76 nucleotide at the CCA-end of the P-site tRNA. The Gln side chain points away from the scissile peptidyl-tRNA ester bond in a pocket formed by the ribose moiety of the P-site tRNA A76 as well as A2451, C2452 and U2506 from the 23S rRNA (*E. coli* numbering used) and as a consequence, is very unlikely to contribute directly to catalysis (Fig. 2D). This correlates well with the mutational analysis showing that most substitutions of the Gln side chain by various residues (including the bulky Trp) only partially affect the catalysis of peptidyl-tRNA hydrolysis *in vitro*, both in bacteria and eukaryotes [65, 66, 69]. The Gln side chain could contribute indirectly and partially to catalysis by participating in the coordination of the nucleophilic water molecule. In the 3.1 Å electron density maps of the 70S termination complex obtained with RF2 and a P-site tRNA substrate analog [68], the carbonyl group from this Gln side chain is located 4.2 Å away from a water molecule, which could correspond to the nucleophilic water (Fig. 2D). Surprisingly, the crystal structures of 70S termination complexes revealed that the main chain amide group of the Gln residue is well positioned to form a hydrogen bond with an oxygen of the tetrahedral transition state intermediate during the hydrolysis reaction and to stabilize it. To support this observation, the replacement of Gln by Pro, which prevents the formation of this hydrogen bond (but could also perturb the backbone of the loop harbouring the GGQ motif), completely abolishes RF1-mediated peptide release activity [16].

Comparison of all the structures of bacterial RFs bound to ribosomes reveals that the Gln side chain adopts a large variety of conformations within the PTC. This flexibility could reflect a loose packing of the Gln side chain in the PTC following peptide release, as all structures but one [68] have been obtained with a deacylated tRNA in the P-site, and hence can be considered as post-release structures. However, it could also result from loose packing of the Gln into the PTC due to the lack of methylation. Indeed, a hydrophobic pocket is located close to the Gln side-chain amide group and the methyl group could fill this hydrophobic cavity and trap this Gln side chain in an optimal orientation within the PTC. Furthermore, molecular dynamics calculation suggests that the absence of the methyl group to the Gln side chain increases the mobility of this side chain in the PTC [67]. It has been shown that the catalytic efficiency in the termination reaction and the affinity for ribosomes of the methylated bacterial RF2 *in vitro* are five-fold and three-fold higher, respectively, than for the unmethylated factor [52, 54]. Similarly, methylation has been estimated to result in a five-fold increase in the efficiency of termination *in vivo* [64].

3.1.4. Physiological role of RF methylation

Physiological studies of RF methylation in bacteria have been performed only in *E. coli*. In gram-positive bacteria, data from systematic gene inactivation show that *ywkE*, encoding the PrmC homolog, is not an essential gene and is not required for normal growth in rich or minimal media [70].

The biological role of translation factor methylation is not always clear and depletion of MTases usually does not cause major phenotypes [71]. In the case of RFs, *prmC* deletion in *E. coli* affects growth, but a distinction must be made depending on whether the strain is K12 or not. The first experiments on the role of PrmC in *E. coli* were done in a K12 background, where the efficiency of termination is already affected due to the *prfB* gene encoding a peculiar RF2 with a Thr at position 246 (RF2T246), shown to be less efficient *in vitro* and *in vivo* [54, 64, 72]. Other strains such as *E. coli* B and C or other *E. coli* isolates have an Ala at position 246 in RF2, and all other bacterial species carry an Ala or Ser at the corresponding position.

In K12 strains, inactivation of *prmC* leads to very poor growth on rich medium. A similar phenotype is observed when RF2(T246) is over-expressed. In both cases, *E. coli*

produces an unmethylated and particularly defective RF2(T246) which is unable to sustain normal cell growth. Suppressors abrogating growth defects of K12 strains with a *prmC* knockout were found to carry mutations in the *prfB* gene, encoding RF2T246A or RF2T246S [5, 56]. The cumulative termination defects described in K12 strains in the absence of RF methylation are reminiscent of recent data showing also that K12 growth is also largely compromised in the presence of a mutation in the *rluD* gene, decreasing RF2 activity [73, 74]. This gene encodes a pseudouridine synthase modifying three positions of helix 69 of 23S rRNA critical for RF activity [75]. In both cases, the decreased rate of termination has been shown to induce transfer-mRNA (tmRNA)-mediated tagging of proteins [56, 74].

prmC inactivation also affected the growth of *E. coli* B strains expressing RF2(A246) on minimal media, especially with poor carbon sources like succinate or acetate. It was suggested that the decreased specific activity of RF1 and RF2(A246) due to their lack of methylation impaired synthesis of proteins required to grow on minimal media [64]. In conclusion, we propose that RF methylation is more specifically required under suboptimal conditions, for growth on minimal media or when specific pathways must be active under stress conditions. In the same vein, it is interesting to note that, for reasons to be determined, *Yersinia pseudotuberculosis* PrmC (VagH) is required for virulence in a mouse infection model, although not for growth in rich liquid media. However, as in B strains of *E. coli*, a *vagH* mutant grew more slowly in a less nutrient rich defined medium [76].

3.2 Eukaryotic RF methylation

3.2.1. Characterization of eRF methylation and identification of the enzyme involved

The perfect conservation of the GGQ motif in RFs from all kingdoms together with the widespread presence of genes encoding potential PrmC homologues in metazoa led to the question as to whether eRF1 was also modified in *S. cerevisiae*. Yeast cytoplasmic eRF1 was found to be methylated *in vivo* on the Gln of its GGQ motif by two independent studies [77, 78]. *S. cerevisiae* genome analysis revealed two genes encoding proteins with significant homology to *E. coli* PrmC, containing a SAM binding site and a conserved NPPY motif involved in methylation catalysis: Mtq1 (YNL063w) and Mtq2 (YDR140w). Mtq1 is localized in mitochondria and methylates *in vivo* the conserved GGQ motif from mitochondrial RF1 (mRF1), which has high sequence similarity to bacterial RF1 [78]. As in

the case of *E. coli* RF1/RF2 and PrmC, modification of mRF1 can be reproduced *in vitro* by adding purified Mtq1 and SAM, showing that Mtq1 is sufficient for catalysis [78].

Methylation of human mitochondrial RF HMRF1L, catalyzed by the HMPrmC enzyme, was also described [79]. Using *in vivo* and *in vitro* approaches, the Mtq2 enzyme was shown to be essential for cytoplasmic eRF1 methylation [77, 78].

Several observations made during the characterization of Mtq2 suggested that it was not acting alone. Only impure preparations of Mtq2 produced from yeast were active in methylation, and with low efficiency [77]. Moreover, expression of Mtq2 alone in *E. coli* led to insoluble protein, which remained inactive after classical solubilization/renaturation protocols [6, 77]. Large scale interactome studies, using the two hybrid system or tandem affinity purification followed by mass spectrometry, identified several proteins potentially interacting with Mtq2 [80, 81]. Among them, Trm112 was a candidate, potentially interacting also with tRNA MTases Trm9 and Trm11. These interactions were later confirmed through biochemical analysis and shown to be essential for tRNA methylation [82-84]. Adding Trm112 to purified Mtq2 from inclusion bodies allowed methylation of eRF1 *in vitro* in the presence of eRF3 (eRF3Ct was used for *in vitro* experiments) and showed that these proteins formed an enzymatically active complex able to methylate eRF1 *in vitro* [6]. Yeast eRF1 methylation is distinct from *E. coli* RF methylation in two respects: (1) to be methylated, eRF1 needs to be part of a ternary complex together with eRF3-GTP, consistent with the fact that these proteins readily form a heterodimer *in vivo*; (2) eRF1 MTase is a complex of two subunits: Mtq2 which carries the catalytic activity and the zinc finger protein Trm112 [6]. Trm112 is conserved in eukaryotes, together with its interaction with several MTases. *In vitro* studies showed that methylation of human and murine eRF1 are performed respectively by HemK2 α /hTrm112 and PRED28 α /mTrm112, and required the presence of eRF3 and GTP [85]. Analysis by mass spectrometry also showed that methylation is present in endogenous human eRF1 and is dependent of the predicted MTase catalytic subunit [86]. However, only 70% of endogenous human eRF1 is methylated in cultured HEK-293T cells. Complete methylation is observed only after ectopic expression of the MTase. Finally, the expression of human HemK2 α can complement the growth defect of yeast strains deleted for *mtq2*, suggesting that it is able to interact *in vivo* with yeast Trm112 as observed *in vitro* [85].

3.2.2. Structure of the Mtq2-Trm112 methyltransferase

Bioinformatics analysis has revealed the existence of proteins with sequence homology to yeast Trm112 within the three kingdoms of life, with molecular weights ranging from 6 kDa in bacteria and archaea to around 15 kDa in eukaryotes. Regions with significant homology are clustered near the amino-terminus (30 residues) and the carboxy terminus (25-35 residues), while a central region composed of 60-70 residues is only found in eukaryotes. Interestingly, a well-conserved CX₃C motif (CX₂C in archaea and prokaryotes) is observed in the N-terminal region together with a CX₂C motif in the C-terminal part, suggesting the presence of a zinc-binding site within most Trm112 proteins, and confirmed by the crystal structure of *S. cerevisiae* Trm112 [6]. The four cysteine residues from *S. cerevisiae* Trm112 bind a zinc atom and residues 1-40 as well as 103-135 fold together as a zinc knuckle domain. In *S. cerevisiae* Trm112, the central region specific for eukaryotic Trm112 proteins folds as a helical domain formed by 3 α -helices.

The crystal structure of the Mtq2-Trm112 complex from the small eukaryotic parasite *Encephalitozoon cuniculi* shows that Trm112 interacts mainly via its zinc knuckle domain with Mtq2, suggesting that this complex also forms in archaea where orthologues of both proteins are present (Fig. 3A; [87]). Mtq2 protein is structurally very similar to the MTase domain from bacterial PrmC, although the PrmC N-terminal helical domain and the linker connecting this domain to the MTase domain appear to be absent from Mtq2 [77]. The structure further reveals that Trm112 is not a structural stand-in for these missing regions since it is located on the side of the Mtq2 active site whereas the PrmC N-terminal domains and the linker are located at the top of the PrmC active site, in the orientation seen in Fig. 2A and 3A. Even though PrmC and Mtq2-Trm112 modify the same motif in class I RFs, the differences are not surprising as the bacterial and eukaryotic RFs are unrelated at the levels of both amino acid sequence and three-dimensional structure.

The structure of the Mtq2-Trm112 complex reveals that large hydrophobic regions located at the surface of both proteins interact upon complex formation, explaining the enhancement of yeast Mtq2 solubility by Trm112 [6]. Trm112 mutants located at the heterodimeric interface abolish Mtq2 enzymatic activity, no doubt by disrupting complex formation [87]. The structure has also brought important clues about the molecular basis for Mtq2 activation by Trm112. This cannot solely be due to solubilisation, as small amounts of

human Mtq2 could be purified in a soluble form, appeared well folded by circular dichroism (MG and VHH unpublished data), but proved to be inactive in the absence of Trm112 [85]. Instead, in the structure, Trm112 interacts with a loop from Mtq2, which in turn contacts the SAM cofactor, suggesting that Trm112 enhances the interaction of Mtq2 with this essential cofactor of the MTase reaction. This was confirmed using the human proteins: Mtq2 alone binds SAM very weakly while Mtq2 incubated with Trm112 or the co-expressed Trm112-Mtq2 complex bind a significantly larger fraction of SAM [87]. Furthermore, SAM binding to Trm9, another MTase activated by Trm112, stabilizes the Trm112-Trm9 complex and probably other complexes formed by Trm112 with MTases.

The Mtq2 active site has been mapped to a highly conserved region surrounding the SAM methyl group. Comparison of the Mtq2-Trm112 structure with the bacterial PrmC-RF1 complex confirms that this region corresponds to the PrmC MTase region which interacts with the loop in PrmC harbouring the GGQ motif. In particular, the (D/N)PPY active site signature, identified as a hallmark of MTases modifying planar amine groups, i.e. N6-adenine [88], N4-cytosine [89] or the N5-Gln side chain [61], superimposes perfectly between bacterial and eukaryotic RF MTases (Fig. 3B). As discussed above, in bacteria this motif is responsible for the binding and correct orientation of the Gln side chain from the GGQ motif in the PrmC active site [62]. Hence, we propose that the same network of interaction as in bacteria is used by Mtq2 to optimally orient the Gln side chain amino group for methyl transfer. In addition, this conserved region is rich in negatively charged residues, as in the bacterial PrmC enzyme. Charge inversion mutants of these acidic residues have shown that this region is important for eRF1 methylation [87]. This is of particular interest as in a model of the yeast eRF1-eRF3-GTP complex inferred from the archaeal Dom34-Hbs1-GTP complex [43], the eRF1 central domain is locked in interaction with the eRF3 GTPase domain and its GGQ motif is surrounded by several conserved Lys and Arg residues. Some of these basic residues are located along the long helix $\alpha 5$ from the eRF1 central domain. Mutation of most of these eRF1 residues does not disrupt interaction with eRF3 but results in complete loss of methylation by Mtq2-Trm112, suggesting their implication in enzyme binding (VHH and MG, unpublished data). In addition, in the eRF1-eRF3-GTP model, the GGQ motif is located near helix $\alpha 4$ of the eRF3 switch I loop, which is enriched in strictly conserved basic residues on its solvent-exposed face. We cannot exclude that these residues interact directly with the

Mtq2-Trm112 active site. Altogether, these observations might rationalize the requirement of eRF3 bound to GTP for eRF1 methylation [77]. First, when bound to eRF3-GTP, the eRF1 central domain might be stabilized and the GGQ motif could then adopt an optimal orientation for entry into the MTase active site. Second, as the switch regions from GTPases are known to adopt radically different conformations depending on the nucleotide bound (GTP or GDP; [90]), the GTP nucleotide may induce a conformation of eRF3 switch I (helix $\alpha 4$) that allows the basic residues from helix $\alpha 4$ to interact directly with Mtq2-Trm112. On the contrary, following eRF1-induced and ribosome-induced GTP hydrolysis, this loop may adopt a completely different structure, incompatible with enzyme binding.

Finally, mutation of solvent-exposed Trm112 residues affect partially the Mtq2 MTase activity without disrupting either Mtq2-Trm112 complex formation or the protein fold (as observed by circular dichroism). This indicates that Trm112 may also be partially involved in direct substrate recognition by the Mtq2-Trm112 complex. Interestingly, the Mtq2-Trm112 complex displays striking structural similarity to RlmA proteins, MTases catalyzing the N¹-methylation of guanine at position G745 (Gram-negative bacteria) or G748 (Gram-negative bacteria) of 23S rRNA [91, 92]. RlmA proteins contain a zinc-binding domain, which was proposed to be involved in substrate recognition and which structurally matches the Trm112 zinc-binding domain [91].

3.2.3. *Physiological role of eukaryotic RF methyltransferases*

Inactivation of yeast *MTQ1* encoding mitochondrial RF (Mrf1) MTase slightly decreases growth only on liquid media containing a non-fermentable carbon source; this is an indicator of its mitochondrial function. As in bacteria, this deletion also affects translation termination in mitochondria, increasing readthrough at a premature stop codon in the mitochondrial *COX2* gene [78]. However, it remains to be established if reduced growth on glycerol is due to inefficient translation termination of essential proteins under these conditions. Depletion of human mitochondrial MTase in HeLa cells leads to a small decrease in mitochondrial translation activity but this phenotype is amplified in the presence of the translation fidelity antibiotic spectinomycin [79]. This observation is reminiscent of the prokaryotic system where PrmC appears to become essential only under certain poor growth conditions or under some stress.

Deletion of the yeast *MTQ2* gene encoding the catalytic subunit of the cytoplasmic eRF1 MTase has more far reaching consequences and multiple phenotypes. The *mtq2Δ* strain is viable but exhibits strong growth defects with a twofold longer generation time on rich media and a cold-sensitive phenotype [77, 78]. It is also sensitive to aminoglycosides like paromomycin and geneticin, which are known to affect translation fidelity through binding to ribosomes (VHH and SF, unpublished data; [78]). The *mtq2Δ* mutant also presents other striking phenotypes such as resistance to the anti-microtubule drugs thiabendazole and benomyl [78]. Clearly, these phenotypes incite many questions and cannot easily be linked to eRF1 methylation defects, raising the possibility of another substrate for Mtq2.

Contrary to the situation in bacteria or yeast mitochondria, the absence of eRF1 methylation in a yeast *mtq2Δ* strain does not significantly decrease the efficiency of translation termination. *MTQ2* deletion does not increase translational readthrough *in vivo* and may even have the opposite effect ([78]; VHH, SF unpublished data). Hence, the growth defect of a *mtq2Δ* strain may be unrelated to termination. In agreement with this, it has been shown that increased stop codon readthrough in eRF1 mutants, *tpa1Δ*, *ett1Δ* strains or [*PSI*⁺] variants does not lead to a reduction in growth rate [93-96]. Since the PTC as well as other functionally crucial regions of ribosomes are extremely well conserved between bacteria and eukaryotes, a conclusion confirmed by the recent high-resolution structure of the yeast ribosome, this result was quite unexpected [27]. This might result from the differences in translation mechanisms between prokaryotes and eukaryotes. So far, the efficiency of translation termination in eukaryotes has only been measured *in vivo*, where other proteins might compensate for the absence of methylation. Alternatively, the termination step could also be so efficient that a decrease might not be detectable in some assays.

Some studies of the murine Mtq2 ortholog (mN6amt1 or PRED28, according to the nomenclature) were performed on the assumption that it was a N⁶-DNA MTase, prior to its identification as an N⁵-Gln-MTase [97]. Two mRNAs resulting from alternative splicing and encoding proteins related to murine RF MTase were identified. The Pred28 α and Pred28 β mRNAs encode respectively for a 23 kDa protein homologous to Mtq2, and a putative one of 14.7 kDa resulting from exon V splicing. Both mRNAs were detected in mouse brain and testis, among other tissues. The longer isoform is very abundant in stem cells and adult tissues. Surprisingly, two different studies on cell cultures after plasmid transfection localized the

GFP-tagged proteins mainly in the nucleus. The smaller form, predicted to be unstable as a large portion of the structural core is absent, was also observed in the nucleus in a stable transfected cell line. Whether this form is also present in natural cell lines is not known. In human, two mRNA species resulting from alternative splicing of exon IV have also been described [97]. If produced in the cell, the shorter protein would not contain the NPPY essential motif for catalysis, and would be inactive in methylation.

Depletion of Pred28 α by RNA interference in murine embryonic stem cells leads to a defect in cell proliferation with an arrest in G1 phase [98]. Similarly, HEK293T human cells in culture with a stable knock down of HemK2 have a two-fold reduction in growth rate [86]. Finally, homozygous mice with disruption of Pred28 α (replacement of exon IV encoding the NPPY motif) are characterized by early embryonic lethality impairing the post-implantation development of mutant embryos. This phenotype is extreme, compared to *S. cerevisiae* where the gene is not essential under standard conditions and growth is only reduced under particular stress conditions.

4. Trm112 interacts with several methyltransferases involved in translation

An increasing number of proteins interacting with and activating enzymes has been described in recent years. For example, the catalytically active conformation of histone acetyltransferase Rtt109 is stabilised by Vps75 [99]. Several MTases exist as holoenzymes, such as the tRNA modification enzymes Trm8-Trm82 [100] and Trm6-Trm61 [101], and the capping MTase enzymes from vaccinia virus and man [102, 103].

The Trm112 protein is particularly interesting since it interacts with and activates three MTases (Mtg2, Trm11 and Trm9) acting on macromolecules involved in translation (Fig.3C). *S. cerevisiae* Trm11 and its archaeal orthologue PAB1283 from *P. abyssi* methylate the guanosine at position 10 in tRNAs to generate m²G10, a modification assumed to be involved in tRNA stability [83, 104]. Trm9 catalyzes the last step of a long series of reactions needed to generate 5-methoxycarbonylmethyl-uridine (mcm⁵U₃₄) at the wobble position of the anticodon loop in some tRNAs (tRNA^{Arg}, tRNA^{Glu},...) in yeast, plants and mammals [82, 105-108]. This Trm9-Trm112 catalyzed modification is very likely to be involved in decoding fidelity, and experimental evidence suggests that this modification is involved in the cellular response to genotoxic stresses in yeast [109, 110]. Indeed, codons recognized by tRNAs

modified by the Trm9-Trm112 complex are over-represented in genes encoding proteins involved in the response to DNA damage, such as RNR1 and RNR3.

Several lines of evidence support the idea that these three MTases compete to interact with Trm112: (i) the catalytic domains of these proteins are predicted to belong to the class I-dependent MTase family and to adopt the same three-dimensional fold; (ii) co-immunoprecipitation assays indicate that over-expression of Trm11 or Mtq2 affects the interaction of Trm9 with Trm112 [84]; (iii) over-expression of Trm11 or Mtq2 results in Trm9 inactivation illustrated by the increased resistance of budding yeast to zymocin (a killer toxin cleaving only those tRNAs that have been fully modified at position U34 by various enzymes including Trm9; [84, 111]); (iv) Trm9 mutants constructed on the basis of a model of the Trm9-Trm112 complex derived from the Mtq2-Trm112 structure and aimed at disrupting the interaction with Trm112, are catalytically inactive and unable to interact with Trm112 *in vivo* [87]. Finally, the interaction of Trm112 with Mtq2 would be fully compatible for Trm112 binding to the other 3 MTases. In the Mtq2-Trm112 complex, the two partners interact via a β -zipper interaction involving hydrogen bonds between main chain atoms from Trm112 strand β 4 and Mtq2 strand β 3 [87]. This mode of interaction is therefore less sensitive to variation of side chains at the positions involved and is well adapted to interaction with proteins of divergent sequence but similar folds. For instance, it is also used by bacterial proteins interacting with the antigen binding fragment (Fab) region of a large panel of antibodies and B-cell receptors [112, 113]. Global quantitative analysis of these proteins in yeast indicates that under normal growth conditions, the 3 MTases together are more abundant than Trm112, indicating that not all molecules may be saturated with Trm112 [114].

Most studies regarding the physiological role of Trm112 have been performed in *S. cerevisiae*. Due to the extremely reduced growth of *trm112* Δ cells, *trm112* is still annotated as an essential gene in the *Saccharomyces* Genome Database. However, recent measurements indicate a generation time of about 6 hours for *trm112* Δ compared to 1.5 hours for a wild type strain in rich liquid medium ([82]; SF and VHH, unpublished data). The *trm112* Δ strain is even more affected than a strain deleted for all genes encoding MTase partners of Trm112 (Mtq2, Trm9, Trm11), suggesting that Trm112 has additional functions in yeast. However, the absence of Mtq2 activity contributes to a large part to this phenotype ([82, 115], SF and VHH, unpublished data). Other potential partners of Trm112 suggested by large scale

interactome studies include Ecm16 and Sfh1, and a reduced function of these proteins may also contribute to the dramatic reduction in growth of *trm112Δ*.

Trm112 orthologs exist in eukaryotic and archaeal kingdoms, and some interactions with eRF1 or tRNA MTase orthologs are also conserved [85, 105, 108]. For instance, *Arabidopsis thaliana* protein SMO2 is a functional analog of yeast Trm112 since its expression in *S. cerevisiae* restores cell growth [116]. As in *S. cerevisiae*, the inactivation of *SMO2* leads to a defect in cell growth. *SMO2* is also required for proper cell division and development, but the mechanisms underlying this phenotype are still unknown.

5. Conclusions

With the progress of proteomics methods such as mass spectrometry and purification of endogenous proteins, it has becoming increasingly evident that post-translational modifications are widely present in all living organisms. These events are known to expand protein sequence complexity and to play crucial roles in various cellular processes and their regulation. Studies concerning the role of these chemical modifications are very much in their infancy, and their impact has yet to be determined. In most cases, it is difficult to predict post-translational modifications only from protein sequence, and identification of the enzyme involved is not always an easy task.

Methylation is one of the most common modifications found on proteins and nucleic acids, although many other targets have been identified. SAM-dependent MTases can be classified in several groups according to their structural folding, the major group adopting the class I, Rossmann-like fold. Their identification in complete genomes is difficult because their primary sequence similarity is very low.

N5-methylation of Gln side chains is an unusual modification, so far found only in ribosomal protein L3 in *E. coli* and related bacteria, and class 1 RFs in bacteria and eukaryotes. Here we review the mechanism of RF methylation and the role of this modification, present on the Gln of the universally conserved and essential GGQ motif. In bacteria, RF methylation clearly stimulates RF function. In eukaryotes, RF methylation is more complex, and the role uncertain. The MTase turned out to be a heterodimeric complex formed by the Mtt2 catalytic subunit and its Trm112 partner. Though Mtt2 was predicted some time ago from *S. cerevisiae* complete genome analysis to be a MTase, much

experimental work was needed to identify its substrate and mechanism of action. Further studies are still necessary to understand more precisely the role of RF modification, particularly in eukaryotic organisms. Some observations suggest that the M_{tq2} MTase has other substrates than eRF1, as yet unidentified. Several other questions are still open: what are the reasons for the essential role of the mammalian MTase? Are the two polypeptides resulting from translation of alternatively spliced mRNA really produced? If so, what are their respective roles? If methylation of eRF1 is also incomplete in cells from a complete organism, does that imply a regulatory mechanism and a more complex biological significance? In addition, HemK2 is encoded on human chromosome 21 and may have a role in the pathology of Down syndrome. Whether or not this pathology is correlated to a deficiency in eRF1 methylation remains to be determined. In *C. elegans*, a HemK2 ortholog has been identified and has been shown to be localized specifically in some parts of the nervous system from the larval stage until adulthood.

Trm112 is necessary for proper folding of M_{tq2} and its activation by improving SAM binding. Surprisingly, Trm112 is more than a simple activator of the M_{tq2} MTase, and can now be considered as an activating platform, insofar as it activates in a similar manner two tRNA MTases. Trm112 is a “hub” protein central to protein synthesis mechanisms, and more studies will be needed to fully decipher its interactions and physiological functions.

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Legends to figures.

Figure 1: Class I release factors.

- A. Structure of the closed conformation of bacterial RF2 protein.** The domains I, II, III and IV are coloured in pink, orange, red and yellow, respectively. The “SPF” motif which interacts with the stop codon and the GGQ motif are depicted as sticks and balls, respectively. PDB code used to generate this figure: 1GQE [4]. With the exception of Fig.3C, all figures were generated using the PYMOL software (<http://www.pymol.org>).
- B. Structure of the bacterial RF2 bound to ribosomes.** The same colour code as in panel A is used. Inset: Representation of the RF2 ribosomal termination complex. The 30S and 50S subunits are coloured in grey and wheat, respectively. The P-site and E-site tRNAs are coloured in light and dark green, respectively. The mRNA is coloured in purple. PDB codes used to generate this figure: 2X9R and 2X9S [68].
- C. Structure of human eRF1.** The N-terminal, central and C-terminal domains are in orange, red and purple, respectively. The “NIKS” motif from the N-terminal domain and the GGQ motif are shown as sticks. PDB code used to generate this figure: 1DT9 [3].

Figure 2: Methylation of bacterial RFs.

- A. Structure of *E. coli* PrmC.** The N-terminal and C-terminal domains are coloured in blue and green. The linker connecting both domains is in light blue. The S-Adenosyl-L-Homocysteine (SAH) ligand is shown as grey sticks. PDB code used to generate this figure: 1NV9 [61].
- B. Structure of the PrmC-RF1 complex from *E. coli*.** The PrmC and RF1 are represented using the same colour codes as panels A and Fig.1A, respectively. The RF1 GGQ motif is shown as balls and sticks. The “PAT” motif contacting the stop codon and the SAH molecule are shown in sticks. PDB code used to generate this figure: 2B3T [62].
- C. Detailed representation of the GGQ binding mode into PrmC active site.** The PrmC MTase domain is shown in green. The RF1 containing the GGQ motif is shown in red and the GGQ motif is highlighted in brown sticks. The hydrogen bonding network coordinating the Gln side chain into the PrmC active site is depicted by black dashed lines. The methyl group (grey sphere) to be transferred from SAM to the Gln side chain has been modelled by substituting SAH by SAM.
- D. Modelling of the GGQ methylation into the peptidyl transferase center.** For clarity, only bases contacting the GGQ motif are depicted. The RF2 loop harbouring the GGQ motif is coloured in purple. The P-site tRNA analog is shown in green. PDB codes used to generate this figure: 2X9R and 2X9S [68].

Figure 3: Eukaryotic RF methyltransferase.

- A. Structure of the Mtq2-Trm112 complex from *E. cuniculi*.** The SAM molecule bound to Mtq2 is shown as grey sticks. A cyan sphere depicts the zinc atom bound to Trm112 and the 4 cystein residues coordinating this zinc are shown as sticks. PDB code used to generate this figure: 3Q87 [87].
- B. Comparison of Mtq2 and PrmC active sites.** PDB code used to generate this figure: 3Q87 and 2B3T [62, 87].
- C. The Trm112 protein is an activator of 3 methyltransferases modifying translation factors.** The tRNA positions modified by Trm9-Trm112 and Trm11-Trm112 are coloured in blue and magenta, respectively.

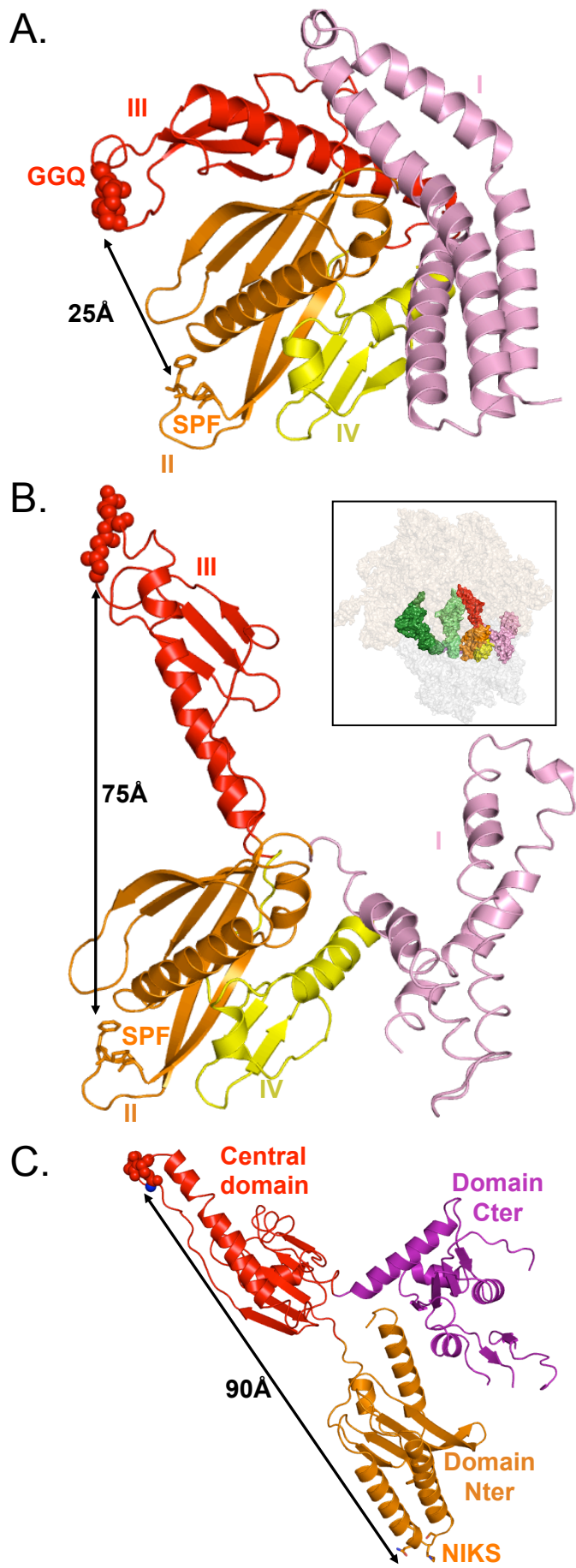


Figure 1

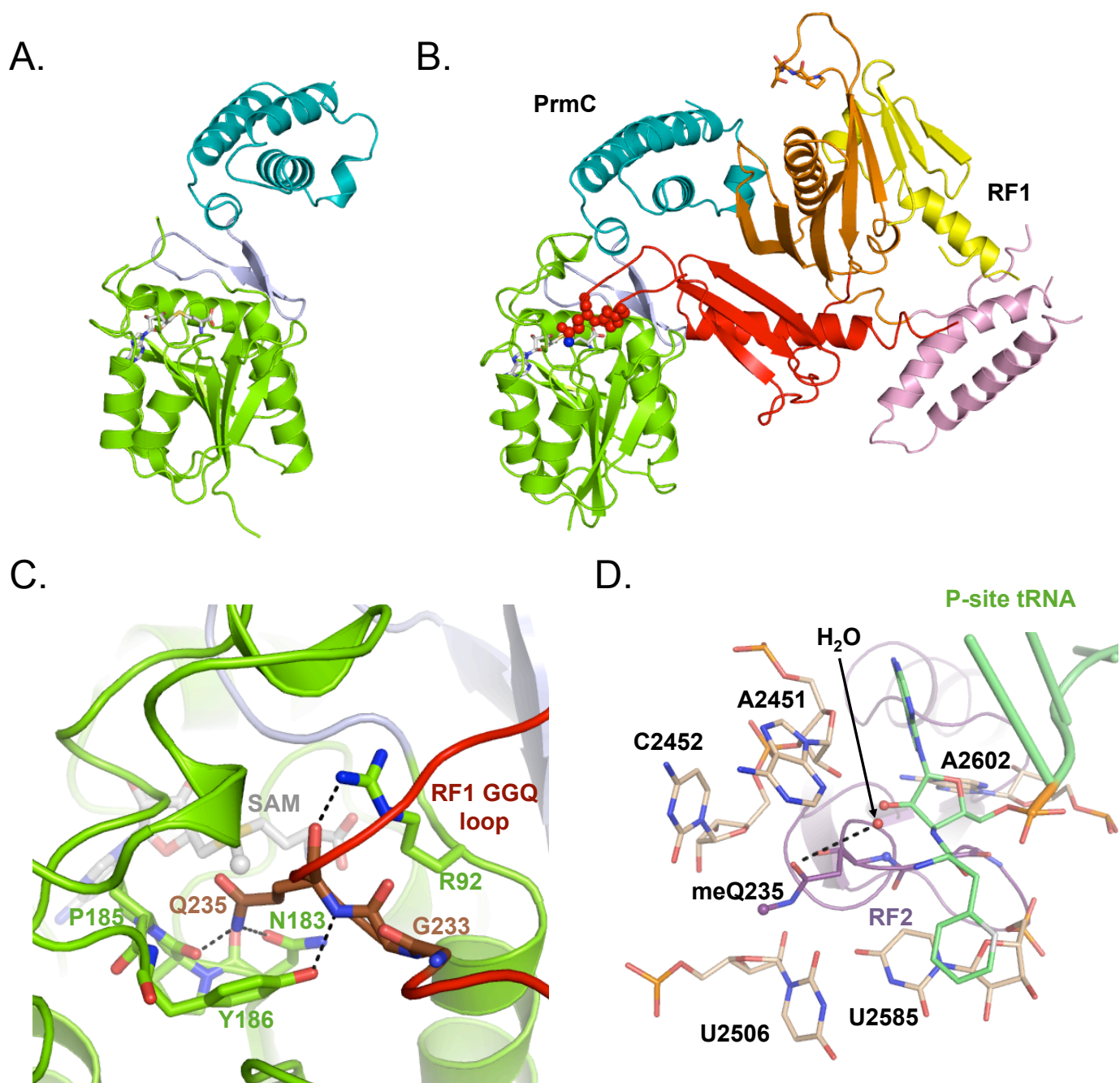


Figure 2

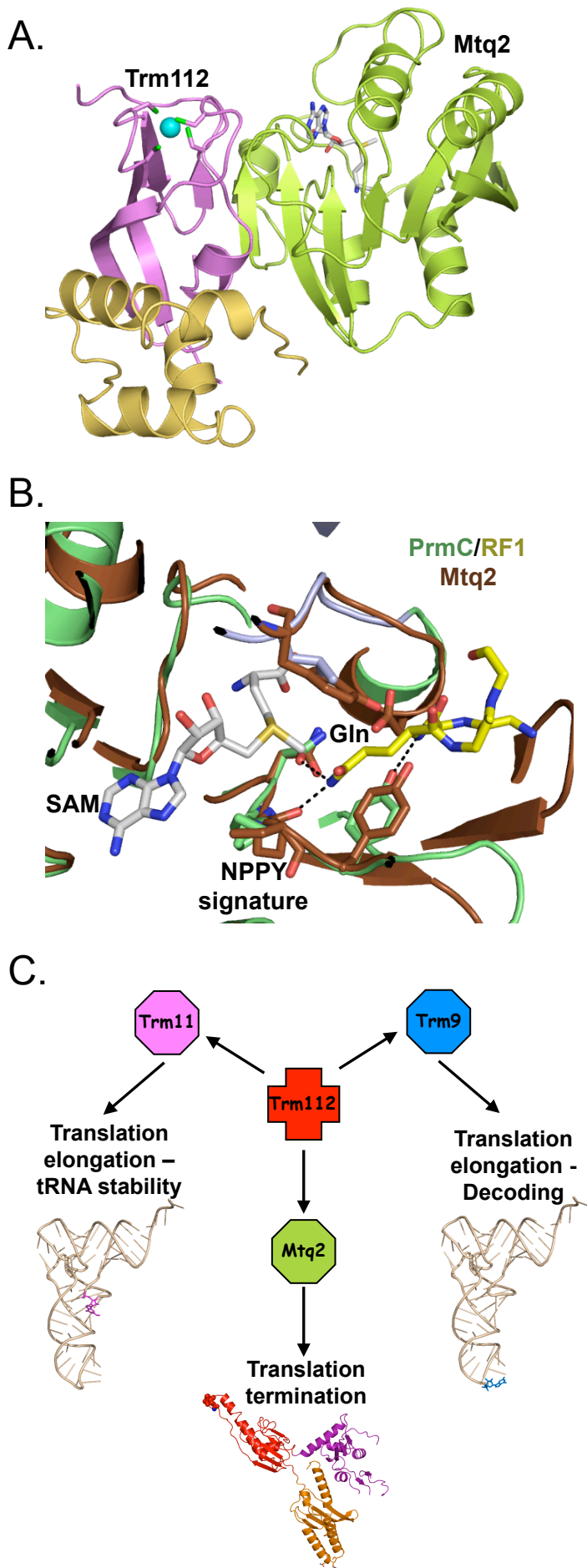


Figure 3

Table 1: Methylated RF and MTases in different organisms

Organisms	Substrate	Methyltransferase
<i>E. coli</i>	RF1, RF2	PrmC
<i>S. cerevisiae</i>	Mrf1 (mitochondrial RF)	Mtq1
	eRF1 + eRF3 + GTP	Mtq2 +Trm112
<i>H. sapiens</i>	HMRF1L (mitochondrial RF)	HMPrmC
	eRF1 + eRF3 + GTP	HemK2 α + hTrm112
<i>M. musculus</i>	eRF1 + eRF3 + GTP	PRED28 α /N6amt1+ mTrm112