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Review

[4Fe-4S]-dependent enzymes in non-redox tRNA thiolation

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ABSTRACT

Post-transcriptional modification of nucleosides in transfer RNAs (tRNAs) is an important process for accurate and efficient translation of the genetic information during protein synthesis in all domains of life. In particular, specific enzymes catalyze the biosynthesis of sulfur-containing nucleosides, such as the derivatives of 2-thiouridine (s^2U), 4-thiouridine (s^4U), 2-thiocytidine (s^2C), and 2-methylthioadenosine (ms^2A), within tRNAs. Whereas the mechanism that has prevailed for decades involved persulfide chemistry, more and more tRNA thiolation enzymes have now been shown to contain a [4Fe-4S] cluster. This review summarizes the information over the last ten years concerning the biochemical, spectroscopic and structural characterization of [4Fe-4S]-dependent non-redox tRNA thiolation enzymes.

1. Introduction

1.1. Modified nucleosides within RNA

Precise decoding of the genetic code is a fundamental process in all living organisms. Transfer RNAs (tRNAs) play a critical role in protein synthesis by translating codons on messenger RNAs (mRNAs) into the corresponding amino acids in the ribosome. All cellular RNAs feature post-transcriptional chemical modifications, which are evolutionarily well conserved [1]. MODOMICS is a database of RNA modifications that provides comprehensive information about RNA modifying enzymes, as well as the chemical structures of modified ribonucleosides, their biosynthetic pathways and the location of modified residues in RNA sequences [1]. Chemical modifications within tRNA are the most numerous and diverse [2]. They stabilize its tertiary structure, introduce recognition determinants and anti-determinants towards RNA-interacting macromolecules and fine-tune the decoding process at the level of both efficiency and fidelity [3–7].

Recently, the dynamic control of post-transcriptional RNA modifications has emerged as a new level of gene expression regulation [8–14]. For example, N6-methyladenosine, the most abundant mRNA internal modification in eukaryotes, has a crucial function in regulating RNA metabolism including export, stability, translation, and decay [15]. Moreover, under stress conditions, tRNA modification reprogramming greatly contributes to cell survival by orchestrating the synthesis of specific proteins vital to stress response [16–20]. Finally, many human pathologies are linked to defects in tRNA modification pathways [20–24].

1.2. Thionucleosides within tRNA

Until now, thionucleosides have been identified only in tRNA [1], at position 8 and 9 in the tRNA core, positions 32, 34 and 37 around the anticodon and position 54 in the T-loop (Fig. 1). Important for controlling the response to cellular stress, tRNA thiolation is negatively regulated in lower eukaryotes under stress, thereby promoting cell survival [25,26]. Although these thionucleosides exhibit diverse cellular functions, they share common biosynthetic strategies [27,28].

The sulfur insertion step is catalyzed by specific thiolation enzymes called TtuI (formerly known as ThiI) for position 8, TtcA for position 32, MnmA in bacteria and Ncs6/CTU1/NcsA in eukaryotes and archaea for position 34, MiaB/MtaB for position 37, TtuA for position 54 (Fig. 1) [29–32].

Sulfur insertion reactions in tRNA can be divided into two classes. One class consists of the insertion of a sulfur atom within an inert C—H bond, such as the reaction catalyzed by methylthiotransferases MiaB and MtaB [33,34]. This high energy-demanding reaction is catalyzed by S-adenosyl-L-methionine (SAM)-radical enzymes. All SAM-radical enzymes catalyzing thiolation reactions contain two iron-sulfur clusters: one [4Fe-4S] cluster is involved in SAM reduction and cleavage, whereas the additional cluster, [2Fe-2S] or [4Fe-4S], is proposed either to function as a source of sulfur atom [35,36] or to activate and transfer an

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exogenous hydrogenosulfide ligand for the thiolation step [37–39]. This mechanistic duality remains a matter of intense debate. The second class of reactions consists of the substitution of an oxygen atom by a sulfur atom, which is a non-redox reaction, in contrast to the first type of reactions. This review will focus on tRNA thiolases that catalyze non-redox thiolations since the mechanisms of SAM-radical tRNA thiolation enzymes have already been extensively covered [36,37,40]. We discuss here about more and more of these enzymes being reported to use a [4Fe-4S] cluster. We illustrate their biochemical and spectroscopic properties gathered over the last ten years with their crystal structures or models calculated with AlphaFold, an artificial intelligence program that has revolutionized the field of structural biology in 2021 by the accuracy of its prediction of proteins three-dimensional models [41].

1.3. [Fe-S] dependent and independent pathways for nonredox tRNA thiolations

In the bacterium *Salmonella enterica serovar Typhimurium*, mutation in the *iscU*, *hscA*, or *fdx* genes involved in the iron-sulfur cluster (ISC) biosynthesis machinery [42] reduced the synthesis of s^2C 2-fold and that of ms^2io^6A 10-fold but not that of s^4U or $(c)mnm^5s^2U$, as shown by High Performance Liquid Chromatography (HPLC)-coupled mass spectrometry (MS) analysis of modified nucleosides after hydrolysis of bulk tRNA from the mutated strains [43]. This led to the suggestion that two principal distinct routes for thiolation of tRNA exist: [Fe-S]-independent enzymes for s^4U and $(c)mnm^5s^2U$ formation and [Fe-S] enzymes for the synthesis of s^2C and ms^2io^6A. Later, after the sulfur mobilization system SUF was discovered as a new [Fe-S] cluster biosynthesis machinery [44], it was shown that, in *Escherichia coli*, the amounts of mnm^5s^2U or s^4U tRNA modifications remained unaffected in the Δ*iscU or Δfdx* strains, independently of the expression of the *suf* operon [45]. This experiment reinforced the hypothesis that the s^2U biosynthetic pathway was [Fe-S]-independent in bacteria. In contrast, ms^2i^6A37 and s^2C32 were recovered when the *suf* operon was overexpressed in the *iscU* or *iscS* deficient strains, indicating that the SUF system could provide [Fe-S] clusters necessary for these thiomodifications [45].

The question as to whether the biosynthesis of [Fe-S] clusters is a prerequisite for the 2-thio modification of uridine in tRNAs in both subcellular compartments of yeast cells was raised in 2007 [46]. The three components of the cytosolic iron-sulfur cluster protein assembly (CIA), Cfd1, Nbp35, and Cia1 [47], were required for 2-thio modification of cytosolic tRNAs, but not of mitochondrial tRNAs. Moreover, the mitochondrial scaffold proteins Isu1 and Isu2 from the ISC system were required for the 2-thio modification of cytosolic tRNAs but not of mitochondrial tRNAs. It was therefore concluded that the 2-thio modification of cytosolic tRNAs, which required both CIA and ISC machineries, is dependent on an [Fe-S] protein, whereas that of mitochondrial tRNAs is [Fe-S] cluster independent. It was shown later that

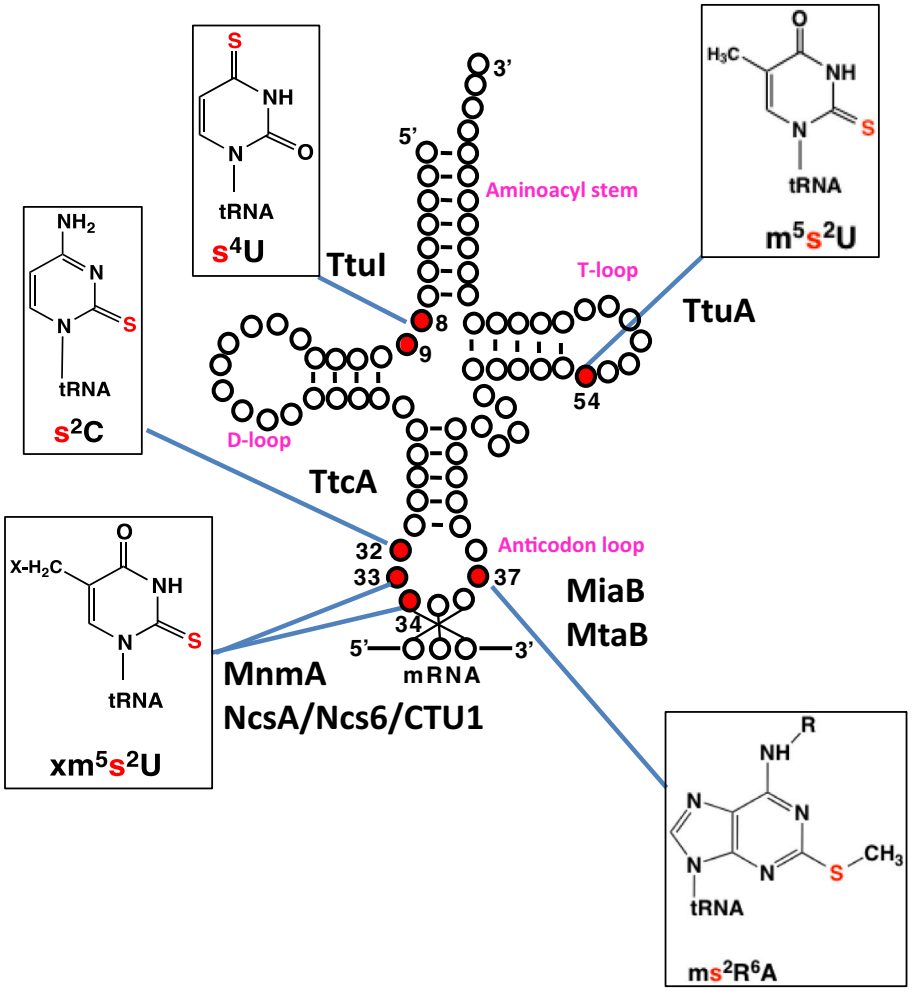

Fig. 1. Position of thiolated nucleosides within tRNA. The name of enzymes catalyzing the specific insertion of sulfur within tRNA is indicated and the thiolated nucleoside is framed: s^2C, 2-thiocytidine; s^4U, 4-thiouridine; m^5s^2U, 5-methyl-2-thiouridine; ms^2A, 2-methylthioadenosine. Thiolation of U34 is catalyzed by MnmA in bacteria and mitochondria, a single protein NcsA in archaea or a complex of two proteins in the cytosol of eukaryotes (called Ncs6p/Ncs2p in yeast and CTU1/CTU2 in humans).

mitochondria play a direct and essential role in cytosolic tRNA thiolation by generating low molecular mass sulfur-containing intermediate species and exporting them to the cytosol, where they were used for tRNA thiolation [48].

1.4. Sulfur transfer pathways from cysteine to the thiolated nucleosides

In all three kingdoms of life, the sulfur atoms that are incorporated into molecules to form iron-sulfur clusters, thiamine, molybdenum cofactor, biotin, or thionucleosides [49–52] mainly originate from the amino acid L-cysteine [53]. In most organisms [54], a pyridoxal-5′-phosphate (PLP)-dependent cysteine desulfurase first mobilizes sulfur *via* formation of a persulfide (-SSH) on its catalytic cysteine residue by desulfurization of L-cysteine into L-alanine [55,56]. The cysteine desulfurase is called IscS in *E. coli* and *S. enterica serovar Typhimurium* [49,50,57,58], Nfs1 in eukaryotes [59], NifZ or YrvO in *Bacillus subtilis* [60,61]. Mitochondrial Nfs1 was shown to be required for 2-thio modification of tRNAs, both in the mitochondria and the cytosol [46].

The terminal sulfur of the persulfide is then presumed to be used directly by the tRNA thiolation enzyme (MnmA from *B. subtilis* (BsMnmA) [61]), *E. coli* TtuI (EcTtuI) [57] and possibly TtuI from *B. subtilis* (BsTtuI) [60]) or be transferred *via* sulfur-carrier proteins through transpersulfuration reactions that generate a persulfide in the last sulfur acceptor (Fig. 3) [28]. Thus, the activity of tRNA thiolation enzymes has usually been studied using radiolabeled cysteine and cysteine desulfurase and by monitoring of incorporation of radioactive sulfur into the enzyme and the tRNA [62]. It is important to note here that the initial step of sulfur transfer catalyzed by cysteine desulfurases is not universal as bacteria mollicules and some archaea lack a cysteine desulfurase [54,63].

A reverse genetic approach combined with mass spectrometry was used to identify the sulfur-transfer system for 2-thiouridine formation in tRNAs in *E. coli* [64] and *S. cerevisiae* [65]. 2-thiouridine formation catalyzed by *E. coli* MnmA (EcMnmA) proceeds through a complex cascade of sulfur carrier proteins known as the Tus pathway [64], which is restricted to certain bacterial species, such as γ-proteobacteria (Fig. 3A) [54]. Five proteins (TusA, the TusBCD complex [66] and TusE) are used to relay sulfur from the IscS-bound persulfide and generate a persulfide on TusE that serves as the sulfur source for tRNA thiolation [64].

Another chemical mechanism generates a thiocarboxylate at the C-terminal glycine of a carrier protein as the last sulfur donor for tRNA thiolation. This thiocarboxylate-mediated sulfur-relay pathway is responsible for U54-tRNA thiolation in *Thermus thermophilus* (Fig. 3B) and for U34-tRNA thiolation in archaea [67] and eukaryotes [65,68,69].

In *T. thermophilus*, s^2U54-tRNA biosynthesis involves a sulfur-transfer chain consisting of the tRNA thiolase TtuA and the proteins TtuB, TtuC and TtuD, with the thiocarboxylate formed on the C-terminal glycine of TtuB being used as the last sulfur donor to TtuA (Fig. 3B) [70–74]. The *ttuA*, *ttuB* and *ttuC* genes are organized as an operon but not the *ttuD* gene. It was proposed that the C-terminal carboxylate on TtuB is activated by the TtuC ATPase, resulting in the formation of an acyl-adenylated TtuB-CO-OAMP intermediate, and that the thiocarboxylation of the latter is performed by sulfur transfer from the persulfide formed on TtuC [71]. The sulfur atom received by TtuC comes from the persulfide formed on the catalytic cysteine of one rhodanese homology domain (RHD) of TtuD [73], itself generated by transfer of a sulfur atom from cysteine by a cysteine desulfurase IscS or SufS. As the final enzyme of this pathway, TtuA catalyzes the transfer of the sulfur atom from TtuB-CO-SH to the tRNA [74,75]. Several organisms like *Thermotoga maritima* do not possess the *ttu* genes (C. Brochier, personal communication) so they likely use another sulfur-carrier pathway to form the thiocarboxylate species or inorganic sulfide as the thiolating agent for s^2U54-tRNA formation.

An equivalent ubiquitination-related pathway is involved in sulfur transfer from cysteine for s^2U34-tRNA thiolation in eukaryotes (yeast [65,69,76–79], nematode [80], plant [81] and human [68,82]), and in archaea [67,83]. The thiocarboxylate that serves as a sulfur source for U34-tRNA thiolation is formed on the C-terminus of the ubiquitin-like protein Urm1 in eukaryotes and archaea (TtuB homologue) through adenylation and sulfur transfer from the E1-ubiquitin activating-like enzyme, called Uba4 in eukaryotes and UbaA in archaea (TtuC homologue). It has been suggested that the prokaryotic sulfur relay enzymes are at the origin of the ubiquitination system that controls a wide range of physiological processes in eukaryotic cells [84].

1.5. Non-redox tRNA thiolation: activation of target position by ATP

TtuI, TtcA, MnmA, Ncs6/CTU1/NcsA and TtuA enzymes catalyze the non-redox substitution of an oxygen atom for a sulfur atom (Fig. 1). All these proteins belong to PP-loop ATP pyrophosphatase (PPase) family [85] possessing a PP(pyrophosphate)-loop motif (SGGKDS) that binds ATP (Fig. 2). ATP binding activates the target nucleoside by formation of an adenylated intermediate (Fig. 4A) so that sulfur insertion takes places specifically at the target position. The introduction of a good leaving group facilitates the subsequent nucleophilic attack of the sulfur donor on the O-adenylated intermediate, after which AMP is released [86,87]. The existence of the adenylated intermediate (Fig. 4A) was revealed when the reaction catalyzed by EcTtuI was performed in the presence of α-[^{32}P]-labeled ATP [87]. The incorporation of radioactivity into tRNA was followed on an electrophoresis gel, and a band with decreased mobility appeared, corresponding to the radiolabeled adenylated intermediate. The nature of this intermediate was confirmed by adding increasing concentrations of unlabeled ATP, which decreased the amount of radiolabeled adenylated tRNA, and by the identification of the intermediate by HPLC/MS. Notably, the adenylated RNA intermediate has been trapped and visualized in the crystal structure of EcMnmA in complex with tRNA [86]. Interestingly, the cysteine desulfurase YrvO was able to transfer [^{35}S]-sulfur from [^{35}S]-L-cysteine to BsMnmA only in the presence of ATP, suggesting that BsMnmA undergoes a conformational change upon nucleotide binding to accept the sulfur atom [61].

1.6. Persulfide-based mechanism

We renamed ThiI proteins that thiolate U8-tRNA (Fig. 1) as TtuI proteins [88,89] because most of these enzymes do not possess the RHD needed for thiamin synthesis. For convenience, we still call the gene encoding EcTtuI as *thiI* because EcTtuI possesses the RHD and is involved in thiamin synthesis. TtuI proteins modify uridine at position 8 in all bacterial and archaeal tRNAs, with s^4U being present in 70 % of tRNAs [90,91]. In addition, s^4U is found at position 9 in tRNAs from some species [1,92]. The target base is buried inside the core of the tRNA structure, which means that a major conformational change of tRNA is needed to make U8 accessible to the enzyme. The product of the TtuI reaction, 4-thiouridine, is a sensor of near UV light: upon irradiation, s^4U8 cross-links with the nearby cytidine at position 13, causing conformational changes that prevent aminoacylation of tRNAs, resulting in accumulation of uncharged tRNAs that triggers stringent responses [2,93].

TtuI proteins are organized into several domains: a N-terminal ferredoxin-like domain (NFLD), an RNA binding domain called THUMP (for THioUridine synthases, RNA Methyltransferases and Pseudo-uridine synthases domain), and a catalytic PPase domain (Fig. 2). TtuI proteins from γ-proteobacteria, such as *E. coli*, and from several archaea contain an additional C-terminal RHD, known to function as a versatile sulfur-carrier [94]. Rhodanese modules possessing a six-amino-acids active-site loop, with a catalytic cysteine at the first position, as in EcTtuI, are known to interact with substrates containing reactive sulfur atoms [95].

Mutagenesis experiments in EcTtuI have shown that two conserved cysteines are crucial for activity: Cys344 from the catalytic domain and Cys456 from the RHD. When Cys456 was mutated into alanine, the

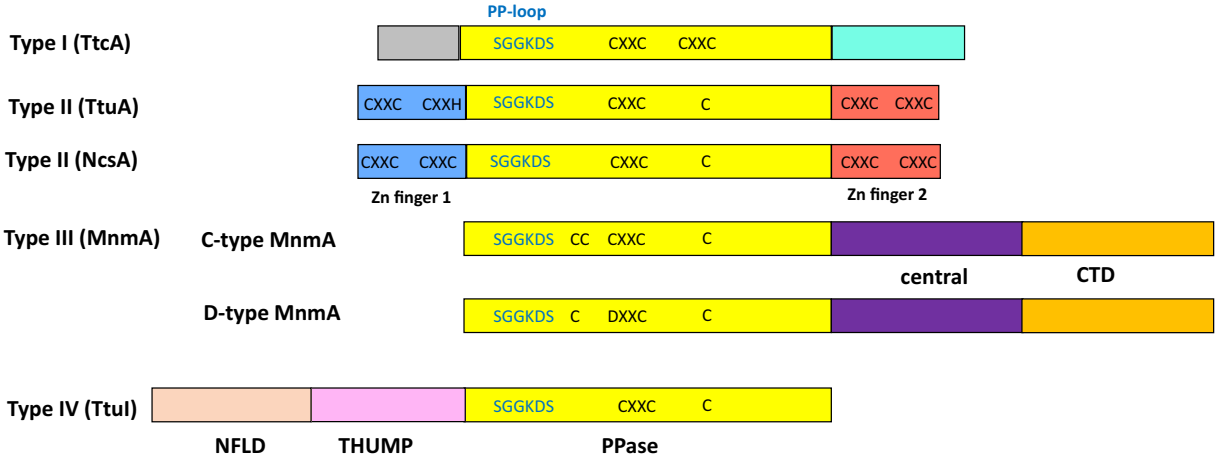

Fig. 2. Conservation of the CXXC + C motif and comparison of the domain organization in [4Fe-4S]-dependent non-redox tRNA thiolases. CTD, C-terminal domain of MnmA proteins; NFLD, N-terminal ferredoxin-like domain; THUMP, THioUridine synthases, RNA Methyltransferases and Pseudo-uridine synthases; PPase, pyrophosphatase.

sulfur transfer assay with the [^{35}S]-cysteine/IscS system did not lead to incorporation of radioactivity into the tRNA product, suggesting that a persulfide is formed on Cys456 during catalysis in the wild-type protein [57,62,96]. Two mechanisms of sulfur transfer for the EcTtuI-catalyzed reaction have been proposed: either the persulfide of Cys456-SSH performs a direct nucleophilic attack on the adenylated intermediate, with Cys344 serving to resolve the disulfide bond formed between the enzyme and the tRNA, or hydrogen sulfide, released from Cys456-SSH with the assistance of Cys344, acts as the nucleophilic species (see Fig. 2 in [97]). Turnover implies that this disulfide is reduced to regenerate the active cysteine residues.

The AlphaFold2 model of EcTtuI shows that the SH group of Cys344 does not point towards the RHD and that the Cα atoms of Cys344 and Cys456 are 15 Å away (see below, Fig. 10A). Although the two cysteines are far from each other in this model, they could form a transient disulfide bond during catalysis since Cys456 is located on a long flexible loop, whose mobility could bring Cys456 closer to Cys344. For simplicity, we call the mechanism that involves two catalytic cysteines, with one of them carrying the persulfide used as a sulfur source for tRNA thiolation, a 'persulfide-based' mechanism.

Two other classes of TtuI proteins, without a RHD, have been identified. One class, represented by TtuI from *Bacillus anthrasis* and *Thermotoga maritima*, contains only one conserved cysteine at the active site (Cys344). Although the crystal structure of both proteins is known [98,99], it is not yet understood how such TtuI enzymes catalyze their reactions. It has been suggested that they may function as a complex with a stand-alone rhodanese protein encoded by a separate gene [97,98]. The third class, which uses an iron-sulfur cluster for catalysis, was discovered only recently [89,100] and will be the subject of Section 2.5.

In bacteria, uridine 34 in the anticodon loop of tRNAs (Fig. 1) is modified into 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U) and 5-carboxymethylaminomethyl-2-thiouridine (cmnm^{5}s^{2}U) by MnmA proteins [101]. Mutations in the *mnmA* gene resulted in severe growth reduction in *E. coli* [58,101] and *S. enterica serovar Typhimurium* [49,102], and in non-viability in *B. subtilis* [103].

Since L-cysteine desulfurase IscS is required for the biosynthesis of s^{2}U34-tRNA in *E. coli* and *S. enterica serovar Typhimurium* [49,50], the transfer of sulfur from [^{35}S]-L-cysteine to unmodified tRNA by the tRNA thiolase was routinely used as a measure of catalytic activity by quantifying the radioactivity in the [^{35}S]-tRNA product bound to DEAE-cellulose filter disks, after incubation in the presence of Mg-ATP, PLP, IscS and dithiothreitol (DTT) as a reductant [58,64]. The first attempts to reconstitute the biosynthesis of s^{2}U-tRNA *in vitro* with this simple system showed very low activity, with large amounts of EcMnmA being required to observe s^{2}U formation (MnmA:tRNA ratio of 1:1) [58]. Later, genetic analysis showed that the EcMnmA biosynthetic pathway not only involves IscS but a more complex cascade of sulfur carrier proteins consisting of TusA, the TusBCD complex and TusE (Fig. 3A) [64]. With all the relay proteins, the sulfur transfer from cysteine to the tRNA was increased 200 fold, but with a noncatalytic amount of enzyme (EcMnmA:tRNA ratio of 2.1:1 [64] or 4:1 [86]). It was proposed that, at the end of the sulfur transfer pathway, a persulfide on the TusE catalytic cysteine would provide sulfur to a catalytic cysteine of EcMnmA, forming a persulfide that could act as the sulfur source for tRNA thiolation [64]. However, using [^{35}S]-L-cysteine and all the relay proteins, EcMnmA was either not labeled [64] or poorly labeled [86], as shown by the analysis on non-reducing SDS-PAGE gels, so it was not clear if the sulfur provided by TusE in the form of a persulfide is accepted directly by EcMnmA.

The mechanism following the formation of a persulfide on TusE proposed by Numata et al. for EcMnmA (see Fig. 5 in [86]) was based on the proximity of three conserved residues (Asp99, Cys101 and Cys199) to the target U34-tRNA, as observed in the crystal structures of EcMnmA in complex with tRNA, and on the inability of the D99A, C102S and C199A mutants to transfer sulfur from [^{35}S]-L-cysteine to the tRNA [86]. It involved an adenylated intermediate, as for TtuI (Fig. 4A), in agreement with the trapping of the adenylated intermediate state in the crystals [86]. The formation of a persulfide on Cys199 would enable Cys199-SSH to act as a nucleophile to attack the C2 position of the adenylated intermediate, followed by the cleavage of the disulfide bond between the enzyme and tRNA by Cys102, with Asp99 acting as a base/acid catalyst to deprotonate/protonate the N3 atom of U34 [86]. This 'persulfide-based mechanism' was analogous to that previously proposed for EcTtuI [97].

2. A new expanding class of tRNA thiolation enzymes operating with a [4Fe-4S] cluster

2.1. C32-tRNA thiolase TtcA: the first biochemically characterized [4Fe-4S]-dependent tRNA thiolation enzyme

As discussed in Section 1.3, genetic studies showed in 2004 that TtcA, which catalyzes thiolation of cytidine 32, near the anticodon loop (Fig. 1), in four tRNAs (tRNA$^{Arg}_{ICG}$ tRNA$^{Arg}_{CCG}$, tRNA$^{Arg}_{mnm5UCU}$, and tRNA$^{Ser}_{GCU}$), - or proteins upstream or/and downstream of TtcA in the s^{2}C-tRNA synthetic pathway - contains an [Fe-S] cluster [43]. The *ttcA* gene was identified by analyzing *E. coli* and *S. enterica serovar Typhimurium* strains

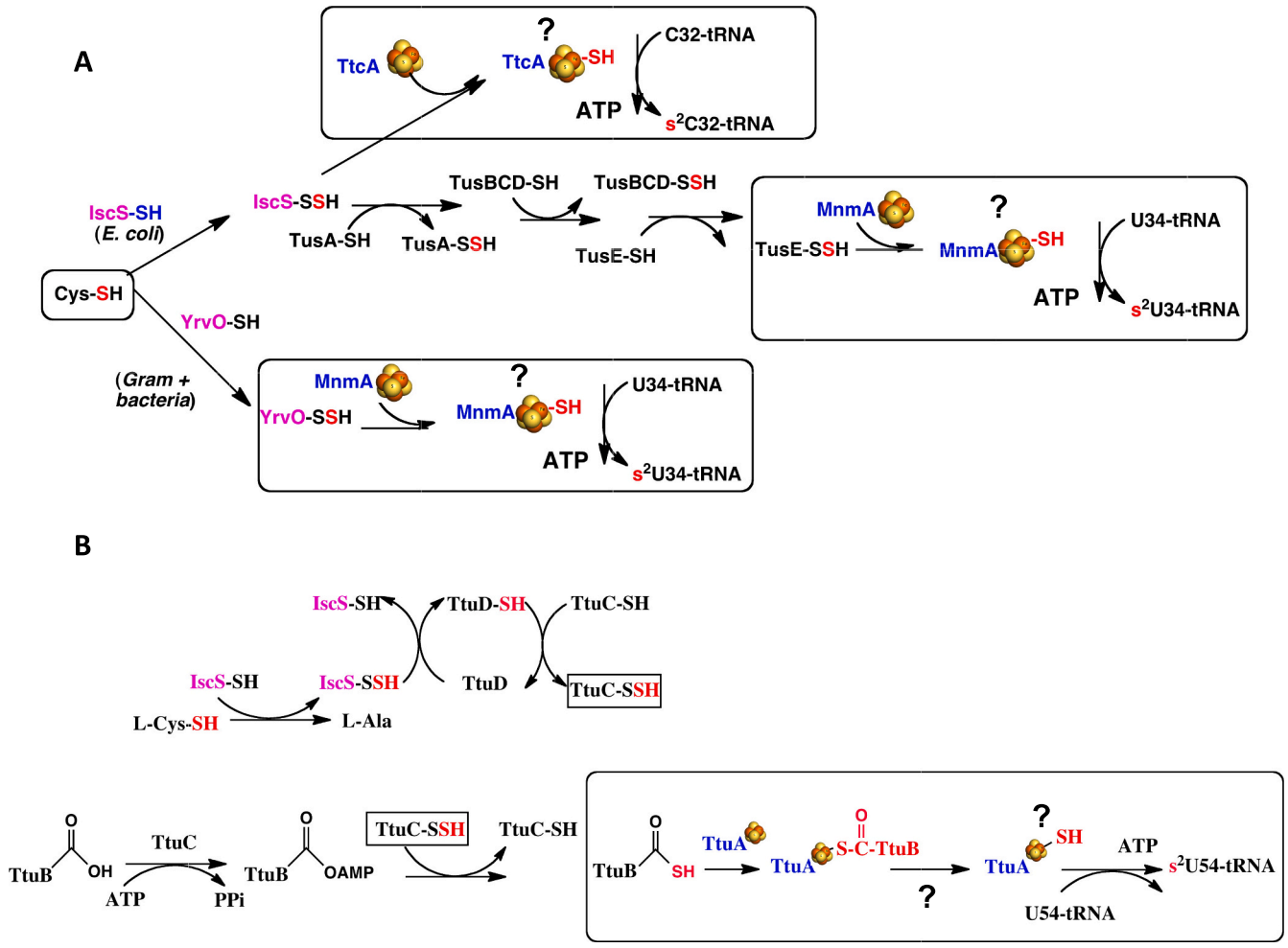

Fig. 3. Sulfur transfer pathways from cysteine to the thiolated nucleosides. **A** Sulfur transfer pathway for s^2C32-tRNA biosynthesis by EcTtcA and s^2U34 biosynthesis by MnmA from two bacteria, *E. coli* and *B. subtilis.* The [4Fe-4S] cluster is shown as orange and yellow spheres. In *E. coli*, the L-cysteine desulfurase activity of IscS is stimulated by the interaction with TusA. The persulfide sulfur of IscS-SSH is attached to TusA, then transferred to TusD within the TusBCD complex. After transfer of the sulfur atom on TusD to TusE, TusE interacts with the MnmA-tRNA complex so that the persulfide sulfur from TusE is used by EcMnmA to thiolate U34-tRNA after its activation by ATP. **B** Sulfur transfer pathway for s^2U54-tRNA biosynthesis by TmTtuA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

deficient in s^2C-tRNAs, leading to the discovery of a new class of thiolation enzymes called the TtcA/TtuA family [104]. The sequence of this class of proteins is characterized by the presence of the PP-loop motif, as well as a conserved CXXC motif in the central region (Fig. 2). The TtcA subclass, present in almost all bacteria, possesses an additional CXXC motif in the PPase domain, whereas the TtuA subclass, which includes almost all family members from eukaryotes and archaea, contains two additional CXXC motifs at the N- and C-termini.

Bulk tRNA was analyzed from *E. coli ttcA* mutant strains encoding a double mutant protein, in which both cysteines in the central CXXC motif were mutated to alanine, and two single mutants, in which each cysteine was mutated to serine [104]. The absence of the s^2C-tRNA modification in all three strains led to the conclusion that the two cysteines in the central CXXC motif are required for s^2C formation in tRNAs. Lack of s^2C-tRNA did not affect the steady-state growth rate, nor did it show any growth disadvantage in a mixed-population experiment with the mutant and wild-type strains. However, several specific steps in the translational decoding process were affected by the absence of s^2C32-tRNA.

The first biochemical characterization of a TtcA enzyme was performed in 2014 [105]. After overexpression in *E. coli*, aerobically-purified EcTtcA was characterized as a dimer of about 70 kDa under reducing conditions in solution, as determined by Size Exclusion Chromatography Multi-Anomalous Light Scattering (SEC-MALS) analysis. It had a reddish color and a UV visible spectrum displaying absorbance bands at 322, 415 and 460 nm, characteristic of a $[2Fe\text{-}2S]^{2+}$ cluster [106]. The Fe content was measured by the Fish method, which uses acid-permanganate to release the complexed iron in solution [107] and the S content by the Beinert method, which consists in denaturing the protein with zinc acetate and sodium hydroxide, adding N-*N*-dimethyl-*p*-phenylenediamine and ferric iron chloride, and following the formation of methylene blue by spectrophotometry [108].

The low Fe and S content in the aerobically-purified protein (respectively 0.16 and 0.21) was increased only by a factor of 1.8 when the protein was purified anaerobically, which is likely due to the limited levels of the iron-sulfur biogenesis machinery in the TtcA overexpression system. Further analysis of the ^{57}Fe-labeled protein, obtained after anaerobic growth in the presence of ^{57}Fe, by Mössbauer spectroscopy [109], indicated a content of 0.25 equivalent of $[2Fe\text{-}2S]^{2+}$ cluster per monomer. This $S = 0$ state of the cluster was confirmed by the absence of signal in electron paramagnetic (EPR) spectroscopy. For increasing the content of [Fe-S] clusters in TtcA, the protein was first treated with EDTA and dithionite to remove the residual cluster, thus obtaining the apo-protein, then treated with a 6-molar excess of ferrous ion and cysteine under reducing conditions (5 mM DTT) in the presence of a cysteine desulfurase, to reconstitute the cluster. The holo-protein thus

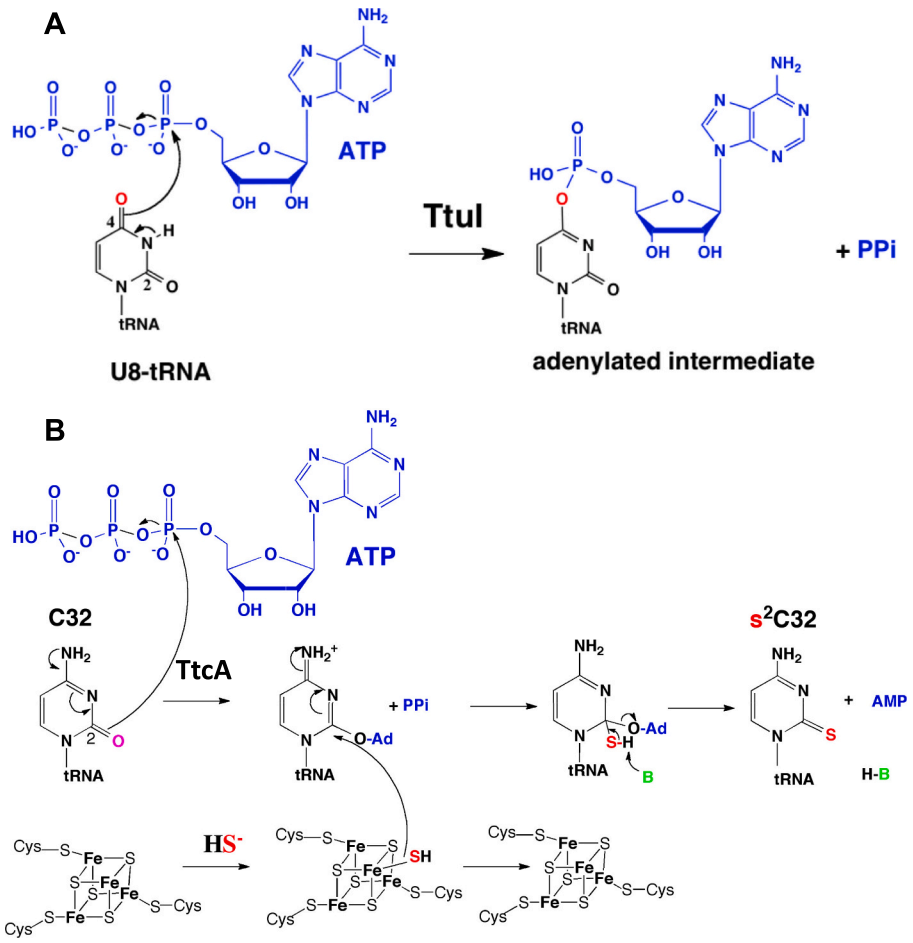

Fig. 4. Proposed mechanism for [4Fe-4S]-dependent tRNA thiolation enzymes. **A** Activation of the target nucleoside with ATP to form an adenylated intermediate. The mechanism is shown for TtuI that targets the C4 position of U8 in tRNA. **B** Proposed mechanism after adenylation of the target base, involving the attack of a [4Fe-5S] cluster intermediate on the adenylated intermediate. The mechanism is shown for TtcA that targets the C2 position of C32 in tRNA. 'Ad' stands for 'adenylate'. After adenylation of C32-tRNA at C2 by ATP and formation of a [4Fe-5S] cluster intermediate, the SH group coordinated by the [4Fe-4S] cluster would perform a nucleophilic substitution of the O-adenosyl monophosphate group at the target nucleoside to generate the thiolated product s^2C32-tRNA and release AMP.

formed had a content of 3.9 Fe and 4 S per monomer. It displayed a UV–visible spectrum with a broad absorption band at around 410 nm and an Electron Paramagnetic Resonance (EPR) spectrum [110] after dithionite reduction (Table 1), both characteristic of a [4Fe-4S] cluster [106]. The Mössbauer spectrum of holo-EcTtcA reconstituted with ^{57}Fe displayed two isomers (Table 2) characteristic of a $[4Fe\text{-}4S]^{2+}$ cluster with two valence-delocalized $[Fe_2S_2]^{+}$ [106,109]. The [4Fe-4S] cluster was highly sensitive to oxygen since the absorbance at 410 nm decreased within 15 min upon air-exposure.

The C32-tRNA thiolation activity of EcTtcA was then investigated by analyzing the hydrolysates of the tRNA product by HPLC after incubation of apo- or holo-enzyme with bulk tRNA isolated from a Δ*ttcA E. coli* strain using cysteine and IscS as the sulfur source. The [4Fe-4S] cluster, ATP and DTT were required for activity [105]. The importance of each of the conserved cysteines of EcTtcA for *in vivo* activity was examined by complementing the Δ*ttcA* strain by plasmids encoding the gene containing the corresponding cysteine-to-alanine mutation, followed by analysis of the nucleoside modifications present in bulk tRNA from each strain. The C32-tRNA thiolation activity was not restored by the mutated *ttcA* genes lacking the cysteines in the first CXXC motif (Cys122 and Cys125), and the last cysteine in the second CXXC motif (Cys213), whereas the C210A-*ttcA* mutant corresponding to the first cysteine in the second CXXC motif retained 50 % activity. Moreover, the C122A, C125A and C213A mutants were purified aerobically and shown to contain much less iron-sulfur cluster than the wild-type enzyme but the purification and characterization of the C210A mutant was not reported. Altogether, it was concluded that Cys122, Cys125 and Cys213 are the ligands of the [4Fe-4S] cluster [105].

Table 1
EPR parameters of [4Fe-4S]-dependent tRNA thiolation enzymes after cluster reconstitution and reduction with dithionite.

	Type of cluster	g_x	g_y	g_z	Temperature (K)	Reference
EcTtcA	Axial		1.9	2.04	10	[105]
TmTtuA	Rhombic	1.890	1.935	2.040	20	[116]
TtTtuA	Rhombic	1.861	1.938	2.064	10	[74]
TtTtuA + TtuB(COSH)						[75]
Conformer 1	Axial	1.900	1.943	2.074	29	
Conformer 2	Axial	1.900	1.943	2.035		
MmNcsA	Rhombic	1.890	1.918	2.033	20	[124]
	Rhombic	1.919	1.944	2.061		

Table 2
Mössbauer parameters of [4Fe-4S]-dependent tRNA thiolation enzymes after cluster reconstitution.

	Type of cluster	δ (mm s^{-1})	ΔE_Q (mm s^{-1})	Γ (mm s^{-1})	Temperature (K)	B (T)	Reference
EcTtcA	Diamagnetic	0.47	1.04		4.2	0.5	[105]
		0.46	1.34				
MmTtuI	Diamagnetic	0.45	1.19	0.37	77.0	0	[89]
		0.45	1.19	0.55	4.2	0.1	[89]
		0.45	1.19	0.49	4.2	5	[89]

The first step of the proposed mechanism for C32-tRNA thiolation by TtcA [105] is the formation of an adenylated intermediate, as in the case of U8-tRNA thiolation by TtuI (Fig. 4A), in agreement with the presence of a PP-loop motif within the sequence and the necessity to add ATP for catalysis *in vitro*. With the hypothesis that the [4Fe-4S] cluster is bound to three cysteine ligands only, the next step of catalysis could involve the transfer of the persulfide sulfur atom of IscS-SSH to the free coordination site of the [4Fe-4S] cluster to form a [4Fe-5S] intermediate (Fig. 5A), perhaps through the formation of a putative cluster-bound IscS-SSH intermediate (Fig. 5B). The release of hydrogenosulfide, potentially *via* a nucleophilic attack of a water molecule or a cysteine residue on the disulfide bond necessitates a reductant, which could be cysteine itself, but this step remains misunderstood. As the final step, the terminal sulfur attached to the [Fe-S] cluster was proposed to act as a nucleophile to attack the adenylated target cytidine, releasing AMP and liberating the s^2C32-tRNA product (Fig. 4B). In the *in vitro* assay, DTT can reduce the persulfide of IscS, generating free sulfide in the medium that can react with the tRNA. Thus, the requirement of DTT for activity suggests that HS^- was the sulfur source for the *in vitro* reaction and that cysteine together with IscS could not serve as the sulfur donor to EcTtcA (Fig. 4B).

The physiological functions of TtcA from *Pseudomonas aeruginosa* (PaTtcA), one of the most opportunistic human pathogens, was examined in 2018 [111]. PaTtcA was expressed in *E. coli* under oxygen-limited conditions. After purification, the UV visible spectrum of PaTtcA revealed a broad absorption band at 420 nm, indicating the presence of a [4Fe-4S] cluster. The absorbance at 420 nm decreased gradually upon exposure to 5–50 mM H_2O_2, suggesting loss of an exposed Fe^{2+} atom and oxidation of the cluster into the $[3Fe\text{-}4S]^+$ state.

The *P. aeruginosa* Δ*ttcA* strain was hypersensitive to oxidative stress. Indeed, it exhibited a 50-fold lower resistance to H_2O_2 treatment and its survival rate against sodium hypochorite (NaOCl) was 8-fold lower than that of the wild-type strain. Complementation of the Δ*ttcA* strain with the site-directed *ttcA* cassettes, in which each of the four conserved cysteines belonging to the two CXXC motifs (Cys115, Cys118, Cys203 and Cys206) was mutated to serine, did not remove the oxidant sensitivity phenotypes, in contrast to the native *ttcA* cassette. It was thus concluded that all four cysteines were required to get a fully functional TtcA that plays a role in the H_2O_2-mediated stress response.

The catalase activity, which is linked to cellular detoxification against H_2O_2 in several pathogenic bacteria and depends on KatA and KatB enzymes in *P. aeruginosa*, was about 40 % lower in the Δ*ttcA* strain than in the wild-type strain [111]. The KatA activity was decreased in the Δ*ttcA* strain and restored by complementation with the native *ttcA* cassette, whereas the KatB activity levels were slightly increased in the Δ*ttcA* strain. Yet, expression of *katA* was approximately three-fold higher in the Δ*ttcA* strain. These results suggested that TtcA contributes to the response against H_2O_2 toxicity by regulating KatA activity at the post-transcriptional level.

Since a fully functional KatA enzyme was shown to be required for

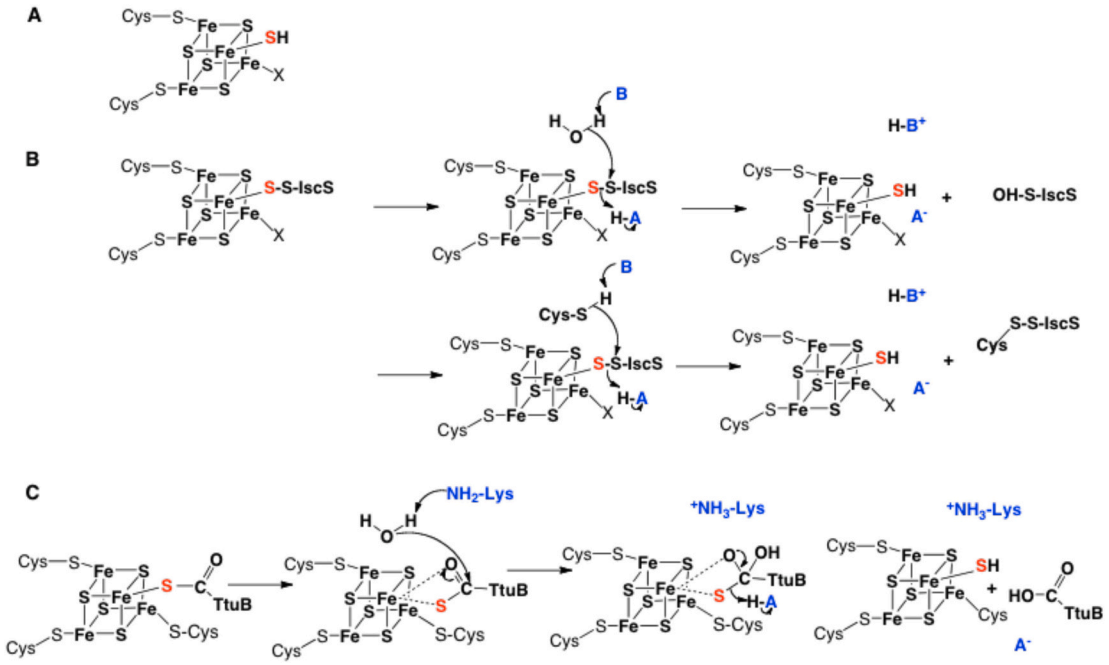

Fig. 5. Potential sulfur intermediates formed during [4Fe-4S]-dependent tRNA sulfuration reactions. **A** [4Fe-5S] intermediate is likely formed during the reaction catalyzed by non-redox [4Fe-4S]-dependent tRNA thiolaton enzymes. X = Cys or Asp. **B** Postulated persulfide intermediate formed through bond formation between the non protein-bonded Fe atom of the [4Fe-4S] cluster and the persulfide attached to cysteine desulfurase (BsMnmA, TtMnmA). The putative attack of the intermediate by a water molecule (top) or a cysteine from the protein (bottom) could release the [4Fe-5S] intermediate. **C** Thiocarboxylate intermediate formed during the biosynthesis of s^2U54-tRNA catalyzed by TtTtuA and proposed mechanism for generation of the [4Fe-5S] intermediate and release of TtTtuB-COOH after water-mediated nucleophilic attack of the thioester function by Lys137 [75].

bacterial virulence [112], the contribution of *ttcA* to the bacterial pathogenicity of *P. aeruginosa* was evaluated using *Drosophila melanogaster* as a pathogen-host model [111]. Feeding the flies with *ΔttcA* cultures resulted in a 2.1-fold increase in fly survival after 24 h, compared with feeding with the wild-type strain cultures. Thus, the *ttcA* deletion attenuated the virulence of *P. aeruginosa* in this model, which likely resulted from reduced ability of the Δ*ttcA* strain to cope with exposure to H_2O_2 during host interactions. The four conserved cysteines of the two CXXC motifs were required for PaTtcA to be fully functional for bacterial pathogenicity. Indeed, feeding the flies with cultures of the Δ*ttcA* strain complemented with the *ttcA* cassettes containing the mutation of each cysteine to serine, did not restore fly survival to the level of the wild-type strain cultures. This suggested that the cluster of PaTtcA is required for successful bacterial infection of the host.

To study adaptation of *P. aeruginosa* towards oxidative stress, the expression patterns of various genes were analyzed using real time reverse transcription polymerase chain reaction upon exposure to different oxidants [111]. H_2O_2 and NaOCl increased *ttcA* expression by 13-fold and 2.5-fold, respectively, in agreement with PaTtcA contributing to protection against oxidative stress. The *oxyR* regulon, which encodes OxyR, the global transcriptional regulator responding to H_2O_2, as well as *katA* and *katB* were induced upon H_2O_2 exposure. Moreover, expression of *ttcA* was 15-fold higher in the Δ*oxyR* strain than in the wild-type strain, independent on the presence of oxidants. Altogether the transcriptional analysis suggested that *ttcA* expression could be regulated by OxyR. Accordingly, electrophoresis mobility shift assays showed that purified OxyR specifically binds to the putative *ttcA* promoter in the presence of DTT, supporting the hypothesis that OxyR could act as a transcriptional repressor of *ttcA* expression in the absence of oxidants. A model of regulation of *ttcA* expression by OxyR was proposed, in which OxyR upregulates *katA* and derepresses *ttcA* expression to increase catalase activities in response to H_2O_2 generated by the host defense mechanisms.

The fact that the four conserved cysteines of the two CXXC motifs of PaTtcA appear to be involved in hydrogen-peroxide mediated stress protection and pathogenicity [111], while only three of them were expected to coordinate the [4Fe-4S] cluster, and were reported to be essential for C32-tRNA thiolation activity of EcTtcA [105], raises the question of the function of the fourth cysteine. In the absence of a crystal structure of a TtcA enzyme till date, the AlphaFold2 model can be used to examine the position and geometry of the four conserved cysteines (Fig. 6A), and the superposition of this model with the crystal structure of *Pyrococcus horikoshii* TtuA (PhTtuA) from the same family (whose crystal structure has been determined in the holo-form; see Section 2.2) used to predict a potential binding mode of the [4Fe-4S] cluster (Fig. 6B). Intriguingly, whereas Cys122 and Cys125 of EcTtcA occupy a position very similar to that of two of the cysteines that coordinate the cluster in PhTtuA (Fig. 6B), it is not easy to predict which of the two other cysteines (Cys210 or Cys213) would be the third ligand, or if both would bind the cluster, which is possible since they belong to a flexible loop and none of them overlap with the third residue coordinating the cluster of PhTtuA. The function of the fourth conserved cysteine thus remains unexplained but it can be hypothesized that it could act as a base to assist departure of AMP (Fig. 4B) or be involved in the release of HS^- (Fig. 5B).

2.2. TtuA, the first structurally characterized [4Fe-4S]-dependent tRNA thiolation enzyme

The TtuA subfamily differs from the TtcA subfamily by the presence of two Zn finger domains at the N- and C-termini (Fig. 2), whose function has not yet been elucidated. The thiolation of U54 in the T-loop of tRNAs, catalyzed by TtuA enzymes (Fig. 1), stabilizes the ternary structure of tRNA in thermophilic bacteria and archaea for growth at high temperature [70,113]. Since we recently summarized the results about TtuA enzymes in a review [114], we will only briefly recall the most relevant information here. First, an *in vivo* study using complementation assays of a Δ*ttuA* strain in the bacterium *T. thermophilus* and analysis of the modified nucleosides in bulk tRNA has shown that the mutation of the three cysteine residues of the CXXC + C motif (Cys130, Cys133, Cys222), aspartate of the SGGKDS motif that binds ATP, and Asp161 from the catalytic domain, significantly impaired the tRNA thiolation activity [115]. Later, the TtuA proteins from *T. thermophilus* (TtTtuA), *P. horikoshii* (PhTtuA) and *T. maritima* (TmTtuA) were overexpressed in *E. coli* and cluster reconstitution assays showed that they were able to bind a [Fe-S] cluster [74,116]. Moreover, characterization of the TtuA enzymes by EPR spectroscopy (Table 1) coupled with biochemical analyses showed that the cluster, in the [4Fe-4S] state, was essential for thiolation of U54-tRNA [74,116]. TtuA enzymes did not thiolate U54-tRNA in the absence of a sulfur source since either Na_2S [74,116] or TtuB-COSH (Fig. 3B) [74] was required for catalysis. This indicated that the sulfur atom incorporated into the nucleoside does not come from the [4Fe-4S] cluster itself. Moreover, the reaction was

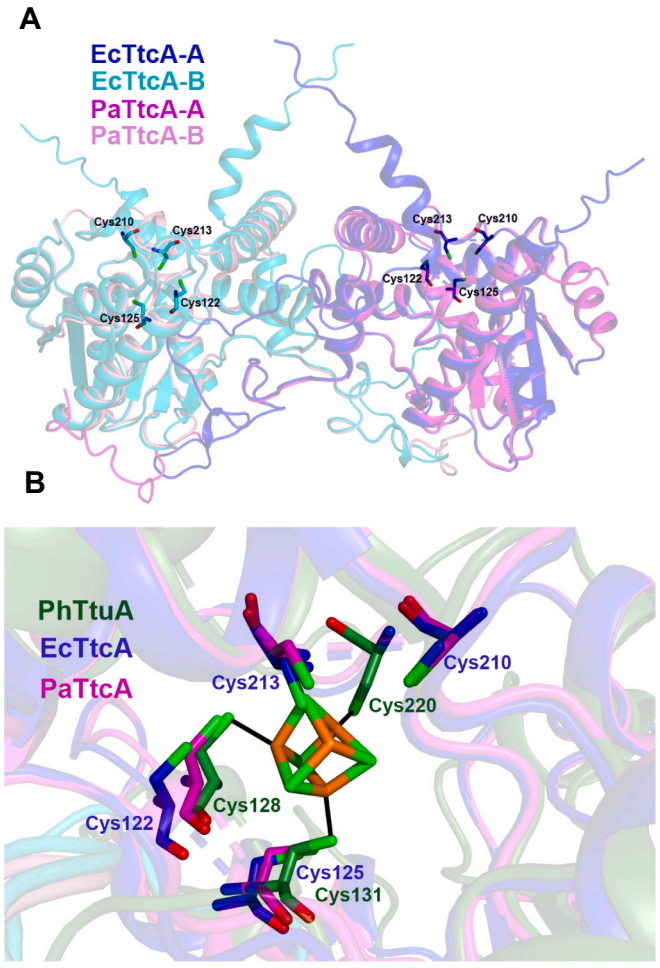

Fig. 6. AlphaFold models of EcTtcA and PaTtcA. **A** The EcTtcA and PaTtcA dimers. The AlphaFold2 models of the dimers of EcTtcA (monomers A and B in blue and cyan, respectively) and PaTtcA (monomers A and B in magenta and pink, respectivley) were superposed on molecules A (rmsd of 0.58 Å for 2707 Cαs). The four conserved cysteines in EcTtcA are labeled (note that Cys210 and Cys213 were wrongly numbered as Cys219 and Cys222, respectively, in [105]). **B** Zoom of the active sites of the AlphaFold2 models of EcTtcA and PaTtcA superposed to the crystal structure of holo-PhTtuA (PDB code 5MKQ). PhTtuA was superposed on one monomer of EcTtcA with an rmsd of 1.965 over 2707 Cα atoms. The cysteines coordinating the cluster in PhTtuA and the four conserved cysteines in EcTtcA are shown as sticks and labeled. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

independent of the oxidation state of the cluster, indicating that the iron-sulfur cluster does not play any redox role in catalysis [74,116].

Several crystal structures of holo-TtuA enzymes were obtained (Table 3, Fig. 7A and B): protein alone [74,116], in complex with an ATP analog [74], the AMP product [116] or TtTtuA in complex with ATP and TtTtuB [75], which supplies the sulfur atom to TtuA (Fig. 3B). These structures have revealed that the [4Fe-4S] cluster is coordinated only by the three cysteines of the CXXC + C motif (Fig. 2), and that the cluster is located inside the catalytic pocket, near the ATP binding site. In the crystal structure of PhTtuA, the presence of an extra electron density on the fourth iron atom, not bound to the protein, was attributed to a labile hydrosulfide ion coming from the cluster reconstitution assay [116]. This indicated the propensity of the non-liganded iron of the cluster to bind the sulfur atom needed for the thiolation reaction, supporting a catalytic mechanism, in which the [4Fe-4S] cluster acts as a Lewis acid to form a [4Fe-5S] intermediate, as proposed for EcTtcA (Fig. 4B). In the three-dimensional model of PhTtuA in complex with tRNA [116], Asp159 (the equivalent to Asp161 in TtTtuA) interacts with the flipped target base, suggesting that this residue plays a crucial role in the specific recognition of U54.

The crystal structure of holo-TtTtuA in complex with the sulfur-carrier protein TtTtuB (Fig. 3B) shows that the two oxygens of the C-terminal carboxylate of TtTtuB bind the fourth nonprotein bound Fe atom of the [4Fe-4S] cluster of TtTtuA (Fig. 7B) [75]. Since the carboxylate mimics the thiocarboxylate that serves as the sulfur donor for the TtuA-catalyzed reaction (Fig. 3B), the structure of the TtTtuA/TtTtuB complex suggests that sulfur transfer from TtTtuB to TtTtuA proceeds *via* direct coordination of the C-terminal thiocarboxylate of TtTtuB to the [4Fe-4S] cluster. The active site of TtTtuA is covered by the Cys222-containing loop upon TtTtuB binding, enabling connection of the active site of TtTtuA to the exterior through two tunnels [74]. The C-terminal loop of TtTtuB is inserted within one of these tunnels, whereas the second tunnel is linked to the positively charged surface that likely serves to bind tRNA. Hence, TtTtuB and the tRNA could simultaneously access the active site. Comparison of the EPR spectrum of holo-TtTtuA alone and holo-TtTtuA in the presence of TtuB-COSH, after reduction by dithionite, was consistent with the COSH group being bound to the cluster (Table 1) [75]. Desulfuration of TtuB-COSH necessitated tRNA adenylation by ATP, with Lys137 playing an essential role for this sulfur mobilization, as shown by mutagenesis. Lys137 is bound to a water molecule that, together with the carboxylate group of TtTtuB, binds to the fourth iron atom of the cluster (Fig. 7B). It is anticipated that Lys137 would deprotonate the water molecule; the hydroxide ion thus generated would act as a nucleophile to attack the carbon of the thiocarboxyl group of TtuB-COSH, releasing TtuB-COOH and generating a [4Fe-5S] intermediate (Fig. 5C) [75]. Thus, the proposed mechanism involves the formation of a [4Fe-5S]-TtuB intermediate and then that of a [4Fe-5S] intermediate that provides the sulfur atom for the reaction [75]. In organisms devoid of TtuB-like proteins such as *T. maritima*, it is very likely that the [4Fe-5S] intermediate is directly formed by the attack of a hydrogenosulfide ion coming from the cellular environment, as proposed for TtcA (Fig. 4B).

Recently, the structure of the cluster of TtTtuA was analyzed by EPR spectroscopy and the enzymatic activity followed over 24 h after oxidation of [4Fe–4S]-TtTtuA into [3Fe–4S]-TtTtuA by ferricyanide under strict anaerobic conditions [117]. [3Fe-4S]-TtuA was formed immediately but it spontaneously transformed back into [4Fe–4S]-TtuA without an additional free Fe source in the solution to reach 50 % [4Fe-4S]-TtuA within 1 h and 77 % [4Fe-4S]-TtuA within 12 h. Just after oxidation of the [4Fe-4S]-TtuA into [3Fe–4S]-TtuA, the metalloenzyme was inactive but its activity was recovered to 48 % after 1 h, and 74 % within 24 h, corresponding to an increase of [4Fe–4S]-TtuA in the solution. These studies unambiguously confirmed that only the [4Fe-4S] state of the cluster is catalytically active and showed that the inactive [3Fe-4S]-TtuA state was highly unstable.

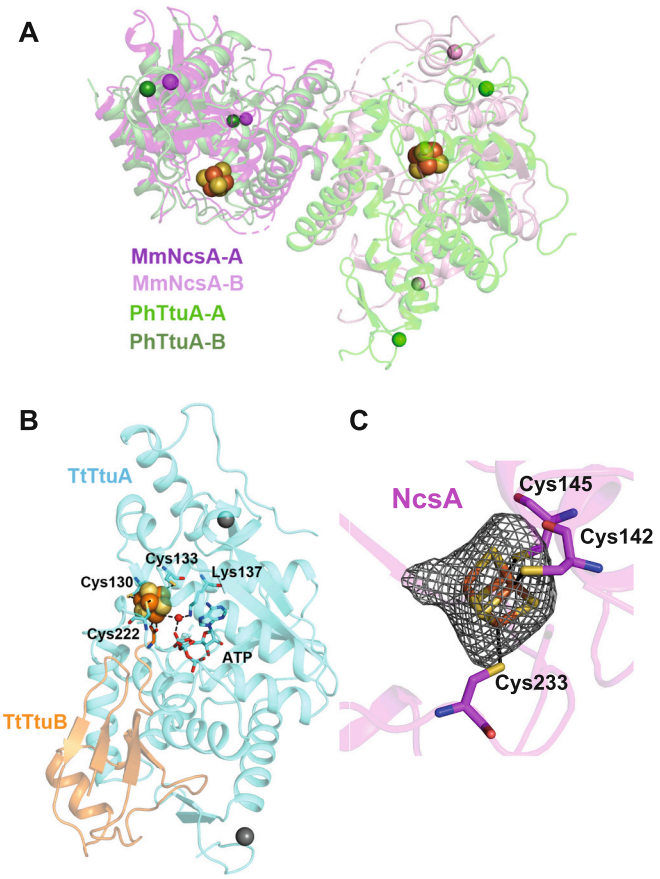

Fig. 7. Crystal structures of several members of the TtuA/NcsA family. **A** Comparison of the crystal structures of the dimers of MmNcsA and PhTtuA. The Zn ions are shown as spheres, colored like the chain to which it belongs. **B** The TtTtuA/TtTtuB complex. The C-terminal carboxylate of TtTtuB (shown as orange sticks) directly binds the [4Fe-4S] cluster of TtTtuA. **C** Catalytic site of MmNcsA showing electron density on the fourth Fe atom that can be attributed to a small anionic ligand.

Table 3
Crystal structures of [4Fe-4S]-dependent tRNA thiolation enzymes in the holo form.

Origin	Enzyme	Resolution (Å)	Space group	N*	PDB code	Reference
P. horikoshii	[4Fe4S]-TtuA	2.5	P 4_3 2_1 2	1	5MKP	[116]
P. horikoshii	[4Fe4S]-TtuA (iron edge)	2.9	P 2_1 2_1 2_1	2	5MKQ	[116]
P. horikoshii	[2Fe-2S]-TtuA + AMP	2.65	P 2_1 2_1 2_1	2	5MKO	[116]
T. thermophilus	[4Fe-4S]-TtuA + AMPPNP	2.75	P 6_1 2 2	1	5B4E	[74]
T. thermophilus	[4Fe-4S]-TtuA	2.7	P 6_1 2 2	1	5B4F	[74]
T. thermophilus	[4Fe-4S]-TtuA/TtuB + ATP	2.2	C 1 2 1	3	5ZTB	[75]
M. maripaludis	[4Fe-4S]-NcsA	2.8	$P2_12_12_1$	2	6SCY	[124]

* N is the number of molecules in the asymmetric unit.

2.3. Thiolation of U34 in tRNA by NcsA/Ncs6/CTU1-type enzymes is dependent on a [4Fe-4S]-cluster, not a [3Fe-4S] cluster

Uridines at position 34 of $tRNA^{Glu}$, $tRNA^{Gln}$ and $tRNA^{Lys}$ are universally converted to 5-methyl-2-thiouridine derivatives in all domains of life [118,119] (Fig. 1). U34 modifications contribute to pre-structuring the anticodon loop of tRNA for optimal translation [120]. Two different kinds of enzymes, both in terms of sequence and structure, are responsible for thiolation of U34-tRNA: MnmA-type enzymes in bacteria [101] and mitochondria [121], and NcsA/Ncs6/CTU1-type enzymes in archaea [54] and the eukaryotic cytosol [118,119,122] (Fig. 2). Genomic analysis has shown that MnmA-type and NcsA/Ncs6/CTU1-type proteins tend not to co-occur [54].

NcsA/Ncs6/CTU1-type enzymes belong to the subfamily of TtuA enzymes (Fig. 2) [104]. They are single proteins in archaea, named NcsA [67,123,124], whereas they are part of a heterodimeric complex in the eukaryotic cytosol, called Ncs6p/Ncs2p in yeast [65,77,125], ROL5/CTU2 in plants [126,127], CTU1/CTU2 (Cytoplasmic ThioUridylases) in higher eukaryotes [65,68,126,128]. The NcsA/Ncs6/CTU1-type enzymes have important cellular functions. For example, archaeal NcsA was shown to be necessary for growth at elevated temperature in *Haloferax volcanii* [67], and the CTU1/CTU2 complex to maintain genome integrity in *Schizosaccharomyces pombe* [128]. U34-tRNA thiolase activity has also been shown to be upregulated in breast cancers [129], promoting the survival and treatment resistance of melanoma cells due to codon-biased translation of metabolic proteins [130].

As early as in 2007, it was known that thiomodification of yeast cytosolic tRNA is an iron-sulfur protein-dependent pathway (Section 1.3) [46]. Several archaeal and eukaryotic U34-tRNA thiolases have only been studied *in vivo*, i. e. under conditions that cannot highlight the involvement of a [Fe-S] cluster, whereas others that were studied *in vitro* showed inefficient 2-thiouridine formation, indicating lack of an important component, presumably a [4Fe-4S] cluster, as discussed below.

Although *S. cerevisiae* Ncs6p and Ncs2p could not be expressed in soluble form, some 2-thiouridine formation was partially reconstituted *in vitro* using a pulled down fraction of tandem affinity purification (TAP)-tagged Ncs6p containing Ncs2p, in the presence of cysteine/cysteine desulfurase Nfs1p, the sulfurtransferases Tum1p, Urm1p and Uba4p together with ATP and $tRNA^{Lys}$ [65]. Moreover, the partially purified CTU1-CTU2 complex from *C. elegans* was shown to be sufficient for tRNA thiolation *in vitro*, as shown by electrophoresis on a gel containing [(N-acryloylamino)phenyl] mercuric chloride (APM), a compound that slows down migration of sulfurated compounds [131], but the efficiency of thiolation was very low [128]. It is likely that the low *in vitro* activity of the eukaryotic U34-tRNA thiolase complexes is due to low cluster amounts in the aerobically purified Ncs6p/CTU1 enzymes.

The most studied NcsA/Ncs6/CTU1-type protein is that from the archaeum *Methanococcus maripaludis* (MmNcsA). MmNcsA was overexpressed in *E. coli* and the anaerobically purified enzyme was shown to be able to incorporate radioactivity from [^{35}S]-Cysteine in the presence of IscS [123]. Hence, it was first suggested that MmNcsA could participate in sulfur transfer using a persulfide mechanism. Moreover, since the radiolabeling of the mutants, in which each cysteine of the CXXC + C motif was substituted to serine, was greatly reduced, it was suggested that these cysteines are essential for the generation or stabilization of a persulfide *in vitro* [123]. Later, the same group showed that, in fact, MmNcsA contained a [3Fe-4S] cluster, so did the maltose-binding protein-tagged human CTU1, as shown by UV–visible and EPR or Mössbauer spectroscopy [100]. However, it was not reported whether the [3Fe-4S] cluster was the catalytically active state for U34-tRNA thiolation by these enzymes [100] or instead was arising from air-degradation of a catalytically active [4Fe-4S] cluster [132].

We recently expressed MmNcsA in *E. coli*, purified the protein aerobically, and performed a cluster reconstitution assay under strict anaerobic conditions. After cluster reconstitution and purification, holo-MmNcsA contained a [4Fe-4S] cluster, with a content of 3.1 Fe and 4.8 S per monomer, and no [3Fe-4S] cluster, as indicated by the UV–visible and EPR spectra (Table 1). Moreover, the [4Fe-4S] cluster together with ATP and Mg^{2+} were essential for U34-tRNA thiolation by MmNcsA using sodium sulfide as a source of sulfur [124]. The crystal structure of holo-MmNcsA, solved at 2.8 Å resolution, showed that it is organized as a dimer and that it displays the same fold as TtuA proteins (Table 3, Fig. 7A) [124]. The [4Fe-4S] cluster was coordinated only by the three conserved cysteines and an extra electron density was present on the fourth non-liganded iron atom, which indicated the propensity of the cluster to bind a fourth exogenous ligand (Fig. 7C). The crystal structure, sulfur content and catalytic activity of MmNcsA were in agreement with a mechanism, where the [4Fe-4S] cluster functions as a Lewis acid to bind a hydrogenosulfide ligand, as depicted for EcTtcA in Fig. 4B.

A model of tRNA bound to MmNcsA can be proposed based on the crystal structure of tRNA-bound lysidine synthetase TilS from *Geobacilllus kaustophilus* [133], which is the closest structural homologue to MmNcsA besides TtuA (Fig. 8). TilS modifies cytidine 34 in tRNAs by adding a lysyl group and is, surprisingly, more similar to MmNcsA than EcMnmA. Superposing the catalytic cores of MmNcsA and TilS that target the same position 34 in tRNAs shows how the two zinc-finger domains of MmNcsA could interact with the D- and T-loops of tRNA (Fig. 8A). In the superposition, the anticodon stem-loop of the tRNA bound to TilS is positioned in the cavity created by the assembly of two MmNcsA monomers without creating any clashes and the flipped C34 target base nicely fits into the MmNcsA active site pocket. Replacing C34 with U34 and superposing TilS from *Aquifex aeolicus* in complex with ATP (PDB code 2E89) to MmNcsA enables to build a simple model of MmNcsA complexed with U34-adenylated tRNA. In this model, the position of the two tRNA molecules relative to the NcsA dimer is in agreement with the complementary electrostatic surface between the highly positively charged putative tRNA binding sites and the negatively charged tRNA substrates (Fig. 8B). Further minimization of the geometry of the MmNcsA/tRNA model with *PHENIX* [134] unveils the potential interactions of the flipped U34 base with MmNcsA (Fig. 8C), which allows us to speculate about the role that several conserved residues could play in catalysis. The U34 flipped base is most likely stabilized by an edge-to-face stacking interaction with His169. Our model also highlights a potential crucial role for Asp173 in orienting the target uridine base by hydrogen binding to the U34 O4 carbonyl atom.

In eukaryotes, the formation of s^2U34-tRNA is performed by the CTU1/CTU2 (or Ncs6p/Ncs2p) complex [127,128]. Anoxically purified recombinant human CTU1 was previously shown to contain a [Fe-S] cluster [100]. Previous attempts to express the human or yeast complex for *in vitro* characterization have been unsuccessful until now [65,69,82]. Hence, in order to get structural insights into these complexes, we compared the AlphaFold2 model of the human CTU1/CTU2 heterodimer with the crystal structure of the MmNcsA homodimer [124]. Not only did CTU1 adopt a fold very similar to that of MmNcsA, but the CTU1/CTU2 complex also formed a heterodimer very close to the MmNcsA homodimer, suggesting that the dimerization mode has been conserved through evolution. Moreover, given the very close superposition of the catalytic site residues of MmNcsA and CTU1, including the cysteines that coordinate the [4Fe-4S] cluster in MmNcsA, we proposed that eukaryotes use the same mechanism for U34-tRNA thiolation as in *M. maripaludis*, mediated by a [4Fe-4S]-dependent enzyme. Sequence comparison indicates that the CTU2/Ncs2p protein partners possess none or only one of the cysteines likely involved in cluster coordination in CTU1/Ncs6p, and most often do not display the terminal zinc finger-binding motifs [124]. Hence, it has been proposed that CTU2/Ncs2p proteins do not have any catalytic activity but provide specificity for tRNA [69]. Moreover, the close sequence identity of the CTU2/Ncs2p proteins to their CTU1/Ncs6p enzymatic partners suggest that the two subunits are evolutionary related. Accordingly, the AlphaFold2 models of CTU1 and CTU2 were very close to each other, confirming that the human U34-tRNA thiolase complex originates from

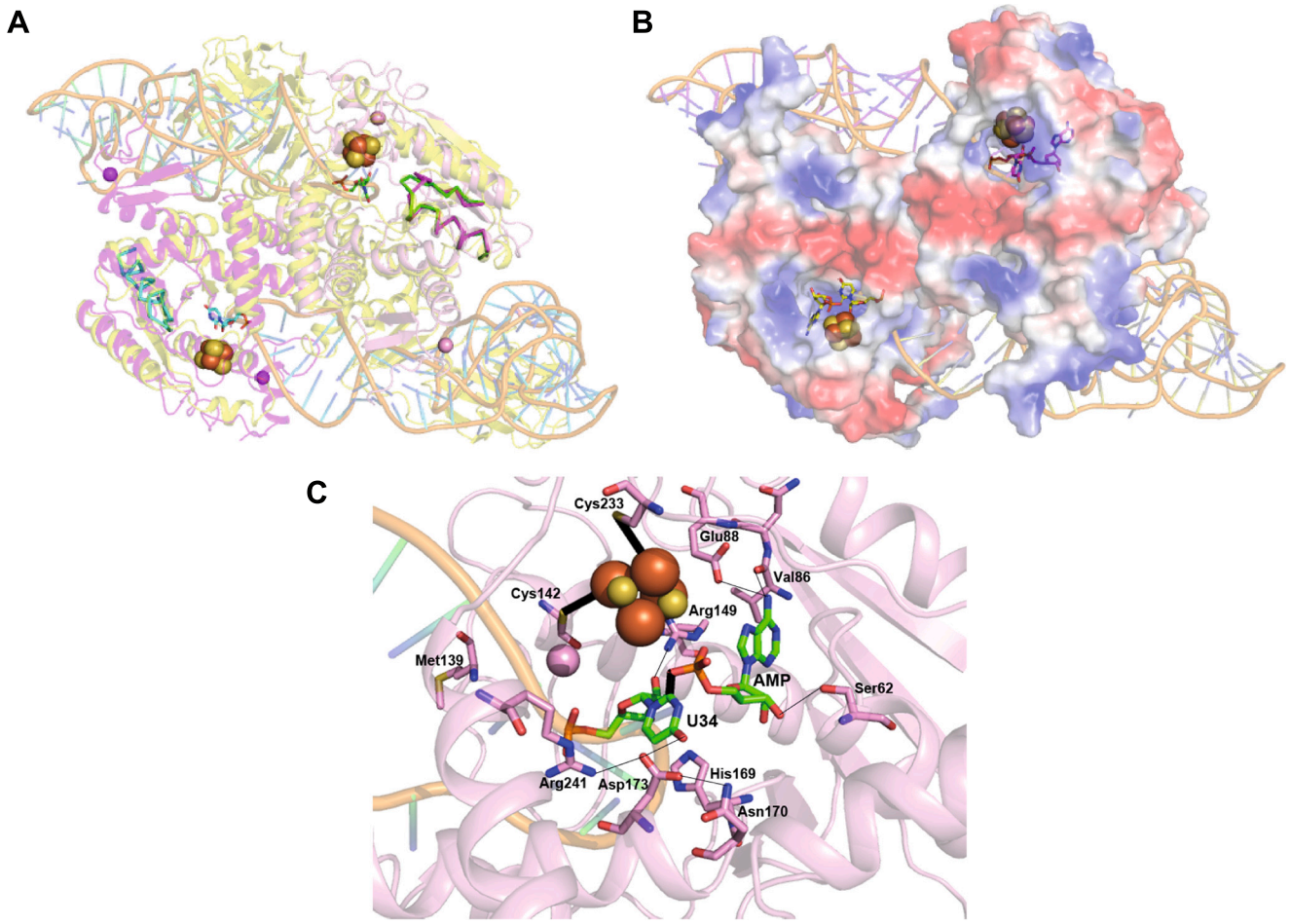

Fig. 8. Model of U34-adenylated tRNA bound to holo-MmNcsA. Superposition of tRNA-bound TilS (PDB code 3A2K) and holo-MmNcsA (PDB code 6SCY). Residues 20–42 of TilS and 51–72 of MmNcsA, corresponding to the ATPase domain (shown as ribbon), were superimposed (rmsd of 0.81 Å for 16 atoms). TilS is shown in yellow with bound tRNA in tan and the flipped C34 nucleotide is shown as green and cyan stick representations. The Zn ions and the iron sulfur clusters as shown as spheres. **B** Same view with MmNcsA shown as its electrostatic surface (negative charge in red, positive charge in blue). **C** Zoom of the potential interactions between the target U34 and the catalytic amino acids of MmNcsA in the model of the MmNcsA/U34-adenylated-tRNA complex. Hydrogen bonding interactions are shown as thin black lines. The position of Asp173 is tightly maintained by hydrogen bonds between its two carboxylate oxygen atoms and the Asn170 ND1 or Arg241 NH1 atoms. The 2'OH ribose group of U34 is most probably recognized by Arg149. Furthermore, Arg205 is correctly positioned to interact with the phosphate group linking U34 and the following nucleotide and Met139 could be involved in a stacking interaction with the base preceding U34. Finally, the 2′OH ribose group of the O-adenylated moiety attached to U34 is most probably recognized by the Ser62 OG atom whereas the NH_2 group at C6 of the adenosine base is hydrogen bonded to the Glu88 OD2 atom and to the main chain carbonyl oxygen atom of Val86. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

gene duplication [124]. It is likely that the eukaryotic complex evolved from a homodimeric archaeal enzyme, with the catalytic subunit CTU1 being conserved, whereas the second subunit CTU2 diverged.

2.4. Thiolation of U34 by bacterial MnmA enzymes: from a 'persulfide-based' to a [4Fe-4S]-dependent mechanism?

Sequence comparisons led Shigi et al. to divide MnmA proteins into two subfamilies [135]: D-type enzymes like EcMnmA, containing a DXXC + C motif, and C-type enzymes like MnmA from *T. thermophilus* (TtMnmA) containing a CXXC + C motif (Fig. 2) [135].

2.4.1. TtMnmA

Sequence comparison of TtMnmA with TtuA enzymes shows alignment of the CXXC + C motif (Fig. 2) [135], the cysteines of which bind a [4Fe-4S] cluster in TtuA (Fig. 7B) [74,116]. In addition, two consecutive cysteines (at positions 55 and 56 in TtMnmA) were found to be conserved in C-type MnmA proteins. In 2020, Shigi et al. reported that, after aerobic purification, a [4Fe-4S] cluster could be reconstituted under anaerobic conditions within TtMnmA, as verified by UV–visible and EPR spectroscopies (Table 1) [135]. Moreover, the cluster was found to be necessary for thiolation of U34 in *in vitro* transcribed $tRNA^{Gln}$ and $tRNA^{Lys}$, in the presence of ATP, and with inorganic sulfide as the sulfur source. Interestingly, this result also indicated that prior modification at C5 of U34-tRNA was not required for 2-thiolation. In addition to inorganic sulfide, cysteine desulfurases IscS and SufS could also provide sulfur from [^{35}S]-cysteine for incorporation into tRNA by holo-TtMnmA in the absence of a reductant, with formation of 0.14 and 0.33 pmole s^2U/pmole MnmA per min, respectively, compared to 0.57 pmole s^2U/pmole MnmA per min with Na_2S as a sulfur source [135]. This result supports the binding of the IscS-SSH persulfide on the nonprotein bonded Fe atom of the [4Fe-4S] cluster of TtMnmA as described in Fig. 5B.

All conserved cysteines of TtMnmA, including those from the CXXC + C motif (Cys105, Cys108, Cys200), were mutated to alanine. The cluster was reconstituted in the mutants, and their Fe content, [Fe-S] cluster stability and thiolation activity were measured *in vitro*. The single mutations of Cys55 and Cys56, as well as the double mutation did not alter cluster binding, and the 2-thiolation activities of the C55A and C55A/C56A mutants were similar to that of the wild-type protein, while

the activity of the C56A mutant being increased by a factor of 1.7. It was thus concluded that both Cys55 and Cys56 were not necessary for 2-thiolation. Furthermore, the C105A, C108A and C200A single mutations decreased the catalytic activity, with the triple mutation completely abolishing it. Each single mutant had a Fe content of around 3.7, similar to that of the wild-type enzyme, while the C105A/C108A/C200A triple mutant contained ~2 mol Fe/mol TtMnmA. The stability of the [Fe-S] cluster was reduced in the mutants, and the UV–visible spectra of the C105A and C108A mutants exhibited a broad shoulder at around 600 nm, in addition to the peak at around 410 nm observed in wild-type TtMnmA, suggesting subtle differences in [Fe–S] clusters between the mutants and the wild-type enzyme. Despite the high amount of [Fe-S] cluster in the triple mutant, which is likely due to Fe binding to the histidine tag, it was proposed from these experiments that Cys105, Cys108 and Cys200 are the ligands of the [4Fe-4S] cluster. It was also shown that mutation of Asp13, which belongs to the PP-loop (SGGVDS) motif, completely abolished the 2-thiolation activity. The equivalent residue binds the ATP phosphate group *via* a magnesium ion in other ATP-pyrophosphatases [136].

2.4.2. *EcMnmA*

The originally proposed mechanism for EcMnmA involved the formation of a persulfide on catalytic Cys199 [86], by analogy with the catalytic mechanism proposed for EcTtuI [97]. An important difference between these two proteins is that EcTtuI contains a RHD that is prone to accept sulfur from a partner protein in the form of a persulfide (see Section 1.6), but EcMnmA does not. We recently reported the crystal structure of [4Fe-4S]-dependent MmNcsA, which catalyzes the same U34-tRNA thiolation reaction as MnmA in archaea (Section 2.3) [124]. Strikingly, the superposition of the catalytic sites of the MmNcsA and EcMnmA crystal structures showed that the three catalytic residues in EcMnmA (Asp99, Cys102 and Cys199) were positioned similarly as the three cysteines that coordinate the [4Fe-4S] cluster in MmNcsA, indicating a geometry compatible with iron-sulfur cluster binding (Fig. 9A). This led us to investigate whether EcMnmA could also be a [4Fe-4S]-dependent enzyme like MmNcsA. We overexpressed EcMnmA and purified the enzyme aerobically [137]. The faint brownish color and the weak absorption at 410 nm after nickel affinity chromatography, which vanished within minutes, suggested the presence of a very air-sensitive

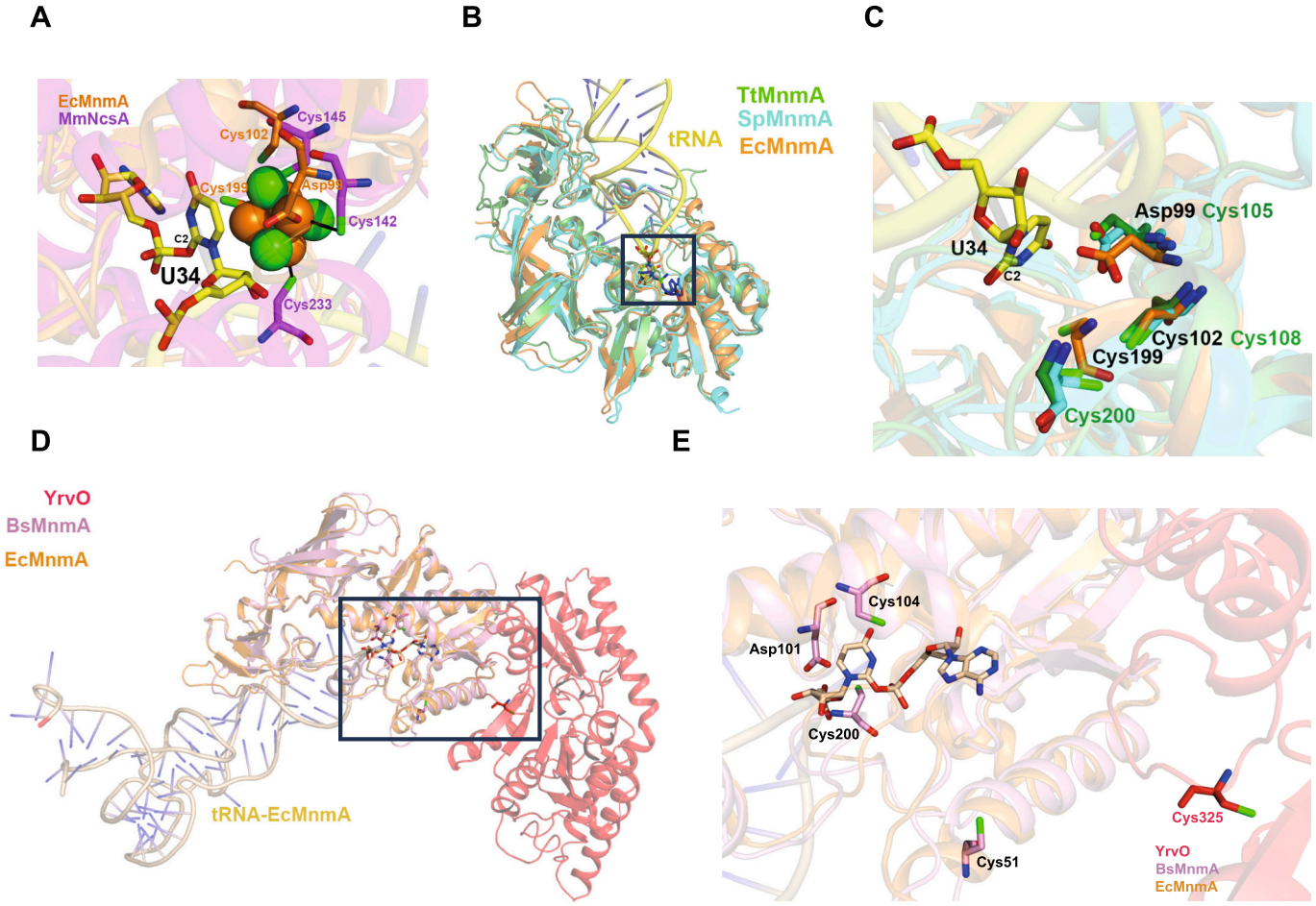

Fig. 9. Crystal structure of EcMnmA and AlphaFold models of MnmA orthologues.
A Superposition of the catalytic domains of EcMnmA (PDB code 2DEU, protein in orange, tRNA in yellow) and MmNcsA (PDB code 6SCY in magenta) using the SSM option in the superpose program of CCP4 (rmsd of 2.37 Å for 123 Cα atoms). The catalytic residues of EcMnmA (Asp99, Cys102 and Cys199) occupy positions equivalent to those of Cys142, Cys145, and Cys233 that bind the [4Fe-4S] cluster in MmNcsA. **B** Overview of the MnmA structures/model showing the MnmA:tRNA interface and the U34-containing loop of tRNA. EcMnmA in complex with adenylated tRNA (PDB code 2DEU, protein in orange, tRNA in yellow) was superimposed onto the AlphaFold2 model of TmMnmA (UNIPROT Q72GX1, in green) with an rmsd of 1.40 Å for 1635 Cαs, and onto SpMnmA in complex with SAM (PDB code 2HMA, in cyan) with an rmsd of 1.01 Å for 1991 Cαs. **C** Zoom of the active sites. The conserved cysteines/aspartate at position 99, 102 and 100 (EcMnmA numbering) are shown as sticks and labels are indicated in black for EcMnmA and in green for TtMnmA. **D** AlphaFold model of the YrvO/BsMnmA complex. BsMnmA from the AlphaFold2 model of the YrvO/BsMnmA complex (YrvO in red and BsMnmA in pink) was superimposed onto EcMnmA from the crystal structure in complex with the adenylated tRNA complex (PDB code 2DEU, protein in orange, tRNA and flipped adenylated U34 in wheat) with an rmsd of 0.79 Å of for 295 Cαs. **E** Zoom of the interacting regions of YrvO and BsMnmA in the AlphaFold2 model of the complex. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[Fe-S] cluster. Indeed, anaerobic cluster reconstitution assays showed that EcMnmA was able to bind a [4Fe-4S] cluster, as shown by UV–visible and EPR spectroscopy (Table 1). While the aerobically-purified protein had the tendency to form a dimer, holo-MnmA was present uniquely as a monomer. Thus, the oligomeric states of apo- and holo-EcMnmA indicated that the cluster does not induce dimerization and suggested that one accessible cysteine can participate in intermolecular disulfide bridge formation in the apo-form. The thiolation activity of EcMnmA was tested using inorganic sulfide as a source of sulfur and an *E. coli* $tRNA^{Glu}_{UUU}$ transcript. After reaction, the tRNA was hydrolyzed into nucleosides and s^2U was quantified by HPLC/MS. The [4Fe-4S] cluster, Mg-ATP and inorganic sulfide, were required for tRNA thiolation by EcMnmA [137].

The *E. coli* Δ*mnmA* strain grew slower than the wild-type strain in the presence of a mild acid stress (*i.e.* growth medium at pH 4.5) and exhibited hypersensitivity to H_2O_2, as monitored by growth in liquid culture [137]. In contrast to the wild-type *mnmA* gene, the D99A-*mnmA*, C102A-*mnmA* and C199A-*mnmA* mutants could not restore U34 thiolation in *in vivo* complementation experiments of the Δ*mnmA* strain. In addition, the double D99A/C102A and the triple D99A/C102A/C199A mutants had a much lower [Fe-S] content than the wild-type enzyme. We thus proposed that Asp99, Cys102 and Cys199 are the ligands of the cluster. Accordingly, the superposition of the AlphaFold2 model of TtMnmA with the crystal structure of EcMnmA in complex with adenylated tRNA (Fig. 9B) shows that the three cysteines that very likely bind the [4Fe-4S] cluster in TtMnmA are positioned very close to the three residues proposed to be the catalytic residues in EcMnmA, at proximity of the target U34 flipped base [86] (Fig. 9C). The fact that EcMnmA uses an aspartate as the third ligand of the cluster, instead of a cysteine, renders the cluster more labile (our unpublished observation). In fact, it is thought that non-cysteine ligation is important for controlling the chemical properties of iron-sulfur clusters as well as their physiological stability [138]. This led us to propose a new [4Fe-4S]-dependent mechanism for EcMnmA, analogous to that drawn for TtcA in Fig. 4B. Moreover, we can speculate that the persulfide sulfur on the last sulfur donor of the sulfur-transfer relay, TusE-SSH, would be transferred to the [4Fe-4S] cluster during the EcMnmA-catalyzed reaction (Fig. 3A). However, the mechanism of liberation of the sulfur atom from the persulfide is not known.

There are several reasons why the [Fe-S] cluster was missed in previous studies of EcMnmA. First, the protein was purified aerobically, i. e. in conditions where degradation of the cluster occurs rapidly. Second, the use of a strain overexpressing EcMnmA may have prevented the assembly of iron-sulfur clusters in significant amount. Finally, the [4Fe-4S] cluster bound by two cysteines and one aspartate is much less stable than that bound by three cysteines. We think that it is likely that some residual cluster was present in the aerobically purified EcMnmA in previous studies, which would account for the low reported catalytic activity [58,64]. It should be stressed that the fact that EcMnmA is a [4Fe-4S]-dependent enzyme remains in full agreement with the reported crystal structures of the EcMnmA/tRNA complexes, in which Asp99, Cys102 and Cys199 have the right geometry to bind a [Fe-S] cluster, and with the loss of catalytic activity when these residues were mutated [86].

The [4Fe-4S]-dependent *in vitro* thiolation activity of EcMnmA raises the question of which [Fe-S] machinery is responsible for cluster biogenesis of EcMnmA. In *E. coli*, the ISC and SUF machineries responsible for the assembly of [Fe–S] clusters are encoded by the *iscRSUA-hscBA-fdx-iscX* and *sufABCDSE* operons, respectively [42]. Deletion of both pathways results in the absence of the biosynthetic apparatus for [Fe-S] cluster biogenesis. Modification of the [Fe-S]-dependent isoprenoid biosynthetic pathway, which supplies essential isopentenyl diphosphate, to an alternative mevalonate-dependent pathway, can offset the indispensability of the [Fe-S] cluster biosynthetic systems so that the resulting Δ*iscUA* Δ*sufABCDSE* strain can grow, albeit very slowly [139,140]. To know if cluster assembly in EcMnmA is necessary for s^2U-tRNA biosynthesis, we quantified the s^2U modification in bulk tRNA from the Δ*iscUA* Δ*sufABCDSE* strain [137]. The mnm^5s^2U/$cmnm^5s^2U$ content was similar in the mutant and wild-type strains, in agreement with previous genetic studies (see Section 1.3).

Several hypotheses, which are currently under investigation, could explain this result: (i) EcMnmA could be matured by one of the several "isolated" [Fe-S] biogenesis factors [141], *i.e.* not part of the ISC or SUF machinery, (ii) EcMnmA could be maturated by a completely new pathway yet to be discovered, or (iii) a [Fe-S]-independent pathway, involving EcMnmA or another enzyme, could overcome the absence of [Fe-S] clusters in the Δ*iscUA* Δ*sufABCDSE* strain.

It was recently confirmed by another group that EcMnmA can bind a [Fe-S] cluster [142] but the [4Fe-4S] cluster was not fully reconstituted after aerobic or anaerobic protein purification, as shown by the Fe contents (2.5 and 2.8 Fe per MnmA monomer, respectively) and the UV–visible spectra. The authors claimed that the cluster inhibited the thiolation activity of EcMnmA *in vitro,* when cysteine, IscS and DTT were used as a source of sulfide. Yet, the quantity of mnm^5s^2U product that was measured by HPLC/MS analysis after digestion of the tRNA product was very low in all enzyme preparations tested so that we think that this experiment is not sufficient to conclude about the role of the cluster in the enzymatic thiolation activity.

2.4.3. BsMnmA

MnmA from the Gram positive bacterium *B. subtilis* (BsMnmA) belongs to the D-type subfamily (Fig. 2) and shares 57 % sequence identity with EcMnmA, with the residues that bind the cluster in EcMnmA being conserved (Asp101, Cys104, Cys200 in BsMnmA). There is also one cysteine, away from the active site (Cys51 in BsMnmA), which is completely conserved in MnmA proteins, suggesting an important function for that residue [135]. In contrast to *E. coli*, there are no *tus* genes in *B. subtilis* and the s^2U34 modification in *B. subtilis* involves an abbreviated pathway requiring only two proteins, BsMnmA and the cysteine desulfurase YrvO (Fig. 3A). Indeed, YrvO was shown to transfer sulfur atom from L-cysteine to MnmA, without the involvement of intermediate sulfur carrier proteins [61]. MnmA and YrvO belong to the genes found to be essential for survival in *B. subtilis* [103], and attempts to inactivate *mnmA* and *yrvO* genes failed. The *ΔiscS* or *ΔmnmA* strains of *E. coli* could only be complemented with *B. subtilis yrvO* and *mnmA* genes together, showing that the partnership between the desulfurase and tRNA thiolation enzyme is highly specific [61]. Aerobically purified BsMnmA showed *in vitro* thiolation activity when bulk tRNA isolated from an *E. coli ΔmnmA* strain was used as a substrate, with the sulfur source coming from sodium sulfide or being generated *in situ* from the YrvO/cysteine system [61]. Quantification of modified nucleosides indicated a large increase of s^2U but almost no change of mnm^5s^2U, compared to the control, indicating that thiolation at C2 precedes modification at C5. However, the assays used far from catalytic amount of enzyme (MnmA:tRNA ratio of 6) and the catalytic efficiency was very low.

Co-expression of BsMnmA mutants, in which one of the conserved cysteines (Cys51, Cys104 or Cys200) was changed to alanine, along with a plasmid coding for *yrvO,* into the *E. coli ΔmnmA* strain, showed that the C51A, C104A and C200A mutants could not synthesize s^2U-tRNA *in vivo*. Cys104 and Cys200 residues are located close to the active site. The corresponding residues in EcMnmA (Cys102 and Cys199), which had been proposed to play catalytic roles [86], have later been shown to coordinate a [Fe-S] cluster [137], supporting the hypothesis that Cys104 and Cys200 could bind a [4Fe-4S] cluster in BsMnmA.

The role of the other conserved cysteine essential for *in vivo* thiolation activity, Cys51, is less clear. One hypothesis is that Cys51 of BsMnmA is important for sulfur transfer between YrvO and BsMnmA. Indeed, persulfide transfer assays with as-purified BsMnmA variants in the presence of [^{35}S]-cysteine, YrvO and ATP showed that the C51A mutant lost its capacity to accept a persulfide from YrvO-SSH [61]. Notably, it was mentioned that, when sulfide was used as a sulfur source

in vitro, the C51A mutant of BsMnmA [61], as well as the equivalent C55A mutant of TtMnmA [135] were fully active for tRNA thiolation. This indicates that Cys51 would not be needed when sulfide or hydrosulfide could directly act as a nucleophile to attack the adenylated intermediate.

The AlphaFold2 model of the BsMnmA/YrvO complex (pTM score of 0.82, pLDDT of 92.9) (Fig. 9D) shows that Cys325 of YrvO, which is presumed to be the catalytic cysteine carrying the persulfide, is very far away (20 Å) from Cys200 of BsMnmA. Furthermore, Cys51 of BsMnmA belongs to an α-helix and is located halfway between Cys325 of YrvO and Cys200 of BsMnmA, its Cα being 18.2 Å away from the Cα of Cys325 and 14 Å away from the Cα of Cys200. Whether the very long flexible loops to which Cys200 of BsMnmA and Cys325 of YrvO belong, near the interface of the BsMnmA/YrvO complex, could adopt appropriate conformations for sulfur transfer *via* Cys51 (Fig. 9E) remains to be established. It is interesting to note that it has previously been shown that the loop containing the catalytic cysteine of cysteine desulfurase NFS1 can move over a distance of 27 Å to reach the cysteines-containing site of the scaffold protein IscU during iron-sulfur cluster assembly [143]. Altogether further experiments are needed to clarify the sulfur transfer mechanism. Remarkably, superposition of the AlphaFold2 model of BsMnmA in complex with YrvO with the tRNA-bound EcMnmA crystal structure (Fig. 9D) indicates that tRNA could bind to the BsMnmA/YrvO complex, suggesting the existence of a ternary complex in *B. subtilis*.

Interestingly, it was recently reported that the levels of s^2U tRNA modification in *B. subtilis* are responsive to sulfur availability in the growth medium [144]. A decrease in sulfur content of the growth media resulted in a delayed growth phenotype accompanied by reduced levels of the YrvO and BsMnmA enzymes, while the levels of intracellular cysteine remained unchanged. This regulation was not at the transcriptional level as the levels of *yrvO* and *mnmA* transcripts remained unchanged. In contrast, the levels of s^4U and other desulfurases (NifS, NifZ and SufS) were not dependent on the sulfur content of growth media. The authors proposed that s^2U in tRNA serves as a marker for sulfur availability in *B. subtilis*.

2.4.4. *SpMnmA*

MnmA from *Streptococcus pneumoniae* (SpMnmA) is also a D-type MnmA protein from another Gram positive bacterium, which shares 68.5 % sequence identity with BsMnmA. The crystal structure of SpMnmA has been deposited in the PDB (PDB code 2HMA) but the related work remains unpublished. The electron density observed at the active was attributed to a SAM molecule. However, according to our analysis, it is better fitted by a degradation product of SAM, 5'deoxy-5'methyl-thioadenosine [145]. This molecule is an analog of AMP, with the methylthio group replacing the phosphate group of AMP, which is in better agreement with an ATP-dependent mechanism than the involvement of SAM. The superposition of the SpMnmA crystal structure with the TtMnmA AlphaFold2 model (Fig. 9B and C) suggests that SpMnmA could be able to bind a [4Fe-4S] cluster, like EcMnmA.

2.5. *[4Fe-4S]-dependent archaeal U8-tRNA thiolase TtuI*

In contrast to EcTtuI (Fig. 10A), TtuI proteins from various archaea, such as methanogenic and Pyrococcales species, do not contain a C-terminal RHD but display a conserved CXXC + C motif (Fig. 2). In 2012, TtuI from *M. maripaludis* (MmTtuI), was produced as a recombinant protein in *E. coli*, purified anaerobically and characterized [91]. Interestingly, the *mmp1354* gene encoding MmTtuI was able to complement an *E. coli* Δ*thiI* strain since it restored s^4U biosynthesis, as revealed by reverse phase HPLC analysis of the nucleosides derived from total tRNA digestion. To know if the cysteine residues from the conserved motif (Cys265, Cys268 and Cys348) were necessary for *in vivo* s^4U formation in *M. maripaludis*, each of them was mutated to alanine and complementation of the *E. coli* Δ*thiI* strain by the mutated gene was examined. The s^4U-tRNA content was 62 % of that of the wild-type in cells expressing the C348A variant, and less than 11 % in cells expressing the C265A or C268A variant [91]. Thus, these results indicated that Cys265 and Cys268 have major contributions to s^4U biosynthesis *in vivo*. Anaerobically purified MmTtuI was able to catalyze s^4U-tRNA formation *in vitro*, under anaerobic conditions, using a *M. jannaschii* $tRNA^{Cys}$ transcript as a substrate, ATP, and Na_2S as a sulfur source, as shown by electrophoresis on an APM-containing SDS-PAGE gel but the catalytic activity was not quantified [91]. Mutation of each of the three conserved cysteines to alanine abolished tRNA thiolation *in vitro*.

Although spectroscopic features indicative of [Fe-S] clusters were not noticed in this first report, the MmTtuI protein that was later produced and purified under anoxic conditions by the same group was brownish in color and contained 2.8 Fe atom per monomer of MmTtuI [100]. The UV visible, EPR and Mössbauer spectra indicated the presence of a $[3Fe\text{-}4S]^+$ cluster. In contrast to the wild-type enzyme, each of the Cys265A, Cys268A and Cys348A mutants contained no detectable iron atoms. The anoxically-purified holo-protein was able to thiolate

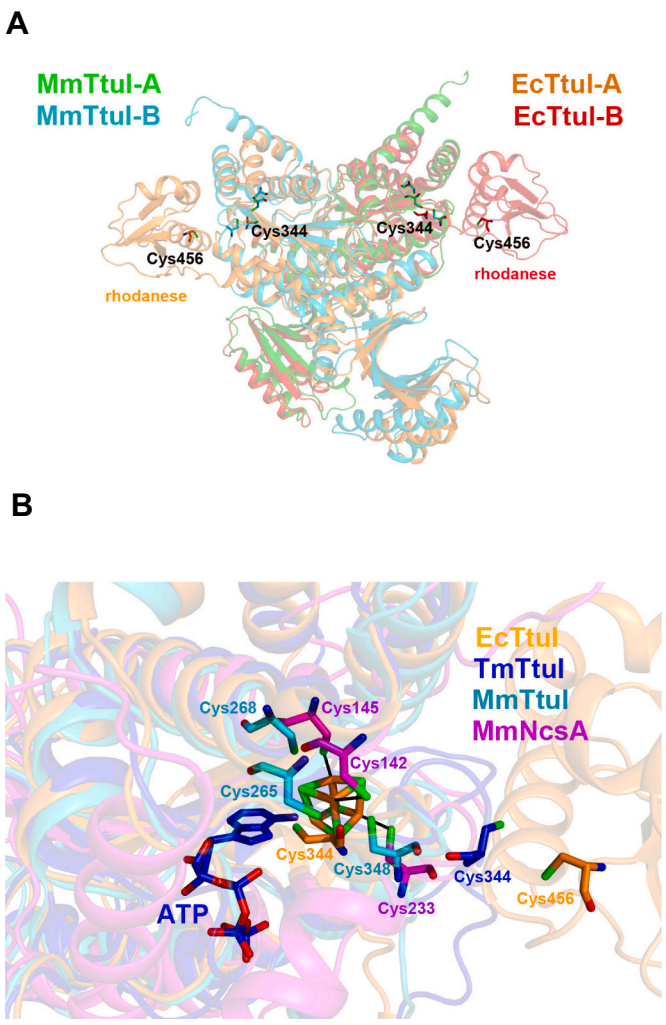

Fig. 10. AlphaFold models of EcTtuI and [4Fe-4S]-dependent MmTtuI. **A** Superposition of the AlphaFold2 models of the dimers of EcTtuI (monomers in orange and red) and MmTtuI (monomers in cyan and green) with an rmsd of 1.37 Å over 260 Cαs. The two catalytic cysteines of EcTtuI are labeled (Cys344 and Cys456 from the RHD) and the cysteines that likely coordinate the [4Fe-4S] of MmTtuI are shown in stick representation. **B** Superposition of the active sites of TmTtuI in complex with ATP (PDB code 4KR7, ATP in blue stick representation), MmTtuI (in cyan), EcTtuI (in orange) and MmNcsA with its [4Fe-4S] cluster (PDB code 6SCY, in magenta). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

M. jannaschii tRNA as a substrate, in the presence of ATP, with Na_2S as the sulfur source, as monitored on an APM-retardation gel. The addition of EDTA inactivated the enzyme, indicating that the cluster was necessary for the thiolation activity.

Intrigued by these contradictory results about MmTtuI and the unlikely possibility that a [3Fe-4S] cluster could act as a catalytic species, since it is usually a degradation product of a [4Fe-4S] cluster and not known to have catalytic properties [146], we recently produced and purified aerobically untagged MmTtuI, as well as *Pyrococcus furiosus* TtuI (PfTtuI), and performed cluster reconstitution under strict anaerobic conditions [89]. The holo-TtuI proteins contained ~3.1 Fe and ~3.1 S atoms per monomer and exhibited a UV–visible spectrum characteristic of a [4Fe-4S] cluster. Further characterization of MmTtuI by SEC-MALS, Mössbauer spectroscopy (Table 2) and biochemistry indicated that holo-MmTtuI is a dimer in solution and that the [4Fe-4S] cluster is essential for tRNA sulfuration using inorganic sulfide as a sulfur source [89]. In the absence of crystals, the superposition of the AlphaFold2 model of apo-MmTtuI (Fig. 10A) and the crystal structures of holo-PhTtuA [89] or holo-MmNcsA [124] (Fig. 10B) indicated that the [4Fe-4S] cluster is very likely coordinated by the three cysteines of the CXXC + C motif, in agreement with the mutagenesis results [100]. Therefore, it appears that it is the [4Fe-4S] state of the cluster, not the [3Fe-4S] state that is the active species for tRNA thiolation by TtuI enzymes [89]. As studied in depth in the case of TtTtuA [117], it is likely that the active [4Fe-4S] cluster is rapidly transformed into the inactive [3Fe-4S] cluster in oxidizing conditions, and its transformation back into the [4Fe-4S] state could account for the residual tRNA thiolation activity.

3. Conclusion

tRNA thiolation enzymes requiring a [Fe-S] cluster for catalysis are a recent discovery. The cluster was missed in early studies [86,91,115] because the cluster is labile under aerobic conditions. Thus, the activity of the tRNA thiolation enzyme purified under aerobic conditions was quantified with an enzyme/tRNA ratio far from catalytic conditions, usually by measuring the sulfur transfer from [^{35}S]-cysteine to the tRNA. It should be remembered that sulfur transfer from cysteine to the thiolation enzyme cannot be seen as a proof of a 'persulfide-based' mechanism. Indeed, cysteines that are able to bind a cluster [89,100,124] have also been shown to accept a sulfur atom from a persulfide [91,123]. Accordingly, formation of a persulfide on a cysteine that coordinates a [Fe-S] cluster is one of the steps towards [4Fe-4S] biogenesis [147,148].

Given that the reaction is a thiolation and that a [4Fe-4S] cluster is involved in catalysis, a fundamental question is raised: does the sulfur that is transferred to the target nucleoside come from the cluster or from an external source? Indeed, in some redox enzymes such as one class of biotin synthases or lipoate synthase, the sulfur atoms of the cluster itself are mobilized, implying destruction and reformation of the metal cofactor [149]. Because sulfur from the cluster can exchange with sulfide present in bulk medium, it is very difficult to know if the cluster acts as substrate or a cofactor, which explains why this issue remains a matter of intense debate in the [Fe-S]-dependent enzymology community. For many [4Fe-4S]-dependent tRNA thiolation enzymes, it has been shown that the enzyme catalyzes several catalytic cycles [74,116,124] and that the cluster does not degrade during reaction time, as followed by UV–visible spectroscopy [124], excluding the possibility of the sulfur atom being provided by the cluster.

Altogether, the results gathered over the last ten years on [4Fe-4S]-dependent tRNA thiolation enzymes support a novel catalytic mechanism, in which the cluster, bound by three amino acids only, acts as a Lewis acid to bind the sulfur donor and catalyze its transfer to the adenylated tRNA substrate although the details of how sulfur insertion occurs within tRNA are still unknown. The function of a [4Fe-4S] cluster acting as a Lewis acid has previously been proposed in desulfuration reactions [150–152] and dehydration reactions [146,153], the case of aconitase being well documented [154]. In the case of tRNA thiolation enzymes, the crystal structures of the holo-form of two different TtuA enzymes and MmNcsA showed that the [4Fe-4S] cluster is bound by three cysteines only [74,75,124], with the fourth nonprotein bonded Fe atom being able to bind a small anionic ligand or the terminal carboxylate group of the TtuB sulfur donor, thought to mimic the thiocarboxylate used as a sulfur source for the TtuA-catalyzed reaction [74,75,124]. These crystal structures provided strong evidence for the existence of a [4Fe-5S] intermediate although direct proof for a role of this intermediate in catalysis is still lacking. Interestingly, two other [4Fe-4S]-dependent sulfuration enzymes were recently proposed to use a [4Fe-5S] cluster as a sulfur source: two sulfur insertases LarE that incorporate sulfur atoms into the precursor of the lactate racemase cofactor [155,156] and a novel type of biotin synthase named Type II BioB that uses an auxiliary [4Fe-4S] cluster instead of the usual [2Fe-2S] cluster [39]. Indeed, the crystal structure of holo-LarE showed that the [4Fe-4S] cluster of LarE was bound to a chloride ion, presumed to mimic a hydrogenosulfide ligand [156], in agreement with native mass spectrometry, which showed that LarE coordinates sulfide at the nonligated iron atom of the [4Fe-4S] cluster, forming a [4Fe-5S] species [155]. In the case of Type II biotin synthase, it was shown by X-ray crystallography that the fifth sulfur atom of a [4Fe-5S] cluster was uniquely positioned for sulfur insertion [39].

Intriguingly, EcMnmA was able to bind a [4Fe-4S] cluster, coordinated by two cysteines and one aspartate [137]. Although it was shown *in vitro* that the cluster is essential for tRNA thiolation using sulfide as a sulfur source, further investigations are required to clarify the cellular functional importance of replacing a cysteine ligand by an aspartate to coordinate the cluster.

Although several crystal structures of tRNA thiolation enzymes have been obtained over the past ten years, only in the case of TtuA and NcsA, the crystal structure of the holo-form is available [74,75,124] (Table 3). More crystal structures of holo-proteins are needed, in particular that of D-type holo-MnmA proteins, which have the peculiarity of using an aspartate as one of the [4Fe-4S] cluster ligands. Moreover, although AlphaFold3 that can predict RNA/protein complexes was released recently, its success is only ~40 % for such complexes at present [157], and there is only one known crystal structure of a tRNA thiolation enzyme in complex with tRNA (EcMnmA), albeit in the apo-form [86]. Future research in the field should focus on obtaining more structures of tRNA/enzyme complexes, which are crucial to understand the specific recognition of the target base. This will also help to improve the AlphaFold predictions. Considering the difficulty in obtaining diffracting crystals of tRNA/protein complexes, the technique that seems to be the most promising to date is cryo-electron microscopy, as evidenced by the recent successful structure determination of several tRNA modification enzymes in complex with tRNA [158–160].

CRediT authorship contribution statement

Sylvain Gervason: Writing – review & editing. **Sambuddha Sen:** Writing – review & editing. **Marc Fontecave:** Writing – review & editing. **Béatrice Golinelli-Pimpaneau:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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References

[1] A. Cappannini, A. Ray, E. Purta, S. Mukherjee, P. Boccaletto, S.N. Moafinejad, A. Lechner, C. Barchet, B.P. Klaholz, F. Stefaniak, J.M. Bujnicki, MODOMICS: a database of RNA modifications and related information. 2023 update, Nucleic Acids Res. 52 (2024) D239–D244.
[2] G.R. Björk, T.G. Hagervall, Transfer RNA modification: presence, synthesis, and function, EcoSal Plus 6 (2014).
[3] T. Suzuki, Biosynthesis and function of tRNA wobble modifications, in: H. Grosjean (Ed.), Fine-Tuning of RNA Functions by Modification and Editing vol. 12, Springer-Verlag, Berlin Heidelberg, 2005, pp. 23–69.
[4] P.F. Agris, F.A. Vendeix, W.D. Graham, tRNA's wobble decoding of the genome: 40 years of modification, J. Mol. Biol. 366 (2007) 1–13.
[5] B. El Yacoubi, M. Bailly, V. de Crecy-Lagard, Biosynthesis and function of posttran-scriptional modifications of transfer RNAs, Annu. Rev. Genet. 46 (2012) 69–95.
[6] M. Helm, J.D. Alfonzo, Posttranscriptional RNA modifications: playing metabolic games in a cell's chemical Legoland, Chem. Biol. 21 (2014) 174–185.
[7] V.Y. Väre, E.R. Eruysal, A. Narendran, K.L. Sarachan, P.F. Agris, Chemical and conformational diversity of modified nucleosides affects tRNA structure and function, Biomolecules 7 (2017).
[8] B.S. Zhao, I.A. Roundtree, C. He, Post-transcriptional gene regulation by mRNA modifications, Nat. Rev. Mol. Cell Biol. 18 (2017) 31–42.
[9] H.J. Chou, E. Donnard, H.T. Gustafsson, M. Garber, O.J. Rando, Transcriptome-wide analysis of roles for tRNA modifications in translational regulation, Mol. Cell 68 (2017) 978–992.e4.
[10] I.A. Roundtree, M.E. Evans, T. Pan, C. He, Dynamic RNA modifications in gene expression regulation, Cell 169 (2017) 1187–1200.
[11] L. Pollo-Oliveira, V. de Crécy-Lagard, Can protein expression be regulated by modulation of tRNA modification profiles? Biochemistry 58 (2019) 355–362.
[12] W. Zhang, M. Foo, A.M. Eren, T. Pan, tRNA modification dynamics from individual organisms to metaepitranscriptomics of microbiomes, Mol. Cell 82 (2022) 891–906.
[13] H. Sun, K. Li, C. Liu, C. Yi, Regulation and functions of non-m6A mRNA modifications, Nat. Rev. Mol. Cell Biol. 24 (2023) 714–731.
[14] M.K. Wang, C.C. Gao, Y.G. Yang, Emerging roles of RNA methylation in development, Acc. Chem. Res. 56 (2023) 43417–43427.
[15] S. Zaccara, R.J. Ries, S.R. Jaffrey, Reading, writing and erasing mRNA methylation, Nat. Rev. Mol. Cell Biol. 20 (2019) 608–624.
[16] C. Yi, T. Pan, Cellular dynamics of RNA modification, Acc. Chem. Res. 44 (2011) 1380–1388.
[17] C.T. Chan, Y.L. Pang, W. Deng, I.R. Babu, M. Dyavaiah, T.J. Begley, P.C. Dedon, Reprogramming of tRNA modifications controls the oxidative stress response by codon-biased translation of proteins, Nat. Commun. 3 (2012) 937.
[18] P.C. Dedon, T.J. Begley, A system of RNA modifications and biased codon use controls cellular stress response at the level of translation, Chem. Res. Toxicol. 27 (2014) 330–337.
[19] L. Endres, P.C. Dedon, T.J. Begley, Codon-biased translation can be regulated by wobble-base tRNA modification systems during cellular stress responses, RNA Biol. 12 (2015) 603–614.
[20] P.C. Dedon, T.J. Begley, Dysfunctional tRNA reprogramming and codon-biased translation in cancer, Trends Mol. Med. 28 (2022) 964–978.
[21] H.H. Shi, P.W. Chai, R.B. Jia, X.Q. Fan, Novel insight into the regulatory roles of diverse RNA modifications: re-defining the bridge between transcription and translation, Mol. Cancer 19 (2020) 78.
[22] J.B. Zhou, E.D. Wang, X.L. Zhou, Modifications of the human tRNA anticodon loop and their associations with genetic diseases, Cell. Mol. Life Sci. 78 (2021) 7087–7105.
[23] L. Wang, S.J. Lin, Emerging functions of tRNA modifications in mRNA translation and diseases, J. Genet. Genomics 50 (2023) 223–232.
[24] Y.S. Wang, E.W. Tao, J. Tan, Q.Y. Gao, Y.X. Chen, J.Y. Fang, tRNA modifications: insights into their role in human cancers, Trends Cell Biol. 33 (2023) 1035–1048.
[25] S. Laxman, B.M. Sutter, X. Wu, S. Kumar, X. Guo, D.C. Trudgian, H. Mirzaei, B. P. Tu, Sulfur amino acids regulate translational capacity and metabolic homeostasis through modulation of tRNA thiolation, Cell 154 (2013) 416–429.
[26] J.R. Damon, D. Pincus, H.L. Ploegh, tRNA thiolation links translation to stress responses in *Saccharomyces cerevisiae*, Mol. Biol. Cell 26 (2015) 270–282.
[27] A. Noma, N. Shigi, T. Suzuki, Biogenesis and functions of thio-compounds in transfer RNA: comparison of bacterial and eukaryotic thiolation machineries, in: H. Grosjean (Ed.), DNA and RNA Modification Enzymes: Structure, Mechanism, Function and Evolution, CRC Press, vol. 27, Taylor & Francis group, Boca Raton London New York, 2009, pp. 392–405.
[28] M. Cavuzic, Y. Liu, Biosynthesis of sulfur-containing tRNA modifications: a comparison of bacterial, archaeal, and eukaryotic pathways, Biomolecules 7 (2017) 27.
[29] N. Shigi, Biosynthesis and functions of sulfur modifications in tRNA, Front. Genet. 5 (2014) 67.
[30] N. Shigi, Recent advances in our understanding of the biosynthesis of sulfur modifications in tRNAs, Front. Microbiol. 9 (2018) 2679.
[31] O. Bimai, S. Arragain, B. Golinelli-Pimpaneau, Structure-based mechanistic insights into catalysis by tRNA thiolation enzymes, Curr. Opin. Struct. Biol. 65 (2020) 69–78.
[32] N. Shigi, Biosynthesis and degradation of sulfur modifications in tRNAs, Int. J. Mol. Sci. 22 (2021) 11937.
[33] H.L. Hernandez, F. Pierrel, E. Elleingand, R. Garcia-Serres, B.H. Huynh, M. K. Johnson, M. Fontecave, M. Atta, MiaB, a bifunctional radical-S-adenosylmethionine enzyme involved in the thiolation and methylation of tRNA, contains two essential [4Fe-4S] clusters, Biochemistry 46 (2007) 5140–5147.
[34] S. Arragain, S.K. Handelman, F. Forouhar, F.Y. Wei, K. Tomizawa, J.F. Hunt, T. Douki, M. Fontecave, E. Mulliez, M. Atta, Identification of eukaryotic and prokaryotic methylthiotransferase for biosynthesis of 2-methylthio-N-6-threo-nylcarbamoyladenosine in tRNA, J. Biol. Chem. 285 (2010) 28425–28433.
[35] S.J. Booker, R.M. Cicchillo, T.L. Grove, Self-sacrifice in radical S-adenosylmethionine proteins, Curr. Opin. Chem. Biol. 11 (2007) 543–552.
[36] O.A. Esakova, T.L. Grove, N.H. Yennawar, A.J. Arcinas, B. Wang, C. Krebs, S. C. Almo, S.J. Booker, Structural basis for tRNA methylthiolation by the radical SAM enzyme MiaB, Nature 597 (2021) 566–570.
[37] F. Forouhar, S. Arragain, M. Atta, S. Gambarelli, J.M. Mouesca, M. Hussain, R. Xiao, S. Kieffer-Jaquinod, J. Seetharaman, T.B. Acton, G.T. Montelione, E. Mulliez, J.F. Hunt, M. Fontecave, Two Fe-S clusters catalyze sulfur insertion by radical-SAM methylthiotransferases, Nat. Chem. Biol. 9 (2013) 333–338.
[38] E. Mulliez, V. Duarte, S. Arragain, M. Fontecave, M. Atta, On the role of additional [4Fe-4S] clusters with a free coordination site in radical-SAM enzymes, Front. Chem. 5 (2017) 17.
[39] J.C. Lachowicz, D. Lennox-Hvenekilde, N. Myling-Petersen, B. Salomonsen, G. Verkleij, C.G. Acevedo-Rocha, B. Caddell, L.S. Gronenberg, S.C. Almo, M.O. A. Sommer, H.J. Genee, T.L. Grove, Discovery of a biotin synthase that utilizes an auxiliary 4Fe-5S cluster for sulfur insertion, J. Am. Chem. Soc. 146 (2024) 1860–1873.
[40] S. Kimura, T. Suzuki, Iron-sulfur proteins responsible for RNA modifications, Biochim. Biophys. Acta 2015 (1853) 1272–1283.
[41] J. Jumper, R. Evans, A. Pritzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Žídek, A. Potapenko, A. Bridgland, C. Meyer, S. A.A. Kohl, A.J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A.W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, Highly accurate protein structure prediction with AlphaFold, Nature 596 (2021) 583–589.
[42] B. Roche, L. Aussel, B. Ezraty, P. Mandin, B. Py, F. Barras, Iron/sulfur proteins bioge- nesis in prokaryotes: formation, regulation and diversity, Biochim. Biophys. Acta 2013 (1827) 455–469.
[43] R. Leipuviene, Q. Qian, G.R. Björk, Formation of thiolated nucleosides present in tRNA from *Salmonella enterica* serovar Typhimurium occurs in two principally distinct pathways, J. Bacteriol. 186 (2004) 758–766.
[44] F.W. Outten, Recent advances in the Suf Fe-S cluster biogenesis pathway: beyond the Proteobacteria, Biochim. Biophys. Acta 2015 (1853) 1464–1469.
[45] M. Bühning, A. Valleriani, S. Leimkühler, The role of SufS is restricted to Fe-S cluster biosynthesis in *Escherichia coli*, Biochemistry 56 (2017) 1987–2000.
[46] Y. Nakai, M. Nakai, R. Lill, T. Suzuki, H. Hayashi, Thio modification of yeast cytosolic tRNA is an iron-sulfur protein-dependent pathway, Mol. Cell. Biol. 27 (2007) 2841–2847.
[47] D.J. Netz, J. Mascarenhas, O. Stehling, A.J. Pierik, R. Lill, Maturation of cytosolic and nuclear iron-sulfur proteins, Trends Cell Biol. 24 (2014) 303–312.
[48] A. Pandey, J. Pain, N. Dziuba, A.K. Pandey, A. Dancis, P.A. Lindahl, D. Pain, Mito-chondria export sulfur species required for cytosolic tRNA thiolation, cell, Chem. Biol. 25 (2018) 738–748.e3.
[49] K. Nilsson, H.K. Lundgren, T.G. Hagervall, G.R. Bjork, The cysteine desulfurase IscS is required for synthesis of all five thiolated nucleosides present in tRNA from *Salmonella enterica serovar typhimurium*, J. Bacteriol. 184 (2002) 6830–6835.
[50] C.T. Lauhon, Requirement for IscS in biosynthesis of all thionucleosides in *Escherichia coli*, J. Bacteriol. 184 (2002) 6820–6829.
[51] R. Shi, A. Proteau, M. Villarroya, I. Moukadiri, L. Zhang, J.F. Trempe, A. Matte, M.E. Armengod, M. Cygler, Structural basis for Fe-S cluster assembly and tRNA thiolation mediated by IscS protein-protein interactions, PLoS Biol. 8 (2010) e1000354.
[52] S. Leimkühler, M. Buhning, L. Beilschmidt, Shared sulfur mobilization routes for tRNA thiolation and molybdenum cofactor biosynthesis in prokaryotes and eukaryotes, Biomolecules 7 (2017) 5.
[53] S. Chatterjee, R.P. Hausinger, Sulfur incorporation into biomolecules: recent advances, Crit. Rev. Biochem. Mol. Biol. 57 (2022) 461–476.
[54] M. Kotera, T. Bayashi, M. Hattori, T. Tokimatsu, S. Goto, H. Mihara, M. Kanehisa, Comprehensive genomic analysis of sulfur-relay pathway genes, Genome Inform. 24 (2010) 104–115.
[55] K.A. Black, P.C. Dos Santos, Shared-intermediates in the biosynthesis of thio-cofactors: mechanism and functions of cysteine desulfurases and sulfur acceptors, Biochim. Biophys. Acta 2015 (1853) 1470–1480.
[56] H. Mihara, N. Esaki, Bacterial cysteine desulfurases: their function and mechanisms, Appl. Microbiol. Biotechnol. 60 (2002) 12–23.
[57] R. Kambampati, C.T. Lauhon, IscS is a sulfurtransferase for the *in vitro* biosynthesis of 4-thiouridine in *Escherichia coli* tRNA, Biochemistry 38 (1999) 16561–16568.

[58] R. Kambampati, C.T. Lauhon, MnmA and IscS are required for in vitro 2-thiouridine biosynthesis in *Escherichia coli*, Biochemistry 42 (2003) 1109–1117.

[59] A. Pandey, R. Golla, H. Yoon, A. Dancis, D. Pain, Persulfide formation on mitochon- drial cysteine desulfurase: enzyme activation by a eukaryote-specific interacting protein and Fe–S cluster synthesis, Biochem. J. 448 (2012) 171–187.

[60] L.J. Rajakovich, J. Tomlinson, P.C. Dos Santos, Functional analysis of *Bacillus subtilis* genes involved in the biosynthesis of 4-Thiouridine in tRNA, J. Bacteriol. 194 (2012) 4933–4940.

[61] K.A. Black, P.C. Dos Santos, Abbreviated pathway for biosynthesis of 2-Thiouridine in *Bacillus subtilis*, J. Bacteriol. 197 (2015) 1952–1962.

[62] R. Kambampati, C.T. Lauhon, Evidence for the transfer of sulfane sulfur from IscS to ThiI during the in vitro biosynthesis of 4-thiouridine in *Escherichia coli* tRNA, J. Biol. Chem. 275 (2000) 10727–10730.

[63] Y. Liu, M. Sieprawska-Lupa, W.B. Whitman, R.H. White, Cysteine is not the sulfur source for iron-sulfur cluster and methionine biosynthesis in the methanogenic archaeon *Methanococcus maripaludis*, J. Biol. Chem. 285 (2010) 31923–31929.

[64] Y. Ikeuchi, N. Shigi, J. Kato, A. Nishimura, T. Suzuki, Mechanistic insights into multiple sulfur mediators sulfur relay by involved in thiouridine biosynthesis at tRNA wobble positions, Mol. Cell 21 (2006) 97–108.

[65] A. Noma, Y. Sakaguchi, T. Suzuki, Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions, Nucleic Acids Res. 37 (2009) 1335–1352.

[66] T. Numata, S. Fukai, Y. Ikeuchi, T. Suzuki, O. Nureki, Structural basis for sulfur relay to RNA mediated by heterohexameric TusBCD complex, Structure 14 (2006) 357–366.

[67] N.E. Chavarria, S. Hwang, S. Cao, X. Fu, M. Holman, D. Elbanna, S. Rodriguez, D. Arrington, M. Englert, S. Uthandi, D. Soll, J.A. Maupin-Furlow, Archaeal Tuc1/Ncs6 homolog required for wobble uridine tRNA thiolation is associated with ubiquitin-proteasome, translation, and RNA processing system homologs, PloS One 9 (2014) e99104.

[68] C.D. Schlieker, A.G. Van der Veen, J.R. Damon, E. Spooner, H.L. Ploegh, A functional proteomics approach links the ubiquitin-related modifier Urm1 to a tRNA modification pathway, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 18255–18260.

[69] S. Leidel, P.G. Pedrioli, T. Bucher, R. Brost, M. Costanzo, A. Schmidt, R. Aebersold, C. Boone, K. Hofmann, M. Peter, Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA, Nature 458 (2009) 228–232.

[70] N. Shigi, Y. Sakaguchi, T. Suzuki, K. Watanabe, Identification of two tRNA thiolation genes required for cell growth at extremely high temperatures, J. Biol. Chem. 281 (2006) 14296–14306.

[71] N. Shigi, Y. Sakaguchi, S. Asai, T. Suzuki, K. Watanabe, Common thiolation mecha- nism in the biosynthesis of tRNA thiouridine and sulphur-containing cofactors, EMBO J. 27 (2008) 3267–3278.

[72] N. Shigi, Posttranslational modification of cellular proteins by a ubiquitin-like protein in bacteria, J. Biol. Chem. 287 (2012) 17568–17577.

[73] N. Shigi, S.I. Asai, K. Watanabe, Identification of a rhodanese-like protein involved in thiouridine biosynthesis in *Thermus thermophilus* tRNA, FEBS Lett. 590 (2016) 4628–4637.

[74] M. Chen, S.I. Asai, S. Narai, S. Nambu, N. Omura, Y. Sakaguchi, T. Suzuki, M. Ikeda-Saito, K. Watanabe, M. Yao, N. Shigi, Y. Tanaka, Biochemical and structural charac- terization of oxygen-sensitive 2-thiouridine synthesis catalyzed by an iron-sulfur protein TtuA, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 4954–4959.

[75] M. Chen, M. Ishizaka, S. Narai, M. Horitani, N. Shigi, M. Yao, Y. Tanaka, The [4Fe-4S] cluster of sulfurtransferase TtuA desulfurizes TtuB during tRNA modification in *Thermus thermophilus*, Commun Biol 3 (2020) 168.

[76] B. Huang, J. Lu, A.S. Bystrom, A genome-wide screen identifies genes required for formation of the wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine in *Saccharomyces cerevisiae*, RNA 14 (2008) 2183–2194.

[77] Y. Nakai, M. Nakai, H. Hayashi, Thio-modification of yeast cytosolic tRNA requires a ubiquitin-related system that resembles bacterial sulfur transfer systems, J. Biol. Chem. 283 (2008) 27469–27476.

[78] A. Jüdes, A. Bruch, R. Klassen, M. Helm, R. Schaffrath, Sulfur transfer and activation by ubiquitin-like modifier system Uba4*Urm1 link protein urmylation and tRNA thiolation in yeast, microb, Cell 3 (2016) 554–564.

[79] M. Termathe, S.A. Leidel, The Uba4 domain interplay is mediated via a thioester that is critical for tRNA thiolation through Urm1 thiocarboxylation, Nucleic Acids Res. 46 (2018) 5171–5181.

[80] S. Kim, W. Johnson, C. Chen, A.K. Sewell, A.S. Bystrom, M. Han, Allele-specific suppressors of lin-1(R175Opal) identify functions of MOC-3 and DPH-3 in tRNA modification complexes in *Caenorhabditis elegans*, Genetics 185 (2010) 1235–1247.

[81] Y. Nakai, A. Harada, Y. Hashiguchi, M. Nakai, H. Hayashi, *Arabidopsis* molybdopterin biosynthesis protein Cnx5 collaborates with the ubiquitin-like protein Urm11 in the thio-modification of tRNA, J. Biol. Chem. 287 (2012) 30874–30884.

[82] M.M. Chowdhury, C. Dosche, H.G. Lohmannsroben, S. Leimkuhler, Dual role of the molybdenum cofactor biosynthesis protein MOCS3 in tRNA thiolation and molyb- denum cofactor biosynthesis in humans, J. Biol. Chem. 287 (2012) 17297–17307.

[83] H.V. Miranda, N. Nembhard, D. Su, N. Hepowit, D.J. Krause, J.R. Pritz, C. Phillips, D. Soll, J.A. Maupin-Furlow, E1- and ubiquitin-like proteins provide a direct link between protein conjugation and sulfur transfer in archaea, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 4417–4422.

[84] M. Hochstrasser, Origin and function of ubiquitin-like proteins, Nature 458 (2009) 422–429.

[85] M. Fellner, R.P. Hausinger, J. Hu, A structural perspective on the PP-loop ATP pyrophosphatase family, Crit. Rev. Biochem. Mol. Biol. 53 (2018) 607–622.

[86] T. Numata, Y. Ikeuchi, S. Fukai, T. Suzuki, O. Nureki, Snapshots of tRNA sulphuration via an adenylated intermediate, Nature 442 (2006) 419–424.

[87] D. You, T. Xu, F. Yao, X. Zhou, Z. Deng, Direct evidence that ThiI is an ATP pyrophosphatase for the adenylation of uridine in 4-thiouridine biosynthesis, Chembiochem 9 (2008) 1879–1882.

[88] R.A. Bender, The danger of annotation by analogy: most "thil" genes play no role in thiamine biosynthesis, J. Bacteriol. 193 (2011) 4574–4575.

[89] N. He, J. Zhou, O. Bimai, J. Oltmanns, J.L. Ravanat, C. Velours, V. Schünemann, M. Fontecave, B. Golinelli-Pimpaneau, A subclass of archaeal U8-tRNA sulfurases requires a [4Fe-4S] cluster for catalysis, Nucleic Acids Res. 50 (2022) 12969–12978.

[90] G. Thomas, A. Favre, 4-Thiouridine triggers both growth delay induced by near-ultraviolet light and photoprotection, Eur. J. Biochem. 113 (1980) 67–74.

[91] Y. Liu, X. Zhu, A. Nakamura, R. Orlando, D. Söll, W.B. Whitman, Biosynthesis of 4-thiouridine in tRNA in the methanogenic archaeon *Methanococcus maripaludis*, J. Biol. Chem. 287 (2012) 36683–36692.

[92] C. Tomikawa, T. Ohira, Y. Inoue, T. Kawamura, A. Yamagishi, T. Suzuki, H. Hori, Distinct tRNA modifications in the thermo-acidophilic archaeon, *Thermoplasma acidophilum*, FEBS Lett. 587 (2013) 3575–3580.

[93] G.F. Kramer, J.C. Baker, B.N. Ames, Near-UV stress in *Salmonella typhimurium*: 4-thiouridine in tRNA, ppGpp, and ApppGpp as components of an adaptive response, J. Bacteriol. 170 (1988) 2344–2351.

[94] R. Cipollone, P. Ascenzi, P. Visca, Common themes and variations in the rhodanese superfamily, IUBMB Life 59 (2007) 51–59.

[95] D. Bordo, P. Bork, The rhodanese/Cdc25 phosphatase superfamily. Sequence-structure-function relations, EMBO Rep. 3 (2002) 741–746.

[96] P.M. Palenchar, C.J. Buck, H. Cheng, T.J. Larson, E.G. Mueller, Evidence that ThiI, an enzyme shared between thiamin and 4-thiouridine biosynthesis, may be a sulfur- transferase that proceeds through a persulfide intermediate, J. Biol. Chem. 275 (2000) 8283–8286.

[97] E.G. Mueller, P.M. Palenchar, C.J. Buck, The role of the cysteine residues of ThiI in the generation of 4-thiouridine in tRNA, J. Biol. Chem. 276 (2001) 33588–33595.

[98] D.G. Waterman, M. Ortiz-Lombardia, M.J. Fogg, E.V. Koonin, A.A. Antson, Crystal structure of *Bacillus anthracis* ThiI, a tRNA-modifying enzyme containing the predicted RNA-binding THUMP domain, J. Mol. Biol. 356 (2006) 97–110.

[99] P. Neumann, K. Lakomek, P.T. Naumann, W.M. Erwin, C.T. Lauhon, R. Ficner, Crystal structure of a 4-thiouridine synthetase-RNA complex reveals specificity of tRNA U8 modification, Nucleic Acids Res. 42 (2014) 6673–6685.

[100] Y. Liu, D.J. Vinyard, M.E. Reesbeck, T. Suzuki, K. Manakongtreecheep, P. L. Holland, G.W. Brudvig, D. Söll, A [3Fe-4S] cluster is required for thiolation in archaea and eukaryotes, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 12703–12708.

[101] M.E. Armengod, S. Meseguer, M. Villarroya, S. Prado, I. Moukadiri, R. Ruiz-Partida, M.J. Garzón, C. Navarro-González, A. Martínez-Zamora, Modification of the wobble uridine in bacterial and mitochondrial tRNAs reading NNA/NNG triplets of 2-codon boxes, RNA Biol. 12 (2014) 1495–1507.

[102] K. Nilsson, G. Jager, G.R. Björk, An unmodified wobble uridine in tRNAs specific for glutamine, lysine, and glutamic acid from Salmonella enterica Serovar typhimurium results in nonviability-due to increased missense errors? PloS One 12 (2017) e0175092.

[103] K. Kobayashi, S.D. Ehrlich, A. Albertini, G. Amati, K.K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S.C. Brignell, S. Bron, K. Bunai, J. Chapuis, L.C. Christiansen, A. Danchin, M. Debarbouille, E. Dervyn, E. Deuerling, K. Devine, S.K. Devine, O. Dreesen, J. Errington, S. Fillinger, S. J. Foster, Y. Fujita, A. Galizzi, R. Gardan, C. Eschevins, T. Fukushima, K. Haga, C. R. Harwood, M. Hecker, D. Hosoya, M.F. Hullo, H. Kakeshita, D. Karamata, Y. Kasahara, F. Kawamura, K. Koga, P. Koski, R. Kuwana, D. Imamura, M. Ishimaru, S. Ishikawa, I. Ishio, D. Le Coq, A. Masson, C. Mauel, R. Meima, R. P. Mellado, A. Moir, S. Moriya, E. Nagakawa, H. Nanamiya, S. Nakai, P. Nygaard, M. Ogura, T. Ohanan, M. O'Reilly, M. O'Rourke, Z. Pragai, H.M. Pooley, G. Rapoport, J.P. Rawlins, L.A. Rivas, C. Rivolta, A. Sadaie, Y. Sadaie, M. Sarvas, T. Sato, H.H. Saxild, E. Scanlan, W. Schumann, J.F. Seegers, J. Sekiguchi, A. Sekowska, S.J. Seror, M. Simon, P. Stragier, R. Studer, H. Takamatsu, T. Tanaka, M. Takeuchi, H.B. Thomaides, V. Vagner, J.M. van Dijl, K. Watabe, A. Wipat, H. Yamamoto, M. Yamamoto, Y. Yamamoto, K. Yamane, K. Yata, K. Yoshida, H. Yoshikawa, U. Zuber, N. Ogasawara, Essential *Bacillus subtilis* genes, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 4678–4683.

[104] G. Jäger, R. Leipuviene, M.G. Pollard, Q. Qian, G.R. Björk, The conserved Cys-X1-X2-Cys motif present in the TtcA protein is required for the thiolation of cytidine in position 32 of tRNA from *Salmonella enterica* serovar Typhimurium, J. Bacteriol. 186 (2004) 750–757.

[105] D. Bouvier, N. Labessan, M. Clemancey, J.M. Latour, J.L. Ravanat, M. Fontecave, M. Atta, TtcA a new tRNA-thioltransferase with an Fe-S cluster, Nucleic Acids Res. 42 (2014) 7960–7970.

[106] S.A. Freibert, B.D. Weiler, E. Bill, A.J. Pierik, U. Mühlenhoff, R. Lill, Biochemical reconstitution and spectroscopic analysis of iron–sulfur proteins, Methods Enzymol. 599 (2018) 197–226.
[107] W.W. Fish, Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples, Methods Enzymol. 158 (1988) 357–364.
[108] H. Beinert, Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins, Anal. Biochem. 131 (1983) 373–378.
[109] M.E. Pandelia, N.D. Lanz, S.J. Booker, C. Krebs, Mössbauer spectroscopy of Fe/S proteins, Biochim. Biophys. Acta 2015 (1853) 1395–1405.
[110] W.R. Hagen, EPR spectroscopy of complex biological iron–sulfur systems, J. Biol. Inorg. Chem. 23 (2018) 623–634.
[111] A. Romsang, J. Duang-Nkern, K. Khemsom, L. Wongsaroj, K. Saninjuk, M. Fuangthong, P. Vattanaviboon, S. Mongkolsuk, *Pseudomonas aeruginosa* ttcA encoding tRNA-thiolating protein requires an iron-sulfur cluster to participate in hydrogen peroxide-mediated stress protection and pathogenicity, Sci. Rep. 8 (2018) 11882.
[112] S.H. Kim, B.Y. Lee, G.W. Lau, Y.H.J. Cho, IscR modulates catalase A (KatA) activity, peroxide resistance and full virulence of *Pseudomonas aeruginosa* PA14, Microbiol. Biotechnol. 19 (2009) 1520–1526.
[113] K. Watanabe, M. Shinma, T. Oshima, S. Nishimura, Heat-induced stability of tRNA from an extreme thermophile, *Thermus thermophilus*, Biochem. Biophys. Res. Commun. 72 (1976) 1137–1144.
[114] J. Zhou, O. Bimai, S. Arragain, L. Pecqueur, B. Golinelli-Pimpaneau, TtuA and TudS, two [4Fe-4S]-dependent enzymes catalyzing non-redox sulfuration or desulfuration reactions, in: S.E. Wiley (Ed.), Encyclopedia of Inorganic and Bioinorganic Chemistry, Wiley, Chichester, UK, 2022, pp. 1–16.
[115] H. Nakagawa, M. Kuratani, S. Goto-Ito, T. Ito, K. Katsura, T. Terada, M. Shirouzu, S. Sekine, N. Shigi, S. Yokoyama, Crystallographic and mutational studies on the tRNA thiouridine synthetase TtuA, Proteins 81 (2013) 1232–1244.
[116] S. Arragain, O. Bimai, P. Legrand, S. Caillat, J.L. Ravanat, N. Touati, L. Binet, M. Atta, M. Fontecave, B. Golinelli-Pimpaneau, Nonredox thiolation in tRNA occurring via sulfur activation by a [4Fe-4S] cluster, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 7355–7360.
[117] M. Ishizaka, M. Chen, S. Narai, Y. Tanaka, T. Ose, M. Horitani, M. Yao, Quick and spontaneous transformation between [3Fe-4S] and [4Fe-4S] iron-sulfur clusters in the tRNA-thiolation enzyme TtuA, Int. J. Mol. Sci. 24 (2023) 833.
[118] N. Ranjan, M.V. Rodnina, tRNA wobble modifications and protein homeostasis, Translation (Austin) 4 (2016) e1143076.
[119] R. Schaffrath, S.A. Leidel, Wobble uridine modifications-a reason to live, a reason to die?!, RNA Biol. 14 (2017) 1209–1222.
[120] P.F. Agris, E.R. Eruysal, A. Narendran, V.Y.P. Vare, S. Vangaveti, S. V. Ranganathan, Celebrating wobble decoding: half a century and still much is new, RNA Biol. 15 (2018) 537–553.
[121] N. Umeda, T. Suzuki, M. Yukawa, Y. Ohya, H. Shindo, K. Watanabe, T. Suzuki, Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs. Implications for the molecular pathogenesis of human mitochondrial diseases, J. Biol. Chem. 280 (2005) 1613–1624.
[122] Y. Nakai, M. Nakai, T. Yano, Sulfur modifications of the wobble U34 in tRNAs and their intracellular localization in eukaryotic cells, Biomolecules 7 (2017) 17.
[123] Y. Liu, F. Long, L. Wang, D. Söll, W.B. Whitman, The putative tRNA 2-thiouridine synthetase Ncs6 is an essential sulfur carrier in *Methanococcus maripaludis*, FEBS Lett. 588 (2014) 873–877.
[124] O. Bimai, P. Legrand, J.L. Ravanat, N. Touati, J. Zhou, N. He, M. Lénon, F. Barras, M. Fontecave, B. Golinelli-Pimpaneau, The thiolation of uridine 34 in tRNA, which contols protein translation, depends on a [4Fe-4S]-cluster in *Methanococcus maripaludis*, Sci. Rep. 13 (2023) 5351.
[125] G.R. Björk, B. Huang, O.P. Persson, A.S. Byström, A conserved modified wobble nucleoside (mcm5s2U) in lysyl-tRNA is required for viability in yeast, RNA 13 (2007) 1245–1255.
[126] R.M. Leiber, F. John, Y. Verhertbruggen, A. Diet, J.P. Knox, C. Ringli, The TOR pathway modulates the structure of cell walls in *Arabidopsis*, Plant Cell 22 (2010) 1898–1908.
[127] M. Philipp, F. John, C. Ringli, The cytosolic thiouridylase CTU2 of *Arabidopsis thaliana* is essential for posttranscriptional thiolation of tRNAs and influences root development, BMC Plant Biol. 14 (2014) 109.
[128] M. Dewez, F. Bauer, M. Dieu, M. Raes, J. Vandenhaute, D. Hermand, The conserved Wobble uridine tRNA thiolase Ctu1-Ctu2 is required to maintain genome integrity, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 5459–5464.
[129] S. Delaunay, F. Rapino, L. Tharun, Z. Zhou, L. Heukamp, M. Termathe, K. Shostak, I. Klevernic, A. Florin, H. Desmecht, C.J. Desmet, L. Nguyen, S.A. Leidel, A. E. Willis, R. Buttner, A. Chariot, P. Close, Elp3 links tRNA modification to IRES-dependent translation of LEF1 to sustain metastasis in breast cancer, J. Exp. Med. 213 (2016) 2503–2523.
[130] F. Rapino, S. Delaunay, F. Rambow, Z. Zhou, L. Tharun, P. De Tullio, O. Sin, K. Shostak, S. Schmitz, J. Piepers, B. Ghesquiere, L. Karim, B. Charloteaux, D. Jamart, A. Florin, C. Lambert, A. Rorive, G. Jerusalem, E. Leucci, M. Dewaele, M. Vooijs, S.A. Leidel, M. Georges, M. Voz, B. Peers, R. Buttner, J.C. Marine, A. Chariot, P. Close, Codon-specific translation reprogramming promotes resistance to targeted therapy, Nature 558 (2018) 605–609.
[131] G.L. Igloi, Interaction of tRNAs and of phosphorothioate-substituted nucleic acids with an organomercurial. Probing the chemical environment of thiolated residues by affinity electrophoresis, Biochemistry 27 (1988) 3842–3849.
[132] J.C. Crack, A.J. Jervis, A.A. Gaskell, G.F. White, J. Green, A.J. Thomson, N.E. Le Brun, Signal perception by FNR: the role of the iron-sulfur cluster, Biochem. Soc. Trans. 36 (2008) 1144–1148.
[133] K. Nakanishi, L. Bonnefond, S. Kimura, T. Suzuki, R. Ishitani, O. Nureki, Structural basis for translational fidelity ensured by transfer RNA lysidine synthetase, Nature 461 (2009) 1144–1148.
[134] P.D. Adams, P.V. Afonine, G. Bunkoczi, V.B. Chen, I.W. Davis, N. Echols, J. J. Headd, L.W. Hung, G.J. Kapral, R.W. Grosse-Kunstleve, A.J. McCoy, N. W. Moriarty, R. Oeffner, R.J. Read, D.C. Richardson, J.S. Richardson, T. C. Terwilliger, P.H. Zwart, PHENIX: a comprehensive Python-based system for macromolecular structure solution, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 213–221.
[135] N. Shigi, M. Horitani, K. Miyauchi, T. Suzuki, M. Kuroki, An ancient type of MnmA protein is an iron-sulfur cluster-dependent sulfurtransferase for tRNA anticodons, RNA 26 (2020) 240–250.
[136] M. Kuratani, Y. Yoshikawa, Y. Bessho, K. Higashijima, T. Ishii, R. Shibata, S. Takahashi, K. Yutani, S. Yokoyama, Structural basis of the initial binding of tRNA(Ile) lysidine synthetase TilS with ATP and L-lysine, Structure 15 (2007) 1642–1653.
[137] J. Zhou, M. Lénon, N. Touati, J.-L. Ravanat, C. Velours, M. Fontecave, F. Barras, B. Golinelli-Pimpaneau, Iron sulfur biology invades tRNA modification: the case of U34 sulfuration, Nucleic Acids Res. 49 (2021) 3997–4007.
[138] D.W. Bak, S.J. Elliott, Alternative FeS cluster ligands: tuning redox potentials and chemistry, Curr. Opin. Chem. Biol. 19 (2014) 50–58.
[139] L. Loiseau, C. Gerez, M. Bekker, S. Ollagnier-de-Choudens, B. Py, Y. Sanakis, J. Teixeira-de-Mattos, M. Fontecave, F. Barras, ErpA, an iron–sulfur (Fe–S) protein of the A-type essential for respiratory metabolism in *Escherichia coli*, Proc. Natl. Acd. Sci. USA 104 (2007) 13626–13631.
[140] N. Tanaka, M. Kanazawa, K. Tonosaki, N. Yokoyama, T. Kuzuyama, Y. Takahashi, Novel features of the ISC machinery revealed by characterization of Escherichia coli mutants that survive without iron-sulfur clusters, Mol. Microbiol. 99 (2016) 835–848.
[141] K. Esquilin-Lebron, S. Dubrac, F. Barras, J.M. Boyd, Bacterial approaches for assembling iron-sulfur proteins, mBio 12 (2021) e0242521.
[142] M. Ogunkola, L. Wolff, E.A. Fenteng, B.R. Duffus, S. Leimkühler, *E. coli* MnmA Is an Fe-S cluster-independent 2-thiouridylase, Inorganics 12 (2024) 67.
[143] N.G. Fox, X. Yu, X. Feng, H.J. Bailey, A. Martelli, J.F. Nabhan, C. Strain-Damerell, C. Bulawa, W.W. Yue, S. Han, Structure of the human frataxin-bound iron-sulfur cluster assembly complex provides insight into its activation mechanism, Nat. Commun. 17 (2019) 2210.
[144] A.M. Edwards, K.A. Black, P.C. Dos Santos, Sulfur availability impacts accumulation of the 2-thiouridine tRNA modification in *Bacillus subtilis*, J. Bacteriol. 204 (2022) e0000922.
[145] C. Desiderio, R.A. Cavallaro, A. De Rossi, F. D'Anselmi, A. Fuso, S.J. Scarpa, Evaluation of chemical and diastereoisomeric stability of S-adenosylmethioninein aqueous solution by capillary electrophoresis, Pharm. Biomed. Anal. 38 (2005) 449–456.
[146] D.H. Flint, R.M. Allen, Iron-sulfur proteins with nonredox functions, Chem. Rev. 96 (1996) 2315–2334.
[147] A.D. Smith, J. Frazzon, D.R. Dean, M.K. Johnson, Role of conserved cysteines in mediating sulfur transfer from IscS to IscU, FEBS Lett. 579 (2005) 5236–5240.
[148] M. Fontecave, S. Ollagnier-de-Choudens, Iron-sulfur cluster biosynthesis in bacteria: mechanisms of cluster assembly and transfer, Arch. Biochem. Biophys. 474 (2008) 226–237.
[149] E.L. McCarthy, S.J. Booker, Destruction and reformation of an iron-sulfur cluster during catalysis by lipoyl synthase, Science 358 (2017) 373–377.
[150] S.I. Tchong, H. Xu, R.H. White, L-cysteine desulfidase: an [4Fe-4S] enzyme isolated from *Methanocaldococcus jannaschii* that catalyzes the breakdown of L-cysteine into pyruvate, ammonia, and sulfide, Biochemistry 44 (2005) 1659–1670.
[151] J. Zhou, L. Pecqueur, A. Aučynaitė, J. Fuchs, R. Rutkienė, J. Vaitekūnas, R. Meškys, M. Boll, M. Fontecave, J. Urbonavičius, B. Golinelli-Pimpaneau, Structural evidence for a [4Fe-5S] intermediate in the non-redox desulfuration of thiouracil, Angew. Chem. Int. Ed. Engl. 60 (2021) 424–431.
[152] J. Fuchs, R. Jamontas, M.H. Hoock, J. Oltmanns, B. Golinelli-Pimpaneau, V. Schünemann, J. Antonio, A.J. Pierik, R. Meškys, A. Aučynaitė, M. Boll, TudS desulfidases recycle 4-thiouridine-5′-monophosphate at a catalytic [4Fe-4S] cluster, Comm. Biol. 6 (2023) 1092.
[153] J.B. Thoden, H.M. Holden, G.A. Grant, Structure of L-serine dehydratase from *Legionella pneumophila:* novel use of the C-terminal cysteine as an intrinsic competitive inhibitor, Biochemistry 53 (2014) 7615–7624.
[154] H. Beinert, M.C. Kennedy, C.D. Stout, Aconitase as iron-sulfur protein, enzyme, and iron-regulatory protein, Chem. Rev. 96 (1996) 2335–2374.
[155] S. Chatterjee, K.F. Parson, B.T. Ruotolo, J. McCracken, J. Hu, R.P. Hausinger, Characterization of a [4Fe-4S]-dependent LarE sulfur insertase that facilitates nickel-pincer nucleotide cofactor biosynthesis in *Thermotoga maritima*, J. Biol. Chem. 298 (2022) 102131.
[156] P. Zecchin, L. Pecqueur, J. Oltmanns, C. Velours, V. Schünemann, M. Fontecave, B. Golinelli-Pimpaneau, Structure-based insights into [4Fe-4S]-dependent sulfur insertase LarE, Protein Sci. 33 (2024) e4874.
[157] J. Abramson, J. Adler, J. Dunger, R. Evans, T. Green, A. Pritzel, O. Ronneberger, L. Willmore, A.J. Ballard, J. Bambrick, S.W. Bodenstein, D.A. Evans, C.C. Hung, M. O'Neill, D. Reiman, K. Tunyasuvunakool, Z. Wu, A. Žemgulytė, E. Arvaniti, C. Beattie, O. Bertolli, A. Bridgland, A. Cherepanov, M. Congreve, A.I. Cowen-Rivers, A. Cowie, M. Figurnov, F.B. Fuchs, H. Gladman, R. Jain, Y.A. Khan, C.M. R. Low, K. Perlin, A. Potapenko, P. Savy, S. Singh, A. Stecula, A. Thillaisundaram, C. Tong, S. Yakneen, E.D. Zhong, M. Zielinski, A. Žídek, V. Bapst, P. Kohli, M. Jaderberg, D. Hassabis, J.M. Jumper, Accurate structure prediction of biomolecular interactions with AlphaFold 3, Nature 630 (2024) 493–500.

[158] L.G. Dolce, A.A. Zimmer, L. Tengo, F. Weis, M.A.T. Rubio, J.D. Alfonzo, E. Kowalinski, Structural basis for sequence-independent substrate selection by eukaryotic wobble base tRNA deaminase ADAT2/3, Nat. Commun. 13 (2022) 6737.
[159] V.M. Ruiz-Arroyo, R. Raj, K. Babu, O. Onolbaatar, P.H. Roberts, Y. Nam, Structures and mechanisms of tRNA methylation by METTL1-WDR4, Nature 613 (2023) 383–390.
[160] N.E. Abbassi, M. Jaciuk, D. Scherf, P. Böhnert, A. Rau, A. Hammermeister, M. Rawski, P. Indyka, G. Wazny, A. Chramiec-Głąbik, D. Dobosz, B. Skupien-Rabian, U. Jankowska, J. Rappsilber, R. Schaffrath, T.Y. Lin, S. Glatt, Cryo-EM structures of the human elongator complex at work, Nat. Commun. 15 (2024) 4094.