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Abstract

In all domains of life, ribonucleic acid (RNA) maturation includes post-transcriptional chemical modifications of nucleosides. Many sulfur-containing nucleosides have been identified in transfer RNAs (tRNAs), such as the derivatives of 2-thiouridine (s^2U), 4-thiouridine (s^4U), 2-thiocytidine (s^2C), 2-methylthioadenosine (ms^2A). These modifications are essential for accurate and efficient translation of the genetic code from messenger RNA (mRNA) for protein synthesis. This review summarizes the recent discoveries concerning the mechanistic and structural characterization of tRNA thiolation enzymes that catalyze the non-redox substitution of oxygen for sulfur in nucleosides. Two mechanisms have been described. One involves persulfide formation on catalytic cysteines, while the other uses a [4Fe-4S] cluster, chelated by three conserved cysteines only, as a sulfur carrier.

Introduction

tRNAs are key players in genetic code decoding, a fundamental process in all living organisms. All tRNAs feature post-transcriptional chemical modifications [1] that stabilize their tertiary structure and fine-tune the decoding process [2,3]. Sulfur, an essential element in life, is present in several cofactors and tRNAs: at positions 8, 9 in the core, 32, 33, 34, 37 around the anticodon and 54 in the T-loop (Figure 1A) [4,5]. The formation of 2-thiouridine (s^2U), 4-thiouridine (s^4U), 2-thiocytidine (s^2C) and 2-methylthioadenosine (ms^2A) is catalyzed by specific enzymes called ThiI, TtcA, MnmA/Ctu1/Tuc1/Ncs6, MiaB and MtaB, TtuA [4,5] acting at positions 8, 32, 34, 37 and 54, respectively (Figure 1A). Because most *thiI* genes play no role in thiamine biosynthesis [6], ThiI is renamed here TtuI for **t**RNA **t**hio**u**ridine I.

There are two main classes of tRNA thiolation reactions. The insertion of sulfur within an inert C-H bond is an [Fe-S]-dependent redox reaction catalyzed by the radical *S*-adenosyl-L-methionine (SAM) methylthiotransferases MiaB and MtaB. Because their structures remain unknown and their mechanisms have recently been reviewed [7,8], this class will not be discussed here. The non-redox substitution of oxygen for sulfur (Figure 1A), is catalyzed by ATP-dependent tRNA thiolases that share a pyrophosphatase (PPase) domain (Figure S1). We review here their crystal structures and catalytic mechanisms in light of research from the last two years showing that several of these enzymes are dependent on a [4Fe-4S] cluster (Table S1).

Sulfur relay system

Formation of persulfides on reactive cysteines

The biosynthesis of sulfur-containing nucleosides involves several proteins that relay sulfur atoms originating from L-cysteine to tRNA [4,9,10]. In most cases, a pyridoxal-5'-phosphate-dependent cysteine desulfurase (IscS/Nsf1, YrvO, Nifz) first uses L-cysteine to form an enzyme-bound cysteine persulfide whose sulfur is next transferred to an acceptor protein [11-15]. This transfer is usually monitored by detecting, upon incubation with [^{35}S]-L-cysteine, radioactive sulfur on the acceptor protein on a non-reducing SDS gel [16]. This labeling experiment monitors the formation of persulfides on reactive cysteines *in vitro* but does not demonstrate that the persulfide on the acceptor is an intermediate in nucleoside thiolation *in vivo.* Indeed, a persulfide adduct was formed on MmTtuI and MmNcs6 [17,18] although these enzymes use a [Fe-S] cluster [19], not a mechanism based on persulfide chemistry (see below). In fact, persulfides are formed on cysteines that ligate an [Fe-S] cluster like persulfides and polysulfides are generated by IscS on the cysteines of protein scaffold IscU during [Fe-S] cluster biogenesis [20,21].

Transporter proteins provide sulfur as a persulfide or thiocarboxylate for tRNA thiolation

tRNA thiolation activity is routinely followed by monitoring [^{35}S] incorporation into tRNA in the presence of [^{35}S]-L-cysteine, cysteine desulfurase and acceptor proteins, but it is rarely quantified. While a cysteine desulfurase and the thiolation enzyme are sufficient for tRNA thiolation by TtuI proteins [13,16] or BsMnmA [15], the L-cysteine sulfur is often relayed to various carrier proteins before the final incorporation into tRNA, as shown for biosynthesis of s^2U34 in *Escherichia coli* [22], eukaryotes [23,24] and archaea, and s^2U54 in *Thermus thermophilus* [25-27, 28••] (Figure S2).

To identify the genes involved in s^2U34-tRNA biosynthesis in *E. coli*, tRNA was extracted from gene-deletion strains and analyzed for modified uridine. This led to the finding that, in addition to IscS and EcMnmA, TusA, a TusBCD complex and TusE are needed for s^2U34-tRNA formation (Figure S2A). This TusABCDE sulfur pathway is mainly restricted to γ-proteobacteria [9].

Other sulfur transfer relays generate a thiocarboxylate at the carboxy-terminal glycine of an ubiquitin-like protein as the ultimate sulfur donor for s^2U34 or s^2U54-tRNA thiolation (Figure S2B) [29,30]. The best-studied sulfur transfer pathway is the TtuABCD pathway for U54-tRNA thiolation in *T. thermophilus* [25-27,28••,31••]. The ultimate step consists in TtTtuA transferring sulfur from TtTtuB-COSH to tRNA [28••,31••] (see below).

U8-tRNA 4-sulfurtransferase TtuI (**t**RNA **t**hio**u**ridine **I**).

s^4U at position 8 in the loop connecting the acceptor and D-stems of bacterial and archaeal tRNAs (Figure 1A) mediates cellular responses to UV stress [32]. In *E. coli* and *Bacillus subtilis*, TtuI and the cysteine desulfurase IscS [33,34] or NifZ [13], respectively, are required for s^4U8-tRNA thiolation. TtuI enzymes have three conserved domains (Figures S1 and 2A). Genomic analysis of the *ttuI* gene family identified two groups [9]: organisms like *E. coli* [35] that possess an additional C-terminal rhodanese-like domain (RHD) and a larger family of organisms that includes *Bacilli* or *Thermotoga maritima* that do not.

One of the first steps of the TtuI mechanism consists in the formation of an O-adenylated tRNA intermediate by adenylation by ATP (Figure 1B) [36]. Site-directed mutagenesis, labeling and chemical trapping experiments, together with mass spectrometry have shown that EcTtuI can accept sulfur from IscS as a persulfide on Cys456 on the RHD [35,37,38]. Mass spectrometry [38] and radiolabeling experiments [35,37] have also revealed the formation of a Cys344-Cys456 disulfide bond in EcTtuI. However, the exact mechanism of sulfur insertion into tRNA by EcTtuI is still unknown. Two possible scenarios involving two catalytic cysteines have been proposed (Figure 1B). Both mechanisms include a nucleophilic attack on the adenylated nucleoside, by either the Cys456 persulfide or hydrogen sulfide, and the formation of a Cys344-Cys456 disulfide bond [32]. Yet, Cys344 was found not to be absolutely required for catalysis [32], which casts doubts on these mechanisms. The hypothesis that EcTtuI could possess an [Fe-S] cluster has previously been dismissed because overexpression in the presence of IscS did not show spectroscopic signature of a cluster [32]. However, given that other residues than cysteine can ligate a [Fe-S] cluster [39] and in view of the expanding number of tRNA thiolation enzymes that are now being shown to use a [4Fe-4S] cluster (see below), anaerobic cluster reconstitution assays should be attempted before definitively ruling out the involvement of a cluster in the reaction. Obtaining the structure of EcTtuI would also be of great help to solve the catalytic mechanism.

TtuI proteins that lack the RHD possess only one conserved cysteine, equivalent to Cys344 in EcTtuI (Figure S1), so that their catalytic mechanism is even more enigmatic. The structures of BaTtuI in complex with AMP [40], TmTtuI in complex with a minimal RNA substrate, in the absence and presence of ATP [41], and PH1313, a truncated TtuI-like protein [42] have been solved (Table S1, Figure 2A and B). In BaTtuI and TmTtuI, a similar homo-dimer is formed, mainly through the PPase domains (Figure 2B). The TmTtuI-RNA-ATP ternary complex (Figure 2C) represents an inactive initial state because flipping out of U8, needed to expose the target base for sulfur insertion, and adenylation were not observed [41]. The role of the two cysteines of TmTtuI (Figure 2C) was probed *in vivo* and *in vitro*, which showed that only Cys344 of TmTtuI has a catalytic role [41]. The active site loop carrying Cys344 is fully disordered in all TtuI structures in the absence of RNA and shows various conformations in the TmTtuI/RNA complex. In the closed conformation, Cys344 is buried, close to ATP, leaving space for U8 to flip (Figure 2D). In addition to anaerobic cluster reconstitution assays, several residues in the active site, besides Cys344, should be mutated to help to solve the mechanism.

Outstandingly, an [Fe-S]-containing TtuI protein was recently uncovered in archaea [19]. The two cysteines of the CXXC motif in the PPase domain of MmTtuI (Figure S1) had previously been shown to be necessary for *in vivo* formation of s^2U in *Methanococcus maripaludis,* and, together with a third conserved cysteine, to be the sites for persulfide formation *in vitro* [17]. In fact, these three cysteines chelate a [Fe-S] cluster [19]. Although spectroscopic data and activity tests indicated that anoxically purified MmTtuI contains a [3Fe-4S] cluster necessary for activity, we believe that the thiolation activity depends on a [4Fe-4S] cluster, like in all other enzymes of the TtcA/TtuA family (Figure S1) (see below). Indeed, we found that MmTtuI purified under strict anaerobic conditions contains the [4Fe-4S] form of the cluster, which was required for catalysis (He and Golinelli-Pimpaneau, unpublished). $[3Fe\text{-}4S]^+$ clusters are known to result from air degradation of $[4Fe\text{-}4S]^{2+}$ clusters [43]. Therefore, some residual [4Fe-4S] cluster is likely present in the anoxically purified enzyme, which could account for its tRNA thiolation activity. The CXXC motif of MmTtuI is conserved in other TtuI proteins from several euryarchaeota and crenarchaeota [17], which therefore most probably operate with a [4Fe-4S] cluster.

C32-tRNA 2-sulfurtransferase TtcA (tRNA-2-thiocytidine A)

TtcA enzymes target cytidine at position 32 near the anticodon in tRNAs (Figure 1A). The [Fe-S]-dependent TtcA/TtuA family was first identified following the characterization of *E. coli* and *Salmonella thyphimurium* strains deficient in s^2C-modified tRNAs [44]. This class is characterized by a CXXC sequence motif in the central region (Figure S1). Analysis of tRNA from mutated strains

indicated that the two cysteines in this motif are required for s^2C formation [42]. Site-directed mutagenesis then confirmed that these cysteines, together with a third conserved cysteine, bind a [4Fe-4S] cluster in TtcA from *E. coli*, which is crucial for *in vitro* C32-tRNA thiolation [45]. A mechanism was proposed in which the sulfur atom from the persulfide of IscS-SSH is first transferred to the accessible coordination site of the [4Fe-4S] cluster. Then, the sulfur attached to the [Fe-S] cluster would act as a nucleophile to attack the adenylated target cytidine, releasing AMP and liberating s^2C32-tRNA. No structure of a TtcA enzyme is known to date.

Thiolation of U34

Sulfuration of U34 at the wobble position of the anticodon in Glu-tRNA, Gln-tRNA and Lys-tRNA (Figure 1A) is conserved in all organisms and guarantees fidelity of protein translation [46]. Lack of s^2U34-tRNA results in severe growth reduction [12,15,18,47-49]. Two distinct enzyme families of the MnmA-types and Ncs6-types catalyze s^2U34-tRNA formation (Figure S1). MnmA-like proteins operate in bacteria [22,50,51] and mitochondria [52], and Ncs6-like proteins in archaea and the eukaryotic cytosol. Eukaryotic Ncs6-like enzymes work as a complex with an Ncs2/Ctu2 protein [47-49]

U34-tRNA 2-sulfurtransferase MnmA (tRNA 2-methylaminomethylthiouridylase A)

The crystal structure of EcMnmA has been determined in three states in complex with tRNA, representing the initial tRNA binding, pre-reaction and adenylated states (Figure 3A-D) [51]. In all structures, U34 is stabilized in a flipped-out conformation by an edge-to-face interaction with His128, conserved in tRNA thiolation enzymes modifying U at the C2 position (Figure S1). The structures show that conserved cysteines Cys102 and Cys199, which are required for *in vitro* thiolation of U34-tRNA, are located near the ATP binding site [51]. In addition, a sulfur transfer experiment using [^{35}S]-Cys in the presence IscS, TusA, TusBCD and TusE showed increased [^{35}S] labeling of the EcMnmA-C102S mutant and diminished labeling of the EcMnmA-C199S mutant compared to wild-type EcMnmA [51]. This suggested a mechanism similar to EcTtuI (Figure 2B), in which Cys199 accepts sulfur as a persulfide from the relay system, whereas Cys102 assists catalysis by forming a covalent linkage with Cys199. Asp99 was hypothesized as the acid/base catalyst that protonates/deprotonates the N3 atom of the target base but His128 is better positioned to play this role (Figure 3B). Asp99 is replaced by Cys99 in a broad range of thermophilic bacteria, leading to the distinction between C-type MnmAs with a CXXC + C motif and D-type MnmAs with a DXXC + C motif [53•]. Remarkably, C-type MnmA from *T. thermophilus* was recently shown to contain a [4Fe-4S] cluster that is essential for catalysis [53•]. Thus C-type MnmA proteins share a catalytic mechanism similar to that of the TtcA/TtuA family (Figure 1C).

U34-tRNA 2-sulfurtransferase Ctu1 (cytosolic 2-thiouridine 1)

Enzymes targeting U34-tRNA in archaea and eukaryotic cytosols belong to the [Fe-S]-dependent TtuA subfamily [54]. This subfamily differs from the TtcA subfamily by the presence of two additional Zn finger domains at the N-termini and C-termini (Figure S1) [44]. Spectroscopic data of MmNcs6 and human Ctu1, purified under anoxic conditions, indicated that they contain a [3Fe-4S] cluster [19]. By contrast, we observed that MmNcs6, after anaerobic reconstitution of the cluster, contains a [4Fe-4S] cluster that is essential for U34-tRNA thiolation (Bimai, unpublished; PDB code 6SCY). The [4Fe-4S] is the active state, not the [3Fe-4S] cluster, in agreement with the fact that there is no known example of a [3Fe-4S] cluster being an active state in bioorganic chemistry and that [3Fe-4S] clusters are the first intermediates appearing upon air-degradation of [4Fe-4S] clusters [43]. The [4Fe-4S] cluster is coordinated by the three conserved cysteines characteristic of the TtcA/TtuA family (Figure 3C) and electron density on the fourth iron atom indicates the propensity of the [4Fe-4S] cluster to bind a small ligand.

U54-tRNA 2-sulfurtransferase TtuA (tRNA-2-thiouridine A)

s^2U at position 54 in the T-loop of tRNAs (Figure 1A) stabilizes its ternary structure in thermophilic bacteria and archaea for growth at high temperature [25]. Spectroscopic and biochemical analyses have shown that TtTtuA, PhTtuA and TtuA from *T. maritima* use a [4Fe-4S] cluster for U54-tRNA thiolation [28••,55••]. Thiolation did not occur in the absence of a sulfur source (Na_2S [28••,55••] or TtTtuB-COSH

[28••]), indicating that the sulfur atom incorporated into the nucleoside does not come from the cluster. The reaction was independent on the oxidation state of the cluster, indicating that this cofactor does not play a redox role in catalysis [28••,55••].
Several crystal structures of PhTtuA and TtTtuA were obtained: without the cluster [56], with the cluster [28,55], with both the cluster and an ATP analog [28••] or the AMP product [55••] (Figures 4A and B). These structures revealed that the [4Fe-4S] cluster is coordinated by 3 cysteines only (Figures 4C and D, which are conserved in the whole TtcA/TtuA family (Figure S1), and that the cluster is located inside the catalytic pocket, near the ATP binding site. The presence of extra electron density on the fourth iron atom of the cluster of PhTtuA, not bound to the protein, was attributed to a labile hydrosulfide ion (Figure 4C) [55••].
Finally, the structure of TtTtuA was determined in complex with TtTtuB (Figure 4E and F), which supplies the sulfur atom to TtTtuA as a thiocarboxylate on its terminal glycine (Figure S2B). In the structure of TtTtuA (with no cluster) in complex with a TtTtuB mutant containing cysteine instead of glycine at C-terminus, the two terminal residues of TtTtuB were disordered [28]. Upon TtTtuB binding, the loop containing Cys222, one of the [Fe-S] cluster ligands, was reorganized to cover the active site of TtTtuA and connect the cluster to the exterior through a positively charged channel corresponding to the tRNA binding site, thus indicating that TtuB and the tRNA can simultaneously access the TtuA active site. Moreover, the structure of [4Fe-4S]-TtTtuA in complex with non-mutated TtTtuB was recently obtained [31••]. Remarkably, it shows how the C-terminus carboxylate of TtuB occupies the free coordination site of the [4Fe-4S] cluster (Figure 4F). The C-terminus carboxylate mimics the C-terminus thiocarboxylate, suggesting that the cluster has a catalytic role and can bind the thiocarboxylate carried by TtuB and deliver its sulfur to the tRNA. This confirms a mechanism for [4Fe-4S]-dependent thiolases in which the [4Fe-4S] cluster acts as a Lewis acid to bind and activate the sulfur of the substrate, playing the role of sulfur carrier (Figure 1C).

Conclusion

Although a general mechanism for tRNA thiolation was initially proposed, in which a persulfide attached to a catalytic cysteine is the sulfur donor for tRNA thiolation [16,51,57], there is increasing evidence that a sulfur-containing species bound to a [4Fe-4S] cluster, ligated to three cysteines only, can be the sulfurating agent [28••,31••,55••, Bimai, unpublished]. According to this finding, the tRNA thiolation enzymes for which a low *in vitro* activity has been detected and/or for which the catalytic residues remain undetermined, should have their mechanism revisited for the possibility that it involves a [4Fe-4S] cluster. Attempts to reconstitute a cluster under anaerobic conditions should be carried out to know if these enzymes can bind a [Fe-S] cluster. Indeed, the existence of [4Fe-4S] clusters has been overlooked in the past because usually only overexpressed proteins are examined, leaving open the possibility that the high level of expression swamps the capacity to generate iron-sulfur clusters. Moreover, enzyme production and activity tests are generally carried out under aerobic conditions in which the cluster is labile. A small amount of [4Fe-4S] cluster could be present in the as-purified protein, producing active enzyme and leading to a misleading conclusion about the mechanism.
In many organisms, thiolation of tRNA nucleosides involves a sulfur-relay system that carries sulfur, first removed from L-cysteine by a cysteine desulfurase, and finally used as a substrate by the tRNA thiolase. Persulfides or thio-carboxylates bound to the C-terminus of a protein carrier are thought to be the ultimate sulfur donor, allowing sulfur transfer in a nontoxic form. A direct transfer from various sulfur donors to the [4Fe-4S] cluster of some tRNA thiolation enzymes has been proposed. For TtcA, the persulfide on cysteine desulfurase may provide its terminal sulfur to the cluster, whereas the [4Fe-4S]-TtTtuA/TtTtuB structure indicates that the thiocarboxylate on the C-terminal glycine of the sulfur carrier protein TtTtuB could attach to the non-ligated iron of the cluster before its desulfurization to give rise to a [4Fe-5S] intermediate [31••]. In organisms living in high sulfide concentration, a hydrosulfide ion from the medium could directly bind the cluster.
Only two crystal structures of tRNA thiolation enzymes have been determined in complex with RNA substrate [41,51] (Table S1). More structures of such complexes are needed to understand the specificity for tRNA and the catalytic mechanism.

Conflict of Interest statement

Nothing declared

References and recommended reading

Papers of particular interest within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest

Acknowledgements.

We thank Marc Fontecave for sharing his enthusiasm for the iron-sulfur field and giving us competent advices all along this work.

This work was supported by the French State Program 'Investissements d'Avenir' (Grants "LABEX DYNAMO", ANR-11-LABX-0011) and the CNRS.

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•• Activity assays and spectroscopic characterization of TtuA from *Pyrococcus horikoshii* and *Thermotoga maritima* indicate that both enzymes use a [4Fe-4S] for thiolating U54-tRNA. The crystal structure of the [4Fe-4S]-containing PhTtuA shows electron density on the fourth non-bonded iron atom of the cluster, suggesting that it could bind the hydrosulfide substrate. A molecular mechanism in which the [4Fe-4S] is used to bind and activate sulfur is proposed.
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Legend Figures

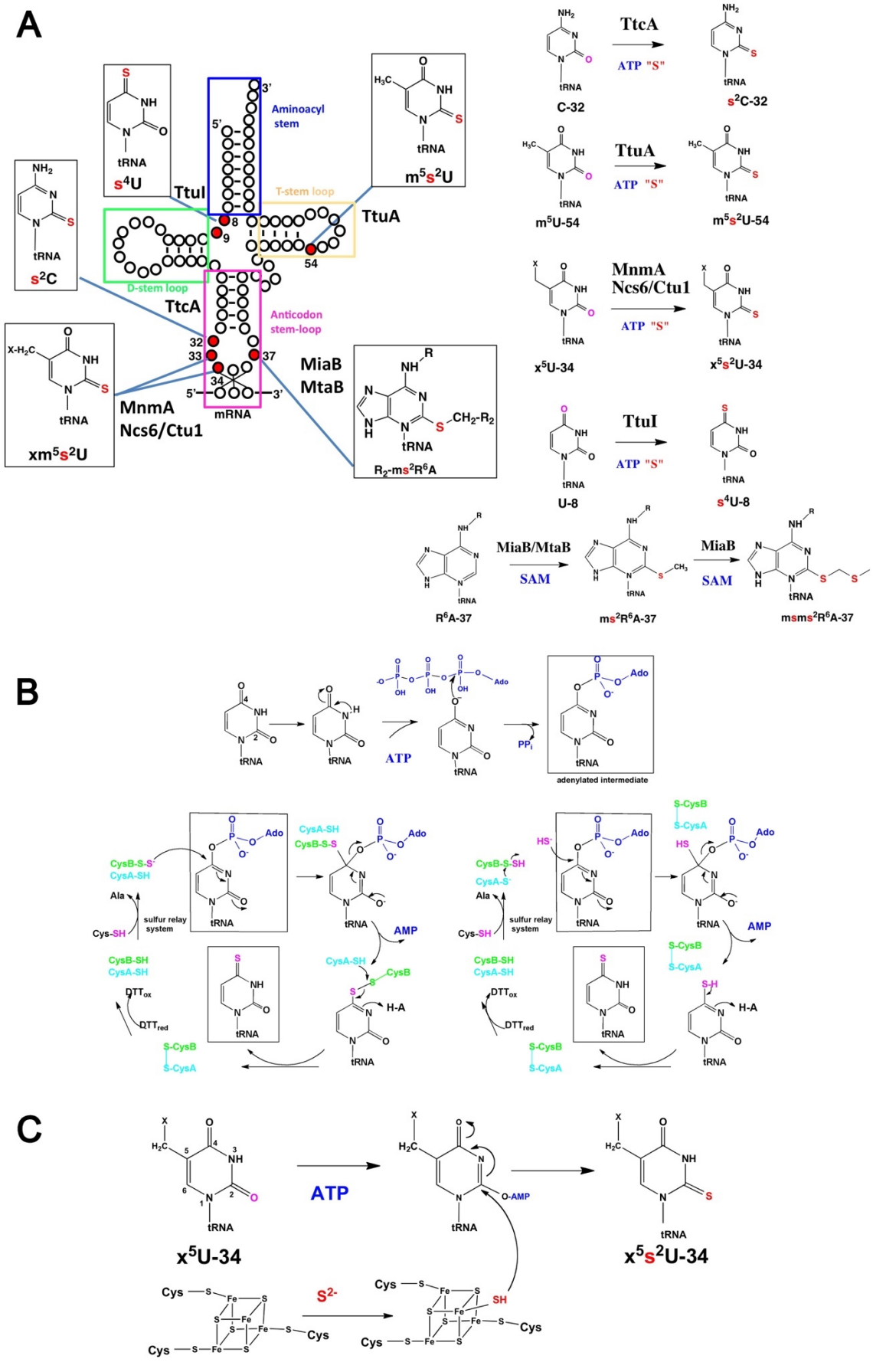
Figure 1: **Reactions catalyzed by tRNA thiolation enzymes and proposed mechanisms.**
A tRNA thiolation enzymes catalyzing sufur insertion in tRNAs. U34 of $tRNA^{Gln}_{UUG}$, $tRNA^{Lys}_{UUU}$ and $tRNA^{Glu}_{UUC}$ is universally thiolated at C2 position and hypermodified by different chemical groups at C5 position depending on the organism [1]. MiaB recognizes i^6A (R=isopentenyl) and catalyzes the formation of ms^2i^6A (R_2=H) and $msms^2i^6A$ (R_2=CH_3-S-) while MtaB recognizes t^6A (R=threonyl carbamoyl) and catalyzes the formation of ms^2t^6A and its cyclic form ms^2ct^6A. **B** Persulfide-based mechanism for U8 or U34 sulfuration proposed for EcTtuI [32] and EcMnmA [51], which involves two catalytic cysteines (CysA and CysB). CysA corresponds to Cys344 in EcTtuI and Cys199 in EcMnmA, while CysB correspond to Cys456 in EcTtuI and Cys102 in EcMnmA. **C** [4Fe-4S]-dependent thiolation mechanism, in which the [4Fe-4S] cluster functions as a sulfur-carrier. The mechanism is depicted for U34-tRNA thiolation. The cluster is bound by three cysteines only. The vacated iron site can bind a sulfur-containing ligand: a thiocarboxylate at the C-terminus of a sulfur-donor protein, a protein-bound persulfide or a hydrosulfide ion from the medium.

Figure 2: Comparison of the crystal structures of U8-tRNA thiolases. A Superposition of the monomers of TtuI proteins. The monomers of BaTtuI (in blue) in complex with AMP (in blue sticks), and TmTtuI (in green) in complex with ATP and Mg^{2+} (red sticks and balls), were superimposed onto PH1313 (in grey) with rmsd of 2.5 Å for 222 Cα atoms and 1.82 Å for 180 Cα atoms, respectively. PH1313 has a truncated PPase domain and lacks several conserved residues, including some in the PP-loop motif (Figure S1). Catalytic Cys344 belongs to loop 342-355 that is disordered in BaTtuI and deleted in PH1313. Cys344 of TmTuI is indicated in red sticks. **B** Superposition of the PPase domains of the BaTtuI and TmTtuI dimers (rmsd of 0.49 Å for 44 Cα atoms). The mini-RNA in complex with TmTtuI is shown in orange. The superposition shows that the dimeric interface is formed even in the absence of RNA and that RNA binding results in a conformational change of the THUMP and NFLD domains relative to the PPase dimeric core. **Insert**. Zoom of the ATP binding site and the dimeric interface between two PPase domains. Superposition of one PH1313 monomer (in grey) with that of BaTtuI and TmTtuI shows that a displacement of the C-terminal helix of PH1313 (in pink) hinders formation of the same dimeric interface as BaTtuI and TmTtuI. **C** General view of the TmTtuI dimer in complex with mini-RNA and ATP. In each subunit, the RNA acceptor-stem is bound by the NFLD and the THUMP domains, and the bulge is recognized by the NFLD. Building a TmTtuI–tRNA model by superimposing the acceptor stems of $tRNA^{Phe}$ and the TtuI-bound truncated RNA results in severe clashes, indicating that full-length tRNA has to adopt a non-canonical conformation upon binding to TtuI. U8 (in blue sticks) is not flipped out and is located away from the ATP binding site (in blue sticks). **D** Zoom of the active site of TmTtuI showing the ATP binding site, including Cys344 and the interface of the PPases domain and the NFLD.

Figure 3. Structures of U34-tRNA thiolases EcMnmA and MmNcs6. A. Superposition of the EcMmnA/tRNA complex in the adenylated state and SpMnmA (in grey) in complex with SAM (in blue sticks) and Mg^{2+} (grey sphere)(rmsd= 1.00 Å for 1896 Cα atoms). U34, U35 and C36 of *E. coli* $tRNA^{Glu}_{UUC}$ are shown as magenta sticks and the adenylated group linked to U34 in orange. U34 is mainly recognized by the PPase domain. Catalytic Cys199 is carried by a 'variable segment' that can adopt different folds. **B** Snapshots of the sequential chemical reactions during EcMnmA catalysis showing U34 recognition by EcMnmA. Top: initial tRNA binding state, middle: pre-reaction state, bottom: adenylated state. In the initial state, the U34 conformation is inactive with respect to adenylation and sulfuration because the O2 thiolation site and the N3 atom are hydrogen bonded to Gln151. In the pre-reaction state, a conformational change of the 'variable segment' closes the active site, which moves U34 close to Cys102 and Cys199 and positions the O2 atom for adenylation by ATP. Cys102 and Cys199 are linked by a disulfide bond in the initial and pre-reaction states. **C** Active site of MmNcs6 seen in the same orientation as EcMnmA. The three cysteines that ligate the cluster in MmNcs6 (Cys142, Cys145, Cys233) are orientated similarly to Asp99, Asp102 and Cys199 in EcMnmA. His169 is oriented similarly to His128 in EcMnmA and could play the same role in catalysis.

Figure 4. Comparison of the structures of [4Fe-4S]-dependent U54 tRNA thiolases. A Superposition of the PhTtuA and TtTtuA dimers. The PPases domains of [Fe-S]-containing (holo)-TtTtuA (monomers in blue and cyan) and holo-PhTtuA (monomers in green and palegreen were superposed (rmsd of 1.5 Å for 44 atoms). AMP and AMPPNP bound to holo-PhTtuA and holo-TtTtuA, respectively, are shown as sticks. The cluster of TtTtuA is shown as spheres (S in yellow, Fe in orange), the Zn atoms of PhTtuA and TtTtuA as grey and black spheres, respectively. **B** Zoom of the active sites of holo-PhTtuA and holo-TtTtuA after superposition. **C and D**: Fobs-Fcalc maps omitting the clusters contoured at 2 σ and 3 σ, for holo-PhTtuA and holo-TtTtuA, respectively, showing that the cluster is bound by three cysteines only. An extra electron density on the fourth, non-protein bonded, iron atom of the cluster of PhTtuA, was attributed to a labile hydrosulfide ion, which could come from inorganic sulfide used for cluster reconstitution. **E**: General view of holo-TtTtuA in complex with TtTtuB (in red) and ATP (in sticks). The structure of holoTtTtuA alone has been superposed and shown in grey. **F**: Zoom of the active site showing that the C-terminal carboxylate group of TtTtuB binds to the fourth, non-protein bonded iron atom of the [4Fe-4S] cluster of holo-TtTtuA.

Figure 1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x⁵U-34
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TtuI
U-8
s⁴U-8
MiaB/MtaB
SAM
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ms²R⁶A-37
msms²R⁶A-37
s⁴U
TtuI
m⁵s²U
Aminoacyl stem
T-stem loop
TtuA
s²C
D-stem loop
TtcA
Anticodon stem-loop
MiaB
MtaB
MnmA
Ncs6/Ctu1
xm⁵s²U
mRNA
R₂-ms²R⁶A
B
ATP
adenylated intermediate
sulfur relay system
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DTT_red
C
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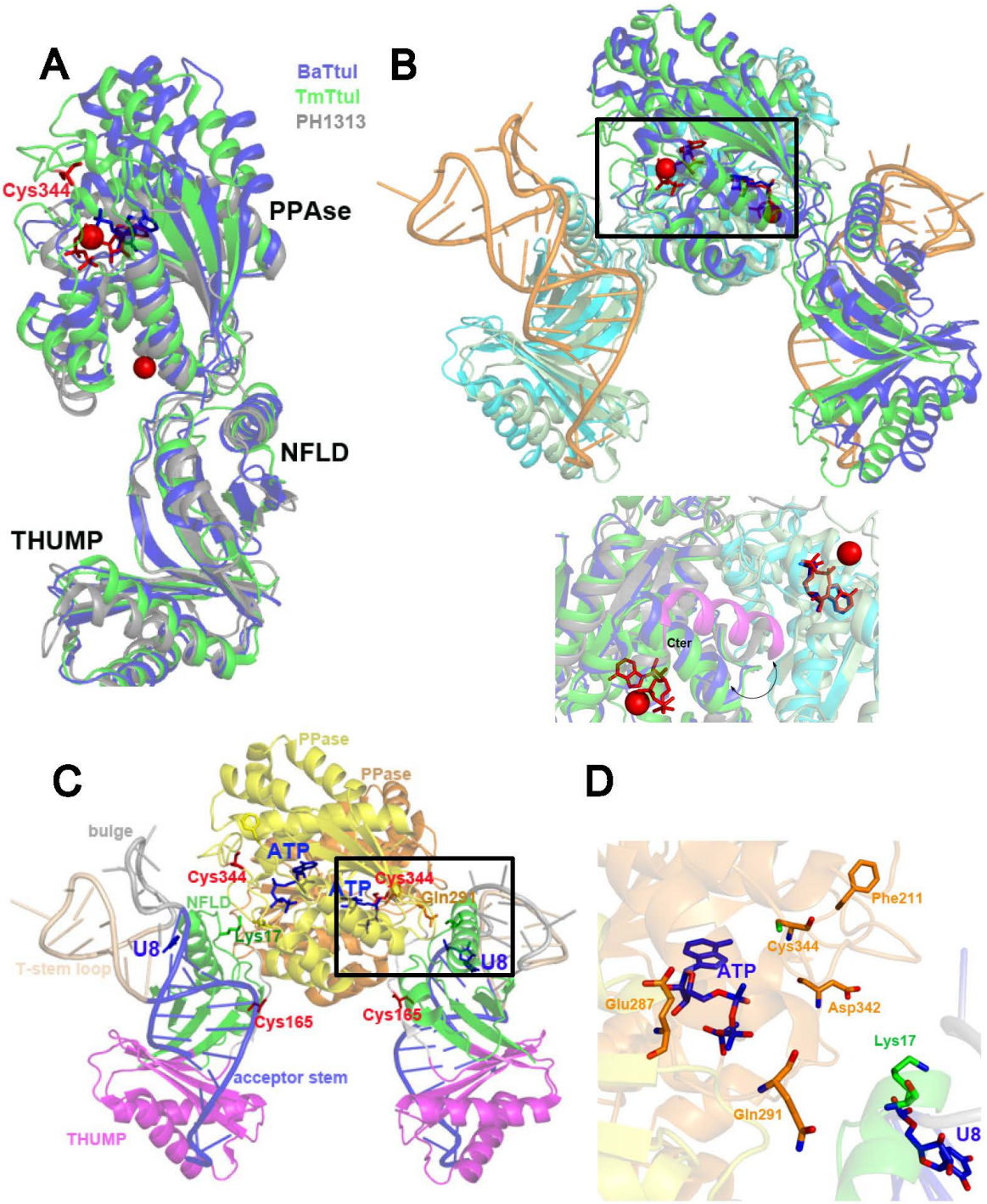
x⁵s²U-34
S²⁻
O-AMP

Figure 2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TmTtuI
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THUMP
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Cter
C
PPase
PPase
bulge
ATP
Cys344
Cys344
ATP
Gln291
NFLD
Lys17
U8
U8
T-stem loop
Cys165
Cys165
acceptor stem
THUMP
D
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Asp342
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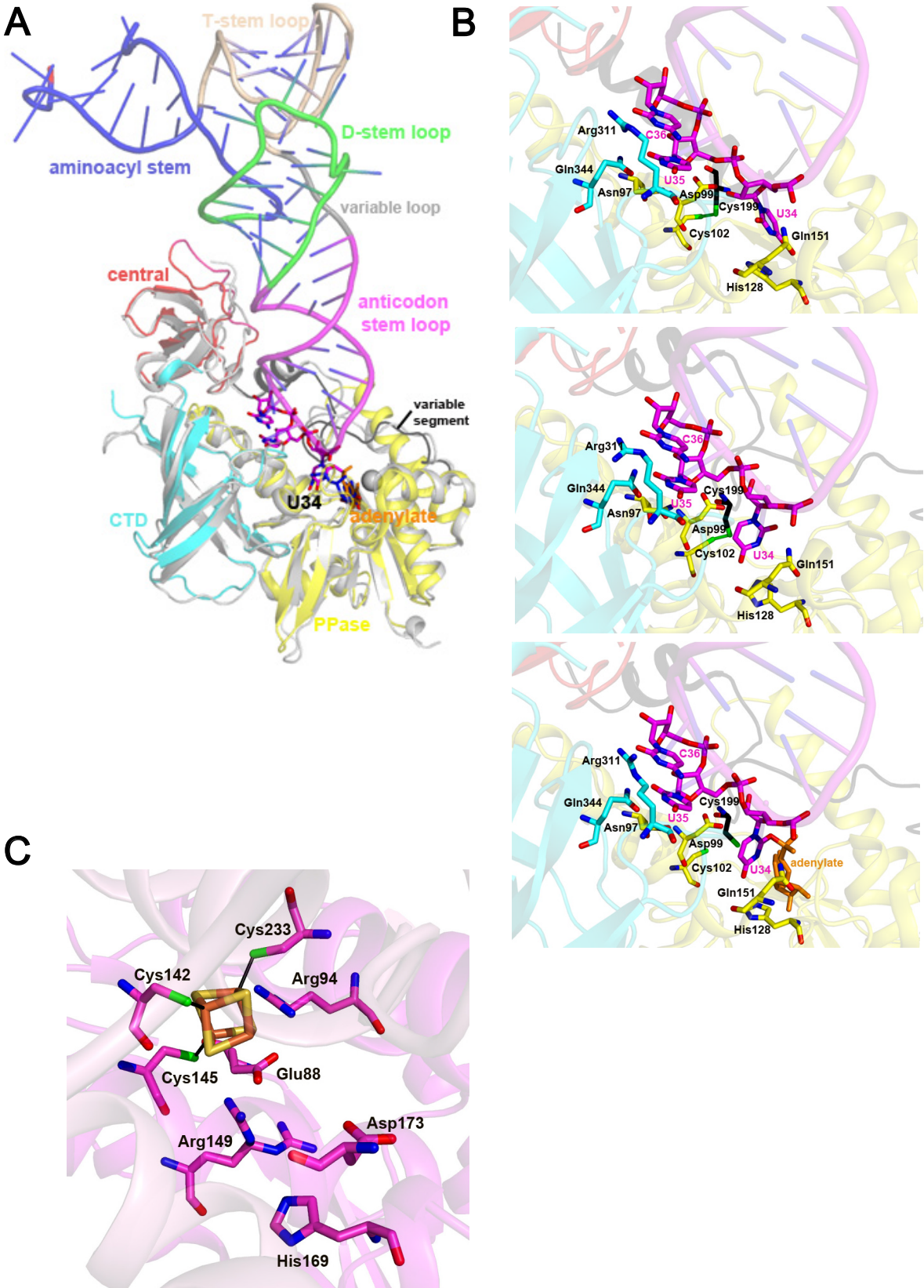
Gln291
U8

Figure 3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D-stem loop
aminoacyl stem
variable loop
central
anticodon
stem loop
variable
segment
U34
adenylate
CTD
PPase
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C36
Gln344
U35
Asn97
Asp99
Cys199
U34
Cys102
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His128
C
Cys233
Cys142
Arg94
Cys145
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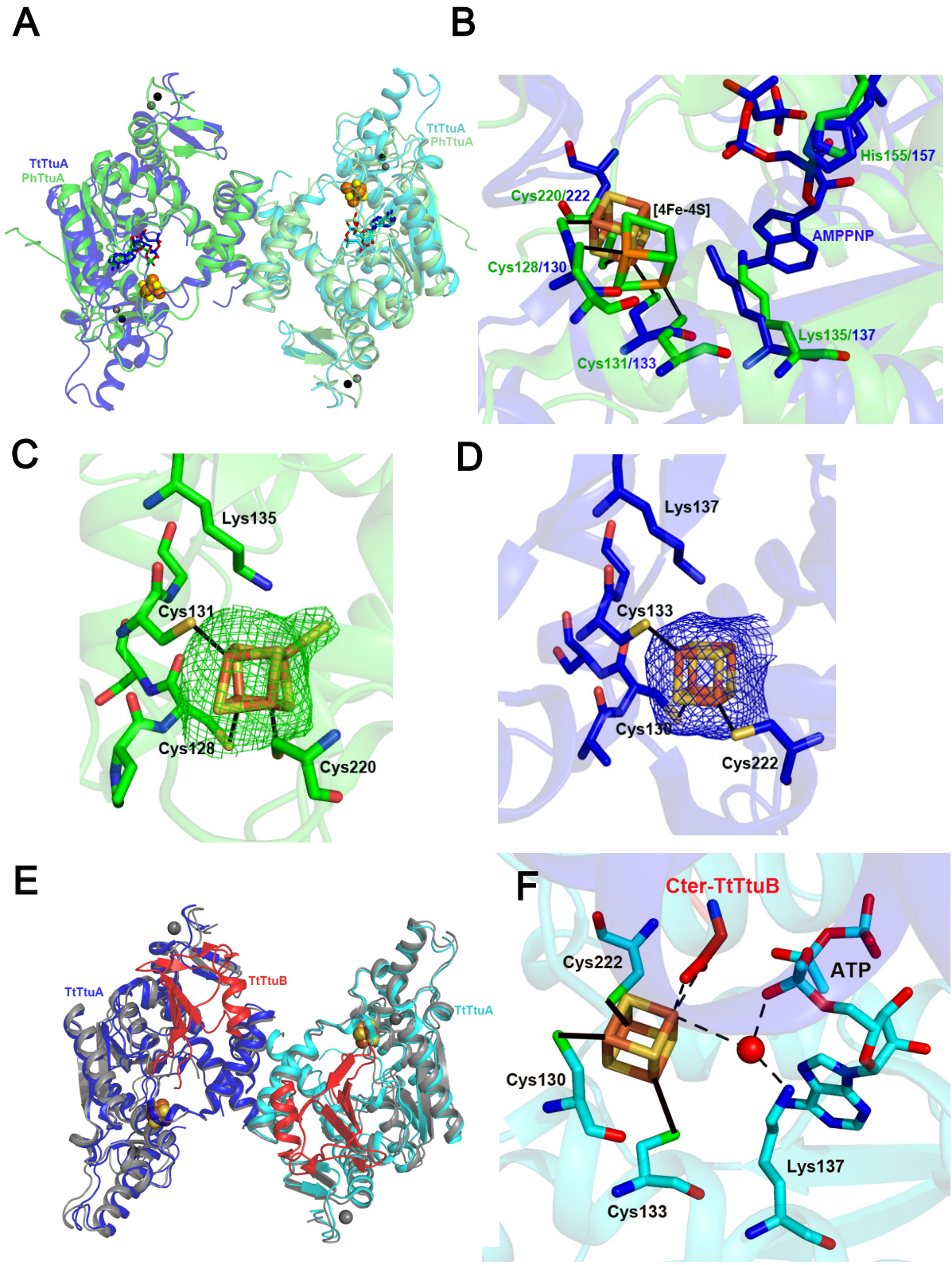
Asp173
Arg149
His169

Figure 4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TtTtuA
PhTtuA
TtTtuA
PhTtuA
B
His155/157
Cys220/222
[4Fe-4S]
AMPPNP
Cys128/130
Lys135/137
Cys131/133
C
Lys135
Cys131
Cys128
Cys220
D
Lys137
Cys133
Cys130
Cys222
E
TtTtuA
TtTtuB
TtTtuA
F
Cter-TtTtuB
Cys222
ATP
Cys130
Lys137
Cys133

Supplementary Figures and Table

Figure S1. **Amino acid sequence alignment of several tRNA thiolases that that have been characterized biochemically or structurally**: TtuA from *Pyrococcus horikoshii* (PhTtuA), *Thermotoga maritima* (TmTtuA) and *Thermus thermophilus* (TtTtuA), TtcA from *Escherichia coli* (EcTtcA), Ncs6 from *Haloferax volcanii Methanococcus maripaludis* (MmNcs6), *Saccharomyces cerevisiae* (ScNcs6), *Homo sapiens* (Ctu1), mitochondrial Mtu1/TrmU, TtuI from *M. maripaludis* (MmTtuI), PH1313, TtuI from *Bacillus anthrasis* (BaTtuI), *B. subtilis* (BsTtuI) and *T. maritima* (TmTtuI). The alignment was performed with Clustal Omega [1] and rendered with ESPript [2]. The secondary structures of PhTtuA and EcMnmA are drawn above and below the alignment, respectively. All enzymes possess an ATP-binding motif indicated as PP-loop. In addition to the common PPase domain (yellow bar), MnmA proteins possess a central domain (red bar) and C-terminal domain CTD (cyan bar); TtuI proteins have an NFLD (N-terminal ferredoxin-like domain) (green bar) and THUMP (pink bar) [3] additional domains. In addition, Ec-TtuI possesses a C-terminal rhodanese-like domain (RHD) (violet bar) [4].
The MnmA- and Ncs6-types families share only 10-15% sequence identity, showing their evolutionary distance. U34-tRNA thiolases are called Ncs6 or NcsA in archaea [5,6], Ncs6p or Tuc1p in yeast [7-9], Ctu1 in nematodes [10], ATPBD3 in humans [11], Rol5 in plants [12,13].
Two clusters of *ttuI* genes have been identified [14]: gamma-proteobacteria such as *E. coli* [15], and archaeal organisms from the *Thermoproteales* or *Thermoplasmatales* orders possess a C-terminal RHD; other proteobacteria, firmicutes such as *Bacilli* and most archaea do not contain the C-terminal extension [14].
Enzymes from the TtcA/TtuA superfamily contain three conserved cysteines (indicated as blue dots) that chelate a [4Fe-4S] cluster. The TtuA and Ncs6 subfamilies also contain two zinc finger motifs at the N- and C-termini whose cysteine/histidine are highlighted by magenta dots. The catalytic cysteines of TtuI and MnmA proteins are shown as green, and orange dots, respectively.
The sequence alignment shows the conservation of two regions outside the PPase-motif: motif 1 comprising residues 138-162 surrounding the strictly conserved Gly154 (PhTtuA numbering) and motif 2 comprising residues 193-208 surrounding the strictly conserved Pro195. First, in motif 1, a histidine, present in all tRNA thiolases targeting the C2 position of uridines, is replaced by Asp/Glu in TtuI enzymes targeting the C4 position of U8. Located at this position, His128 in EcMnmA could act as an acid/base catalyst to deprotonate/protonate N3 of U34-tRNA and/or to stabilize the flipped-out conformation of U34 (Figure 3B). The equivalent residue Glu287 in TmTtuI is in close proximity to the ATP ribose in the TmTtuI/RNA/ATP complex (Figure 2D). Hence, the conserved His/Asp/Glu residue in motif 1 could act as an acid/base catalyst and/or could be involved in the stabilization of the flipped-out conformation of the target base in all tRNA thiolation enzymes, with the conserved glycine enabling its flexibility. Second, the conserved K/R/FPL/F/V/I sequence in motif 2 could participate in the correct positioning of this His/Asp/Glu residue as Phe168 in EcMnmA makes van der Waals interactions to His128 together with Leu155 [16]. The equivalent residue is a conserved Arg/Lys in other tRNA thiolases.

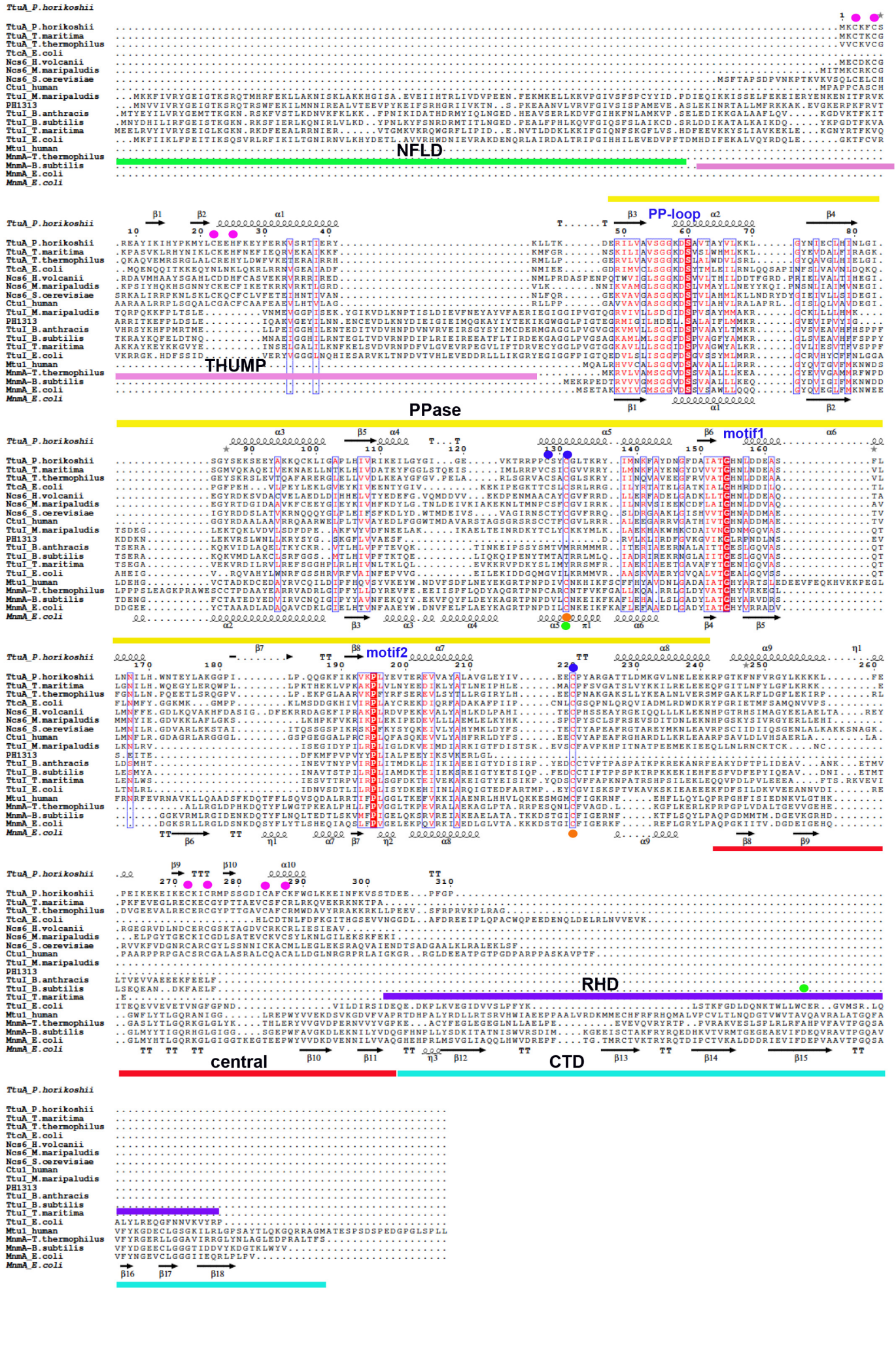
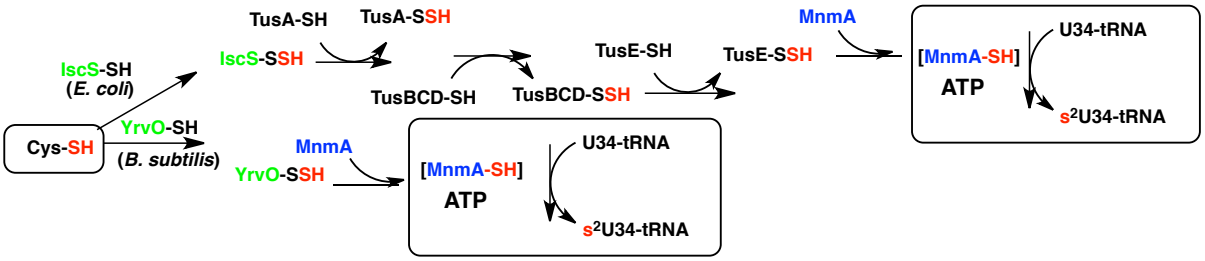

NFLD
THUMP
PPase
motif1
motif2
RHD
central
CTD
PP-loop

Figure S2. Sulfur relay system for the biosynthesis of thionucleosides. In the first step, a cysteine desulfurase (in green) receives sulfur (in red) as a persulfide on a catalytic cysteine. In the last step, sulfur (in the form of a persulfide (A) or a thiocarboxylate (B) bound to a carrier protein) is used as a substrate by the tRNA thiolation enzyme (in blue). **A** The TusABCSD sulfur relay for U34-tRNA thiolation by EcMnmA. The transfer of [^{35}S] from [^{35}S]-IscS to TusA, but not to the TusA-C19S mutant, identified Cys19 as the sulfur acceptor on TusA. Cys78 of TusD and Cys108 of TusE were crucial for s^2U34-tRNA formation *in vivo*, implying their involvement in persulfide relay [17]. It was proposed that the IscS/TusA complex [18] first stimulates IscS activity [19]. After the transfer of the persulfide sulfur of IscS to TusA, sulfur transfer from TusA to TusD is stimulated by the binding of TusE to the TusBCD complex [17]. After sulfur transfer from TusD to TusE, the interaction of TusE with the MnmA-tRNA complex leads to U34-tRNA thiolation. Because EcMnmA was not labeled [19] or poorly labeled [16] in the [^{35}S]-sulfur transfer experiment, it is not clear if the sulfur provided by TusE as a persulfide is accepted directly by EcMnmA or not. This uncertainty is represented as [MnmA-SH]. **B** The TtuABCSD sulfur relay for s^2U54-tRNA thiolation by TtTtuA [20-24] (top) and its equivalent for s^2U34-tRNA thiolation in eukaryotes (bottom). **Top.** The *ttuA, ttuB* and *ttuC* genes are organized as an operon in *T. thermophilus* but not the *ttuD* gene. It was proposed that, first, TtTtuD enhances the activity of cysteine desulfurase (IscS or SufS) and receives sulfur as a persulfide on the catalytic cysteine of one of its RHD [23]. The C-terminal glycine of the ubiquitin-like protein TtTtuB is activated by an ATPase named TtTtuC, resulting in the formation of acyl-adenylated TtTtuB-COAMP [21]. The thiocarboxylation of this intermediate is likely performed by the persulfide bound on TtTtuC. Finally, TtuA is involved in the transfer of the sulfur atom from TtTtuB-COSH to the tRNA [24,25]. **Bottom.** In *S. cerevisiae,* s^2U34-tRNA biosynthesis in the cytosol starts with cysteine desulfurase Nfs1 giving sulfur as a persulfide to a cysteine of the C-terminal RHD of Tum1 [9,26,27]. Once persulfurated, Tum1 likely transfers the sulfur to the RHD of the activating enzyme Uba4 (TtuC homologue). Uba4 adenylates the C-terminus of the ubiquitin-like protein Urm1 (TtuB homologue), and transfers sulfur onto Urm1 to generate a C-terminal thiocarboxylate. Recently, the details of the interaction between Uba4 and Urm1 has been revealed, involving the formation of a thioester between the C-terminal carboxylate of Urm1 and Cys225 of Uba4 [28]. Urm1 is then transferred to the RHD domain of Uba4 via a persulfide generated on Cys397 of Uba4. A subsequent reductive cleavage regenerates Uba4 and releases Urm1 thiocarboxylated at its C-terminus. This thiocarboxylate is finally utilized for 2-thiouridine formation mediated by the tRNA modifying enzyme.
Sulfur carrier proteins involved in such a ubiquitination-related pathway have been identified in yeast [8,9,26,27,29], nematode [30], plant [31] and human [11,32], in the archaeum *H. volcanii* [6,33] and predicted in other archaeal organisms using comparative genomic analysis [14,34].

A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TusBCD-SSH
TusE-SH
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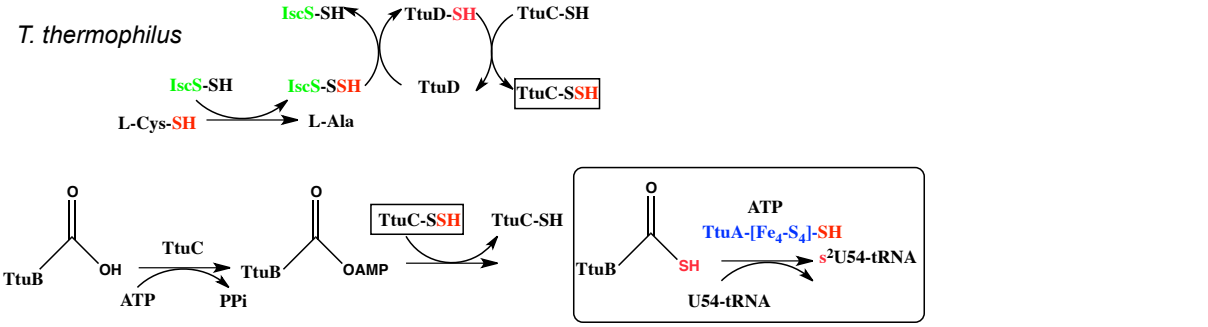
B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L-Ala
TtuB
OH
TtuC
ATP
PPi
TtuB
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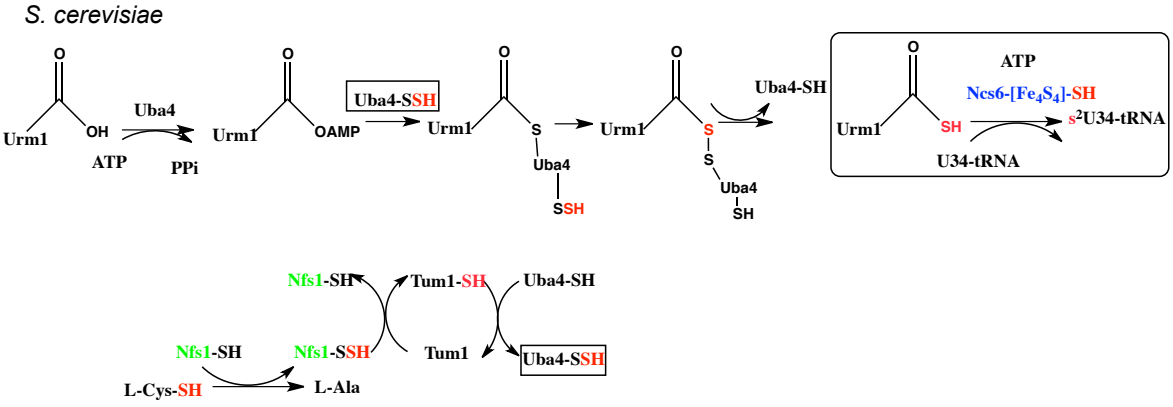
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U34-tRNA

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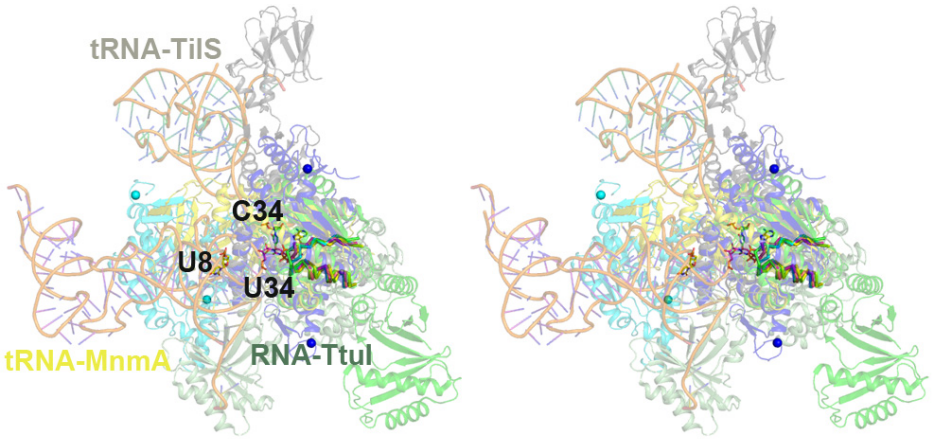
L-Cys-SH
L-Ala

Figure S3: Superposition of the structures of several RNA modification enzymes solved in complex with RNA. **A** Stereoview of the superposition of the PPase cores (shown as ribbons) of GkTilS, (in grey), TtTtuA (monomers in cyan and blue), MmNcsA (monomers in pink and magenta) and TmTtuI (in green and palegreen), EcMnmA (in yellow). RNAs bound to GkTilS, EcMnmA and TmTtuI are shown as tan cartoons. U8 of RNA from TmTtuI and the flipped C34 and U34 target bases of tRNA bound to GkTilS and EcMnmA are represented as sticks. All enzymes catalyzing the replacement of oxygen for sulfur in tRNA, as well as tRNA lysidine synthetase [35] use ATP to activate their target nucleoside. Hence, the ATP binding sites are well conserved (Figure S1) and the superposition of the TtuI/ATP, MnmA/ATP and TtuA/AMPPNP structures shows the same location of the cofactor, with the same H-bonding interactions to the PP-loop motif. However, the tRNA substrates are bound very differently, even for tRNA modifying enzymes targeting the same position such as tRNA lysidine synthetase from *Geobacillus kaustophilus* (GkTilS, PDB code 3A2K) that adds lysine to C34 in tRNAs [35] and EcMnmA that targets U34.



B Comparison of the electrostatic surfaces of GkTilS, PhTtuA, TmTtuI, MmNcs6 and EcMnmA calculated with *PYMOL/APBS* colored by the electrostatic potential from red (negative) to blue (positive). The proteins have been superposed on their PPase core (indicated as green ribbons) and are shown in the same orientation. ATP, as shown in the TmTtuI structure, is shown as sticks in all figures to highlight the ATP binding site. The [4Fe-4S] clusters in PhTtuA and MmNcsA are indicated as spheres. When present, tRNA is shown in cartoon representation with the target base drawn as sticks. This comparison highlights the great variety of charged surfaces used to bind the RNA substrate, indicating that unique tRNA binding modes are probably used by each enzyme.

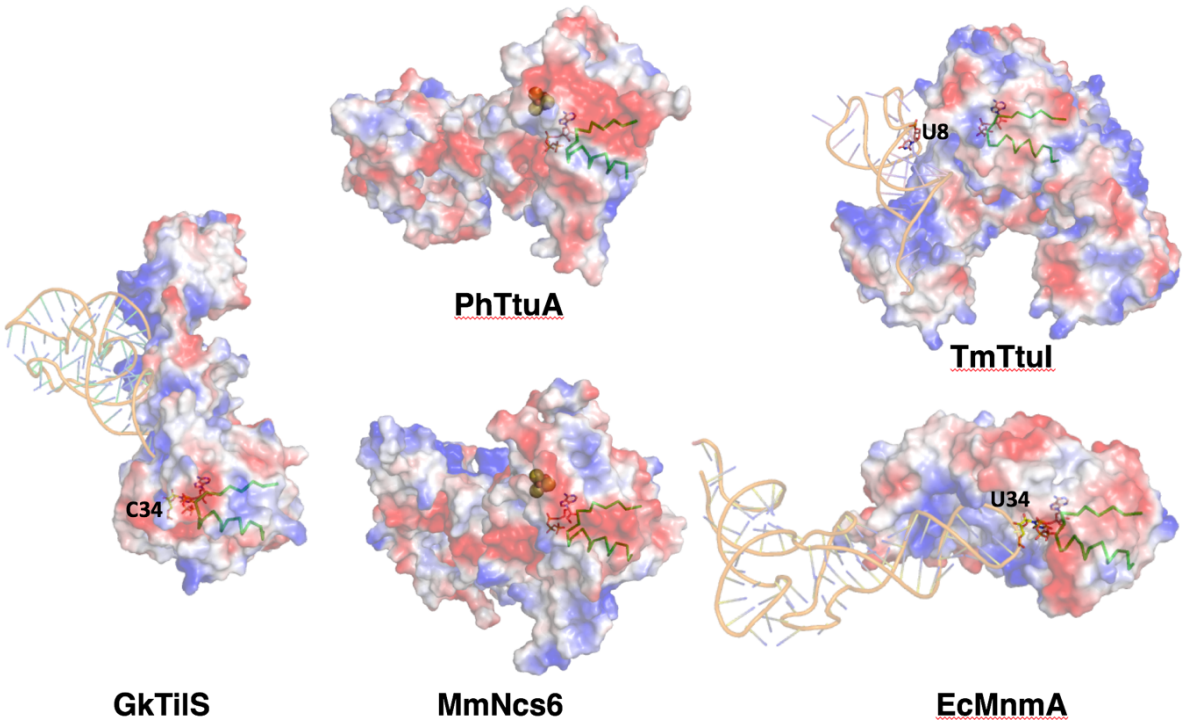


Table S1: Structures of non-redox tRNA thiolation enzymes.

PDB code	Enzyme + ligands	Position of modification	organism	abbreviation	Resolution (Å)	reference
4KR6	TtuI + mini-RNA	U8	*Thermotoga maritima*	TmTtuI	2.85	[36]
4KR7	TtuI + mini-RNA + ATP	U8	*T. maritima*	«	3.42	[36]
4KR9	TtuI + mini-RNA	U8	*T. maritima*	«	3.5	[36]
2C5S	TtuI + AMP	nd	*Bacillus anthracis*	BsTtuI	2.5	[37]
1VBK	PH1313	nd	*Pyrococcus horikoshii*	PH1313	1.9	[38]
2DER	MnmA + $tRNA^{Glu}$ (initial binding state)	U34	*Escherichia coli*	EcMnmA	3.1	[16]
2DET	MnmA + $tRNA^{Glu}$ ((pre-reaction state)	U34	*E. coli*	«	3.4	[16]
2DEU	MnmA + $tRNA^{Glu}$ (adenylated state)	U34	*E. coli*	«	3.4	[16]
2HMA	MnmA + SAM	nd	*Streptococcus pneumoniae*	SpMnmA	2.41	Kim et al., unpublished
6SCY	[4Fe-4S]Ncs6	U34	*Methanococcus maripaludis*	MmNcs6	2.8	Bimai et al., unpublished
3VRH	Apo-TtuA	U54	*Pyrococcus horikoshii*	PhTtuA	2.1	[39]
5MKP	[4Fe4S]TtuA	U54	*P. horikoshii*	«	2.5	[40]
5MKQ	[4Fe4S]TtuA (iron edge)	U54	*P. horikoshii*	«	2.9	[40]
5MKO	[2Fe-2S]TtuA + AMP	U54	*P. horikoshii*	«	2.65	[40]
5B4E	[4Fe-4S]TtuA + AMPPNP	U54	*Thermus thermophilus*	TtTtuA	2.75	[24]
5B4F	[Fe-S]TtuA	U54	*T. thermophilus*	«	2.7	[24]
5GHA	TtuA-(G65S)TtuB complex	U54	*T. thermophilus*	«	2.5	[24]
5ZTB	[4Fe-4S]TtuA-TtuB complex + ATP	U54	*T. thermophilus*	«	2.2	[25]

nd :not determined

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