# CELLULAR AND MOLECULAR FUNCTIONS OF TRANSFER RNA MODIFYING ENZYMES

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**Cellular and Molecular Functions of Transfer RNA Modifying Enzymes**

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**Abstract**

All transfer RNAs (tRNAs) are highly modified. Several modifications are found within the tRNA elbow, including the universally conserved 5-methyluridine ($m^5U$) 54 and pseudouridine (Ψ) 55 modifications in the T arm and 7-methylguanosine ($m^7G$) 46 in the tRNA variable loop, formed by the bacterial tRNA modifying enzymes TrmA, TruB, and TrmB, respectively. Despite conservation of these enzymes throughout all domains of life, they are not essential for bacterial growth in ideal conditions. The overall goals of this thesis were to investigate molecular determinants for tRNA modification by these enzymes *in vitro* and determine cellular roles for these modifications *in vivo*. Specific chapters address (I) the preferential tRNA modification status for modification by TrmA, TruB, and TrmB, (II) the mechanism of tRNA binding by TrmB, and (III) the cellular roles for TrmA and TruB. Taken together, these studies reveal the mechanistic basis for TrmA and TruB acting in the early stages of tRNA maturation and provide justification for using an unmodified tRNA substrate for the rapid-kinetic analysis of TrmB binding tRNA, which reveals that prior S-adenosylmethionine binding is necessary for stable and rapid tRNA binding by TrmB. Additionally, residues distant from the active site within TrmB were identified to contribute for tRNA binding. Finally, tRNA sequencing, proteomics, and biochemical studies uncover that TrmA and TruB fine-tune global tRNA function by enhancing further tRNA modification, aminoacylation, and protein translation. In summary, this thesis provides insight into the tRNA binding and modification activity of three highly conserved tRNA modifying enzymes and identifies cellular roles of TrmA and TruB for tRNA modification, folding and protein synthesis, thus explaining why these enzymes are so highly conserved. This research sets the stage for studying the mechanisms and functions of further tRNA modifying enzymes, in addition to modification enzymes that target other species of RNAs.

**Contributions of Authors**

Chapters 3 and 4 of this thesis were written as book chapters that describe in detail the methods I used in Chapters 5 and 6. Chapter 3, titled “Partially modified tRNAs for the study of tRNA maturation and function,” was conceptualized and written by myself and Ute Kothe as a chapter for Methods in Enzymology Volume 658 (RNA Modification Enzymes), published in July 2021. Chapter 4, “Fluorescent labeling of tRNA for rapid kinetic interaction studies with tRNA-binding proteins,” was conceptualized and written by Ute Kothe and me for Methods and Enzymology Volume 690 (Enzymes in RNA Science and Biotechnology), which is currently in press.

Chapter 5 of this thesis, “tRNA elbow modifications affect the tRNA pseudouridine synthase TruB and the methyltransferase TrmA,” was published in the journal *RNA* in 2020 with myself as first author and Ute Kothe as corresponding author. This research was conceptualized by Ute Kothe and myself. I performed and analyzed all experiments and wrote the first draft of the manuscript. As supervisor, Ute Kothe was involved with data analysis, manuscript editing, and funding.

Chapter 6, “Molecular mechanism of tRNA binding by the *Escherichia coli* N7 guanosine methyltransferase TrmB,” was published in the *Journal of Biological Chemistry* in 2023 with myself, Kieran Meadows, and Ute Kothe as authors. This project was conceptualized by Ute Kothe and myself. As first author, I performed and analyzed the majority of the experiments and wrote the first draft of the manuscript. Under my supervision, Kieran prepared three TrmB variants and performed and analyzed the stopped-flow data shown in Figure 6.6. As supervisor, Ute Kothe contributed to data analysis, manuscript editing, and funding.

Chapter 7, “Modifications in the T arm of tRNA globally determine tRNA maturation, function and cellular fitness” is a collaborative project with Tao Pan’s lab at the University of Chicago and Richard Fahlman at the University of Alberta. As first author, I performed and analyzed the majority of the experiments. Tao Pan and his lab members Christopher Katanski, Mateusz Halucha, and Noah Pena performed and analyzed the MSR-seq experiments from total

RNA samples I prepared, shown in Figure 7.2, Figure 7.3B, C, F, Figure A2.1, and Figure A2.2A. From cellular extract I prepared and separated on an SDS PAGE, Richard Fahlman performed mass spectrometry for proteome comparison, shown in Figure 7.5, Figure A2.7, and Figure A2.8. Ute Kothe and I together conceptualized this project, analyzed data together with our collaborators, and wrote and edited the manuscript.

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**Abbreviations**

| | |
|---|---|
| Ψ | pseudouridine |
| 5-IAF | 5-iodoacetamidofluorescein |
| aaRS | aminoacyl tRNA synthetase |
| aa-tRNA | aminoacyl-tRNA |
| $acp^3U$ | 3-(3-amino-3-carboxypropyl)uridine |
| APM | [p-(N-acrylamino)-phenyl]mercuric chloride) |
| ASL | anticodon stem-loop |
| ATP | adenosine triphosphate |
| ADP | adenosine diphosphate |
| BONCAT | biorthogonal noncanonical amino acid tagging |
| D | dihydrouridine |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleic acid triphosphate |
| DPM | decays per minute |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EF-Tu | Elongation factor thermal unstable |
| EF-G | Elongation factor G |
| GTP | guanosine triphosphate |
| GDP | guanosine diphosphate |
| HEPES | *N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid |
| HPLC | high performance liquid chromatography |
| iPPase | inorganic pyrophosphatase |
| IPTG | isopropyl β-D-1-thiogalactopyranoside |
| KO | knockout |
| L-AHA | L-azidohomoalanine |
| LB | lysogeny broth |
| LC/MS | liquid chromatography mass spectrometry |
| lncRNA | long non-coding RNA |
| $m^5U$ | 5-methyluridine |
| $m^7G$ | 7-methylguanosine |
| miRNA | microRNA |
| mRNA | messenger RNA |
| $ms^2i^6A$ | 2-methylthio-6-isopentyladenosine |
| MS | mass spectrometry |
| MSR-seq | multiplex small RNA sequencing |
| NGS | next generation sequencing |
| NMR | nuclear magnetic resonance |
| NTP | nucleotide triphosphate |
| $OD_{600}$ | optical density at 600 nm |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PDB | protein data bank |
| PLP | pyridoxal 5'-phosphate |
| PMSF | phenylmethanesulfonylfluoride |
| pre-tRNA | precursor tRNA |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rRNA | ribosomal ribonucleic acid |
| RTD | rapid tRNA decay |
| $s^4U$ | 4-thiouridine |
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosylmethionine |
| SDS | sodium dodecyl sulfate |

| | |
|---|---|
| SEC | size exclusion chromatography |
| snoRNA | small nucleolar RNA |
| snRNA | small nuclear RNA |
| TBE | Tris/Borate/EDTA buffer |
| TCA | trichloroacetic acid |
| Tris | tris(hydroxymethyl)aminomethane |
| tRNA | transfer RNA |
| UTP | uridine triphosphate |
| UV | ultraviolet |
| WT | wildtype |

# Chapter 1 – Introduction
## Roles of tRNA modifying enzymes in the biogenesis and functions of tRNAs

## 1.1 Preface

Chapter 1 has been written as an early draft of a review intended for publication that summarizes the roles that tRNA modifying enzymes play throughout the tRNA lifecycle. This initial draft has been written by myself and edited by Dr. Ute Kothe.

## 1.2 Abstract

Transfer RNAs (tRNAs) are the most highly modified cellular RNAs, both by the proportion of nucleotides that are modified in tRNAs in addition to the remarkable diversity in modification chemistry observed in tRNAs. tRNAs have two general clusters of modifications. The first is within the anticodon stem-loop; these modifications are often essential within cells for protein translation. Roles for the second cluster of tRNA modifications, in the so-called tRNA elbow, are generally not essential in cells, but have nonetheless been highly conserved throughout all domains of life. In addition to forming modifications, many tRNA modifying enzymes have either been demonstrated or hypothesized to additionally play an important role in folding tRNA. In this review, we summarize the known roles for tRNA modifying enzymes through the “lifecycle” of a tRNA molecule. Here, we describe how tRNA modification and folding by tRNA modifying enzymes enhance tRNA maturation, tRNA aminoacylation, and protein synthesis, ultimately impacting cellular phenotypes and disease.

## 1.3 Introduction

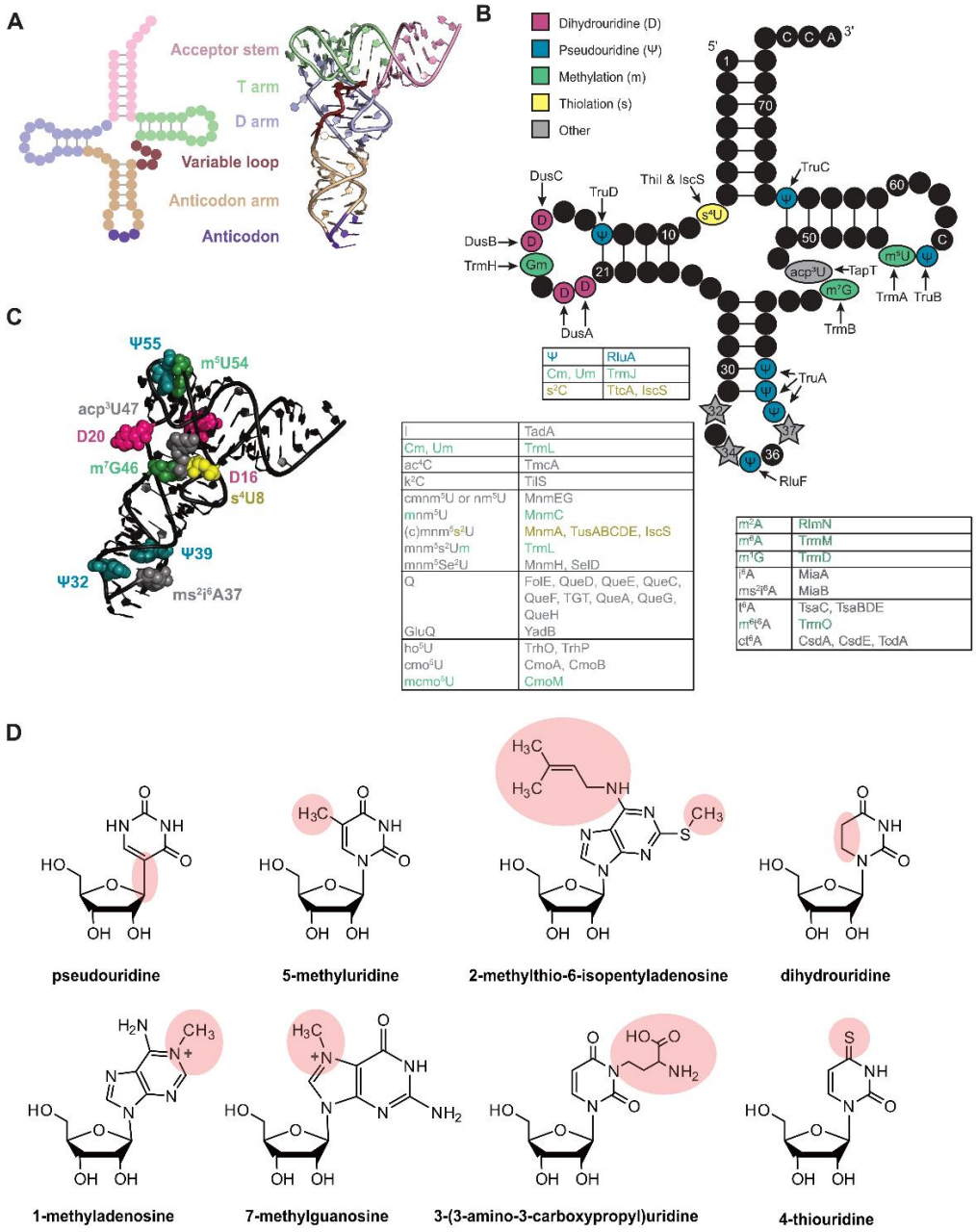
Transfer RNAs (tRNAs) are essential molecules that act as physical adapters between the genetic code and functional proteins in every cell. Almost all tRNAs feature a cloverleaf secondary structure that folds into an L-shaped tertiary structure (Figure 1A). Decades of ongoing research has established that all tRNAs contain modifications that play a variety of roles in tRNA structure and function, introduced by a diverse set of dedicated tRNA modifying enzymes (1-4). Underlying the abundance and energy investment of tRNA modification within

cells, genes encoding tRNA modifying enzymes can account for 1% of the protein coding genes in an organism. For example, the model organism *Escherichia coli* has ~4300 protein coding genes, of which 59 encode enzymes involved in the formation of 28 tRNA modifications (Figure 1B). Highlighting the importance of tRNA modification, mutations within tRNA sequences that prevent modification or within the genes encoding tRNA modifying enzymes have been implicated in a growing number of diseases (5-7).

Within the L-shaped three-dimensional tRNA structure, two clusters of tRNA modifications are apparent (Figure 1C) (1,8). The first cluster of tRNA modifications is found within the tRNA anticodon stem-loop. In this region, modifications often play direct functional roles during protein translation (9,10). For this reason, several modifications found within this region are essential for cell viability. The second cluster of tRNA modifications is evident in the elbow of the L-shaped tertiary structure, which is also referred to as the tRNA body (8). In general, modifications within this region fine-tune tRNA structure and stability (1).

Modifications to tRNA are often simple reactions, such as isomerization of the uracil base to form pseudouridine, reduction of uracil to form dihydrouridine, methylation at various atoms of the nucleobase or ribose sugar, or exchange of oxygen for sulfur during thiolation; however, some reactions are more complex and involve more than one enzyme, forming bulky hypermodifications (Figure 1D). Most often, tRNA hypermodifications are found within the tRNA anticodon stem-loop, especially at position 34, which is the wobble position that base pairs to the nucleotide at the third position of an mRNA codon during translation, and the conserved purine at position 37, which is adjacent to the anticodon. Certain modifications to the tRNA elbow are found in many tRNAs across all domains of life, suggesting a conserved function. These include dihydrouridine (D) at various positions within the D arm, 5-methyluridine ($m^5U$, also known as ribothymidine, rT) 54, pseudouridine (Ψ) 55, 1-methyladenosine ($m^1A$) 58, 2'-O-methylguanosine (Gm) 18, and 7-methylguanosine ($m^7G$) 46. In contrast, modifications within the tRNA anticodon stem-loop are often found in only a handful of tRNAs and display more variation between different organisms, suggesting possible roles for certain modifications in environmental

adaptation. In the model organism *Escherichia coli*, all tRNA modifications are thought to be mapped, and with the identification of the TapT enzyme in 2019, all enzymes responsible for *E. coli* modifications have been identified (11-13). In contrast, complete mapping of tRNA modifications in most organisms has yet to be accomplished and novel modifications remain to be discovered.




*Caption on next page.*

**Figure 1.1 tRNA structure and modification. (A)** Cloverleaf secondary structure (left) and L-shaped tertiary structure (right) typical of the majority of tRNAs. **(B)** Locations and identities of all modifications in *E. coli.* Multiple different modifications can occur at positions 32, 34, and 37 in different tRNAs, as shown in tables. **(C)** Modifications within *E. coli* $tRNA^{Phe}$ as a typical tRNA to demonstrate clusters of modifications in the tRNA elbow and in the tRNA anticodon stem-loop. Modifications are coloured as per the legend in panel B. **(D)** Examples of some of the modifications found within tRNAs. Atoms that constitute the modification are indicated in red.

The majority of tRNA modification enzymes have been found to be non-essential for cells grown in ideal laboratory conditions; however, many cellular phenotypes have been identified for tRNA modification enzyme knockouts in stress conditions, suggesting an importance for these enzymes in maximizing cellular fitness. Moreover, deletion of two or more modification enzymes oftentimes results in more severe growth defects, emphasizing the likely redundant nature of tRNA modifications and modifying enzymes. In addition to the catalytic role of modifying tRNAs, certain tRNA modification enzymes may additionally play an important role in folding tRNAs, acting as tRNA chaperones (14-16). Thus, tRNA modifying enzymes not only affect the modification status of tRNAs, but also tRNA folding.

tRNA interacts with a variety of factors during its lifecycle (Figure 2, Figure 3) and tRNA modifications affect several of these interactions. tRNAs must be different enough to be distinguished by certain factors including aminoacyl-tRNA synthetases, yet similar enough to universally bind other factors such as EF-Tu and the ribosome. In this review, we summarize precise roles for tRNA modifications that have been identified by *in vivo* deletion studies or with purified components *in vitro*. In particular, we focus on how modifications affect tRNA maturation and function during translation.

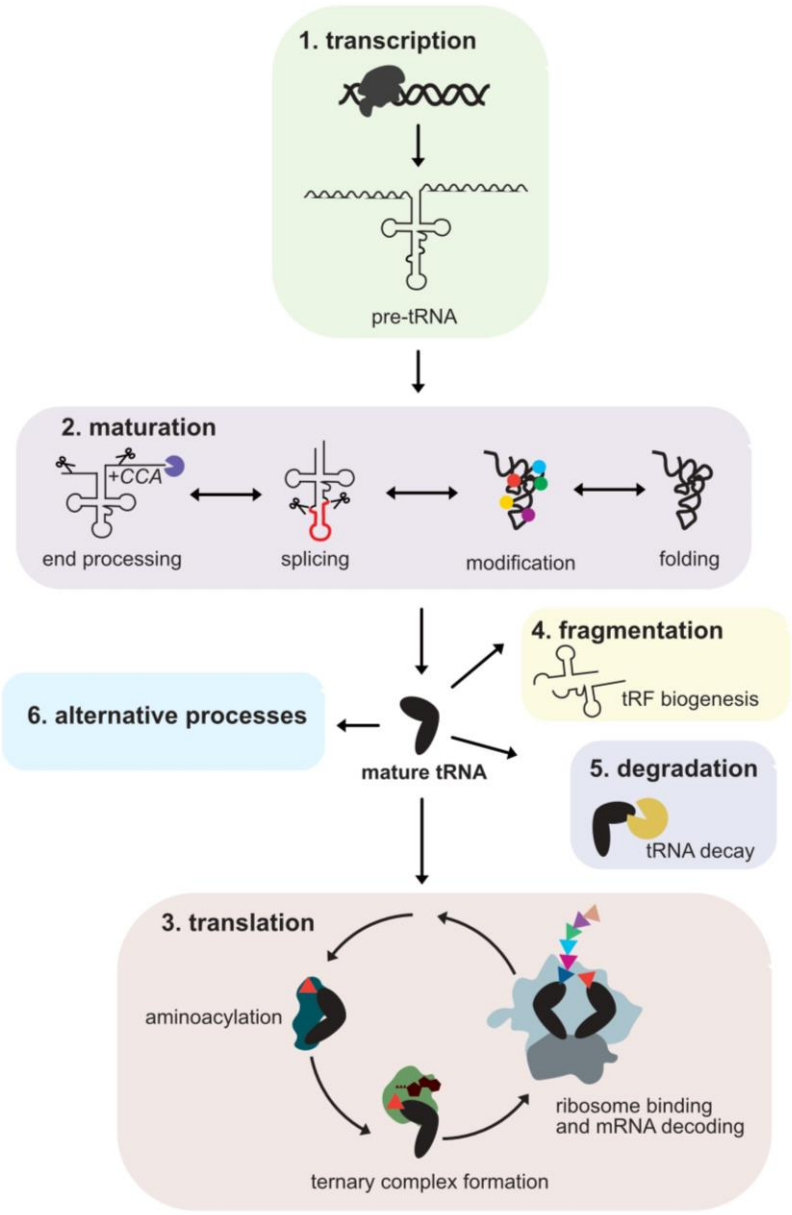



**Figure 1.2. General lifecycle of a tRNA.** Following transcription of pre-tRNA by RNA polymerase (1), tRNA undergoes several maturation steps (2), including 5' and 3' end-processing, intron splicing, modification of many nucleotides, and folding, giving rise to a mature tRNA. Mature tRNA is then aminoacylated to form aa-tRNA, which then binds EF-Tu•GTP, forming the ternary complex for delivery to the ribosome (3). Various ribonucleases target tRNA, cleaving tRNA to form functional tRNA fragments (4) or to degrade aberrantly matured tRNA (5). Finally, tRNAs also participate in a variety of non-translation alternative processes.

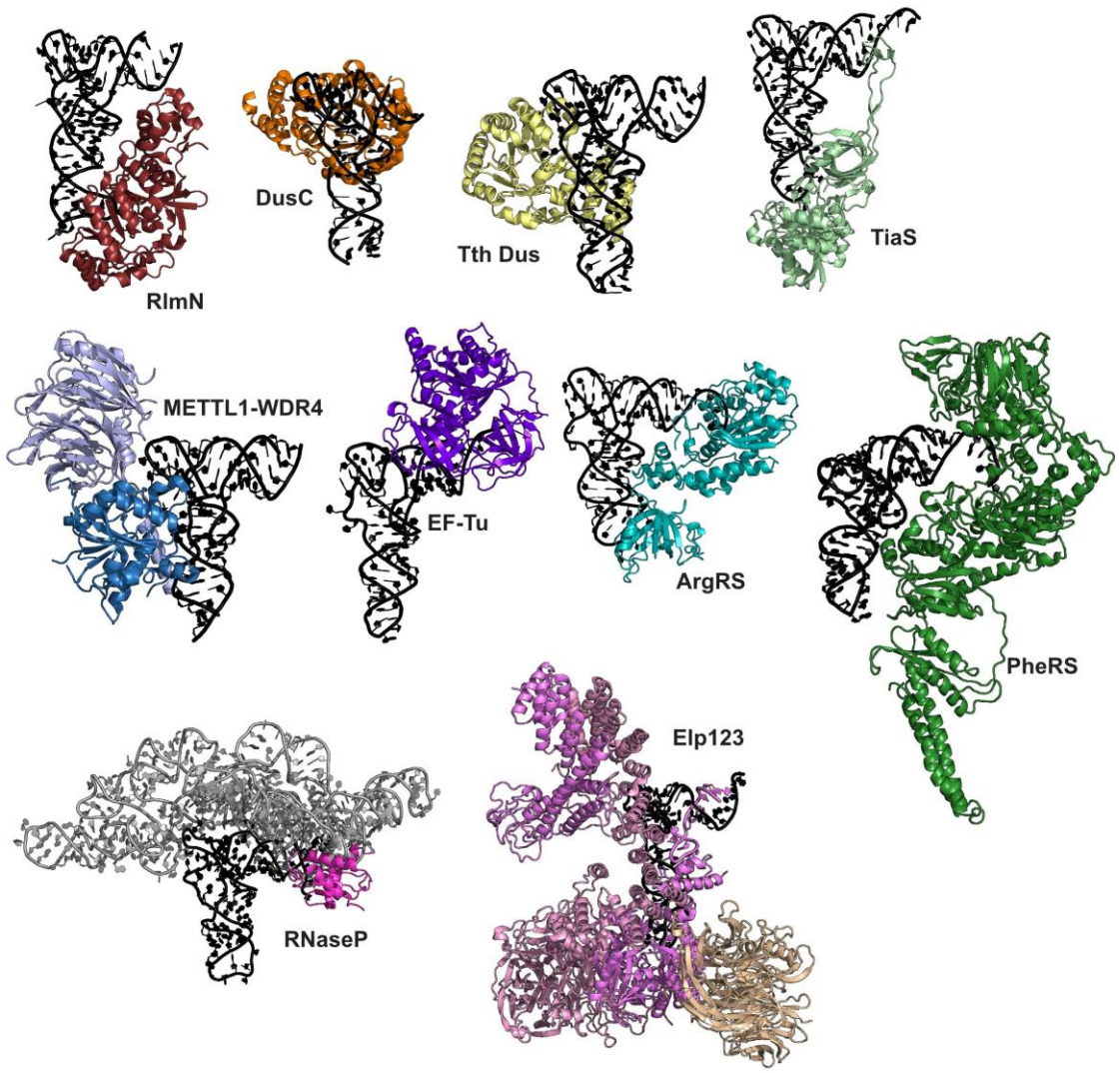



**Figure 1.3. Examples of enzymes bound to tRNA.** In all cases, tRNA is coloured black and is in roughly the same orientation to demonstrate how different enzymes target different surfaces of the tRNA. Associated PDB accession numbers: RlmN: 5HR7, DusC: 4YCP, TTh Dus: 3B0V, TiaS: 3AMT, METTL1-WDR4: 8EF0, EF-Tu: 1B23, ArgRS: 5BD3, PheRS:1EIY, RNaseP: 3Q1Q, Elp123: 8ASW.

### 1.4 tRNA maturation

tRNA is transcribed as a precursor (pre)-tRNA containing 5' leader and 3' trailer sequences. The 5' and 3' extensions are removed by various endo- and exoribonucleases. The precise pathway and collection of enzymes used for tRNA end-trimming depends on the organism and identity of the tRNA being processed (17,18). The tRNA genes from many organisms (except for Gram-negative bacteria) lack the 3'-CCA tail necessary for aminoacylation. As such, this sequence must be post-transcriptionally added by the tRNA nucleotidyltransferase (19). Certain tRNAs contain an intron, which is typically present within the tRNA anticodon stem-loop. Eukaryotic and archaeal tRNA introns require several enzymes to excise the intron and repair the tRNA; however, the details of these reactions differ between organisms (20-22). Bacterial tRNAs rarely contain introns, but when present they are self-spliced without the need for proteins (23).

The relative order of the maturation events described above generally begins with 5' processing, then 3' processing (including CCA addition, if applicable), followed by tRNA splicing if necessary (2,24,25); however, tRNA processing events do not seem to be strictly temporally defined, and the order can vary depending on the identity of the tRNA being processed and the organism. In eukaryotes, the subcellular localization of tRNA processing components largely dictates the tRNA processing order, as tRNAs are transcribed in the nucleolus, and subsequently encounter different processing enzymes as they move to the nucleus and cytoplasm, followed by potential retrograde import to the nucleus and re-export from the nucleus (2,26). Again, the details of protein localization can differ between organisms. For example, whereas the tRNA splicing occurs in the nucleus in *Xenopus* oocytes and human cells (27,28), the tRNA splicing machinery is instead localized to the cytoplasmic surface of mitochondria in yeast (29).

Ribose and base modifications to tRNA can occur at all steps of tRNA maturation. Like the tRNA processing steps, the intracellular localization of the responsible modifying enzyme plays a role in whether a modification will occur during the early or late steps of tRNA maturation, and in many cases, it may be that a tRNA is modified by a given enzyme at the first available

opportunity within the cell, rather than in a strict order (30). Decades ago, it was first shown that many tRNA modifications occur prior to tRNA end-processing and tRNA intron splicing in *Xenopus* oocytes (27,31,32). Whereas several tRNA modification enzymes are insensitive to the presence or absence of tRNA introns, others absolutely rely on intron presence (or absence) for catalysis, as summarized in (33). For example, pseudouridylation of U35 in $tRNA^{Tyr}$ by Pus1 within various eukaryotes absolutely requires intron presence but does not require the intron to be of a certain size or sequence (34-36), and C5-methylation of yeast $tRNA^{Phe}$ at position 40 requires an intron, but is not sensitive to the presence of the D or T arms (37). In contrast to these examples of tRNA modifying enzymes being sensitive to tRNA processing status, to the best of our knowledge, there is no known reliance of the tRNA end-processing or the tRNA splicing machineries on the tRNA modification status. Whether any preferences of tRNA processing enzymes for a certain (sub)set of tRNA modifications exists has yet to be examined. For this reason, we will focus on the effect of tRNA modifications on the activities of tRNA modifying enzymes in the sections below.

*1.4.1 Early tRNA modifications affect the activity of later acting tRNA modifying enzymes*

Approximately 10% of all nucleosides within tRNA are post-transcriptionally modified by a large set of dedicated tRNA modifying enzymes. Each tRNA contains its own unique set of modifications, but certain modifications, including $m^5U54$, Ψ55, $m^7G46$, and D at several positions, are found in many tRNAs (8). As discussed above, it has been long evident that tRNA modification is at least somewhat ordered, with certain modifications appearing prior to tRNA end processing and splicing and others forming only after the pre-tRNA is processed. However, it is unlikely that tRNA modification follows a strict sequential order, as many tRNA modifying enzymes can effectively modify *in vitro* transcribed (i.e. unmodified) tRNA. Several instances of tRNA modifications positively and negatively affecting the activity of tRNA modifying enzymes have been uncovered and have been referred to as “modification crosstalk,” “modification circuits,” or “modification networks” (38-42). Determining the relative temporal order of tRNA

modification is a challenging task because tRNA modifications are introduced quickly, due to difficulties in purifying specific tRNA isoacceptors, obstacles of quantitatively distinguishing sites of modification (e.g. distinguishing Ψ32 vs Ψ55), and the need for sophisticated expertise and equipment to monitor many chemically distinct nucleotides in one experiment. Most often, modification crosstalk has been uncovered by extracting total tRNA or a specific tRNA isoacceptor from deletion strains lacking one (or more) tRNA modification enzymes to monitor for potential changes in the steady-state abundance of other modifications in response, typically using high performance liquid chromatography-mass spectrometry (LC/MS) or various next-generation sequencing (NGS) techniques (43). In other cases, *in vitro* studies have been used to determine how the presence/absence of modifications affects tRNA binding and modification by modifying enzymes; this approach can determine the mechanisms behind tRNA modification crosstalk. Known instances of tRNA modifications positively and negatively affecting the activities of tRNA modifying enzymes that have been examined using these and similar assays are summarized in Table 1. As several reviews have explained modification circuits with an emphasis on tRNA anticodon stem-loop modifications (38-42), we summarize here recent techniques that have advanced our knowledge regarding the temporal placement of tRNA modifications with a focus on particular modifications within the tRNA variable and T loops: $m^7G46$, $m^5U54$, Ψ55, and $m^1A58$.

Supporting early studies in *Xenopus* oocytes (27), several new studies have identified that T arm modifications, in particular $m^5U54$ and Ψ55, generally tend to be among the earliest introduced during tRNA maturation. Recently, a time-resolved NMR assay was developed to follow the modification of a stable-isotope labeled *in vitro* transcribed $tRNA^{Phe}$ molecule within yeast cell extract (44). This experiment identified that Ψ55 is introduced early and quickly into tRNA, followed by $m^5U54$ (over a long period of time) and $m^7G46$ (over a short period of time), followed by $m^2G10$, $m^5C49$, and D16. Finally, $m^1A58$ was the last modification to be detected within $tRNA^{Phe}$ ($m^2{}_2G26$ and $m^5C40$ were not observed within the incubation timeframe). Although this experiment is able to detect many modifications at one time in a cell-like environment, disadvantages include loss of cellular compartmentalization, meaning tRNA may

encounter (cytoplasmic) enzymes sooner than it would within the cell. An interesting finding on this study is the apparently late introduction of the $m^1A58$ modification. Considering the enzymes responsible for $m^1A58$ (Trm6/Trm61) are localized to the nucleus (45), it would be surprising for $m^1A58$ to be one of the last modifications to be introduced. Moreover, methylation of A58 in initiator tRNA is well-known to be vital to protect initiator $tRNA^{Met}$ from nuclear decay (45-47), again suggesting this modification to be introduced early. To reconcile these differences, the interdependence of the early modifications $m^5U54$ and Ψ55 on $m^1A58$ was examined using yeast knockouts (44), and the effect of these early modifications on Trm6/Trm61 activity was studied with partially modified tRNAs *in vitro* (48). Interestingly, presence of $m^5U54$ and Ψ55 increased $m^1A58$ content in bulk tRNA in the cell and previous introduction of Ψ55 was necessary for efficient formation of $m^1A58$ in *in vitro* transcribed elongator tRNA, but not initiator tRNA (44,48). *In vitro*, $m^5U54$ additionally stimulated Trm6/Trm61 activity (48). Importantly, as both Trm2 ($m^5U54$) and Pus4 (Ψ55) are localized to the nucleus in yeast, it is feasible that these enzymes modify tRNA prior to Trm6/Trm61. In contrast, in human cells, loss of $m^1A58$ has been found to decrease $m^5U$/$m^5Um$ content within $tRNA^{Lys}$ and $tRNA^{Phe}$ (49) and seems to be among the first modifications introduced into human $tRNA^{Phe}$ (50), as will be further discussed below.

Although mass spectrometry has long been used to detect tRNA modifications, to examine the temporal order of tRNA modification, nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) was recently developed (50). This assay uses stable isotope pulse-chase labeling of human cell cultures to observe tRNA modification in newly-transcribed tRNA. Using $tRNA^{Phe}$ as a model, within the tRNA elbow, Ψ55 was again found to be very quickly incorporated in tRNA, followed by $m^5U54$, $m^1A58$, and $m^5C49$, whereas $m^7G46$ was introduced slowly. In the anticodon stem-loop, $m^1G37$ is introduced quickly and slowly converted to yW37, and Cm and Gm are introduced following $m^1G37$ (50). Thus, compared to the time-resolved NMR study, $m^1A58$ is introduced quite early, but still potentially could be stimulated by the presence of $m^5U54$/Ψ55. Moreover, whereas $m^7G46$ was introduced relatively early and quickly in the prior study, the latter study suggests a later introduction of this variable loop modification.

Finally, recent advances within the field of tRNA sequencing have made strides towards distinguishing many tRNAs modifications (51,52). In comparison to the experiments described above, NGS approaches are advantageous as high-throughput studies, which allow for study of all tRNA species without extracting specific isoacceptors. Unlike Illumina sequencing, Nanopore sequencing can directly sequence RNA without the need for cDNA preparation and additionally can distinguish several modifications from canonical nucleotides during this process. Recently, using Nano-tRNA-seq recapitulated the requirement of the yeast *pus4* gene (catalyzing Ψ55) for efficient $m^5U54$ and $m^1A58$ formation (51). As Nanopore is a single molecular method that can determine the presence of modifications within individual tRNAs, this technique is likely to uncover more tRNA modification networks, as has recently been accomplished for rRNA (43,51,53). In contrast to Nanopore, Illumina RNA sequencing requires short reads (<300 base pairs) and thus RNA is usually fragmented prior to sequencing. Because of this step, Illumina sequencing is generally regarded to not be a single molecule method, and only the proportion of modifications in a population can be reported. Since tRNAs are small (76 – 90 nucleotides), the entire length can be covered by a single Illumina sequencing read, especially due to advances in overcoming tRNA folding and bulky modifications during cDNA preparation. Thus, a computational pipeline termed single-read analysis of crosstalks (SLAC) was developed to treat Illumina tRNA sequencing reads in a “pseudo-single molecular manner” to examine interdependencies between tRNA modification, aminoacylation, and fragmentation (54). Application of this technique to previously-published Illumina tRNA sequencing datasets confirmed several modification crosstalks in tRNA, and identified some new interdependencies, several of which involve the $m^1A58$ modification (54).

Taken together, these studies have identified several instances of interdependencies between the introduction of tRNA modifications, particularly in yeast and humans. Similarities between studies strongly includes the fast and early introduction of Ψ55 during tRNA maturation; however, more work remains in order to determine if differences in studies are a result of using different techniques, or if species-specific differences exist. Moreover, while the results above determined the presence of tRNA modification circuits, we are still lacking an understanding of

the mechanisms that give rise to these interdependences. A few studies have addressed this by using partially-modified *in vitro* transcribed tRNA and purified enzymes and comparing the binding and affinity of these enzymes for unmodified and partially modified tRNA (48,55,56). By studying the binding and modification preferences of the bacterial enzymes responsible for $m^7G46$, $m^5U54$, and Ψ55 (TrmB, TrmA, and TruB, respectively), it was uncovered that TruB has a tighter affinity and faster modification rate for unmodified tRNA compared to tRNA that contains one or more additional modifications (56), suggesting the early introduction of Ψ55 is likely to also occur in bacteria. The affinity of TrmA was increased for tRNA containing Ψ55; however, methylation of this Ψ55 tRNA was slower than unmodified tRNA. Thus, it is plausible that $m^5U54$ also acts early, but potentially following Ψ55 introduction in bacteria. *In vivo* studies will need to be accomplished to determine the cellular situation. Finally, corroborating the introduction of $m^7G$ somewhere during the middle of tRNA maturation, TrmB did not have a binding or modification preference for tRNA containing or lacking modifications (56), and *in vivo* studies suggest a requirement of $m^7G46$ formation for later incorporation of $acp^3U47$ (13). Thus, more work is still needed to fully understand the hierarchical order of tRNA modification within the tRNA maturation pathway.

**Table 1.1 Interactions between tRNA modifications and tRNA modifying enzymes.**

| Initial modification (enzyme) | Affected modification (enzyme) | +/- * | Organisms | Evidence | Ref. |
|---|---|---|---|---|---|
| $m^1A58$ (TRM6/TRM61) | $m^5U54$ (TRMT2A) | + | *Homo sapiens* (HEK293FT cell line) | LC/MS of total, purified $tRNA^{Phe}$, and purified $tRNA_3^{Lys}$ extracted from *TRM6* mutant cell lines | (49) |
| $m^1A58$ (TrmI) | $s^2m^5U54$ (TtuABC, IscS) | + | *Thermus thermophilus* | LC/MS and enzymatic assays of bulk tRNA extracted from a *ΔtruB* strain. | (57) |
| Ψ55 (Pus4) | $m^5U54$ (Trm2) and $m^1A58$ (Trm6/Trm61) | + | *S. cerevisiae* | Mass spectrometry of bulk and purified $tRNA^{Phe}$ from a *Δpus4* strain | (44) |
| Ψ55 (TruB) | Gm18 (TrmH) $m^5s^2U54$ (TtuABC, IscS) $m^1A58$ (TrmI) | - | *T. thermophilus* | LC/MS and enzymatic assays of bulk tRNA extracted from a *ΔtruB* strain. | (58) |
| Ψ55 (TruB) | $m^5U54$ (TrmA) | + | *E. coli* | Increased affinity of TrmA to Ψ55 tRNA *in vitro* | (56) |
| $m^5U54$ (Trm2) | $m^1A58$ (Trm6/Trm61) | + | *S. cerevisiae* | Mass spectrometry of bulk and purified $tRNA^{Phe}$ from a *Δtrm2* strain | (44) |
| $m^5U54$ (TrmA), $s^4U8$ (ThiI), $m^7G46$ (TrmB) | Ψ55 (TruB) | - | *E. coli* | Decreased affinity and activity of TruB for singly modified tRNA *in vitro* | (56) |
| $m^7G46$ (TrmB) | $acp^3U47$ (TapT) | + | *E. coli* | Mass spectrometry of bulk tRNA extracted from a *ΔtrmB* strain; primer extension to examine specific tRNAs | (13) |
| $m^7G46$ (TrmB) | Gm18 (TrmH) $m^1G37$ (TrmD) and several others that were not validated | + | *T. thermophilus* | LC/MS and enzymatic assays of bulk tRNA extracted from a *ΔtrmB* strain | (59) |
| $i^6A37$ (MiaA) | (C/U)m34 (TrmL) | + | *E. coli* | LC/MS of bulk and specific tRNAs from *ΔmiaA* strains; *in vitro* activity assays | (60,61) |
| $t^6A37$ (TsaBDE) | L34 (TilS) | + | *E. coli* | Faster (~13-fold) L34 formation for tRNA transcripts containing $t^6A37$ | (62) |
| $i^6A37$ (Tit1) or $t^6A37$ (Tcd1/2) | $m^3C32$ (Trm140) | + | *S. cerevisiae* *S. pombe* | tRNA-HySeq and LC/MS or primer extension of RNA from wildtype and knockout strains; tRNA pulldowns in knockout strains; *in vitro* methylation assays | (63,64) |

| | | | | | |
|---|---|---|---|---|---|
| $m^1G37$ (TrmD) | $mcmo^5U34$ (CmoM) | + | *E. coli* | Faster *in vitro* methylation of $tRNA^{Pro}_{UGG}$ carrying $m^1G37$ (despite lower affinity) | (55) |
| Q34 (Tgt) | $m^5C38$ (Dnmt2) | + | *S. pombe* *Dictyostelium discoideum* | *In vivo* bisulfite sequencing for wt and knockout strains | (65) |
| Cm32 and Gm34 (Trm7 and Trm732 or Trm734) | yW37 (yeast, Tyw1-4) or o2yW37 (humans, unknown) | + | *S. cerevisiae* *S. pombe* Humans | LC/MS and biochemical characterization of tRNA purified from knockout strains | (66-68) |
| $m^3U32$ (Trm140) | I34 (ADAT2/3) | + | *T. brucei* | *In vitro* inosine formation is more efficient for tRNAs that contain $m^3C32$ | (69) |
| One or more of the full modification set | D20 (Dus2) | + | *S. cerevisiae* | Faster oxidation of purified Dus2 when mixed with $tRNA^{Leu}$ containing modifications compared to the *in vitro* transcript; tighter binding of Dus2 to modified vs. unmodified tRNA | (70) |

* + indicates modification whose presence is stimulated by the enzyme identified in the first column. - indicates a modification whose presence is repressed by the enzyme identified in the first column.

*1.4.2 Many tRNA modifying enzymes affect tRNA folding and structural dynamics*

Although the vast majority of tRNAs adopt the canonical L-shaped tertiary structure in the absence of any modifications, tRNA modifying enzymes have been shown to enhance tRNA folding and structural dynamics through two independent processes: **(1)** the chemistry of the modified nucleotide altering tRNA structure or dynamics or **(2)** the act of the tRNA modifying enzyme binding and accessing its target base locally unfolds the tRNA structure, providing potentially misfolded tRNA a second chance at correctly folding. Each of these topics has been recently reviewed elsewhere (14-16). Below, we will briefly summarize how tRNA modifying enzymes impact tRNA structure.

Only very few tRNAs require modification in order to adopt the canonical tRNA structure and these instances primarily involve mitochondrial tRNAs, which often feature an atypical secondary structure, sometimes lacking canonical tRNA features such as the D and/or T arms (71). In particular, unmodified human mitochondrial $tRNA^{Lys}$ primarily adopts an extended hairpin conformation secondary structure, but introduction of $m^1A9$ shifts the structural equilibrium to the

canonical L-shape fold by disrupting a base-pair between A9 and U64 (72). Similarly, unmodified mitochondrial $tRNA^{Asp}$ exists in several conformations *in vitro*, but presence of its native modifications ($m^1A9$, $m^1G10$, Ψ27, and Q34) stabilizes the canonical cloverleaf structure (73), and $m^1A9$ has been shown to be important for the overall structure of T arm-less mitochondrial tRNAs from nematodes (74). Finally, $m^{2,2}G$ at different positions within the tRNA D arm functions to restrict the folding of tRNAs that can fold into multiple conformations (75,76).

In contrast to the examples above, whereas the vast majority of tRNAs do not require any modifications to adopt the canonical L-shaped tertiary structure (77), various biophysical techniques have demonstrated how modifications subtly affect tRNA structure and thermodynamics (78). UV melting curves have indicated a loss of tRNA thermal stability in the absence tRNA modifications; for example, several tRNA body modifications collectively contribute to stability of $tRNA^{Ser}$ (79), NMR studies have shown the contribution of $m^1A58$ for folding of the tRNA elbow for yeast $tRNA_i^{Met}$ (48), circular dichroism (CD) spectrometry has demonstrated Ψ has enhanced base-stacking propensity compared to uridine (80), and molecular dynamics (MD) simulations have shown various hypermodifications at position 37 result in different nucleotide glycosidic and backbone conformations compared to unmodified tRNA (81). Thus, modifications play subtle, but important roles to enhance the structure and dynamics of tRNA.

In addition to the role of modifications to augment tRNA structure, the enzymes that modify tRNAs themselves influence tRNA folding independently of their modification activity. Following decades-old research showing expression of catalytically-inactive TruB rescues the co-culture growth defect of *ΔtruB E. coli* (82), TruB was the first modification enzyme proven to be a tRNA chaperone (15). Upon binding tRNA, TruB disrupts tertiary interactions between the D and T arms, thus providing a potentially misfolded tRNA multiple chances at refolding and obtaining its correct fold (15). Subsequently, bacterial TrmA was additionally characterized to be a tRNA chaperone (14). So far, *E. coli* TrmA and TruB have been the only tRNA modifying enzymes characterized as tRNA chaperones; however, likely several more tRNA chaperones are yet to be

discovered (16), including TrmA and TruB's homologs. For example, the expression of catalytically-inactive Trm2 rescues accumulation of mature $tRNA^{Ser}$ variant (83). Further tRNA modification enzymes likely to have tRNA chaperone activity include other tRNA enzymes that disrupt tRNA structure in order to access their target base thus giving the tRNA a second chance at forming interactions, such as ArcTGT, which remodels tRNA into the "lambda (λ)" form in order to access its target base (84). Finally, a catalytically-inactive variant of the methyltransferase Trm1 can rescue pre-tRNA maturation in *Schizosaccharomyces pombe* and functions redundantly with the tRNA chaperone La, suggesting Trm1 may also act as a tRNA chaperone (85,86)

*1.4.3 tRNA modifications affect tRNA cellular stability*

In general, tRNAs are thought to be very stable within cells, with half-lives similar to that of ribosomal RNA (2). However, tRNAs are subjected to different quality control mechanisms during their lifecycle, wherein defective (pre-)tRNAs are repaired or degraded. These mechanisms have been primarily studied in *S. cerevisiae*, where two main tRNA decay pathways have been identified: nuclear surveillance and rapid tRNA decay (RTD). In tRNA nuclear surveillance, pre-$tRNA_i^{Met}$ lacking $m^1A58$ or elongator pre-tRNAs with unprocessed 3' ends are bound by the TRAMP complex, whose Trf4 subunit polyadenylates the tRNA 3' end, followed by degradation via 3'-to-5' exonucleolytic cleavage by Rrp6, which is part of the nuclear exosome (87). Whereas the nuclear surveillance pathway primarily monitors pre-tRNAs in the nucleus, the RTD pathway instead acts predominantly on mutation-containing or hypomodified cytoplasmic tRNAs with unstable acceptor/T stems and uses different machinery to degrade tRNAs in the 5'-to-3' direction. Here, Met22 and the exonucleases Xrn1 and Rat1 work together to degrade tRNAs with acceptor stems that leave the 5' tRNA termini exposed.

Although both described tRNA pathways act on hypomodified tRNAs, the exonucleases involved in these processes most likely do not act on the tRNA due to hypomodification, but rather are hypothesized to recognize their substrates based on an aberrant or unstable tRNA

fold, thus explaining why not all hypomodified tRNAs are degraded and why the RTD pathway is most active in yeast grown at high temperatures, where hypomodified tRNAs are likely destabilised. Interestingly, overexpression of a single specific tRNA is often sufficient to suppress phenotypes associated with RTD. For example, yeast lacking the methyltransferases *trm8* and *trm4* are slow-growing at high temperatures (88). Although both methyltransferases have a wide range of substrate tRNAs in yeast, strikingly, examination of tRNA abundance revealed only $tRNA^{Val}_{(AAC)}$ levels are decreased, and the phenotype associated with this strain can be suppressed by overexpressing $tRNA^{Val}_{(AAC)}$. This trend has also been demonstrated for other tRNA modification combinations with other examples listed in Table 1.2. Thus, it may be that not all tRNAs require all modifications; instead, certain modifications may be more important in specific tRNAs than others (89).

In contrast to yeast, the mechanism of bacterial tRNA degradation is less studied. Work in *Vibrio cholerae* has demonstrated that several different tRNAs lacking elbow modifications including $s^4U8$, $m^5U54$, and Ψ55 are subject to decay by the RNA degradosome, which is comprised of the helicase RhlB, Enolase, the polynucleotide phosphorylase PNPase, and RNase E (90). This mechanism appears to be broader in comparison to tRNA decay in yeast, as the degradasome seems to target a number of hypomodified tRNAs, in contrast to the only one or a few specific tRNA targets in the absence of modification enzymes in yeast (Table 1.2). However, more work is required to advance the field of bacterial tRNA degradation.

**Table 1.2 tRNA modifications known to influence the cellular stability of one or more tRNAs**

| **Modification enzyme** (modification) | **Affected tRNA(s)** | **Organism, exacerbating conditions, mechanism (if known)** | **Ref.** |
|---|---|---|---|
| $m^7G46$ (Trm8/Trm82) and $m^5C48/49$ (Trm4) | $tRNA^{Val}_{(AAC)}$ and $tRNA^{Cys}_{(GCA)}$ to a smaller extent | *S. cerevisiae*, heat stress (37°C); RTD | (88) |
| $m^7G46$ (Trm8/Trm82) and D47 (Dus3) | $tRNA^{Val}_{(AAC)}$ and $tRNA^{Cys}_{(GCA)}$ to a smaller extent | *S. cerevisiae,* heat stress (37°C); RTD | (88) |
| $m^7G46$ (Trm8/Trm82) and Ψ13 (Pus7) | $tRNA^{Val}_{(AAC)}$ | *S. cerevisiae,* heat stress (37°C); RTD | (88) |
| $m^7G46$ (Trm8/Trm82) | $tRNA^{Tyr}_{(GUA)}$ and $tRNA^{Pro}_{(AGG)}$ to a smaller extent | *S. pombe*, heat stress (38°C, RTD) | (91) |
| $m^7G46$ (METTL1/WDR4) and $m^5C48/49$ (NSUN2) | $tRNA^{Val}_{(AAC)}$ | *H. sapiens* (HeLa cell line) exposed to 5-fluorouracil; mechanism unknown | (92) |
| $m^7G46$ (TrmB) | $tRNA^{Phe}$, $tRNA^{Ile}$ | *T. thermophilus*, heat stress (80°C) | (59) |
| $ac^4C12$ (Tan1) and Um44 (Trm44) | $tRNA^{Ser}_{(CGA)}$ and $tRNA^{Ser}_{(UGA)}$; $tRNA^{Leu}_{(GAG)}$ to a smaller extent | *S. cerevisiae*, particularly at high temperatures; exacerbated when grown in glycerol; RTD | (93) |
| $m^{2,2}G26$ (Trm1) and $m^5C48/49$ (Trm4) | $tRNA^{Ser}_{(CGA)}$ and $tRNA^{Ser}_{(UGA}$ | *S. cerevisiae*, particularly upon heat stress; RTD | (94) |
| $m^1A58$ (Trm6/Trm61) | pre-$tRNA_i^{Met}$ | *S. cerevisiae* (RTD and nuclear surveillance) and *S. pombe* (RTD), particularly in heat stress | (45-47) |
| $m^7G46$ (METTL1/WDR4) | Several METTL1 substrate tRNAs | *H. sapiens* (glioblastoma multiforme cell line LNZ308) | (95) |
| $s^4U8$ (ThiI) | Several ThiI substrate tRNAs | *Vibrio cholerae* during stationary phase; bacterial RNA degradosome (RNase E) | (90) |
| $s^4U8$ (ThiI) and $m^5U54$ (TrmA) | $tRNA^{Tyr}$ | *Vibrio cholerae* during stationary phase; bacterial RNA degradosome (RNase E) | (90) |
| $s^4U8$ (ThiI) and Ψ55 (TruB) | $tRNA^{Tyr}$ | *Vibrio cholerae* during stationary phase; bacterial RNA degradosome (RNase E) | (90) |

### 1.5 tRNA modifications during canonical tRNA function: protein translation

Following tRNA biogenesis, most tRNAs execute their canonical role in bringing amino acids to the ribosome. As the majority of tRNAs are elongator tRNAs, the process for an elongator tRNA will be described here and the specific case of initiator tRNA is addressed in the sections below. First, tRNAs are aminoacylated by their cognate aminoacyl-tRNA synthetase, to form aminoacyl-tRNA. Bacterial elongation factor Tu (EF-Tu, (eEF1A in eukaryotes) bound to

GTP then binds an aminoacyl-tRNA, to form the EF-Tu•aa-tRNA•GTP ternary complex for aa-tRNA delivery to the ribosomal acceptor (A) site. The process of aminoacyl-tRNA binding to the ribosome for mRNA decoding will be discussed in more detail in section 1.5.4. Next, a peptide bond is formed between the elongating polypeptide chain attached to the tRNA in the peptidyl (P) site and the amino acid attached to the A site tRNA, leaving uncharged tRNA in the P site and peptidyl-tRNA in the A site. Subsequently, bacterial elongation factor G (EF-G, eEF2 in eukaryotes) catalyzes translocation, wherein the uncharged tRNA in the P site moves to the ribosomal exit (E) site and the new peptidyl tRNA moves to the P site, leaving the A site empty for binding of a new EF-Tu•aa-tRNA•GTP ternary complex. When the ribosome encounters a stop codon (UAG, UGA, or UAA), bacterial release factors 1 or 2 (RF1 or RF2) recognize UAG and UAA codons or UGA and UAA codons, respectively, and bind the ribosome. In either case, the ester bond attaching the newly formed protein to peptidyl-tRNA is hydrolyzed, and release factor 3 (RF3) helps RF1 or RF2 dissociate from the ribosome.

Unsurprisingly, tRNA modifications within the anticodon or adjacent to the anticodon within the tRNA ASL have been shown to play a variety of direct and sometimes essential roles during translation, which will be discussed below. However, tRNA modifications within the tRNA elbow can also play roles during this process. Here, we summarize known roles for tRNA modifications during tRNA aminoacylation, elongation/initiation factor binding, translation initiation, and translation elongation. Presumably, tRNA modifications do not play a role in translation termination, which will not be discussed further.

### *1.5.1 tRNA modifications affect tRNA aminoacylation*

Each functional tRNA relies upon a cognate aminoacyl-tRNA synthetase (aaRS) for the covalent attachment of the appropriate amino acid, forming an aa-tRNA. To maintain the fidelity of protein synthesis, it is paramount that aminoacyl-tRNA synthetases charge only their cognate tRNA, and not other similar tRNA species. Although not a common theme in tRNA aminoacylation, there are cases where tRNA modifications act as determinants or

antideterminants for aminoacyl-tRNA synthetases, which we will describe below. Moreover, *in vitro* aminoacylation of unmodified tRNAs often tends to be inefficient (96), suggesting a role for modifications in fine-tuning the affinity of tRNA for its cognate synthetase or enhancing the esterification reaction. Finally, as discussed above, proper tRNA folding is a pre-requisite for tRNA charging; thus, the tRNA chaperone activity of certain modification enzymes is likely to increase the proportion of aminoacylated tRNAs in cells (14-16,97).

In Table 3, we have summarized tRNA modifications that are known to affect the activity of certain aminoacyl-tRNA synthetases. In all cases, these effects have been determined by comparing the steady-state kinetic parameters for aminoacyl-tRNA synthetases with native (fully modified), unmodified, or partially modified tRNAs *in vitro*.

**Table 1.3 tRNA modifications affect aminoacylation**

| Modification (enzyme) | Affected aaRS | Affected tRNA | Organism | Evidence/mechanism | Ref. |
|---|---|---|---|---|---|
| mnm$^5$s$^2$U34 (MnmE,MnmG; MnmA) | GluRS<br>LysRS<br>GlnRS | tRNA$^{Glu}$<br>tRNA$^{Lys}$<br>tRNA$^{Gln}$ | *E. coli* | *In vitro* aminoacylation kinetics | (98-102) |
| L34 (aka k$^2$C, TilS) | IleRS | tRNA$^{Ile}_{CAU}$ | bacteria | tRNA$^{Ile}_{CAU}$ lacking L34 is efficiently aminoacylated by MetRS and not IleRS. L34 modification serves as a positive determinant for IleRS and a negative determinant for MetRS | (103) |
| | MetRS | tRNA$^{Ile}_{CAU}$ | bacteria | | |
| I34 (ADAT2/3) | IleRS | tRNA$^{Ile}_{IAU}$ | *S. cerevisiae* | Introduction of only I34 increases the $k_{cat}$ of aminoacylation 12-fold compared to an unmodified transcript | (96) |
| t$^6$A37 (TsaBDE) | IleRS | tRNA$^{Ile}_{GAU}$ | *E. coli* | Faster aminoacylation (~25-fold) of tRNA$^{Ile}_{GAU}$ *in vitro* by *E. coli* IleRS when t$^6$A37 is present | (62) |
| agm$^2$C34 (TiaS) | IleRS | tRNA$^{Ile}_{agm2CAU}$ | archaea | Analog of lysidine; similarly is responsible for Ile identity | (104,105) |
| m$^1$G37 (TrmD) | ProRS | tRNA$^{Pro}_{CGG}$ | *E. coli* | 17-fold reduction in catalytic efficiency for tRNA$^{Pro}_{CGG}$ | (106) |

| | | | | | |
|---|---|---|---|---|---|
| | | | | aminoacylation by ProRS | |
| $m^1G37$ (Trm5) | ArgRS | $tRNA^{Asp}$ | *S. cerevisiae* | methylation prevents misacylation of $tRNA^{Asp}$ by ArgRS by reducing $v_{max}$ and increasing $K_M$ | (107) |
| Ψ35 (Pus1) | TyrRS | $tRNA^{Tyr}$ | *S. cerevisiae* | *In vitro* aminoacylation kinetics | (108) |
| Ψ34/Ψ36 (Pus1) | IleRS | $tRNA^{Ile}_{\Psi A\Psi}$ | *S. cerevisiae* | 40-fold decrease in catalytic efficiency of transcript compared to native tRNA (additional native tRNA modifications may also be involved) | (96) |
| yW37 (Trm7) | PheRS | $tRNA^{Phe}$ | *S. cerevisiae and S. pombe* | Decreased cellular aminoacylation for *Δtrm7* cells compared to wildtype when grown in minimal media | (54,109) |
| $m^1A9$ | PheRs MetRS | mt-$tRNA^{Phe}$ mt-$tRNA^{Met}$ | *Ascaris suum* | Decreased *in vitro* aminoacylation for at least these two T-armless tRNAs | (74) |

*1.5.2 Translation initiation: $t^6A37$ restricts codon reading by initiator tRNA*

Unlike EF-Tu/eEF1A, which binds all elongator aa-tRNAs, initiation factor 2 (IF2, bacteria; eIF2, eukaryotes) is specific to binding only initiator tRNAs (fMet-$tRNA^{fMet}$, bacteria; Met-$tRNA_i^{Met}$, bacteria). *In vitro* transcribed mammalian $tRNA_i^{Met}$ has the ability to be aminoacylated and form a complex with eIF2•GTP, but a kinetic comparison of these interactions has yet to be examined (110). Although modifications to $tRNA_i^{Met}$ do not appear to affect binding to eIF2, in plants and fungi, presence of a 2′-O-phosphoribosyl modification of the $tRNA_i^{Met}$ ribose at position 64 formed by Rit1 acts as a steric block to initiator tRNA from binding eEF1A (111,112). Absence of this modification allows Met-$tRNA_i^{Met}$ to bind eEF1 *in vivo* (113). In contrast, the sequence of vertebrate $tRNA_i^{Met}$ itself acts to prevent elongation factor binding in a modification-independent manner (114). In bacteria, IF2 first binds the ribosome to recruit fMet-$tRNA^{fMet}$. To the best of our knowledge, all studies of bacterial translation initiation *in vitro* have

used native fMet-tRNA$^{fMet}$ (115), and no studies examining the role of modifications for bacterial initiation have been conducted.

As mentioned above, no direct roles for tRNA modifications in bacterial translation initiation have yet been identified. Mammalian unmodified tRNA$_i^{Met}$ can effectively form 43S pre-initiation and 48S initiation complexes *in vitro,* and fully-assembled 80S ribosomes produce methionylpuromycin at similar speeds *in vitro* with unmodified and modified initiator tRNAs (110). Thus, modifications to tRNA$_i^{Met}$ do not seem to be essential for translation initiation; however, at least one tRNA modification works to fine-tune this step. Several lines of *in vivo* evidence suggest the highly-conserved t$^6$A37 modification is important for restricting translation initiation to AUG codons in eukaryotes (116-118). Interestingly, t$^6$A37 is found in eukaryotic initiator tRNAs, but is noticeably absent in bacterial tRNA$^{fMet}$, even though this modification is present in nearly all other tRNAs with an ANN anticodon in bacteria. Unlike eukaryotic tRNA$_i^{Met}$, which rarely decodes non-AUG codons, bacterial tRNA$^{fMet}$ effectively decodes GUG and UGG codons, supporting the role for the presence (or absence) of this modification for start codon selection (117).

*1.5.4 Several roles for tRNA modifications during translation elongation*

Before delivery to the ribosome, elongator tRNAs must form a ternary complex with EF-Tu/eEF1A. Since EF-Tu/eEF1A must bind virtually all aa-tRNAs regardless of sequence, it is unsurprising that mutations in the tRNA sequence that do not perturb tRNA tertiary structure do not greatly affect Phe-tRNA$^{Phe}$ affinity for EF-Tu•GTP (119). In a similar manner, Phe-tRNA$^{Phe}$ lacking modifications binds EF-Tu•GTP with only an approximately 1.5-fold lower affinity compared to fully modified Phe-tRNA$^{Phe}$ (120). Likewise, affinities of unmodified Gly-tRNA$^{Gly}$, Val-tRNA$^{Val}$, Ala-tRNA$^{Ala}$, and Gln-tRNA$^{Gln}$ binding EF-Tu are no more than three-fold lower compared to their modified counterparts (121-123). Presumably, EF-Tu•GTP displays a similar lack of preference between other modified and unmodified aa-tRNA species, and eEF1A probably behaves in a similar manner to EF-Tu. As EF-Tu only contacts the tRNA acceptor stem

and T arm (Figure 1.3), which don't harbour many modifications, likely, the small increases in affinity observed for elongation factors in binding modified tRNAs can be attributed to tRNA modification enzymes fine-tuning the L-shaped tRNA tertiary structure and tRNA dynamics. For the special case of selenocysteine translation at UGA codons, the selenocysteine tRNA-specific elongation factor (SelB) is specific for binding only Sec-tRNA$^{Sec}$ (124). The affinities of *apo* SelB for modified and unmodified Sec-tRNA$^{Sec}$ are almost identical, suggesting SelB does not require modifications for tRNA binding (125).

Once part of a ternary complex, tRNAs bind to the ribosome in a complex, multistep process. In brief, a translating ribosome reversibly samples aa-tRNA•EF-Tu•GTP ternary complexes, forming initial binding complexes. If the ribosome identifies a cognate codon:anticodon pair, GTPase activation and subsequent GTP hydrolysis for EF-Tu is triggered, followed by the release of inorganic phosphate. Dissociation of EF-Tu may proceed or follow accommodation of aa-tRNA in the A site, which is then followed either by proofreading, wherein aa-tRNA may be ejected from the ribosome, or rapid peptide bond formation (126,127). The next step of elongation is translocation, which prepares the ribosome for another round of elongation (Figure 1.4).

tRNA modifications have been shown to play a multitude of roles during this process, including aiding tRNA binding to the ribosome, enhancing decoding mRNA codons, restricting decoding to only cognate codons, preventing proofreading at cognate codons, aiding in translocation, and enhancing or supressing various forms of ribosomal frameshifting, and several of these processes have been reviewed extensively elsewhere (9,10,128). In the paragraphs below, we will detail general roles for tRNA modifications in translation and the approaches used to study these. Roles for specific modifications are summarized in Table 1.4.

Of course, all of the processes affected by tRNA modifications can influence translation (e.g. modifications that lead to lower concentrations of cellular tRNA or decreased aminoacylation), but modifications themselves additionally play roles in translation. Traditional *in vivo* experiments to examine the role of tRNA modifications on translation generally compare a

tRNA modifying enzyme knockout to its corresponding wildtype strain, (e.g. pulse-labeling, translation reporter experiments, and examination of single protein expression by Western blotting) are often unable to distinguish between a direct role of a modification in translation and the effects of the modification for a previous step of tRNA maturation or preparation for translation. In contrast, *in vitro* assays using purified ribosomes and translation components and differently-modified tRNA can often demonstrate direct roles for tRNA modifications in translation and narrow down the affected step in translation. As many tRNA modification enzymes modify only select tRNAs and the codon content of mRNAs often plays a role in fine-tuning translation efficiency, it is important to examine how codons are affected differently by the loss of a tRNA modification enzyme. High-throughput experiments to examine translation such as ribosome profiling (129,130) can distinguish mRNA codons (and thus their corresponding tRNAs) that are most affected by modification enzyme deletion and provide clues into how translation is affected by examining whether ribosome pausing occurs when the codon is in the A site or P site. Additionally, ribosome-bound tRNA capture (131) can be used to identify which tRNAs are bound to ribosomes, whether they are in the A or P site, which modifications ribosome-bound tRNAs contain, and how this compares to the overall cellular tRNA population. Further, by comparing the proteome between a tRNA modifying enzyme knockout and its corresponding wildtype, codon-bias can be examined for up- and down-regulated proteins to identify codons that are potentially affected by tRNA modification (132).

Since non-Watson-Crick base-pairs are tolerated at the “wobble” position between the third nucleotide of a codon and the first nucleotide (N34) of a tRNA anticodon, modifications are very frequently found at the wobble position in tRNAs to restrict, enhance, or rarely, change decoding. In particular, uridines at position 34 are modified at a high frequency and have been extensively studied and reviewed in (10). Modifications adjacent to the anticodon, including at the conserved purine 37, often play roles in stabilizing the codon:anticodon base-pairing interaction by enhancing base-stacking (133). Although the impact of many anticodon stem-loop modifications on translation have been studied, whether tRNA modifying enzymes that target nucleotides outside of the tRNA anticodon (i.e. in the tRNA elbow) directly contribute to

translation remains unknown. Potentially, by enhancing the structure, folding, and dynamics of the tRNA elbow, these modifications may promote tRNA movement through the ribosome. Supporting this, several tRNA elbow modifications have been indirectly implicated in protein synthesis (Table 1.4).

**Table 1. 4 Examples of tRNA modifications and translation elongation**

| **Modification** (enzymes) | **Codons** and/or tRNAs affected**;** organism | **Experiments** | **Summary/mechanism** | **Ref.** |
|---|---|---|---|---|
| **mcm$^5$s$^2$U34** (ELP and URM1 pathway) | AAA, GAA, and CAA | Yeast proteome analysis, tandem-codon translation reporter assays, *in vitro* translation assays | Downregulated proteins in *URM1* deletion strains are enriched in these codons and tandem-codon reporter assays have confirmed defects in translation. *In vitro* assays show s$^2$U and mcm$^5$ modifications enhance A site binding | (134) |
| **s$^2$U** moiety of mcm$^5$s$^2$U34 (URM1 pathway) | tRNA$^{Lys}_{UUU}$ reading AAA codon | *In vitro* translation kinetics | Optimizes several steps subtly, increasing translation speed: **(1)** increase of codon recognition complex stability (6-fold decrease in $k_{off}$); **(2)** 7-fold increase in rate of Pi release/EF-Tu rearrangement; **(3)** 3-fold reduction in rate of of tRNA rejection (proof-reading); **(4)** 2-fold increase of clockwise ribosome rotation | (135) |
| **Ψ35** (RluF) | tRNA$^{Tyr}_{GUA}$ | Tandem- codon translation reporter assays | Decreased translation efficiency through multiple Tyr (TAY) codons | (136) |
| **m$^7$G46** (TrmB) | tRNA$^{Phe}_{GAA}$ reading UUU and UUC, tRNA$^{Asp}_{GUC}$ reading GAC and GAU codons | Tandem-codon translation reporter assays | Decreased translation efficiency through multiple tandem codons | (137) |

| | | | | |
|---|---|---|---|---|
| **mnm$^5$U34** (MnmE/G), **m$^1$G37** (TrmD), **t$^6$A37** (TsaBCDE) | tRNA$^{Glu}_{UUC}$, tRNA$^{Pro}_{UUC}$; both tRNA$^{Ile}_{GAU}$; and tRNA$^{Asn}_{GUU,}$ respectively | *In vitro* translation (PURExpress system) | *In vitro* translation can be supported using *in vitro* transcribed tRNAs, if these tRNAs contain these modifications | (138) |
| **Cm32 and Gm34** (Trm7) | tRNA$^{Phe}_{GAA}$ reading UUU | Polysome profiling, tRNA overexpression | Decreased translation of tRNA$^{Phe}$ | (67,139, 140) |
| **yW37** (Tyw2-4) | tRNA$^{Phe}_{GAA}$ reading UUU | *In vitro* translation kinetics | More efficient binding to the ribosomal A site | (133) |
| **t$^6$A37 and mnm$^5$U34** | tRNA$^{Lys}$ ASL | *In vitro* ribosomal A site binding and translocation | More efficient ribosomal A site binding and translocation of tRNA$^{Lys}$ ASL | (141) |
| **cmo$^5$U34** | tRNA$^{Val1}$ ASL reading GUU | *In vitro* ribosomal A site binding and translocation | Modification is required for translocation at GUU, but not GUA codons | (141) |
| **Ψ55** (TRUB1) | Global mitochondrial translation | Comparison of specific protein abundance by Western blot | Decreased abundance of several mitochondrial proteins | (142) |
| **m$^7$G** (TrmB) | Global translation, *Thermus thermophilus* under heat stress | $^{35}$S-pulse labeling | Decreased global translation | (59) |
| **Ψ55** (TruB) | Global translation, *Thermus thermophilus* under cold stress | $^{35}$S-pulse labeling | Decreased global translation | (58) |

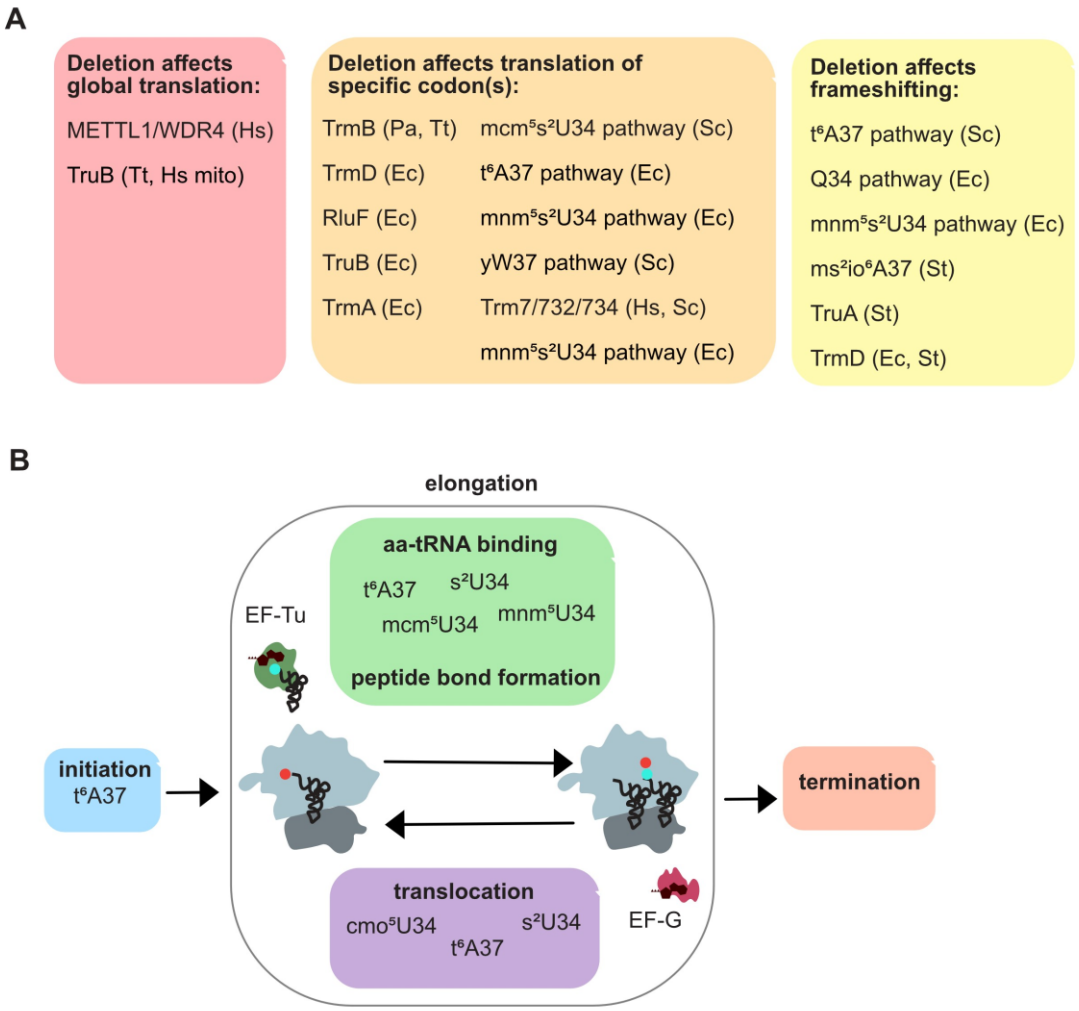



**Figure 1.4. Summary of the roles of modifications during different steps of translation. (A)** tRNA modifying enzymes whose absence *in vivo* affects global translation, specific codon translation, or frameshifting. Model organism used in each study is indicated in parenthesis. Hs: *Homo sapien*, Tt: *Thermus thermophilus*, Pa: *Pseudomonas aeruginosa*, Ec: *E. coli*, Sc: *Saccharomyces cerevisiae,* St: *Salmonella typhimurium*. **(B)** General schematic of the overall steps of translation. Modifications found to play roles within each step are highlighted within coloured boxes.

### 1.6 Conclusions and perspectives

In summary, we have described here how tRNA modifying enzymes affect tRNAs at all stages of the tRNA lifecycle by modifying and folding tRNAs. Together, the research summarized here suggests that deletion of a single tRNA modification enzyme can affect more than the loss of a single modification; instead, the entire tRNA modification status can be altered and the tRNA folding and structural dynamics may be affected. Thus, hypomodified and potentially misfolded tRNA may be targeted for degradation, affecting cellular tRNA abundance. Alternatively, the hypomodified tRNA may be improperly recognized by its interacting factors, which may result in altered tRNA aminoacylation and/or function during translation. In some cases, tRNA modifications may act redundantly to each other, masking effects in translation. In other cases, by affecting the translation of one or more codons, resulting cellular proteome changes explain the cellular phenotypes and diseases observed when tRNA modification is affected.

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# Chapter 2:
# Objectives

## 2.1 Objectives

As described in Chapter 1, all tRNAs contain several modifications that play various roles throughout the tRNA lifecycle. Particularly within the tRNA elbow, many tRNA modifications and their dedicated enzymes are highly conserved throughout all domains of life. Moreover, several tRNA elbow modifications are found in the majority or even all tRNA isoacceptors, rather than only a subset of tRNA species. Despite decades of tRNA research, due to the wealth of tRNA modification enzymes catalyzing diverse chemical reactions at different target nucleobases or sugars at various positions within the tRNA, the molecular mechanisms for several tRNA modifying enzymes have yet to be uncovered. Furthermore, contrasting the abundance and conservation of these modifications and modifying enzymes, in many cases, tRNA elbow modifications are non-essential under ideal and different stress conditions, thus leading to the question: why are these enzymes and modifications so highly conserved?

In this Ph.D. thesis, I primarily focus on the mechanisms and functions of three highly conserved tRNA modifying enzymes: the bacterial methyltransferases TrmA and TrmB, and the pseudouridine synthase TruB. The human homologs for these enzymes are TRMT2A/B, METTL1, and TRUB1/2, respectively. TrmA and TruB form $m^5U54$ and Ψ55 at adjacent positions within the T loop of every *E. coli* tRNA, and their homologs similarly introduce these modifications in the majority of elongator tRNAs throughout all domains of life. Notably, TrmA and TruB have two functions during tRNA maturation, as they additionally fold tRNA independently of their modification activity (1,2). In most organisms, $m^7G46$ is a common feature of tRNAs with short variable loops, formed by TrmB or one of its homologs. This thesis is divided into three experimental chapters wherein each chapter is a manuscript that examines the mechanism and/or function of one or more of the TrmA, TruB, and TrmB enzymes. The motivation underlying each manuscript and the objectives addressed in each are detailed below.

Since previous studies of TrmA, TruB, and TrmB have used unmodified tRNA as a substrate (2), whether these enzymes prefer to modify tRNA with a certain modification status remains unexplored. Since TrmA and TruB are the only two enzymes to modify all tRNAs, I

hypothesized that these enzymes are likely to form $m^5U54$ and Ψ55 early during tRNA maturation. Since no other modification is additionally present in all TrmA and TruB substrate tRNAs, it is unlikely that another modification acts as a determinant for modification by these enzymes. Moreover, as tRNA chaperones, having TrmA and TruB act early in tRNA maturation would seemingly be advantageous, ensuring tRNA is properly folded at an early stage. In contrast, as $m^7G46$ formed by TrmB is present in the majority of (but not all) tRNAs, I hypothesized that this enzyme may act later during tRNA maturation. The only additional modifications shared by all *E. coli* TrmB substrates are $m^5U54$ and Ψ55; therefore, presumably, if TrmB recognizes any prior modifications, it would likely be those introduced by TrmA and TruB. By quantitatively determining whether these enzymes prefer to bind and modify an unmodified tRNA or a tRNA that already contains several modifications, predictions can be made for which enzymes are likely to act early in tRNA maturation (on a mostly unmodified tRNA) and which are more likely to act in the later stages. Thus, the objectives of **Chapter 5** were:

I. to develop a system to prepare tRNA with a single modification (described in **Chapter 3**), in order to compare
II. the binding affinity and
III. the reaction initial velocity of TrmA, TruB, or TrmB for unmodified tRNA versus partially modified tRNA.

Whereas the binding of TrmA and TruB to tRNA has been well-studied using structural (3,4) and biochemical (1,2,5) approaches, the molecular determinants and mechanism for tRNA binding by TrmB are not well understood as no structure for bacterial TrmB in complex with tRNA exists. Although site-directed mutagenesis has revealed several TrmB residues necessary for methylation activity (6), it is unknown which of these residues are involved in tRNA binding and which are involved in catalytic activity. Additionally, diverse organisms including *Pseudomonas aeruginosa* and the parasitic fungus *Colletotrichum lagenarium* have been shown to display a hydrogen peroxide sensitivity in the absence of the *trmB* gene (7), but whether this phenotype

exists for the model organism *E. coli* remains unknown. With the goals of investigating the impact of TrmB for bacterial fitness and to gain an understanding of how conserved TrmB residues and the methyl donor SAM contribute to tRNA binding, the objectives of **Chapter 6** were to:

I. investigate the potential for a growth phenotype for *E. coli* lacking the *trmB* gene under stress conditions (hydrogen peroxide).
II. prepare a tRNA with a single modification, as described in **Chapter 4**, to subsequently introduce an iodoacetamide fluorescein-derivative internally within tRNA.
III. examine the interaction of fluorescent tRNA with wildtype and variant TrmB proteins with single-amino acid substitutions in the presence and absence of the methyl donor SAM using rapid-kinetic binding assays.
IV. compare the affinity of radiolabeled tRNA for wildtype and variant TrmB.

Although TrmA and TruB are highly conserved and $m^5U54$ and Ψ55 are abundant in tRNAs throughout all domains of life, paradoxically, deletion of these enzymes results in only modest phenotypes (8,9). Wondering why tRNA modification and folding by these enzymes is so conserved, the final experimental chapter of my thesis asks the question: what are the cellular roles of TrmA and TruB in bacteria? In collaboration with Tao Pan at the University of Chicago and Richard Fahlman at the University of Alberta, we addressed the following objectives by comparing wildtype *E. coli* with *E. coli* lacking the *trmA* and/or the *truB* genes in **Chapter 7:**

I. Determination of the effect of TrmA and TruB on:
    a. cellular tRNA abundance
    b. aminoacylation proportion
    c. and further modification by additional tRNA modifying enzymes for all cellular tRNAs.
II. Examination of how deletion of *trmA* and/or *truB* affects translation at various levels:
    a. global translation using a pulse labeling assay (BONCAT)
    b. codon-specific translation using tandem codon reporter assays

III. Investigation of how translational alterations in the absence of *trmA* and/or *truB* affect the *E. coli* transcriptome and proteome in order to determine changes in protein translation.

Taken together, the studies presented in this thesis advance our knowledge regarding the molecular mechanisms and cellular functions of universally conserved tRNA modifying enzymes that modify the elbow region of a majority of tRNAs. Thus, this research uncovers that the early-acting tRNA modification enzymes TrmA and TruB are vital for the maturation and aminoacylation of every tRNA and sheds light on why these enzymes are so highly conserved across all domains of life.

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**Chapter 3:**
**Partially modified tRNAs for the study of tRNA maturation and function**

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## 3.1 Preface

The text within this chapter is a manuscript titled, “Partially modified tRNAs for the study of tRNA maturation and function” that was published in *Methods in Enzymology* Volume 658 (RNA Modification Enzymes) pages 225-250 on July 14, 2021 with Sarah K. Schultz and Ute Kothe as authors (https://doi.org/10.1016/bs.mie.2021.06.007) (1). Dr. Ute Kothe and I conceptualized this manuscript as a detailed overview of the Kothe lab’s procedures for preparing partially modified tRNA. I wrote the first draft of the manuscript, and Dr. Ute Kothe and I both edited this manuscript. The original formatting has been changed to fit requirements for this thesis.

## 3.2 Abstract

Transfer RNA (tRNA) is the most highly and diversely modified class of RNA in all domains of life. However, we still have only a limited understanding of the concerted action of the many enzymes that modify tRNA during tRNA maturation and the synergistic functions of tRNA modifications for protein synthesis. Here, we describe the preparation of *in vitro* transcribed tRNAs with a partial set of defined modifications and the use of partially modified tRNAs in biochemical assays. By comparing the affinity and activity of tRNA modification enzymes for partially modified and unmodified tRNAs, we gain insight into the preferred pathways of tRNA maturation. Additionally, partially modified tRNAs will be highly useful to investigate the importance of tRNA modifications for tRNA function during translation including the interaction with aminoacyl-tRNA synthases, translation factors and the ribosome. Thereby, the methods described here lay the foundation for understanding the mechanistic function of tRNA modifications.

### 3.3 Introduction

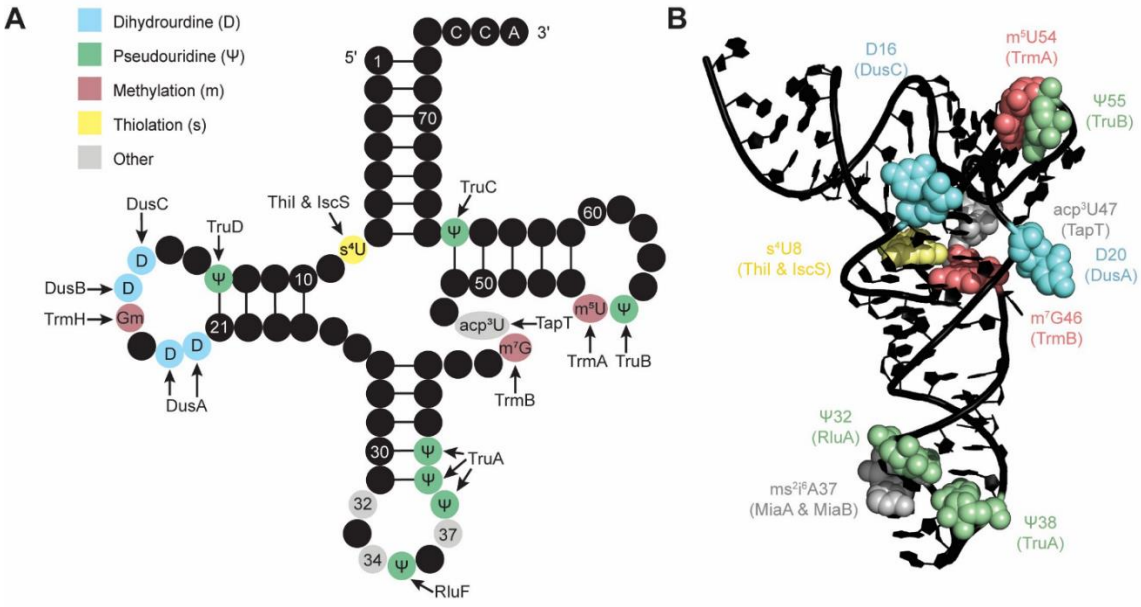
Over one hundred tRNA modifications have been identified to date, making it the most impressive RNA to study the mechanism and function of RNA modification. The locations and identities of tRNA modifications have been fully mapped in a handful of model organisms including *Escherichia coli,* where tRNAs contain 28 different modifications formed by 59 enzymes (Figure 1A) (2). As the most highly modified class of RNA, tRNAs contain a median of 8 modifications per molecule, which account for about 10% of a tRNA's 76 – 90 nucleotide sequence (3). Two clusters of tRNA modifications are evident within the tRNA tertiary structure which likely play different roles during protein synthesis (Figure 1B). The largest diversity of tRNA modification is found within the anticodon stem-loop, particularly at the wobble position 34 and the conserved purine 37 that is located at the 3' site of the anticodon (Figure 1A, Table 1). Specific anticodon stem-loop modifications are generally found in only a handful of specific tRNAs where they play various roles during mRNA decoding (4,5). The second cluster of modifications is found within the tRNA elbow, and these modifications are often found in many tRNA species where they in general contribute to tRNA stability (6-8) and folding (9-11). For example, pseudouridine (Ψ) 55 and 5-methyluridine ($m^5U$) 54 are present in every *E. coli* tRNA and these positions are commonly modified throughout all domains of life (3). TruB homologs introduce Ψ55 in nearly every elongator tRNA in all organisms, and position 54 is frequently methylated by TrmA homologs in eukaryotes and Gram-negative bacteria or by TrmFO homologs in Gram-positive bacteria (12). Archaea feature diverse modifications at U54 that stabilize their tRNA structure (13). Other common modifications within the tRNA elbow include 4-thiouridine ($s^4U$) 8 and 2'-O-methylguanosine (Gm) 18 in bacteria, and 7-methylguanosine ($m^7G$) 46 and dihydrouridines (D) at positions 16, 17, 20, and 20a across all domains of life (Figure 1A) (3). Although not present in *E. coli*, 1-methyladenosine ($m^1A$) 58 is also a common tRNA elbow modification in many organisms.

Despite the vast number of modification enzymes that interact with tRNA during maturation, we remain uncertain about the mechanisms and functions of tRNA modifications, in particular how they synergistically promote tRNA maturation and protein synthesis. Mechanistic

studies for many tRNA modification enzymes have been accomplished using *in vitro* transcribed, thus completely unmodified, tRNA. Although these studies demonstrate many tRNA modification enzymes are active on unmodified tRNA, completely unmodified tRNA may not be the preferred substrate of the enzymes. In fact, several studies have demonstrated that tRNA modification is likely not a random process. Injection of yeast pre-tRNA$^{Phe}$ into *Xenopus* oocytes revealed tRNA modification order is strictly correlated with tRNA end-trimming and intron removal (14,15). Recently, modification of *in vitro* transcribed tRNA$^{Phe}$ in yeast cellular extract revealed a preferred order of modification with Ψ55 being formed first followed by m$^5$U54 and m$^7$G46 (16). Interestingly, some human tRNAs contain Ψ54 rather than m$^5$U54, and at least *in vitro*, m$^1$A58 is advantageous for Ψ54 introduction by Pus10 (17). Work in human cells has shown a similar trend for early introduction of the Ψ55 modification (18). Similarly, we have shown that *E. coli* TruB has a lower affinity and reduced activity for tRNAs containing certain modifications, and the tRNA affinity and activity of *E. coli* TrmA is also modulated by other tRNA modifications suggesting that TruB and TrmA are likely to act in the early stages of *E. coli* tRNA maturation (19). In addition, the presence of m$^7$G46 has been shown to be advantageous for the later introduction of acp$^3$U47 by TapT in *E. coli* (20). In *Thermus thermophilus*, temperature-specific networks of tRNA modifications have been reported that stabilize tRNA in the thermophilic bacterium at different temperatures (21). Taking these studies together, most enzymes that modify the tRNA elbow do not rely on the previous introduction of a certain modification for activity; however, a preferential hierarchy of tRNA modification order seems to exist.

In contrast to the elbow region, tRNA modification circuits exist in the tRNA anticodon loop, wherein certain modifications are required for the activity of later-acting tRNA modification enzymes. Five well-characterized instances have been reviewed recently (22,23). For example, the conversion of m$^1$G37 to wybutosine (yW) 37 in tRNA$^{Phe}$ is surprisingly absent in *Saccharomyces cerevisiae* lacking the enzyme responsible for ribose methylations at positions 32 and 34, suggesting that ribose methylations are a prerequisite for the formation of yW37 (24). Interestingly, this modification circuit is conserved in *Schizosaccharomyces pombe* and humans (25,26). A second example of a conserved modification circuit is queuosine (Q) 34-driven

formation of $m^5C38$ in *S. pombe, D. discoideum* and humans, wherein loss of the Q34 modification is associated with loss of the $m^5C38$ modification *in vivo*, and Q34 presence stimulates, but is not strictly required for, C38 methylation *in vitro* (27,28). Modification circuits may be most prevalent in the tRNA anticodon loop because early modifications can act as sequence or structural (anti)determinants for later-acting modification enzymes to ensure that each tRNA contains the correct set of modifications despite the sequence and structural constraints of a functional anticodon loop (22). Modifications within the anticodon loop play a variety of functional roles including modulating interaction with aminoacyl tRNA synthetases (29-31), determining codon specificities for certain tRNAs (32), preventing frameshifting (33), optimizing elongation speed (28,34-37), and fine-tuning translational fidelity (38-40). Thus, despite the large collection of anticodon loop modification enzymes (Figure 3.1A, Table 3.1), it is particularly important that each enzyme modifies only their appropriate substrate tRNAs such that each mature tRNA contains the correct set of tRNA anticodon loop modifications.




*Caption on next page.*

**Figure 3.1. Modification of E. coli tRNA. (A)** Location of all *E. coli* tRNA modifications mapped onto the tRNA secondary structure. For tRNA positions that only contain one modification, the modification is shown using the standard, abbreviated tRNA modification nomenclature (41), and modifications are classified by colour according to their chemistry. Enzyme(s) responsible for each modification are shown with arrows. For tRNA positions that contain a variety of modifications (positions 32, 34, and 37), the modifications and the responsible enzymes are outlined in Table 3.1. **(B)** Location of modifications in *E. coli* $tRNA^{Phe}$ highlighted as coloured spheres within the crystal structure of unmodified $tRNA^{Phe}$ (PDB 3L0U) (42). Modifications and the enzyme(s) responsible for each modification are indicated. The colour scheme for modification type is the same as panel A.

Here, we describe a method to generate partially modified tRNAs that contain one or more specific modifications. These partially modified tRNAs are required to dissect the mechanisms and pathways of tRNA function from maturation to protein synthesis. First, as illustrated here, we can use partially modified tRNAs in biochemical and biophysical quantitative studies to gain a mechanistic understanding of the preferred order of all the different tRNA modification enzymes. Second, the partially modified tRNAs can also be tested regarding their interaction with other factors such as CCA-adding enzyme, aminoacyl-tRNA synthetases, initiation and elongation factors and of course the ribosome itself (43-47). Thereby, we can dissect the role of modifications and sets of modifications for the critical functions of tRNAs during all stages of protein synthesis.

**Table 3.1. *E. coli* tRNA anticodon loop modifications at positions 32, 34, and 37 and the enzymes responsible for their catalysis.** Related modifications are grouped, wherein the first line represents a common initial modification and later lines represent further modification(s).

| Position | Modification | Enzyme(s) |
|---|---|---|
| 32 | Ψ | RluA |
| | Cm, Um | TrmJ |
| | $s^2C$ | TtcA, IscS |
| 34 | I | TadA |
| | Cm, Um | TrmL |
| | $ac^4C$ | TmcA |
| | $k^2C$ | TilS |
| | $cmnm^5U$ or $nm^5U$<br>$mnm^5U$<br>$(c)mnm^5s^2U$<br>$mnm^5s^2Um$<br>$mnm^5Se^2U$ | MnmE, MnmG<br>MnmC<br>IscS, TusA, TusB, TusC, TusD, TusE, MnmA<br>TrmL<br>MnmH, SelD |
| | Q<br>GluQ | FolE, QueD, QueE, QueC, QueF, TGT, QueA, QueG, QueH<br>YadB |
| 37 | $m^2A$ | RlmN |
| | $m^6A$ | TrmN6 |
| | $i^6A$<br>$ms^2i^6A$ | MiaA<br>MiaB |
| | $t^6A$<br>$m^6t^6A$<br>$ct^6A$ | TsaB, TsaC, TsaD, TsaE<br>TrmO<br>CsdA, CsdE, TcdA |
| | $m^1G$ | TrmD |

### 3.4 Partial modification of *in vitro* transcribed tRNA

To determine the role of tRNA modifications for further tRNA maturation and tRNA function during translation, a collection of tRNAs with different modification statuses should be prepared. The activity and affinity of any tRNA-interacting factor can then be compared for differently modified tRNAs. In general, generation of partially modified tRNA can be accomplished by two different approaches. In the first approach, a specific tRNA isoacceptor can be purified from a knockout strain wherein the gene encoding the modification enzyme of interest has been deleted or from an organism that lacks the modification of interest (27,37,48-52). This method provides a

substrate tRNA for the modification enzyme of interest that contains all native modifications except for the modification being studied. This approach may be required to even detect activity for certain modification enzymes that require modifications to be present for activity, such as yeast Dus2 (48). However, it is difficult to narrow down which tRNA modification(s) are required for enzyme activity, as this would require preparation of a vast set of double knockout strains that lack not only the enzyme responsible for the modification being studied, but also additional modification enzyme(s). Moreover, in the absence of a sensitive mass spectrometry set-up, it is difficult to know the exact modification content within tRNA purified from cells, as not all modifications are always present at stoichiometric levels (18,53,54).

Alternatively, partially modified tRNA can be generated *in vitro* by incubating *in vitro* transcribed, unmodified tRNA with selected purified modification enzyme(s). Thereby, tRNA modifications within the tRNA can be selected and introduced in a controlled manner. This method easily allows for preparation of tRNA containing only a single modification or defined combinations of modifications. Depending on the planned study, this approach also allows preparation of modified, radiolabelled tRNA; for example, tRNA containing tritium ($^{3}H$) at the C5 proton of every uridine can be used in tritium release assays to monitor further tRNA modifications (55). As with tRNAs extracted from cells, it is critical to quantify the presence of the introduced tRNA modifications. This is feasible with a variety of different methods, as explained below. In order to introduce each tRNA modification of interest, the respective enzyme must be overexpressed and purified. For the vast number of modifications present in *E. coli*, cloning of the respective gene can be avoided as each open reading frame encoding a protein in *E. coli* has been cloned for overexpression with an N-terminal hexahistidine tag for affinity purification (56).

Here, we will describe the workflow for the generation of partially modified tRNAs *in vitro* using either non-radioactive or radioactively-labelled *in vitro* transcribed tRNA (Figure 3.2). For example, [5-$^{3}H$]tRNA is necessary if the modified tRNA will be used in tritium release activity assays (described in Section 3.5.1) and is also useful for nitrocellulose filtration binding assays

(described in Section 3.6). Likewise, $^{32}$P-labelled tRNA can be used in subsequent thin-layer chromatography (TLC) assays to detect tRNA modifications (57). In contrast, non-radioactive modified tRNA is necessary for the use in trichloroacetic acid (TCA) precipitation assays (described in Section 3.5.2). Whether or not the tRNA is radioactive and which enzyme is introducing the modification will determine how the extent of modification is determined. For [5-$^{3}$H]tRNA that is being modified by either TrmA or a pseudouridine synthase, the extent of modification introduced into the tRNA can be determined during the modification reaction itself and confirmed after modified tRNA purification, as described below. In contrast, if the tRNA is non-radioactive, then the percent modification of the tRNA can be determined by performing a small-scale reaction using the appropriate radioactive component in parallel under the same conditions. Alternatively, approaches such as high-performance liquid chromatography (HPLC), mass spectrometry or nuclear magnetic resonance (NMR) can allow quantification of the modification content (16,58-60).

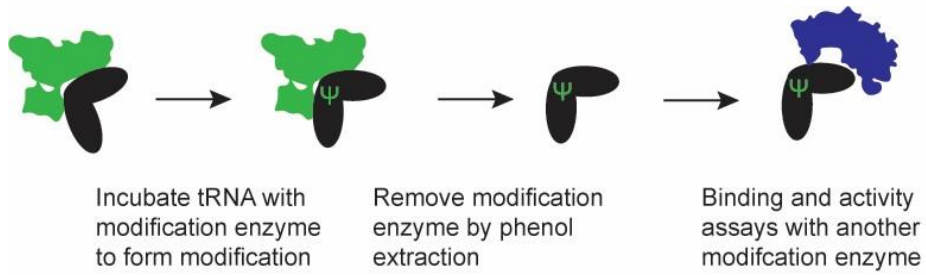



**Figure 3.2. Preparation of modified tRNA.** *In vitro* transcribed tRNA is incubated with a purified modification enzyme of interest, such as the pseudouridine synthase TruB. After modification by TruB to form Ψ55 tRNA, the enzyme is removed by phenol extraction. The resulting Ψ55 tRNA is then used in activity and binding assays with tRNA interaction partners, e.g. other tRNA modification enzymes.

The preparation of the uniformly, internally tritium-labeled RNA has been described in detail (61). In brief, template DNA for the tRNA of interest including a T7 promoter for *in vitro* transcription is prepared by PCR, and the PCR product is included in a standard *in vitro* transcription reaction to prepare non-radioactive tRNA. For the preparation of radioactive tRNA, the reaction includes the desired radiolabelled NTP. Subsequently, template DNA is removed by DNase I digestion, and the tRNA can be purified by a variety of different methods such as size exclusion chromatography or disposable anion exchange gravity chromatography (19,61).

### *3.4.1 Materials*

1. *In vitro* transcribed tRNA (non-radioactive, or radioactive, e.g. with a tritium label at the C5 of each uridine base for use in tritium release assays described in Section 3.5.1)
2. Purified tRNA modification enzyme(s) to introduce each modification of interest
3. Cofactor(s) appropriate for the modification enzyme of interest
4. TAKEM$_4$ buffer (50 mM Tris-HCl pH 7.5, 70 mM $NH_4Cl$, 30 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$) or other suitable reaction buffer
5. Phenol and chloroform
6. 3 M sodium acetate pH 5.2
7. Ethanol
8. Centrifuge
9. UV spectrometer

### *3.4.1 Method*

1. Refold purified *in vitro* transcribed tRNA for five minutes at 65°C in TAKEM$_4$ buffer followed by slowly cooling at room temperature for at least ten minutes to ensure the tRNA is properly folded.

2. Incubate refolded tRNA with the modification enzyme of interest and any necessary cofactors (e.g. *S*-adenosylmethionine for SAM-dependent methyltransferases). To ensure the modification is introduced within the tRNA at a high stoichiometry, single-turnover conditions with enzyme excess are preferred. For example, 1.6 μM tRNA can be incubated with 5 μM of the purified enzyme in a 7 mL reaction volume. If the modification enzyme requires cofactor(s) (such as SAM), the concentration of the cofactor should be high (for example, 50 μM). The reaction is incubated at the temperature optimal for the enzyme (i.e. 37°C for *E. coli* enzymes) for at least two hours.
3. Optional: to determine the extent of modification in the case of modifying tritium-labeled tRNA with a pseudouridine synthase or C5-methyltransferase, aliquots of the reaction can be quenched and used in the tritium release assay, as described in Section 3.5.1.
4. Following tRNA modification, heat inactivate enzyme(s) by incubating the reaction at 85°C for fifteen minutes to prevent enzyme binding to tRNA during RNA extraction.
5. To remove the modification enzyme(s), phenol extraction is performed by adding one volume of phenol, vortexing, and centrifugation at 4500*g* for 15 minutes at room temperature. Transfer the aqueous phase to a new tube, and repeat phenol extraction. To remove phenol, one volume of chloroform is added, vortexed, and centrifuged as above.
6. After transferring the extracted RNA to a new tube, 0.1 volumes of 3 M sodium acetate are added along with three volumes of ethanol. Precipitate RNA overnight at -20°C.
7. Collect tRNA by centrifugation at 4500*g* for 45 minutes at 4°C. Remove the ethanol supernatant and wash precipitated RNA with 70% ethanol. Centrifuge as before to collect the washed tRNA. Resuspend the modified tRNA in Milli-Q water.
8. The modified tRNA concentration can be determined in one of two ways:
    a. Absorbance at 260 nm using the extinction coefficient appropriate for the tRNA being used (e.g. 500,000 $M^{-1}cm^{-1}$ for *in vitro* transcribed *E. coli* $tRNA^{Phe}$ (62))

b. Comparison of the radioactive tRNA specific activity in dpm/pmol before and after modification. Whereas the specific activity in dpm/pmol/U should remain unchanged during tRNA modification, tritium release upon modification will mean there is one less radioactive uridine in the tRNA sequence upon modification. This can be accounted for to determine the new dpm/pmol and thus the new concentration of the tRNA. This method might be necessary if cofactors that absorb in the UV range were included in the preparative reaction, if these were carried through to the final product. Alternatively, the partially modified tRNA can be purified by chromatography or gel extraction to remove cofactors.

9. Finally, the extent of modification within the partially modified tRNA is determined:
    a. In the case of the preparation of tritium-labeled tRNA containing either $m^5U$ or Ψ modification, the extent of modification can be determined during the initial reaction, as described in Step 3. To further confirm the extent of modification, the purified partially modified tRNA can then be used in a single-turnover activity assay with the enzyme originally used to modify tRNA. After a long incubation (two hours), if any modification is detected on the partially modified tRNA, then that percentage can be assumed to be the percent tRNA left unmodified in the preparative modification reaction.
    b. The method above is not suitable for all modifications, for example in the case of TrmB modification to prepare $m^7G46$ tRNA, since this reaction does not result in the release of tritium from [5-$^3$H]tRNA. In this case, a small-scale modification reaction under the same conditions is performed in parallel to the large scale preparative reaction, but including radioactive [methyl-$^3$H]SAM in the reaction to monitor methylation progress.
    c. Depending on the type of modification, any other quantitative tRNA modification detection assay can be utilized such as HPLC, TLC or mass spectrometry to determine the extent of modification (57,58,63-65).

### 3.5 tRNA modification activity assays with partially modified tRNAs

To understand the tRNA modification preferences of modification enzymes, enzyme activity assays can be used to determine if a given modification enzyme has a preference towards the modification status of a tRNA. Herein, the activity of an enzyme acting on unmodified tRNA can be compared to tRNA that is partially modified. Under multiple-turnover (substrate excess) conditions, the initial velocity ($v_o$) of the reaction can be determined, which is influenced by all reaction steps including tRNA binding, possible conformational changes, catalysis, and product release. Additional experiments can provide insight into which step(s) precisely are affected by tRNA modification status. For example, under single-turnover conditions using a quench-flow apparatus, the effect of a tRNA modification on the rate of the catalytic step can be determined. Moreover, as described in Section 3.6, the nitrocellulose filtration assay can be used to determine the effect of tRNA modifications on the affinity of a modification enzyme.

Since tRNA modifications are diverse in chemistry, a variety of different activity assays are available to detect tRNA modification enzyme activity. Some techniques are specific to only certain chemical reactions, whereas other techniques such as HPLC coupled with either UV detection or mass spectrometry can detect nearly any tRNA modification that has an available standard (58). However, these techniques require access to specialized equipment and expertise. Here, we will describe two simple, radioactive activity assays that can each be used with multiple modification enzymes. First, the tritium release assay detects the activity of pseudouridine synthases and uridine C5 methyltransferases (55). Second, the TCA precipitation assay monitors the activity of SAM methyltransferases as well as other transferases that transfer a functional group from a radioactively labelled cofactor to tRNA (66,67).

*3.5.1 Tritium release assay*

The tritium release assay is a quantitative assay that is used to detect the activity of pseudouridine synthases (such as *E. coli* TruB, TruA, and RluA) and uridine C5 methyltransferases (such as *E. coli* TrmA) (9,61). Upon modification by these enzymes, the proton at C5 is released from the modified uridine into the solution (Figure 3.3A). Pseudouridine synthases cleave the N-C glyosidic bond to the uracil base such that after rotation of the uracil base, C5 of the uracil base can form a C-C glycosidic bond with the ribose sugar while the proton associated with C5 is released into solution (68). Similarly, during tRNA modification by TrmA, transfer of the SAM methyl group to C5 of uracil results in release of the C5 proton into solution (69,70). The tritium release assay quantifies the release of the C5-associated proton by replacement of this proton with the radioactive isotope, tritium. Thus, as mentioned in Section 3.4, a prerequisite for the tritium release assay is tRNA that has been *in vitro* transcribed in the presence of [C5-$^{3}$H]UTP.

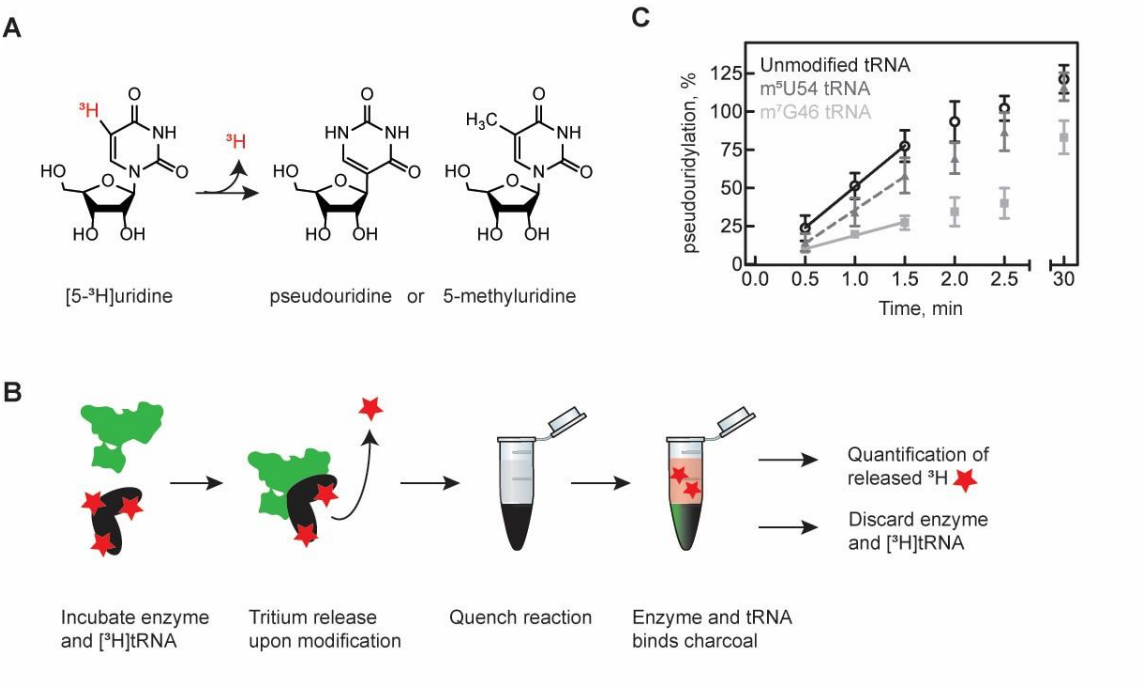



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**Figure 3.3. Tritium release assay to detect pseudouridylation or uridine C5 methylation.** **(A)** Upon modification by TruB or TrmA, the target uridine is either isomerized to pseudouridine or methylated to 5-methyluridine. During this process, the C5-associated tritium is released. **(B)** Overview of the tritium release assay. Here, TruB is incubated with [5-$^{3}$H]tRNA. Upon Ψ55 formation, a tritium is released into the solution. At different time points, the reaction is quenched in 0.1 M HCl that contains 5% (w/v) activated charcoal. While the released tritium remains in solution, the TruB enzyme and [5-$^{3}$H]tRNA bind to the charcoal and are removed by centrifugation. After repeating this step and removing charcoal by filtration through glass wool (not shown), the tritium released into the solution is quantified by scintillation counting. **(C)** Example tritium release data comparing the initial velocity of 10 nM TruB acting on 600 nM unmodified tRNA (open circles) with m$^{5}$U54 tRNA (grey triangles, dashed line) and m$^{7}$G46 tRNA (light grey squares). Reactions were performed in triplicate with the error bars representing standard deviation. The initial velocities are determined by linear regression showing that m$^{7}$G reduces the modification velocity by TruB significantly.

To monitor time courses of tRNA modification, the amount of tritium released from tRNA can be quantified by quenching the reaction in 0.1 M HCl containing 5% activated charcoal at different time points (Figure 3.3B). The enzyme and radioactive tRNA will bind to the activated charcoal and are subsequently removed by centrifugation. To ensure complete removal of radioactive tRNA, the resulting supernatant is then added to fresh 0.1 M HCl 5% activated charcoal and centrifuged again. The final supernatant containing the released tritium is filtered through glass wool to ensure removal of charcoal and the supernatant is then subjected to scintillation counting.

This assay can be used under multiple-turnover conditions to compare the initial velocity of a tRNA modification enzyme modifying unmodified tRNA to that of a partially modified tRNA (Figure 3C). Additionally, with the help of a quench flow apparatus, single turnover conditions can

be used to compare the rate of the catalytic step ($k_{\Psi}$ for TruB or $k_{methyl}$ for TrmA) in the presence or absence of tRNA modifications (9,61).

*3.5.1.1 Materials*

1. [5-$^{3}$H]uridine labeled tRNA, either unmodified or partially modified as described in Section 2
2. Purified pseudouridine synthase or C5 methyltransferase
3. 5% (w/v) Norit A (activated charcoal) in 0.1 M HCl
4. 1 mL pipette tips containing glass wool
5. Microcentrifuge
6. 6 mL scintillation vials
7. EcoLite Scintillation Cocktail (MP Biomedicals)
8. Liquid scintillation analyzer

*3.5.1.2 Method*

1. Refold tRNA in TAKEM$_4$ buffer as described earlier.
2. For multiple turnover assays, incubate 600 nM [$^{3}$H]tRNA with 10 nM enzyme at the desired temperature (37°C for *E. coli* enzymes). For methyltransferases, SAM is included at a final concentration of 50 μM. Reaction volumes will depend on the volume and number of time points to be removed. Each reaction time point should contain more than 1000 disintegrations per minute (dpm) of $^{3}$H that can be released upon tRNA modification to yield a robust signal.
3. At desired time points, remove a constant volume of the reaction and quench in 1 mL 0.1 M HCl containing 5% activated charcoal. Vortex and incubate for 10 min or longer.
4. After collecting each time point, samples in 0.1 M 5% activated charcoal are centrifuged at 10,000 *g* for two minutes. Transfer 0.8 mL of the supernatant to a microcentrifuge tube

containing 0.5 mL 0.1 M HCl 5% activated charcoal for a second extraction. Incubate for at least ten minutes, centrifuge as before.

5. Filter 1 mL of the resulting supernatant through a 1 mL pipette tip containing glass wool to remove the activated charcoal.
6. Count 0.8 mL of the filtered supernatant in 4 mL scintillation cocktail. Additionally, count 2 µL of the reaction directly in triplicate as a control to confirm the total activity of the reaction. Determine the radioactivity in each sample in dpm by scintillation counting.
7. Assuming that the original reaction volume removed at each time point was low (less than 50 µL, step 3), the tritium extraction results in scintillation analysis of only 49.2% of the removed reaction volume. Using this factor, the amount of $^{3}H$ released in the analyzed reaction volume can be calculated, and the percent tRNA modification can be determined by comparing the released tritium to the total activity of the reaction divided by the number of uridines per RNA (as only the one modified uridine releases tritium). Alternatively, the amount of tritium released in dpm can be converted into the amount of tRNA modification formed (in pmol) with the help of the specific activity of the radioactive tRNA (in dpm/pmol/U). Plotting the concentration of tRNA modification formed over time allows for the determination of the initial reaction velocity by linear regression of the early time points.
8. Optional: The experiment can be repeated with differently modified tRNA to determine the effect of different modifications on the activity of pseudouridine synthases or C5 methyltransferases. Also, a titration with tRNA at constant low enzyme concentration will allow determination of Michaelis-Menten parameters ($K_M$, $k_{cat}$).

*3.5.2 TCA precipitation assay*

The TCA precipitation assay is a simple and versatile assay that can be used to detect the activity of any enzyme that transfers a functional group from a cofactor to tRNA, if a radioactive version of the cofactor is commercially available or can be prepared wherein the radiolabel is

present at the functional group. This assay can be used for all SAM-dependent tRNA methyltransferases using radioactive SAM with the methyl group containing either a $^{14}C$ label or $^{3}H$ label (Figure 3.4A). Additionally, this assay has been used to detect the formation of N6-threonylcarbamoyladenosine (67) and N6-isopentenyladenosine (66) using radiolabeled L-threonine or 7,7-dimethylallyl pyrophosphate, respectively. It is also suitable to follow tRNA aminoacylation using radioactive amino acids (71).

Upon modification by an enzyme that transfers a radioactive cofactor into non-radioactive tRNA, the tRNA will be radioactive and can be precipitated onto Whatman paper filters that have been pre-soaked in 5% trichloroacetic acid (TCA, Figure 3.4B). Washing the filters with 5% TCA will remove unincorporated radioactive cofactor, and a final wash with ethanol will remove the TCA and any enzyme. Thus, the extent of modification of the tRNA precipitated on the filter can be determined by scintillation counting. Below we will explain the use of this assay to detect the activity of SAM-dependent methyltransferases such as *E. coli* TrmA and TrmB.

*3.5.2.1 Materials*

1. Whatman filter paper, cut into 0.5 x 1 cm rectangles, pre-soaked in 5% (w/v) TCA and dried before use
2. *In vitro* transcribed tRNA (non-radioactive)
3. [methyl-$^{3}H$]SAM (Perkin Elmer) and non-radioactive SAM (NEB) to prepare a suitable stock solution (e.g. 50 µM and 600 dpm/pmol)
4. Purified methyltransferase
5. 5% (w/v) TCA
6. Ethanol
7. Oven (set to 65°C)
8. 20 mL scintillation vials
9. EcoLite Scintillation Cocktail (MP Biomedicals)
10. Liquid scintillation analyzer

*3.5.2.2 Method*

1. Incubate folded $tRNA^{Phe}$ with a tRNA methyltransferase. For example, for multiple-turnover conditions, 10 nM enzyme, 600 nM tRNA, and 50 μM [methyl-$^3$H]SAM (600 dpm/pmol) may be incubated in a 60 μL total reaction volume. Quench the reaction at desired time points by removing 10 μL of the reaction and spotting onto the pre-soaked filter paper.
2. After air-drying the filter papers for at least 10 minutes, remove unincorporated radioactive SAM from the filter papers by washing no more than 10 filters in ~40 mL 5% TCA in a 50 mL tube with rocking for 5 minutes. Discard 5% TCA. Repeat twice.
3. To remove TCA and protein, wash filters once with ~40 mL ethanol.
4. Dry filters in an oven at 65°C for a half hour. Then add filters to 4 mL scintillation cocktail and vortex well. Count each filter to determine the radioactivity in dpm. In addition, count the reaction directly in triplicate as a control.
5. To determine the extent of tRNA methylation at each time point, the radioactivity in dpm for each time point will be divided by the theoretical dpm value expected for 100% modification. To calculate the theoretical dpm value of 100% modification, the final specific activity of the [methyl-$^3$H]SAM (dpm/pmol) in the reaction will be multiplied by the spot volume (μL) and the concentration of tRNA in the reaction (μM). To ensure correct pipetting, the specific activity can be confirmed using the 2 μL direct reaction counts. As with the tritium release assay, the concentration of tRNA modification formed (in μM) can be plotted against time to determine the initial velocity in multiple turnover experiments.
6. Optional: This experiment can be repeated with differently modified tRNA to determine the effect of different modifications on the activity of tRNA methyltransferases or to determine Michaelis-Menten parameters by using increasing substrate concentrations.

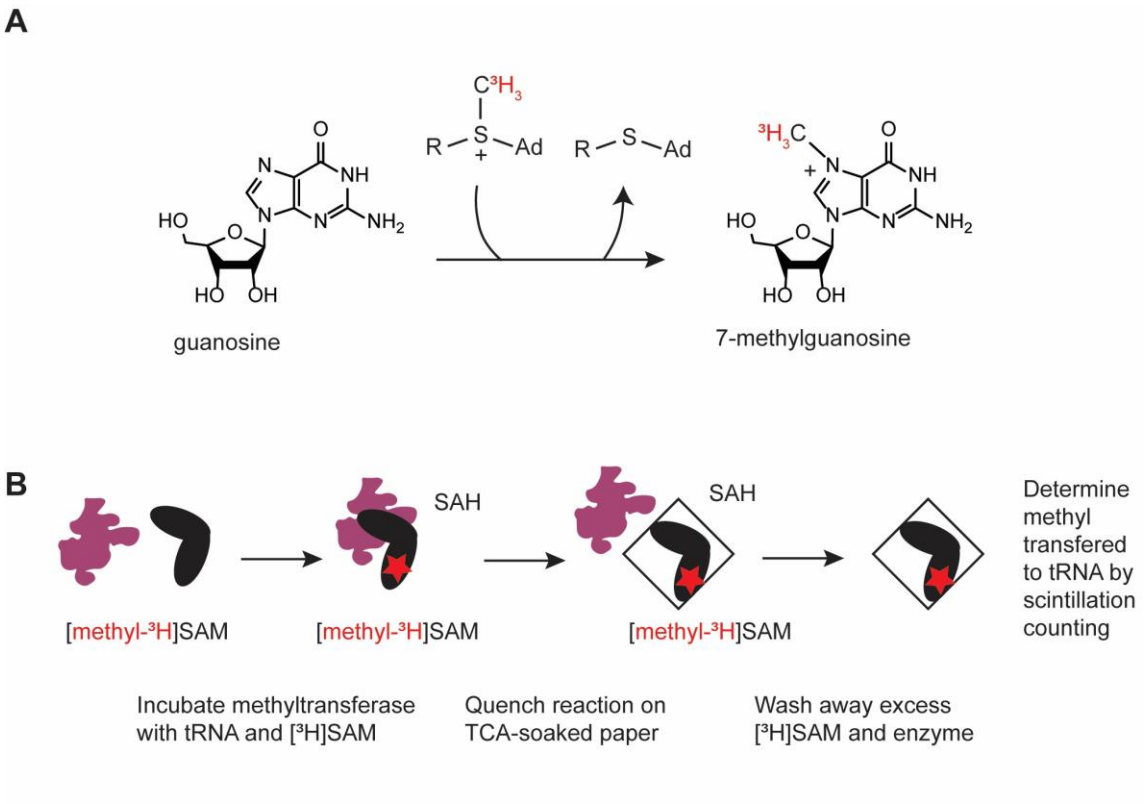



**Figure 3.4. TCA precipitation assay to monitor tRNA methylation. (A)** Methylation by TrmB forms 7-methylguanosine from guanosine in the presence of [methyl-$^3$H]SAM. Upon methylation, the radioactive methyl group is transferred from the cofactor to the tRNA. **(B)** Overview of the TCA precipitation assay. In this case, TrmB is incubated with non-radioactive tRNA and [methyl-$^3$H]SAM. After transfer of the radioactive methyl onto the tRNA, the reaction is spotted onto TCA-soaked Whatman paper to precipitate the tRNA. After drying the filters, unincorporated [methyl-$^3$H]SAM is removed by washing three times with 5% (w/v) TCA. After a final wash with ethanol, the filters are dried, and the amount of radioactive methyl incorporated is determined by scintillation counting.

### 3.6 Determining tRNA affinity with the nitrocellulose filtration assay

The nitrocellulose filtration assay is a simple and sensitive method to detect radiolabeled tRNA binding to proteins through the protein's high affinity to the nitrocellulose filter (Figure

3.5A). In brief, tRNA bound to protein will be retained on the filter and detected by scintillation counting whereas unbound tRNA is washed off the nitrocellulose membrane (47). In a variation of this assay, the unbound tRNA can be captured on a second positively charged membrane placed underneath the nitrocellulose filter (44).

This assay can be applied to any protein that binds tRNA such as tRNA modifying enzymes, aminoacyl-tRNA synthetases, and translation factors. To measure substrate rather than product binding to an enzyme, either a catalytically inactive enzyme can be used or the cofactors necessary for tRNA modification catalysis can be omitted. Alternative binding assays can instead be used, such as electrophoretic mobility shift assays (EMSA) with radiolabeled or fluorescent tRNA, titrations with fluorescently labeled tRNA in a fluorescence spectrometer, or isothermal calorimetry (72).

A prerequisite to the nitrocellulose filtration assay is radioactive tRNA. The tRNA can contain any type of radiolabel; for example, the internally tritium-labeled tRNA used in the tritium release assay is also useful in the filter binding assay. Alternatively, tRNA can be labelled with $^{32}$P either through end-labelling with [γ-$^{32}$P]ATP by T4 polynucleotide kinase or through body-labelling by using any [α-$^{32}$P]NTP within the *in vitro* transcription reaction. Using $^{32}$P-labelled RNA also allows quantifying the tRNA retained on the nitrocellulose filter by phosphorimaging; however, the short half-life of $^{32}$P limits the lifetime of the resulting radioactive tRNA (44). As with the activity assays described above, the affinity of a protein can first be determined for unmodified tRNA and then compared to that of differently modified tRNAs (Figure 3.5B).

### *3.6.1 Materials*

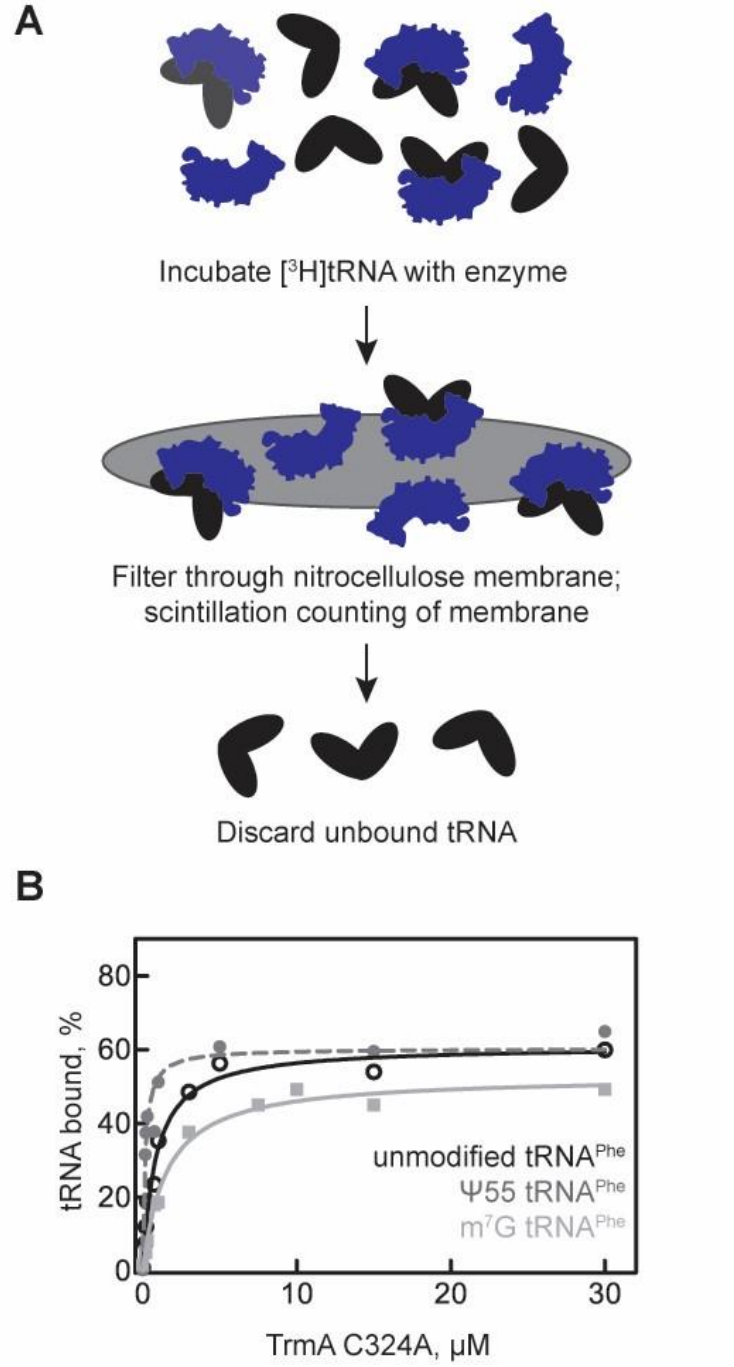
1. Radioactive (modified) tRNA either internally labeled (by using [C5-$^{3}$H]UTP or any [α-$^{32}$P]NTP during *in vitro* transcription) or end-labeled with [γ-$^{32}$P]ATP
2. Purified tRNA-binding protein (e.g. an inactive variant of a modification enzyme)
3. Filter binding apparatus (or dot blot apparatus) and vacuum pump

4. Nitrocellulose filters
5. TAKEM$_4$ buffer or other suitable reaction buffer
6. 20 mL scintillation vials
7. EcoLite Scintillation Cocktail (MP Biomedicals)
8. Liquid scintillation analyzer

*3.6.2 Methods*

1. Refold tRNA as above in TAKEM$_4$ buffer.
2. Prepare a series of protein dilutions with increasing final concentrations in TAKEM$_4$ buffer. Generally, 8 – 10 protein concentrations are used for each titration, with one sample containing no enzyme to monitor the background of radioactive tRNA binding the nitrocellulose membrane. The protein concentrations used in each titration may have to be optimized according to the affinity for tRNA.
3. Add refolded tRNA to each protein dilution at 1-minute intervals. The final tRNA concentration is kept constant across all enzyme dilutions at a low concentration (10-40 nM) that is at least 3-fold lower than the lowest protein concentration used. The volume of each reaction and the concentration of tRNA used should provide counts >1000 dpm for each data point. For example, a tRNA with a specific activity of ~5000 dpm/pmol is used at a concentration of 40 nM in a 25 µL reaction.
4. After incubation for ten minutes at the desired temperature, filter each reaction through a nitrocellulose filter under vacuum on a filter binding apparatus. After spotting the reaction mixture onto the filter, quickly wash with 1 mL of ice-cold buffer to remove transiently bound tRNA. Repeat for each enzyme/tRNA mixture.
5. Dissolve each filter in 10 mL scintillation cocktail in a scintillation tube and scintillation count.

6. Count 2 – 10 µL of the tRNA dilution directly three times to confirm the specific activity (in dpm/µL) of the tRNA dilution. This value is used to determine the activity of 100% tRNA binding (in dpm).




*Caption on next page.*

**Figure 3.5. Nitrocellulose filtration assay to monitor tRNA binding. (A)** Overview of the nitrocellulose filtration assay. The tRNA binding protein, in this case, TrmA, is incubated with radioactively labeled tRNA. After 10 minutes, the mixture is spotted onto a nitrocellulose membrane under vacuum and quickly washed with ice-cold buffer. The protein binds to the nitrocellulose membrane with a high affinity. Only tRNA that is bound to TrmA remains bound to the membrane. The amount of radiolabelled tRNA retained on the membrane is determined by scintillation counting. **(B)** Example filter binding data comparing the affinity of catalytically inactive TrmA C324A that is deficient in covalent bond formation with 20 nM unmodified tRNA (open circles), Ψ55 tRNA (grey circles, dashed line), and $m^7$G46 tRNA (light grey squares). Fitting of the data to a hyperbolic function yields the dissociation constant, $K_D$, which reveals that the presence of Ψ55 increases the affinity of TrmA for tRNA more than 4-fold, whereas the presence of $m^7$G46 reduces the affinity of TrmA for tRNA ~2-fold.

7. Determine the percent tRNA bound for each protein concentration by dividing the activity of the tRNA bound by that of the theoretical 100% tRNA binding value, determined above. Percent tRNA bound is plotted against protein concentration and fit to a hyperbolic equation to determine the dissociation constant ($K_D$):

$$Bound = Bound_{max} \times [enzyme]/(K_D + [enzyme])$$

8. Optional: The experiment can be repeated for a differently partially modified tRNA to determine the impact of tRNA modifications on the binding of the given modification enzyme.

### 3.7 Future perspectives

Recent research has highlighted that tRNA modification is most likely not a random process. Within the anticodon stem loop, several tRNA modification circuits are evident, and

within the tRNA elbow, there is a preferential hierarchy of tRNA modification order. However, the preferred pathway of tRNA maturation and modification across the entire tRNA remains unknown as the preferences of many tRNA modification enzymes remain to be studied. Complementing *in vivo* studies, the biochemical approach using partially modified tRNAs described here allows for quantitative dissection of tRNA modification pathways and provides mechanistic insight into the target tRNA selection as well as catalysis of tRNA modification enzymes (19). Given the large number of tRNA modification and modification enzymes, much insight can be gained in future from such approaches.

Possibly even more important, partially modified tRNAs are ideally suited to uncover the function of RNA modifications for protein synthesis. Whereas unmodified tRNAs have been demonstrated to be less efficient than fully modified tRNAs during *in vitro* translation (44,46,73,74), the contributions of individual modifications or groups of modifications for protein synthesis often remain unclear. In particular, the function of tRNA elbow modifications during translation is typically not known and may be covered by synergies between different modifications. To gain a better understanding of the individual and collective roles of tRNA modifications, it will be highly useful to study the interaction of partially modified tRNAs with aminoacyl-tRNA synthetases, translation factors and the ribosome. In conclusion, partially modified tRNAs are a critical tool to identify the evolutionarily conserved functions of tRNA modifications for tRNA maturation and protein synthesis.

### 3.8 Acknowledgements

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**Chapter 4:**
**Fluorescent labeling of tRNA for rapid kinetic interaction studies with tRNA-binding proteins**

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## 4.1 Preface

This text was conceptualized by myself and Dr. Ute Kothe as a methods chapter describing the Kothe lab's procedures for partially modifying and introducing an internal fluorophore within *in vitro* transcribed tRNA. This manuscript was published in *Methods in Enzymology* Volume 692 (Enzymes in RNA Science and Biotechnology) on May 30, 2023, pages 103-126 (https://doi.org/10.1016/bs.mie.2023.05.007) with the title, "Fluorescent labeling of tRNA for rapid kinetic interaction studies with tRNA-binding proteins" with Sarah K. Schultz and Ute Kothe as authors (1). I wrote the first draft of this manuscript, which was edited by Dr. Ute Kothe and myself. This manuscript has been reformatted for use in this thesis.

## 4.2 Abstract

Transfer RNA (tRNA) plays a critical role during translation and interacts with numerous proteins during its biogenesis, functional cycle and degradation. In particular, tRNA is extensively post-transcriptionally modified by various tRNA modifying enzymes which each target a specific nucleotide at different positions within tRNAs to introduce different chemical modifications. Fluorescent assays can be used to study the interaction between a protein and tRNA. Moreover, rapid mixing, fluorescence stopped-flow assays provide insights into the kinetics of the tRNA-protein interaction in order to elucidate the tRNA binding mechanism for the given protein. A prerequisite for these studies is a fluorescently-labeled molecule, such as fluorescent tRNA, wherein a change in fluorescence occurs upon protein binding. In this chapter, we discuss the utilization of tRNA modifications in order to introduce fluorophores at particular positions within tRNAs. Particularly, we focus on *in vitro* thiolation of a uridine at position 8 within tRNAs using the tRNA modification enzyme ThiI followed by labeling of the thiol group with fluorescein. As such, this fluorescently-labeled tRNA is primarily unmodified, with the exception of the thiolation modification to which the fluorophore is attached and can be used as a substrate to study the binding of different tRNA-interacting factors. Herein, we discuss the example of studying the

tRNA binding mechanism of the tRNA modifying enzymes TrmB and DusA using internally fluorescein-labeled tRNA.

### 4.3 Introduction

Transfer RNAs (tRNAs) interact with several cellular factors throughout tRNA biogenesis, during their role in translation and when they are degraded. Among the many tRNA-interacting factors is a large group of tRNA modifying enzymes, which generally accounts for at least one percent of the protein coding genes within a genome (2,3). tRNA is both the most densely and diversely modified class of RNA, with a median of eleven modifications per tRNA sequence and over ninety chemically different modifications appearing within tRNAs throughout all domains of life (4). tRNA modifications and their corresponding enzymes are currently best studied in the model organism *Escherichia coli*. The tRNA modification set is thought to be completely mapped in this organism, including 59 proteins involved in the formation of 28 tRNA modifications (3-5). Several modifications found in *E. coli* tRNAs are well-conserved across all domains of life, suggesting such modifications may play conserved roles in tRNA biology. Accordingly, modified tRNAs outperform their unmodified transcribed counterparts in *in vitro* translation assays (6-12) and mutations within human genes encoding certain tRNA modifying enzymes have been implicated in different diseases (13-15). On the other hand, other modifications are found in only specific groupings of organisms, suggesting possible roles for these modifications for environmental adaptations (16-18).

Despite the vast number of tRNA modifications and tRNA modifying enzymes already identified, full mapping of tRNA modifications has been accomplished for only a few model organisms. As such, novel modifications and enzymes remain to be discovered today (19,20) and the mapping of known modifications still remains to be accomplished for many organisms (21). The discovery of new tRNA modifications and advancement in tRNA modification mapping has been facilitated primarily by improvements to mass spectrometry and sophisticated next generation sequencing techniques (22-27).

Despite the abundance and importance of tRNA modification enzymes, in many cases we lack a clear understanding of how these enzymes recognize and bind their substrate tRNAs and identify and orient only their target nucleotide for modification. Moreover, as tRNA is a highly-structured molecule, many tRNA modification enzymes must locally remodel tRNA in order to access their target base (28-34). In at least two cases (TrmA and TruB), tRNA modifying enzymes have been demonstrated to provide tRNAs a second chance at proper folding (28,29,35), and this tRNA chaperone activity appears to be the primary cellular function of these enzymes (36-38). Thus, investigations into the mechanism of modification enzymes binding tRNAs is vital to obtain a clear understanding of the cellular functions of modification enzymes. Moreover, studies of tRNAs with its interacting enzymes provides a platform for understanding cellular effects of tRNA modification enzyme malfunction.

In addition to the many interactions of tRNA with modifying enzymes during tRNA biogenesis, there are several other proteins that bind to tRNA during its lifecycle (Figure 1) (39). While some of these interactions are well studied such as formation of the EF-Tu•GTP•tRNA ternary complex (40), other tRNA binding partners are less well characterized. Moreover, the role of tRNA modifications for the association with other proteins remains often unknown. During tRNA biogenesis, the pre-tRNA is processed at the 5′ and 3′ ends into its mature form by several different enzymes (41) which may include the help of the La protein as a tRNA chaperone (42). In addition, tRNAs can contain introns within the anticodon arm which are removed by a tRNA-specific splicing machinery (43). During its functional cycle, tRNA is charged with a specific amino acid by an aminoacyl-tRNA synthetase (44) and then binds to EF-Tu / eEF-1 which delivers it to the ribosome where tRNA moves from the A to the P and eventually E site (45). Lastly, tRNAs can be site-specifically cleaved generating tRNA-derived fragments (tRFs) with various functions (46) and quality control pathways involving multiple factors will result in tRNA degradation (47).

In this chapter, we discuss the use of fluorescent labelling techniques of tRNA, focusing on the example of thiol modification at tRNA position 8. We then discuss the use of fluorescent

tRNA to study the mechanisms of tRNA binding in real-time using the rapid-kinetic technique stopped-flow.

### 4.4 Fluorescent labelling of tRNA using modifications

Several methods to fluorescently label various positions within RNA molecules exist. In general, RNAs can either be uniformly labeled during *in vitro* transcription through addition of fluorescent nucleotide triphosphates or a fluorescent label can be incorporated at various specific positions within the RNA by chemical and/or enzymatic means. For fluorescence interaction studies, a single label at a known and desired position is often required to obtain a clear signal for mechanistic analysis. In general, 3' end labeling of periodate-oxidized RNA is a simple method to fluorescently label RNA (48); however, labeling at the 3' end may not provide a change in fluorescence when mixed with the tRNA binding enzyme being studied. In these cases, an internal fluorescent label is desirable. One such way to introduce an internal fluorescent label is custom chemical RNA oligonucleotide synthesis wherein a fluorescent nucleoside analog is included internally. In this case, a common choice is 2-aminopurine (49,50), which has been frequently used in the context of studying the binding of different modification enzymes to tRNA or rRNA (29,51-54). 2-aminopurine and other nucleoside derivatives are advantageous as their structure is not bulky, as large fluorophores may interfere with RNA structure and interactions. However, chemical synthesis can be expensive and RNA synthesis length may be limited such that two RNAs may need to be ligated together to achieve the full-length RNA of interest (29,51).

Alternatively, the chemistry of several naturally occurring tRNA modifications can be exploited for several purposes, including to introduce an internal fluorescent label within RNA (55-57). For example, labelling strategies have been described for dihydrouridines (58-61), nucleobase thiolations including 4-thiouridine, 2-thiouridine, and 2-thiocytosine (62-65), queuosine (66), and 3-(3-amino-3-carboxypropyl)uridine (67-69). Although not extensively used, the potential for fluorescent labeling has been described for 7-methylguanosine (70). Finally, the wybutosine modification at position 37 of eukaryotic $tRNA^{Phe}$ is intrinsically fluorescent, and thus

offers a possible method for monitoring binding of ligands to $tRNA^{Phe}$ without labeling (71); alternatively, the chemistry of this base is also amenable to fluorescent labeling (58). Thus, this collection of tRNA modifications amenable to fluorescent labeling provides a number of internal labeling possibilities at different positions within the tRNA (Figure 4.1).

Typically, the above labelling strategies have been used on native tRNAs that have been extracted and purified from cells. Although these native, fully modified, mature tRNAs are powerful for studies of tRNAs on the ribosome during protein translation, native tRNAs are generally not appropriate for studying enzymes that act during tRNA maturation. In particular, studies of tRNA modification enzymes should utilize a substrate tRNA that lacks the modification the enzyme in study produces. Moreover, as certain tRNA modifications are known to be introduced relatively early in tRNA biogenesis and may be hindered by the presence of subsequently-introduced modifications (72-75), utilizing an unmodified or mostly unmodified tRNA is appropriate in many cases. Thus, by starting with *in vitro* transcribed tRNA, one can modulate which tRNA modifications are present and introduce only those that are desired, for example, for introducing a fluorescent label or to mimic a tRNA at a certain stage of tRNA maturation (76). Moreover, use of partially-modified tRNA rather than fully-modified tRNA limits any potential side-products; for example, the initial step of the fluorescence labeling of dihydrouridine modifications is borohydride treatment, but this affects the $m^7G$ base in addition to the targeted dihydrouridine residues (70). Similarly, as many tRNAs contain several identical modifications at different positions (e.g. dihydrouridine), introducing the tRNA modification *in vitro* allows one to limit the fluorescent label to only a specific position in cases when different enzymes are responsible for modifying different positions (e.g. dihydrouridine synthases).

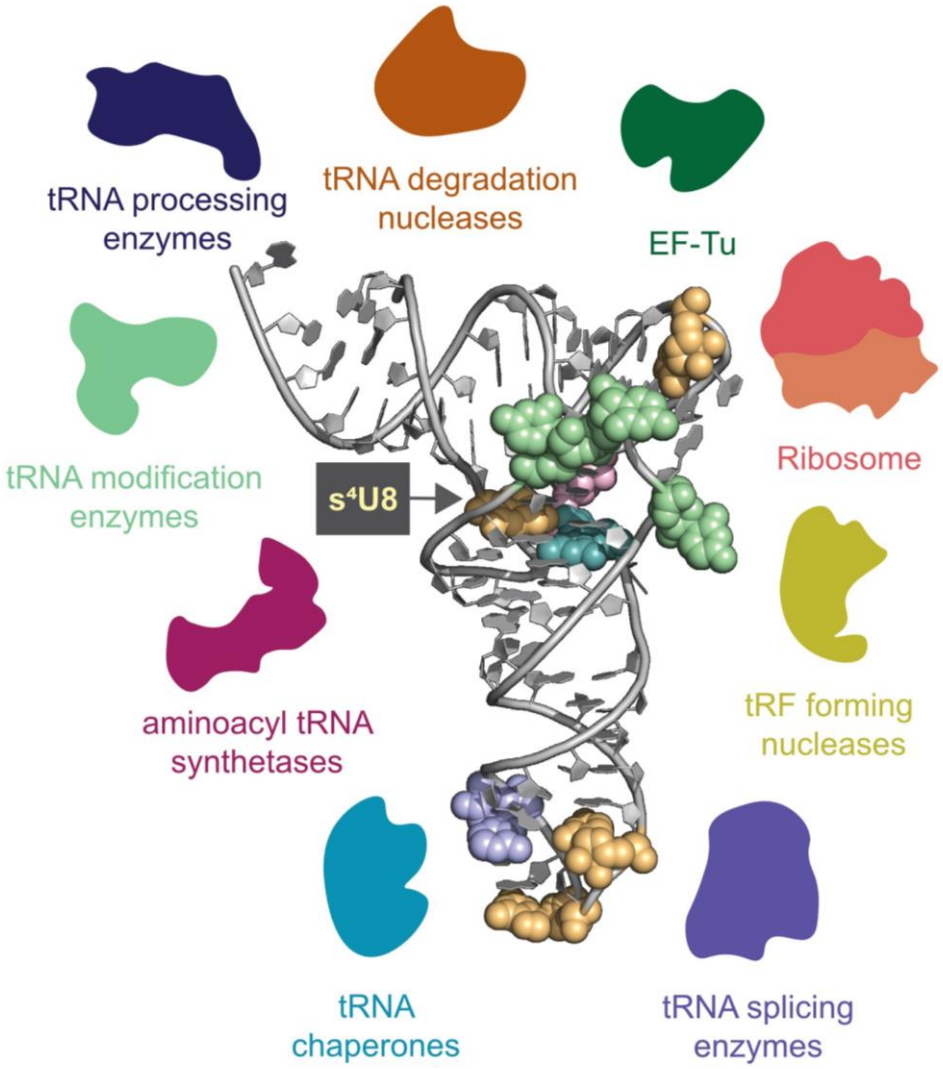



**Figure 4.1. Potential positions for fluorescent tRNA modification labeling and tRNA interacting factors.** Crystal structure of unmodified *E. coli* $tRNA^{Phe}$ (77) showing several possible fluorescent labelling positions at modification sites: dihydrouridine (D; green), thiolations ($s^2U$, $s^2C$, or $s^4U$; yellow), wybutosine (yW), 3-(3-amino-3-carboxypropyl)uridine ($acp^3U$; teal), and 7-methylguanosine ($m^7G$; pink) as spheres. As this chapter focuses on $s^4U8$, its location is marked. Several different tRNA-interacting protein classes surround the tRNA structure, demonstrating the variety of proteins whose tRNA binding mechanism can be examined using rapid-kinetic assays with fluorescent tRNA.

In this chapter, we will focus on using tRNA modification enzymes to modify tRNA, followed by subsequent chemical fluorescent labeling. In particular, we will focus on the example of ThiI-dependent tRNA thiolation, followed by fluorescein labelling; however, similar principles can be used for introducing different modifications and fluorophores into tRNAs of interest.

### *4.4.1 Preparation, purification, and quantification of s4U8 tRNA*

Thiolation of tRNA is common in bacteria at U8 and sometimes U9 within the acceptor stem where it is introduced by ThiI, which is also called TtuI (78,79) (Figure 4.2B). Prior to modification, a thiol group is transferred from free L-cysteine to the cysteine desulfurase IscS in the presence of the cofactor pyridoxal 5'-phosphate, followed by transfer to ThiI. ThiI then thiolates tRNA in an ATP-dependent manner (Figure 4.2A). For *in vitro* enzyme turnover, a reductant such as dithiothreitol is required (80,81).

In *E. coli*, ThiI modifies the majority of tRNA species, but the exact identity of all modified tRNAs remains disputed and may reflect a cell growth dependence of this modification (4,26,82). Besides presence of U8, ThiI does not appear to have sequence constraints for modifying tRNA and instead recognizes the overall tRNA tertiary structure, including the 3'-NCCA terminus (83). This provides the opportunity for thiolation of a wide variety of tRNA sequences *in vitro*; for example, thiolation of eukaryotic tRNAs that do not typically encounter ThiI.

Below, we detail the modification of *in vitro* transcribed tRNA assuming already expressed and purified ThiI and IscS enzymes and already *in vitro* transcribed tRNA by standard techniques (84-88). To validate the success of tRNA thiolation, we describe the use of a simple polyacrylamide gel method to detect tRNA thiolation without requiring high performance liquid chromatography (HPLC) or mass spectrometry systems (89) (Figure 4.2C).

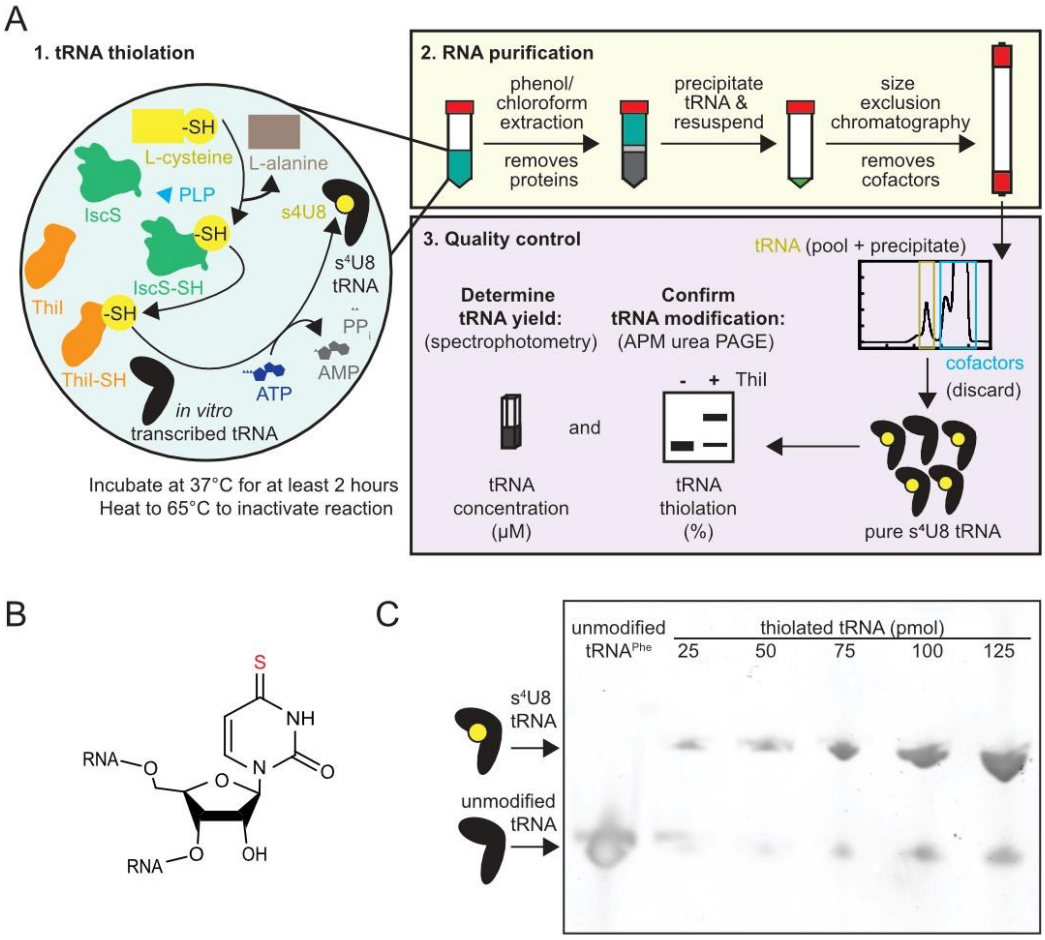



**Figure 4.2. Overview of tRNA thiolation by ThiI. (A)** Schematic of tRNA thiolation and purification. (1) *In vitro* thiolation cascade wherein a thiol group is first transferred from free L-cysteine to the cysteine desulfurase IscS in the presence of PLP cofactor, followed by transfer to the tRNA modification enzyme ThiI, and finally to tRNA in an ATP-dependent manner, thus forming s$^4$U8-tRNA. (2) Initial purification of s$^4$U8-tRNA$^{Phe}$ by phenol and chloroform extractions to remove the ThiI and IscS enzymes. After precipitation to reduce the volume, small molecule cofactors (PLP, L-cysteine, ATP, and their corresponding products) are removed by size exclusion chromatography. (3) APM urea PAGE is used to validate tRNA thiolation, and absorbance at 260 nm is used to validate tRNA recovery. **(B)** Structure of the s$^4$U8 modification, which features a sulfur replacing an oxygen atom at C4. **(C)** Example APM urea PAGE, wherein 81 ± 1% of the tRNA preparation is thiolated.

*4.4.1.1 Materials*

1. *In vitro* transcribed tRNA that can be modified by ThiI
2. Purified *E. coli* ThiI and IscS enzymes
3. Cofactors for ThiI and IscS: ATP, pyridoxal 5′-phosphate (PLP), and L-cysteine
4. Dithiothreitol
5. TAKEM$_4$ buffer (50 mM Tris-HCl pH 7.5, 70 mM $NH_4Cl$, 30 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$) or other suitable reaction buffer
6. Phenol and chloroform
7. 3 M sodium acetate, pH 5.2
8. Ethanol or isopropanol
9. [(N-acryloylamino)phenyl]mercuric chloride (APM), urea, 19:1 acrylamide/bis-acrylamide solution, and standard polyacrylamide gel electrophoresis supplies
10. Centrifuge
11. UV spectrometer
12. Fast protein liquid chromatography (FPLC) system and Superdex 75 (10/300 GL) or similar size exclusion column

*4.4.1.2. Procedure*

1. Refold *in vitro* transcribed tRNA by heating to 65 °C for five minutes in TAKEM$_4$ buffer and subsequently place tRNA at room temperature to cool for at least ten minutes.
2. In order to thiolate the tRNA at position 8, incubate refolded tRNA with purified ThiI and IscS enzymes in addition to PLP, ATP, and L-cysteine in TAKEM$_4$ buffer containing 1 mM DTT and 0.07 U/µL RiboLock RNase inhibitor. To facilitate near-stoichiometric amounts of tRNA modification, a low tRNA concentration, relatively high enzyme and cofactor concentrations, and long incubation times are preferred. For example, we incubate 5 µM tRNA with 3 µM each of ThiI and IscS in the presence of 40 µM PLP, 4 mM ATP, and 500 µM L-cysteine in a 20 mL reaction volume at 37 °C for at least two hours.

3. To ensure tRNA is not lost during phenol chloroform extraction, the reaction can be stopped by heating to at least 65 °C for fifteen minutes in the presence of EDTA in order to minimize tRNA hydrolysis.
4. In order to remove enzymes from the reaction, one volume of phenol is added to the reaction after cooling, vortexed, and centrifuged for 15 minutes at 5000*g*. Transfer the aqueous layer to a new tube and repeat. To remove residual phenol, add one volume of chloroform, vortex, centrifuge as before, and transfer the aqueous layer to a fresh tube. To reduce the volume, add 0.1 volumes of 3 M sodium acetate (pH 5.2) and 0.9 volumes isopropanol and incubate at -20 °C for at least sixteen hours. Collect precipitated tRNA by centrifugation at 5000*g* for at least 45 minutes at 4 °C. Remove supernatant, wash precipitated RNA with 70% ethanol and repeat centrifugation.
5. Since thiolation reaction cofactors are likely to co-precipitate with tRNA, further purification is necessary. It is vital to remove L-cysteine prior to fluorescent labelling as L-cysteine is present in high concentration and contains a thiol group, which will react with the iodoacetamide-conjugated fluorophore. Thus, the precipitated RNA is dissolved in a small (maximum 500 µL) volume of water and injected onto a size exclusion column equilibrated with TAKEM$_4$ buffer to separate the tRNA from small cofactors.
6. Pool the tRNA-containing fractions together and add 0.1 volumes 3 M sodium acetate (pH 5.2) and 3 volumes of ethanol to precipitate for at least 16 hours at -20 °C. Centrifuge at 5000*g* for at least 45 minutes. Remove supernatant, wash precipitated RNA with 70% ethanol, and resuspend in a small volume of water (~20-50 µL). Store the s$^4$U8-tRNA at -20°C.
7. Optional: determine the yield of tRNA recovered from the purification steps above by measuring the absorbance at 260 nm.
8. To determine the proportion of tRNA that was thiolated, a 10% polyacrylamide gel containing 7 M urea and 20 µM[(N-acryloylamino)phenyl]mercuric chloride (APM) is used (89). Herein, increasing amounts (~25-125 pmol for staining with ethidium bromide) of

$s^4U8$ tRNA is analyzed, and the proportion of thiolated tRNA (slow migrating) is compared to the faster-migrating unmodified tRNA by densitometry (e.g. ImageJ).

*4.4.2 Fluorescent labeling of thiols in tRNA*

The reactivity of thiols to the alkylating agent iodoacetamide is well-known and highly exploited for different techniques within protein and RNA biochemistry. Fluorescent labeling of the $s^4U8$ modification with an iodoacetamide derivative of fluorescein was first described in 1982 (62). Since then, different fluorophore iodoacetamide conjugants have been used to label tRNA, including BODIPY® 507/545 dyes (63). Here, we describe labeling of $s^4U8$-tRNA with 5-iodoacetamidofluorescein (5-IAF); however, a similar procedure can be applied to any iodoacetamide-conjugated dye (Figure 4.3). Similarly, this method can be applied to other RNA thiolations, such as 2-thiouridine (formed by *E. coli* MnmA and IscS in certain tRNAs at U34 and by *Thermus thermophilus* TtuA and TtuB in certain tRNAs at U54) and 2-thiocytosine (formed by *E. coli* TtcA and IscS at position 32) (78,90-92).

*4.4.2.1. Materials*

1. $s^4U8$-tRNA prepared as described above
2. Iodoacetamide-conjugated fluorophore (e.g. 5-iodoacetamidofluorescein, 5-IAF; Sigma-Aldrich I9271)
3. Dimethyl sulfoxide (DMSO)
4. Phenol and chloroform
5. 3 M sodium acetate (pH 5.2) and ethanol
6. Centrifuge
7. UV spectrometer

*4.4.2.2. Procedure*

1. Dissolve 5-IAF fluorophore in DMSO to a concentration of 17 mM.
2. To fluorescently label $s^4$U8-tRNA, we incubate 60 µM $s^4$U8-$tRNA^{Phe}$ with 3.2 mM 5-IAF in 12 mM HEPES-KOH (pH 8.2) buffer containing a final concentration of 80% (v/v) DMSO in a final reaction volume of about 1 mL. To facilitate accessibility of the $s^4$U8 base within the folded tRNA structure, the reaction is incubated at 65°C for at least four hours in the dark.
3. Following incubation, the excess non-polar fluorescent dye can be removed by successive phenol reactions. First, dilute the above reaction at least two-fold with water in order to lower the DMSO concentration, which will facilitate cleaner separation between the aqueous and organic phases. Next, add one volume phenol, vortex for ten seconds and centrifuge at 5000*g* for twenty minutes. Remove the aqueous (top) layer and transfer to a new tube. Repeat until the organic (bottom) phase is no longer yellow colored.
4. To remove traces of phenol, add one volume of chloroform, vortex for ten seconds and centrifuge as before. Remove aqueous layer and repeat twice.
5. Precipitate the resulting fluorescein-$s^4$U8-tRNA by addition of 0.1 volumes 3 M sodium acetate (pH 5.2) and 3 volumes ethanol. Incubate at -20°C for at least 16 hours.
6. Collect RNA by centrifugation and resuspend in water.
7. Measure fluorescein-$s^4$U8-tRNA absorbance at 260 and 492 nm to determine RNA concentration and fluorescein concentration, respectively. Note, different dilutions of fluorescein-$s^4$U8-tRNA are likely necessary for accurate measurement at each wavelength. Determine tRNA concentration using the appropriate extinction coefficient (e.g. 500,000 $M^{-1}$ $cm^{-1}$ for $tRNA^{Phe}$) and determine the concentration of fluorescein using an extinction coefficient of 80,000 $M^{-1}$ $cm^{-1}$. The percent tRNA labelled can be calculated by dividing the fluorescein concentration by the final tRNA concentration and multiplying by 100%. We generally obtain a fluorescein labelling efficiency between 2-15%.

8. Optional: Steps 3-6 can be exchanged for an alternative purification method that allows for purification of fluorescent tRNA from unlabeled tRNA, in addition to removing unbound fluorophore (93).
9. Store fluorescein-s$^4$U8-tRNA at -20 °C and away from light exposure to prevent photobleaching.

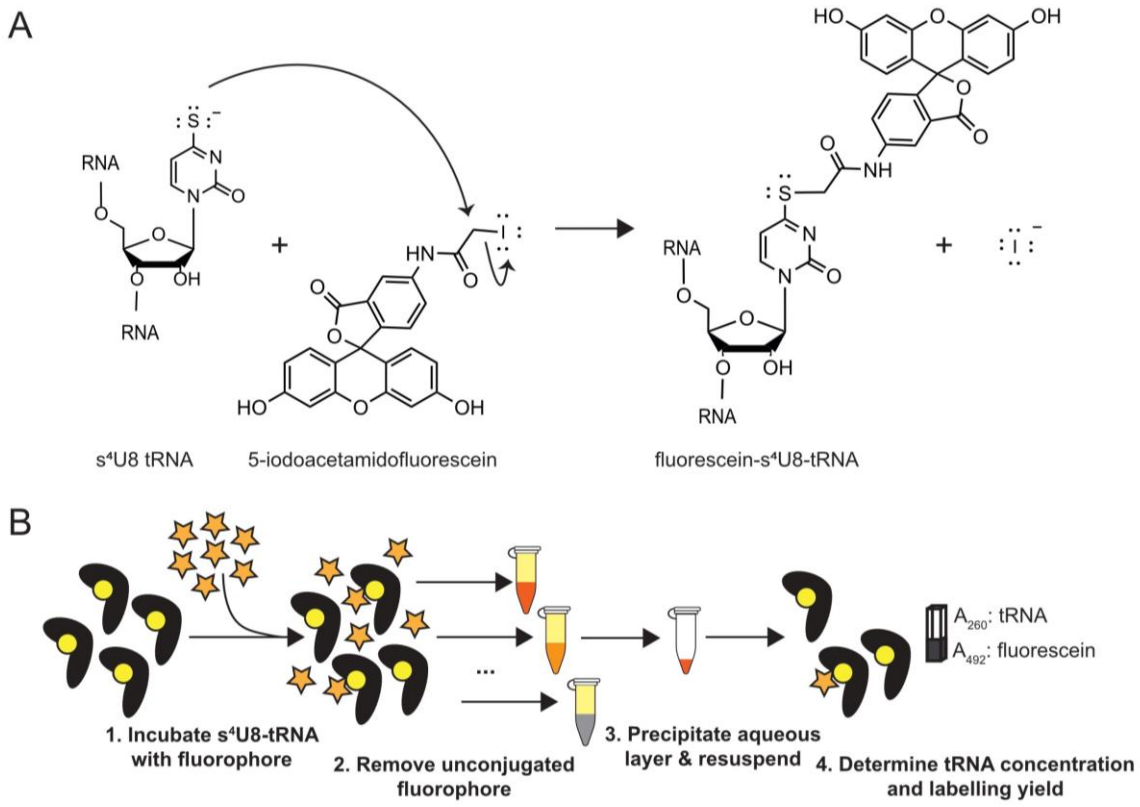



**Figure 4.3. Fluorescent labelling of thiol residues in tRNA.** Reaction mechanism **(A)** and experimental scheme **(B)** for labeling thiols in tRNA. Purified s$^4$U8-tRNA is incubated with 5-iodoacetamidofluorescein (5-IAF) to label thiolated tRNA. Excess hydrophobic fluorophore is then removed by successive phenol extractions until the phenol layer is no longer yellow. Following chloroform extraction, tRNA is precipitated and resuspended in water. The tRNA concentration and labeling efficiency is determined by spectrophotometry.

### 4.5. Rapid-kinetic stopped-flow assays

Rapid mixing of tRNA and protein within a stopped-flow apparatus (Figure 5.4A) allows for observation of binding between the proteins and tRNA in real-time. A kinetics experiment provides a different and more detailed insight into the interaction between the enzyme and tRNA compared to equilibrium binding techniques to determine the dissociation constant ($K_D$), including nitrocellulose filtration experiments, electrophoretic mobility shift assays (EMSAs), equilibrium fluorescence measurements, and microscale thermophoresis (MST). Particularly, stopped-flow measurements provide insights into the mechanistic steps of binding and the speed of binding.

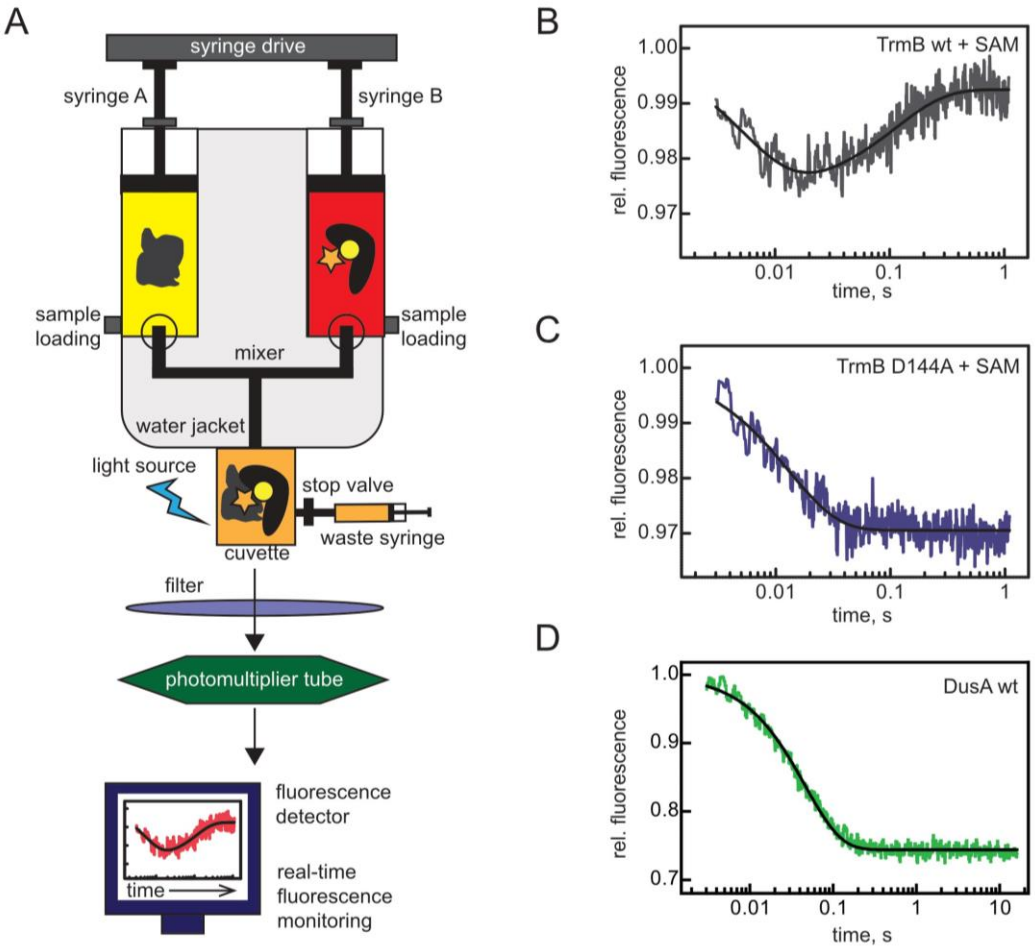
In the stopped-flow instrument, tRNA and protein are loaded into separate syringes (A and B) and then rapidly mixed with the help of a syringe drive to synchronize the reaction start (Figure 5.4A). The protein-tRNA mixture flows into a cuvette and the liquid flow is stopped with a dedicated valve. The entire system is equilibrated to a desired temperature with a water jacket such that temperature-dependence of enzyme kinetics can be recorded. Importantly, mixing takes only approximately 3 ms allowing the observation of fast reactions. In the cuvette, the sample is excited with the help of a light source (typically a xenon lamp) and a monochromator. The light emitted by the sample is detected with the help of a long pass filter, to remove excitation light, and a photomultiplier tube. This setup allows the observation of reaction kinetics in the ms to min time range. Typically, the experiment is repeated 5-15 times by mixing fresh components to record several time courses.

In order to gain insight into the reaction mechanism, it is typically useful to follow the reaction under single-turnover, pre-steady-state conditions where the enzyme is in at least 3-fold excess of the substrate such that all substrates are simultaneously interacting with the enzyme. Under these conditions, the recorded time courses usually display an exponential increase or decrease in fluorescence, and often multiple phases of different kinetics or amplitudes can be observed for different reaction steps such as substrate binding, conformational changes or product release. For a detailed description of pre-steady-state enzyme kinetics, we recommend

dedicated books (94,95). Here, we will focus on the example of catalytically-deficient methyltransferase TrmB D144A (88) binding to fluorescein-s$^4$U8-tRNA$^{Phe}$ in the procedure below.

*4.5.1 Materials*

1. Fluorescein-s$^4$U8-tRNA prepared as described above
2. tRNA binding enzyme (e.g. TrmB)
3. TAKEM$_4$ or other suitable reaction buffer
4. Stopped-flow apparatus (e.g. from Kintek or Applied Photophysics)
5. Disposable syringes and needles




*Caption on next page.*

**Figure 4.4. Stopped-flow studies of tRNA modification enzymes interacting with fluorescein-s$^4$U8-tRNA$^{Phe}$. (A)** Schematic of a stopped-flow apparatus for the rapid mixing of a tRNA modification enzyme (syringe A) and fluorescein-s$^4$U8-tRNA$^{Phe}$ (syringe B). Time courses for the rapid mixing of 15 μM wildtype TrmB **(B)** or catalytically deficient TrmB D144A **(C)** with 1 μM fluorescein-s$^4$U8-tRNA$^{Phe}$ in the presence of 50 μM SAM. For the wildtype enzyme, data was fit with a double exponential equation to determine apparent rate constants, $k_{app1}$: 180 $s^{-1}$ and $k_{app2}$: 9 $s^{-1}$. For the catalytically deficient enzyme, data was fit with a single exponential equation revealing an apparent rate of 77 $s^{-1}$ (88). **(D)** Time course for the rapid mixing of 1.7 μM DusA with 0.5 μM fluorescein-s$^4$U8-tRNA$^{Phe}$ in the absence of cofactors. Single exponential fitting of the data yields an apparent rate of 22 ± 0.2 $s^{-1}$.

*4.5.2 Procedure*

Here, we focus on a generalized stopped-flow procedure. For details on operating the stopped-flow apparatus, consult the appropriate instrument manual.

As much as possible, keep fluorescein-s$^4$U8-tRNA in the dark during the experiment to prevent photobleaching.

1. Prepare a fluorescein-s$^4$U8-tRNA$^{Phe}$ dilution in TAKEM$_4$ buffer. The tRNA dilution concentration should be two-fold higher than the experimental tRNA concentration to account for the two-fold dilution upon mixing in the stopped-flow apparatus. Similarly, dilute the tRNA methyltransferase TrmB to double the desired experimental concentration in TAKEM$_4$ buffer and store on ice until use. To ensure pre-steady-state conditions the enzyme concentration should be at least three-fold higher than that of the tRNA. The fluorescent tRNA concentration used will depend on the quantum yield of the fluorophore and the labelling efficiency of the fluorescent tRNA stock in use. We find that a 1 μM final fluorescein-s$^4$U8-tRNA$^{Phe}$ concentration provides a robust signal in our

experiments, but this is likely to be variable between different fluorescent RNA preparations, the fluorophore and the equipment in use.

2. Refold tRNA by heating the dilution to 65°C for five minutes and letting cool at room temperature for at least ten minutes.
3. Prepare and equilibrate the stopped-flow as per the users' manual. Confirm the monochromator is set to a wavelength suitable to excite the fluorophore used and that the desired filters are inserted to select longer wavelengths for emission measurements. Ensure the water jacket is at the appropriate temperature.
4. Centrifuge both samples to remove any bubbles or potential solids/precipitates that may interfere with fluorescence measurement. Load each stopped-flow syringe carefully with a disposable syringe, without introducing bubbles.
5. Set data collection parameters, including the length of time fluorescence will be monitored for the mixing event. Ensure stopped-flow valves are set such that the contents of each syringe can enter the mixer.
6. Repeat step four several times to gather replicate data for the given experimental condition.
7. To analyze the data, plot the fluorescence against time (e.g. Figure 4.4). Ensure any time points earlier than the dead time of the instrument (about 3 ms) are removed from analysis. Determine the best fit for the kinetic data using a program such as TableCurve 2D. For wildtype TrmB binding tRNA in the presence of SAM, a two-exponential equation is appropriate (Figure 4.4B):

$$Y = Y_{\infty} + Amp_1 \times \exp\left(-k_{app1} \times t\right) + Amp_2 \times \exp(-k_{app2} \times t)$$

In contrast, for catalytically deficient TrmB D144A binding tRNA in the presence of SAM or for wildtype DusA binding tRNA in the absence of cofactors (Figure 4), a one-exponential equation is appropriate:

$$Y = Y_{\infty} + Amp \times \exp(-k_{app} \times t)$$

However, the equation used for fitting will vary for different enzymes, conditions, etc., reflecting kinetic differences.

8. To gain understanding into the kinetic mechanism of the reaction, it is useful to determine the concentration dependence of the given reaction. In this case, the mixing reaction is monitored for increasing concentrations of TrmB D144A and a constant concentration of fluorescein-s$^4$U8-tRNA$^{Phe}$. If $k_{app}$ is not concentration-dependent (e.g. Figure 4.5A), then it is likely to relate to a unimolecular event, such as a conformational change. If $k_{app}$ increases with increasing enzyme concentrations (e.g. Figure 4.5B), $k_1$ can be determined by plotting $k_{app}$ against enzyme concentration and fitting with a linear equation:

$$k_{app} = k_1 \times [enzyme\ concentration] + k_{-1}$$

9. The above equation can also be used to estimate $k_{-1}$ for the reaction. If desired, $k_{-1}$ can be validated with chase experiments, which allows for monitoring of fluorescein-s$^4$U8-tRNA$^{Phe}$ dissociation from TrmB in this example. Herein, the enzyme•fluorescein-s$^4$U8-tRNA$^{Phe}$ complex is pre-formed under equilibrium conditions and loaded into one stopped-flow syringe. Concentrations for the enzyme•fluorescein-s$^4$U8-tRNA$^{Phe}$ complex may be similar to those used in the mixing experiments described above (for example, 1 μM fluorescein-s$^4$U8-tRNA$^{Phe}$ and 3 μM modification enzyme, such as TrmB). Similar to above, solutions loaded into the syringes must be double the final concentration. In the second syringe, load an excess concentration of non-fluorescent tRNA$^{Phe}$ (for example, to obtain a final concentration of 20 μM). Upon mixing the non-fluorescent tRNA with the fluorescein-tRNA-containing complex, the fluorescent tRNA will dissociate from TrmB and will be replaced by the non-fluorescent tRNA. Thus, exponential fitting of the resulting time course will provide the apparent rate of dissociation of tRNA from TrmB.

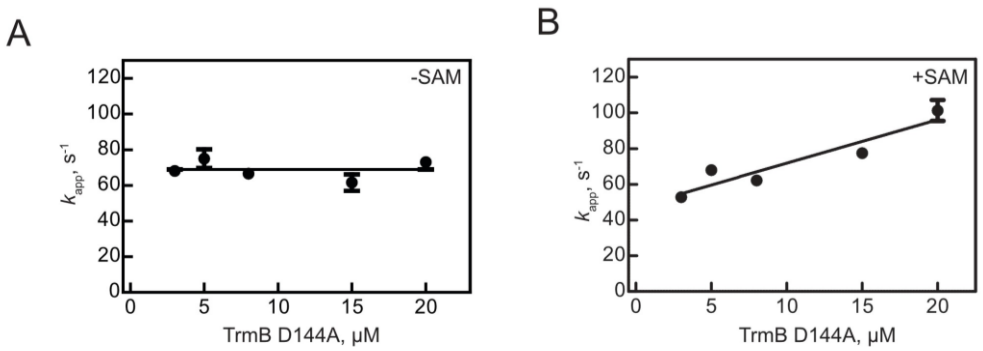



**Figure 4.5. Concentration-dependence of tRNA binding by catalytically deficient TrmB D144A in the presence and absence of the cofactor SAM.** Rapid mixing of TrmB D144A and fluorescein-s$^4$U8-tRNA$^{Phe}$ was repeated in the absence **(A)** or presence **(B)** of SAM with increasing TrmB D144A concentrations in order to determine the concentration-dependence of the interaction. In the absence of SAM, the association of TrmB D144A to tRNA is not concentration-dependent, and the horizontal line represents an average $k_{app}$ of 69 ± 5 $s^{-1}$. In the presence of SAM, the $k_{app}$ increases with increasing TrmB D144A concentrations. Thus, linear fitting reveals a slope representing the association rate constant ($k_1$) equal to 2.4 ± 0.5 μM $s^{-1}$ (88).

### 4.5 Conclusions and future directions

Some proteins like EF-Tu / eEF1 as well as the ribosome have to interact with all tRNAs in a uniform manner independent of the tRNA's differences in sequence, structure, modification and amino acid (8,96). Other proteins, particularly aminoacyl-tRNA synthethases, are highly specific in their interaction with tRNAs (44). Similarly, tRNA modification enzymes modify their tRNA substrates with high specificity, choosing only the appropriate tRNA isoacceptors and the correct position within the tRNA. With a large pool of tRNA modification enzymes targeting nucleosides at different positions throughout the tRNA structure, sometimes having to access nucleobases buried within the folded tRNA, presumably each enzyme utilizes a different mechanism to bind tRNA appropriately for tRNA modification. Most likely, other tRNA-interacting

proteins even distinguish tRNAs based on their modification status such as tRNA modifying enzymes that depend on prior modifications (75). Likewise, proteins involved in tRNA degradation target in particular tRNAs that lack modifications that likely affects their structure and stability (47). For all these tRNA-interacting proteins, fluorescence experiments such as stopped-flow studies can be used to gain a mechanistic understanding into tRNA binding, but a prerequisite to these studies is a tRNA containing an appropriate fluorescent label.

Here, we described the potential for exploiting the chemistry of RNA modifications for fluorescently labeling tRNA, particularly using *in vitro* partially modified tRNAs that contain only the desired modification(s). This approach for fluorescent labeling of tRNA can be applied to studies of additional tRNA modifying enzymes and other tRNA-interacting factors, such as tRNA processing enzymes, EF-Tu, and the ribosome (Figure 1), in addition to variants of these components in order to understand how mutations may influence tRNA function. Moreover, this approach of selecting only certain modification(s) to introduce within the tRNA under study allows for dissection of how tRNA modifications affect tRNA function and protein interactions.

The strategy described here to generate fluorescently labelled tRNA opens avenues for other fluorescence studies beyond the rapid kinetic stopped-flow assays. For example, the fluorescent tRNAs can be utilized in equilibrium binding assays to determine the affinity of a tRNA-protein interaction, to assess binding to protein variants harboring amino acid substitutions or to evaluate the specificity of tRNA-binding proteins. For these experiments, a standard fluorescence plate reader or a fluorescence spectrometer will be sufficient. Similarly, the fluorescent tRNA allows for anisotropy measurements to quantify the affinity even in the absence of a fluorescence change upon tRNA-protein interaction. Moreover, the fluorescent tRNA is also suitable for microscale thermophoresis assays to characterize the interaction with a protein. In addition to the described stopped-flow assays, detailed kinetic insight into the interaction of tRNA with a protein can also be gained through single-molecule fluorescence studies if a suitable instrument is available (97). For this purpose, the tRNA should be labelled with dyes like Cy3 or

Cy5 that are very bright. Finally, fluorescent tRNAs can even be electroporated into living cells to track their function within their normal biological environment (98).

In conclusion, the tRNA fluorescent labelling strategy described here is highly versatile for several reasons: (1) it can be adopted to the labelling of other modification sites in the tRNA, (2) it allows for the preparation of unmodified and partially modified tRNAs to investigate the effect of RNA modifications on the interaction with other proteins, (3) it is suitable to be used in studies of numerous tRNA-binding proteins that are critical for tRNA maturation, protein synthesis and tRNA degradation, and (4) it enables a large number of different fluorescence assays beyond the described stopped-flow experiments including standard biochemical, advanced biophysical and cellular studies.

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**Chapter 5:**
**tRNA elbow modifications affect the tRNA pseudouridine synthase TruB and the methyltransferase TrmA**

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## 5.1 Preface

This chapter is a form of a publication titled, “tRNA elbow modifications affect the tRNA pseudouridine synthase TruB and the methyltransferase TrmA” by Sarah Kai-Leigh Schultz and Ute Kothe that was accepted in the journal *RNA* May 8, 2020, volume 26, pages 1131-1142 (http://www.rnajournal.org/cgi/doi/10.1261/rna.075473.120) (1) that has been formatted for this thesis. To maintain consistency throughout the thesis, the reference style has been changed. Dr. Ute Kothe and I wrote this manuscript together. As first author of this manuscript, I was involved in project conceptualization, methodology including performing all experiments, preparing figures, writing of the initial draft, and editing of the manuscript. Dr. Ute Kothe was involved with conceptualization, methodology, supervision, and funding acquisition. We thank three anonymous reviewers for their feedback on this manuscript during the revision process, resulting in the form it appears now.

## 5.2 Abstract

tRNAs constitute the most highly modified class of RNA. Every tRNA contains a unique set of modifications, and Ψ55, $m^5U54$, and $m^7G46$ are frequently found within the elbow of the tRNA structure. Despite the abundance of tRNA modifications, we are only beginning to understand the orchestration of modification enzymes during tRNA maturation. Here, we investigated whether pre-existing modifications impact the binding affinity or catalysis by tRNA elbow modification enzymes. Specifically, we focused on the *Escherichia coli* enzymes TruB, TrmA, and TrmB which generate Ψ55, $m^5U54$, and $m^7G46$, respectively. tRNAs containing a single modification were prepared, and the binding and activity preferences of purified *E. coli* TrmA, TruB, and TrmB were examined *in vitro*. TruB preferentially binds and modifies unmodified tRNA. TrmA prefers to modify unmodified tRNA, but binds most tightly to tRNA that already contains Ψ55. In contrast, binding and modification by TrmB is insensitive to the tRNA modification status. Our results suggest that TrmA and TruB are likely to act on mostly unmodified tRNA precursors during the early stages of tRNA maturation whereas TrmB

presumably acts on later tRNA intermediates that are already partially modified. In conclusion, we uncover the mechanistic basis for the preferred modification order in the E. coli tRNA elbow region.

### 5.3 Introduction

Transfer RNA (tRNA) is the most highly and most diversely modified class of RNA, containing over 100 chemically distinct modifications and a median of twelve modifications per individual tRNA (2). In some organisms, more than 1% of the genome encodes tRNA modification enzymes, highlighting the energetic cost all cells invest in tRNA modification (3) whereas in many organisms including humans the full set of tRNA modification enzymes is not even known yet (4). The model organism *Escherichia coli* contains over 25 different modifications, with each tRNA containing between three and 13 modifications (2).

Two clusters of tRNA modifications are evident in the tRNA tertiary structure. The first cluster of tRNA modifications is found within the tRNA anticodon loop. In general, these modifications are bulky, complex, and each modification is only found within a few isoacceptor tRNAs per organism. Several tRNA anticodon modifications have been shown to play important roles during translation (5). The second cluster of tRNA modifications is found within the tRNA elbow, a region formed by long-range base-pairing interactions between the D and T loops.

In contrast to anticodon loop modifications, tRNA elbow modifications are less diverse. For example, 5-methyluridine ($m^5U$) 54 (also known as ribothymidine 54) and pseudouridine (Ψ) 55 are found within every tRNA in *E. coli*, where they are introduced by TrmA and TruB, respectively (Fig. 3.1A and B). Additionally, $m^5U54$ and Ψ55 modifications are found widely within elongator tRNAs throughout all domains of life, including in humans, where these modifications are introduced by the TrmA and TruB homologs TRMT2A and PUS4 in the cytoplasm. Another abundant tRNA elbow modification is 7-methylguanosine ($m^7G$) 46 (Fig. 5.1A and B), which is introduced by TrmB in *E. coli* and is frequently found within tRNAs containing short variable loops

(6). TrmB is also widely conserved, with METTL1 as its human homolog. Within bacteria, 4-thiouridine ($s^4U$) 8 is a common modification and is catalyzed by ThiI, which requires sulfur transfer by IscS (Fig. 5.1A and B; (7)). tRNA elbow modifications are generally considered to be important for promoting tRNA structure and cellular stability (8,9); however, modifications within the tRNA elbow also have roles during translation. Specifically, *E. coli* TruB has been found to be important for the translation of consecutive arginine CGA codons (10), and *Pseudomonas aeruginosa* TrmB is important for the expression of genes enriched in phenylalanine and aspartate codons (11). In addition to the direct stabilizing effects of tRNA modifications on tRNA structure, two tRNA elbow modification enzymes, namely TruB and TrmA, have been described as tRNA chaperones that help tRNA to adopt its overall L-shaped fold independent of their modification activity (12,13).

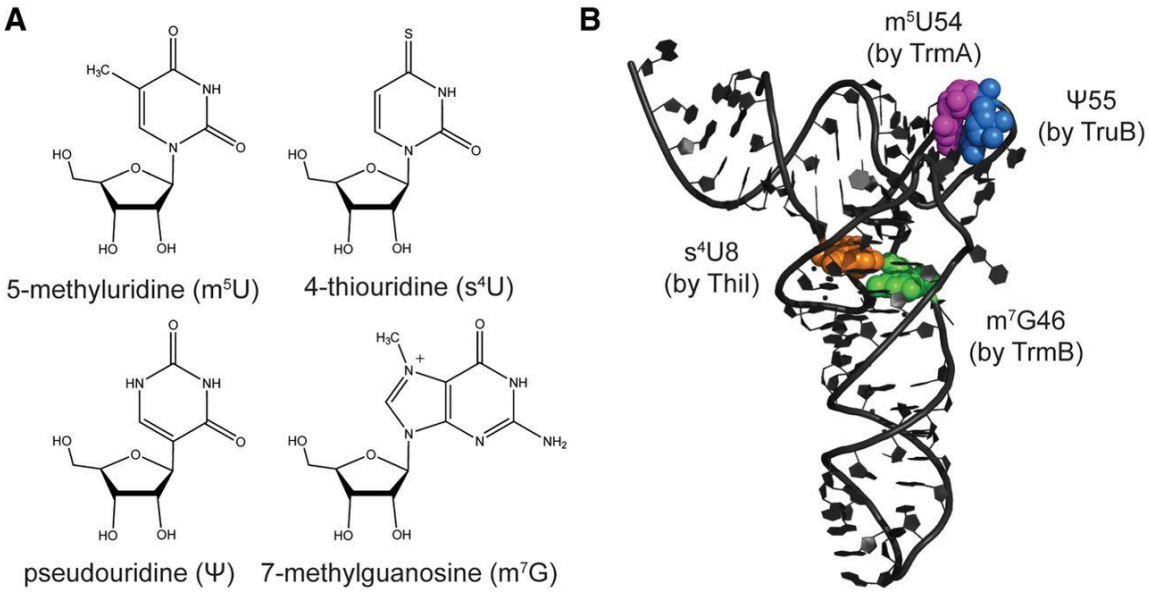



**Figure 5.1 Structures of selected tRNA elbow modifications and their locations within *E. coli* $tRNA^{Phe}$. (A)** Structures of the 4-thiouridine, 7-methylguanosine, 5-methyluridine, and pseudouridine modifications. **(B)** The locations of these modifications within the tRNA elbow are highlighted as spheres within the crystal structure of unmodified $tRNA^{Phe}$ (PDB 3L0U; (14)). The enzyme responsible for each modification is indicated next to the respective modification site.

Here, we are specifically focusing on the pseudouridine synthase TruB, and the two methyltransferases TrmA and TrmB. TruB utilizes a catalytic aspartate residue in conjunction with a conserved arginine and a second-shell aspartate residue to catalyze the isomerization of uridine to pseudouridine (15). Specifically, the catalytic aspartate attacks the 2′ oxygen of the ribose to form a glycal intermediate and to break the N-C glycosidic bond. Upon rotation of the uracil base, a new C-C glycosidic bond is formed and the covalent bond to the catalytic aspartate is broken (16). TrmA and TrmB both use S-adenosylmethionine (SAM) as the methyl donor. During tRNA methylation by TrmA, a catalytic cysteine residue forms a covalent bond with C6 in the uracil ring enabling methyl transfer from SAM to the base. Subsequently, a catalytic glutamate residue acts a general base abstracting a proton from the uracil ring, allowing the covalent bond to the catalytic cysteine to be broken (17). The catalytic mechanism of TrmB methylating G46 is not known yet, but a conserved aspartate residue (D144 in *E. coli*) is essential for catalysis (6). It has been suggested that the catalytic aspartate acts as a general base deprotonating G46 such that the base can attack the SAM cofactor for methyl transfer (18).

Despite the abundance and importance of tRNA modifications, we are still in the early stages of understanding the multistep process through which tRNA is modified. From previous mechanistic studies, it is known that the majority of tRNA modification enzymes, including TrmA, TruB, TrmB, and ThiI, are active on *in vitro* transcribed, unmodified tRNA (7,11-13,19); however, completely unmodified tRNA may not be the preferred substrate of these enzymes *in vivo*. Recent reviews have emphasized that the order of tRNA modification is likely not random (20-22). In particular, five so-called “tRNA modification circuits” within the tRNA anticodon loop have been well described where introduction of a certain modification strictly depends on the prior presence of another modification (20). Within the tRNA elbow, temperature-specific networks of tRNA modifications have been reported in *Thermus thermophilus* (23). Additionally, a sequential formation of tRNA elbow modifications has been described recently for the *Saccharomyces cerevisiae* system, wherein the modification of *in vitro* transcribed yeast $tRNA^{Phe}$ by yeast cell extract was monitored over time. Ψ55 was found to be fully introduced within the tRNA population prior to the appearance of any other modification, followed by $m^5U54$ and $m^7G46$,

then $m^2$G10, D16, and $m^5$C49, and finally $m^1$A58 (24). This study provides strong evidence that modifications in the tRNA elbow region are not introduced randomly, but the molecular determinants of the sequential modification order remain unclear.

Therefore, the goal of this study was to examine the impact of tRNA modification status on the *in vitro* binding and catalytic activity of *E. coli* tRNA elbow modification enzymes TruB, TrmA, and TrmB. Specifically, by conducting quantitative biochemical experiments, we aimed to not only identify the preferred tRNA substrate for these enzymes, but to also characterize the molecular basis for the ordered modification of tRNA in the elbow region. To accomplish this, we prepared *in vitro* transcribed tRNA that contained a single modification for use in enzyme binding and activity assays. Here, we report that TruB prefers to bind and modify tRNA that does not yet contain any modifications, whereas TrmA prefers to modify unmodified tRNA, but has a binding preference for tRNA already containing the Ψ55 modification. This proof-of-principle study suggests tRNA elbow modification enzymes are sensitive to the modification status of their substrate tRNAs, explaining why the order of tRNA elbow modification is not completely random.

### 5.4 Results

In order to determine the effect of a pre-existing tRNA modification on the affinity and activity of another tRNA modification enzyme, we used in vitro transcribed *E. coli* $tRNA^{Phe}$ as a model substrate. Singly-modified $tRNA^{Phe}$ was prepared by incubation with purified *E. coli* TrmA, TruB, TrmB, or ThiI and IscS. Each singly-modified tRNA contained one modification at a level of at least 78% (see Materials and Methods). Unmodified tRNA as well as singly-modified tRNAs were subsequently used in binding and activity assays in order to compare whether TrmA, TruB, or TrmB prefer binding and/or modifying tRNA with a certain modification status.

*5.4.1 Binding and activity preferences of TruB*

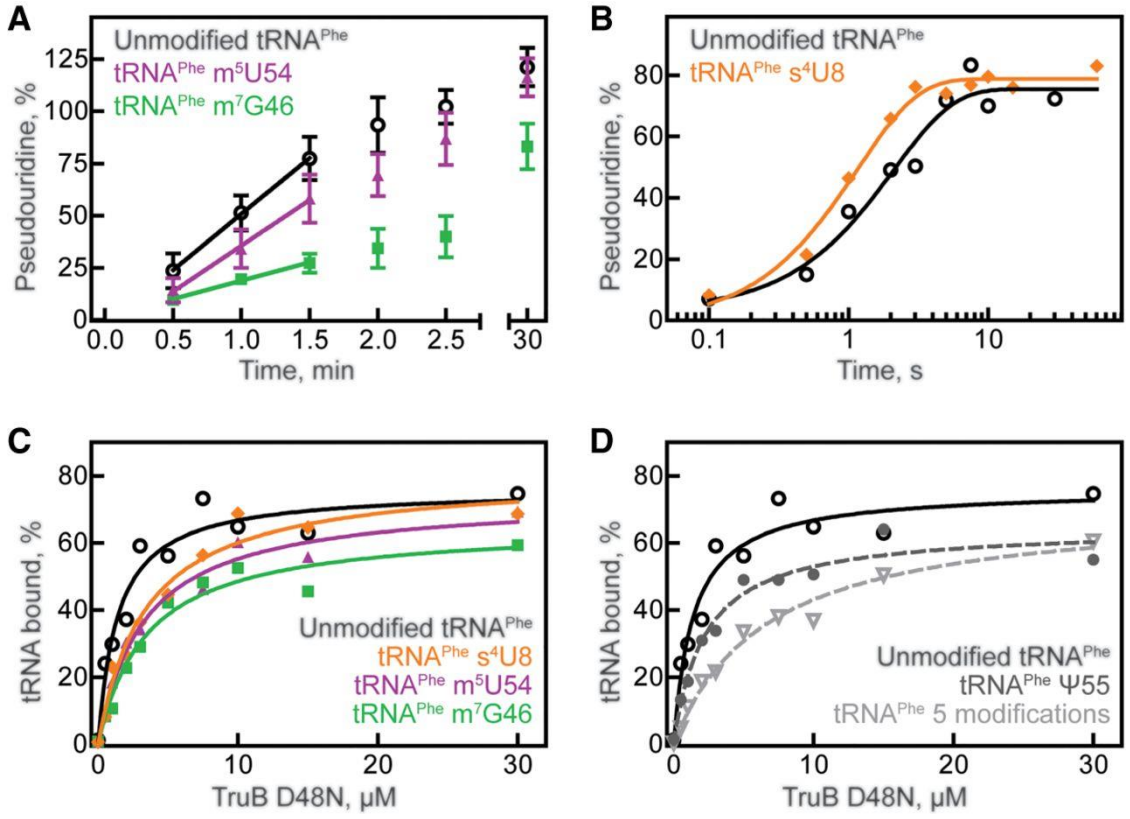
To assess whether TruB prefers to bind and modify unmodified tRNA or tRNA already containing a single modification, we prepared tritium-labeled $tRNA^{Phe}$ containing $s^4U8$, $m^7G46$, or $m^5U54$. First, we compared the activity of TruB under multiple-turnover conditions with unmodified tRNA and tRNA containing $m^7G46$ or $m^5U54$ using tritium release assays. At a tRNA concentration of 600 nM (near its Michaelis constant, $K_M$, 550 nM), 10 nM TruB has an initial velocity of approximately 4.7 nM $s^{-1}$ for pseudouridylating unmodified tRNA (Table 5.1; Fig. 5.2A), as previously reported (19). Introduction of the adjacent $m^5U54$ modification did not significantly affect the initial rate of TruB for tRNAPhe; however, incorporation of $m^7G46$ into the tRNA lowered the initial velocity of pseudouridylation by TruB almost three-fold to 1.6 nM $s^{-1}$ (Table 5.1; Fig. 5.2A). The presence of $s^4U8$ did not significantly affect single-turnover TruB rate (Table 5.2; Fig. 5.2B).

**Table 5.1. Average multiple-turnover initial velocities ($v_0$, nM $s^{-1}$) for TruB and TrmA modifying different singly-modified tRNAs.** Initial velocities are provided with standard deviation as determined in at least three independent experiments. Initial velocities cannot be determined for a tRNA that already contains the respective modification made by TruB or TrmA as indicated by NA (not applicable).

| $tRNA^{Phe}$ Modification | TruB | TrmA |
|---|---|---|
| Unmodified | 4.7 ± 0.6 | 4.5 ± 0.9 |
| $m^7G46$ | 1.6 ± 0.5 | 2.4 ± 0.6 |
| $m^5U54$ | 3.8 ± 0.6 | NA |
| Ψ55 | NA | 2.7 ± 0.7 |

**Table 5.2. Average single-turnover apparent rates for TruB ($k_{\Psi}$, $s^{-1}$), TrmA ($k_{methyl}$, $s^{-1}$), and TrmB ($k_{app}$, $s^{-1}$) modifying different singly-modified tRNAs.** Apparent rates are provided with standard deviation as determined in at least three independent experiments. For certain tRNAs, single-turnover apparent rates were not determined (ND), whereas formation of the same modification cannot be determined (not applicable, NA).

| $tRNA^{Phe}$ Modification | TruB | TrmA | TrmB |
|---|---|---|---|
| Unmodified | 0.5 ± 0.3 | 0.08 ± 0.03 | 0.2 ± 0.02 |
| $m^5U54$ | ND | NA | 0.3 ± 0.05 |
| Ψ55 | NA | 0.06 ± 0.02 | 0.2 ± 0.05 |
| $s^4U8$ | 0.7 ± 0.3 | 0.09 ± 0.03 | ND |




*Caption on next page.*

**Figure 5.2. tRNA modification and binding preferences of TruB. (A)** Multiple-turnover modification by 10 nM TruB incubated with 600 nM unmodified $tRNA^{Phe}$ (open circles), $tRNA^{Phe}$ $m^5U54$ (purple triangles), or $tRNA^{Phe}$ $m^7G46$ (green squares). Time courses were performed at least in triplicate, and initial velocities were determined by linear regression (see Table 5.1). **(B)** Single-turnover modification by 5 μM TruB incubated with 1 μM unmodified $tRNA^{Phe}$ (open circles) or $tRNA^{Phe}$ $s^4U8$ (orange diamonds). Here, we show one representative time course, but each time course was performed in triplicate and the apparent rate of each reaction was determined by fitting with a one-exponential equation, summarized in Table 5.2. Binding of catalytically inactive TruB D48N to 20 nM **(C)** unmodified $tRNA^{Phe}$ (open circles), $tRNA^{Phe}$ $m^5U54$ (purple triangles), $tRNA^{Phe}$ $m^7G46$ (green squares), $tRNA^{Phe}$ $s^4U8$ (orange diamonds), or to **(D)** $tRNA^{Phe}$ Ψ55 (gray circles, dashed line), or $tRNA^{Phe}$ containing $s^4U8$, Ψ32, Ψ38, $m^7G46$, $m^5U54$, and Ψ55 (light gray inverted triangles, dashed line), unmodified $tRNA^{Phe}$ (same as shown in C) determined by nitrocellulose filtration. One representative curve each of at least three replicates is shown. The data were fit with a hyperbolic equation to determine the dissociation constant, $K_D$ (see Table 5.3).

In order to reveal whether the decreased speed of TruB modification for tRNA containing $m^7G46$ was caused by a decrease in the binding affinity of TruB for this tRNA, dissociation constants ($K_D$) were determined using nitrocellulose filtration assays. The catalytically inactive TruB D48N variant was used in order to measure the affinity of TruB for its substrate tRNA, rather than for its product, $tRNA^{Phe}$ Ψ55. As reported previously, the dissociation constant of TruB D48N for unmodified tRNA is 1.4 μM (Table 5.3; Fig. 5.2C; (19)) and thus slightly lower than the dissociation constant of TruB wild-type for pseudouridylated tRNA (2.4 μM) (13). When $m^7G46$ is present within the tRNA, the affinity of TruB D48N is reduced approximately two-fold with a dissociation constant of about 3.1 μM (Table 5.3; Fig. 5.2C), in accordance with the decrease in TruB initial velocity for tRNA $m^7G46$ (Table 5.1; Fig. 5.2A). Interestingly, the presence of either $s^4U8$ or $m^5U54$ also lowered the affinity of TruB D48N for $tRNA^{Phe}$ approximately two-fold (Table

5.3; Fig. 5.2C), despite the fact these modifications did not significantly lower the initial velocity of TruB modification (Table 5.1; Fig. 5.2A and B). TruB D48N binding to $tRNA^{Phe}$ Ψ55, the product of TruB, was found to be similar to TruB D48N binding to its unmodified substrate $tRNA^{Phe}$. In summary, the presence of $s^4U8$, $m^7G46$, or $m^5U54$ (but not Ψ55) lowered the affinity of TruB D48N, highlighting that $s^4U8$, $m^7G46$, and $m^5U54$ significantly and negatively affect TruB binding to tRNA (Table 5.3; Fig. 5.2D).

**Table 5.3. Average dissociation constants ($K_D$, μM) for TruB, TrmA, and TrmB binding to differently modified tRNAs.** Dissociation constants ($K_D$) are provided with standard deviation as determined in at least three independent experiments. Selected dissociation constants were not determined (ND).

| $tRNA^{Phe}$ Modification(s) | TruB | TrmA | TrmB |
|---|---|---|---|
| Unmodified | 1.4 ± 0.3 | 0.9 ± 0.2 | 4.5 ± 1.9 |
| $m^5U54$ | 3.4 ± 1.4 | 2.3 ± 0.7 | 3.7 ± 1.1 |
| Ψ55 | 2.3 ± 0.9 | 0.2 ± 0.1 | 3.8 ± 1.7 |
| $m^7G46$ | 3.1 ± 0.8 | 1.9 ± 0.6 | 3.1 ± 1.3 |
| $s^4U8$ | 3.2 ± 0.9 | 0.8 ± 0.2 | ND |
| $m^5U54$, Ψ55 | ND | ND | 3.7 ± 0.5 |
| Ψ32, Ψ38, $m^7G46$, $m^5U54$, Ψ55 | 6.4 ± 1.4 | ND | ND |

Next, we wanted to examine whether the presence of multiple modifications further decreased the affinity of TruB for tRNA. Therefore, [$^3$H]$tRNA^{Phe}$ containing five modifications, namely two pseudouridines in the anticodon loop (Ψ32 and Ψ38 introduced by RluA and TruA), as well as $m^7G46$, $m^5U54$, and Ψ55 was prepared. The affinity of TruB for this tRNA was found to be further decreased compared to the singly-modified tRNA variants with a $K_D$ of about 6.4 μM (Table 5.3; Fig. 5.2D), further suggesting TruB prefers to bind to tRNA prior to modification by other enzymes. Taken together, these results indicate that the binding of TruB to tRNA is sensitive to the presence of single $s^4U8$, $m^7G46$, and $m^5U54$ modifications, with $m^7G46$ additionally lowering the reaction velocity of pseudouridylation by TruB.

*5.4.2 Binding and activity preferences of TrmA*

Similarly, we tested the impact of prior modifications on the TrmA enzyme using singly-modified, tritium-labeled $tRNA^{Phe}$ in multiple-turnover tritium release assays. When acting on unmodified $tRNA^{Phe}$, the initial velocity of TrmA-catalyzed methylation is about 4.5 nM $s^{-1}$ (Table 5.1; Fig. 5.3A). The initial velocity of TrmA modification is reduced about two-fold when either $m^7G46$ or Ψ55 are present, to 2.4 nM $s^{-1}$ and 2.7 nM $s^{-1}$, respectively (Table 5.1; Fig. 5.3A). It is interesting that the initial velocity of TrmA is slower when Ψ55, the modification introduced by TruB, is present, because the affinity of TruB was reduced when the modification introduced by TrmA, $m^5U54$, was present within $tRNA^{Phe}$ (Table 5.3; Fig. 5.2C), suggesting previous TruB modification negatively affects modification by TrmA and vice versa.

To further examine the impact of the adjacent Ψ55 modification on catalysis by TrmA, the apparent rate of tRNA methylation by TrmA was compared for unmodified $tRNA^{Phe}$ and $tRNA^{Phe}$ Ψ55 using tritium release assays under single-turnover conditions in a quench-flow apparatus. In the absence and presence of the Ψ55 modification, the apparent rate was found to be about 0.08 and 0.06 $s^{-1}$, respectively, suggesting that Ψ55 modification does not significantly affect catalysis by TrmA (Table 5.2; Fig. 5.3B). As observed with TruB, the presence of $s^4U8$ also did not affect the apparent rate of TrmA (Table 5.2; Fig. 5.3B).

Next, we examined the influence of tRNA modification status on TrmA tRNA binding by nitrocellulose filter binding. Here, the TrmA C324A variant was used because this variant is deficient in catalysis and because it cannot form a covalent bond between enzyme and substrate (25). The affinity of TrmA C324A for unmodified $tRNA^{Phe}$ was found to be about 0.9 μM (Table 5.3; Fig. 5.3C). The presence of the $m^7G46$ modification lowered the affinity of TrmA C324A for $tRNA^{Phe}$ two-fold to a dissociation constant of 1.9 μM (Table 5.3; Fig. 5.3C), consistent with the observation that this modification results in a reduction of the initial velocity of TrmA (Table 5.1; Fig. 5.3A). Similarly, we assessed the affinity of TrmA C324A for the product tRNA $m^5U54$ and determined a reduced affinity compared to unmodified tRNA (Table 5.3; Fig. 5.3D). In other words, the affinity of TrmA C324A for $tRNA^{Phe}$ $m^7G46$ is similar to the affinity of TrmA

C324A for tRNA $m^5U54$. The presence of $s^4U8$ did not affect TrmA C324A binding (Table 5.3; Fig. 5.3D), in agreement with the lack of effect of this modification on TrmA activity (Table 5.2; Fig. 5.3A).

Interestingly, the presence of the Ψ55 modification strongly improved the affinity of TrmA C324A for tRNA Ψ55 almost five-fold compared to unmodified $tRNA^{Phe}$ (Table 5.3; Fig. 5.3C), contrasting the two-fold decrease in TrmA activity for $tRNA^{Phe}$ Ψ55 (Table 5.1; Fig. 5.3A). Given that Ψ55 does not affect the rate constant of catalysis by TrmA (Table 5.2; Fig. 5.3B) and Ψ55 increases the affinity of TrmA for $tRNA^{Phe}$ (Table 5.3; Fig. 5.3C), the multiple-turnover activity of TrmA is surprisingly slow on $tRNA^{Phe}$ Ψ55 compared to unmodified tRNA (Table 5.1; Fig. 5.3A). As further discussed below, these findings suggest that Ψ55 must hinder another reaction step such as product release during tRNA modification by TrmA.

### *5.4.3 Binding and activity preferences of TrmB*

Finally, we examined whether or not the methyltransferase TrmB is sensitive to the modification status of tRNA. In order to examine the single-turnover apparent rate for TrmB modifying tRNAs with different modification statuses, we measured radioactive methyl incorporation from tritium-labeled SAM into nonradioactive tRNA under single-turnover conditions. TrmB was found to modify $tRNA^{Phe}$ containing no modifications at a rate of about 0.2 $s^{-1}$, and the rate of modification was similar for $tRNA^{Phe}$ containing either $m^5U54$ or Ψ55 (Table 5.2; Fig. 5.4A), suggesting TrmB does not have an activity preference for either unmodified or tRNA that is partially modified in the T loop.

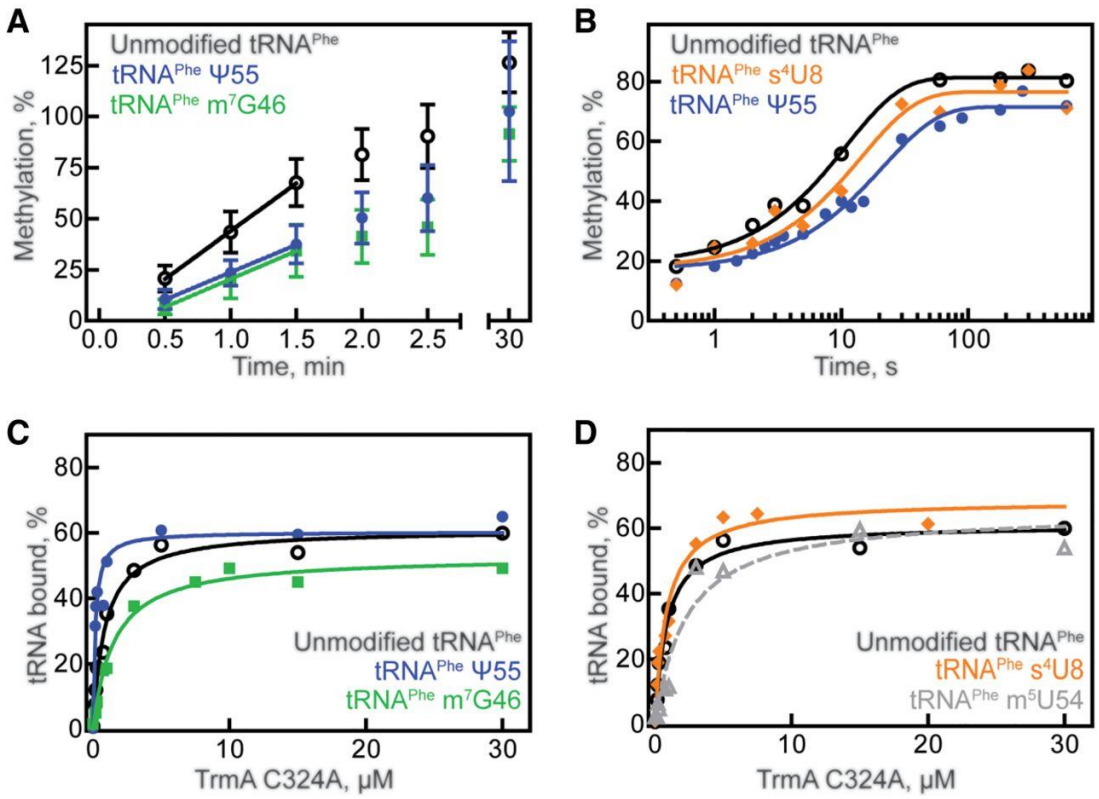



**Figure 5.3. tRNA modification and binding preferences for TrmA. (A)** Multiple-turnover modification reaction of 10 nM TrmA incubated with 600 nM unmodified $tRNA^{Phe}$ (open circles), $tRNA^{Phe}$ $m^7G46$ (green squares), or $tRNA^{Phe}$ Ψ55 (blue circles). Time courses were performed at least in triplicate. Initial velocities are summarized in Table 5.1. **(B)** Single-turnover modification by 5 μM TrmA and 50 μM SAM rapidly mixed with 1 μM unmodified $tRNA^{Phe}$ (open circles), $tRNA^{Phe}$ $s^4U8$ (orange diamonds), or $tRNA^{Phe}$ Ψ55 (blue circles). Here, individual representative time courses are displayed, and the average apparent rates from three replicates is summarized in Table 5.2. Catalytically inactive TrmA C324A variant binding to 20 nM **(C)** unmodified $tRNA^{Phe}$ (open circles), $tRNA^{Phe}$ $m^7G46$ (green squares), $tRNA^{Phe}$ Ψ55 (blue circles), or to **(D)** unmodified $tRNA^{Phe}$ (black circles, same as shown in C), $tRNA^{Phe}$ $s^4U8$ (orange diamonds), or $tRNA^{Phe}$ $m^5U54$ (gray triangles, dashed line). The average $K_D$ from at least three replicates for each experiment is summarized in Table 5.3.

Subsequently, we determined the affinity of TrmB for unmodified tRNA and tRNA containing either $m^5U54$ or Ψ55 which may differ even though no changes in the modification rate were observed. Here, wild-type TrmB was used, but the cofactor SAM was omitted from the reaction such that TrmB is not capable of forming product. The affinity of TrmB for $tRNA^{Phe}$ containing either $m^5U54$ or Ψ55 was similar to that of TrmB binding to unmodified $tRNA^{Phe}$, characterized by a $K_D$ of approximately 3–4 μM (Table 5.3; Fig. 5.4B). Similarly, the presence of both $m^5U54$ and Ψ55 modifications together did not impact the affinity of TrmB for $tRNA^{Phe}$ (Table 5.3; Fig. 5.4C). Like several other tRNA modification enzymes, TrmB was found to bind its product (tRNA $m^7G46$) with a similar affinity as its substrate tRNA (Table 5.3; Fig. 5.4C). Our results point toward an insensitivity of TrmB binding and activity to the tRNA modification status in the T loop.

## 5.5 Discussion

In this study, we examined the effects of single modifications within $tRNA^{Phe}$ on the activity and affinity of tRNA modification enzymes TruB, TrmA, and TrmB. We report here that the tRNA binding and modification activities of TruB and TrmA are sensitive to the modification status of the substrate tRNA. In contrast, none of the modifications examined here affected either tRNA binding or activity by TrmB. In the following, we will first discuss the molecular basis of how prior tRNA modifications affect (or do not affect) the molecular and kinetic mechanism of TruB, TrmA, and TrmB (Fig. 5.5A) before considering the cellular consequences and preferential modification order for tRNA maturation.

### *5.5.1 Impact of modifications on tRNA binding and catalysis by other modification enzymes*

Of the tested enzymes, TruB is most sensitive to the presence of other modifications in tRNA. We observed that $m^7G46$ decreases the initial reaction velocity of pseudouridine formation by TruB and decreases the affinity of TruB for tRNA (Tables 5.1, 5.3). In addition, the

modifications $m^5U54$ and $s^4U8$ also decrease the affinity of TruB for tRNA although the formation of pseudouridine is not affected (Tables 5.1, 5.2, 5.3). Based on the structure of TruB with an unmodified T arm (PDB ID 1K8W), only $m^5U54$ will be in contact with TruB whereas $s^4U8$ and $m^7G46$ are predicted to be distant from TruB (26). Within the unmodified T arm in the TruB structure, U54 forms a base pair with A58 and is contacted by the thumb loop in TruB (residues 120 to 149). Specifically, the side chain of Lysine 130 and the backbone oxygen of Alanine 128 interact with each other and are in very close proximity of C5 of U54 (3.6 and 3.8 Å, respectively) leaving no space for an additional methyl group (Fig. 3.5B). The steric clash between the methyl group of $m^5U54$ and Lysine 130 explains the decreased affinity of TruB for tRNA with $m^5U54$ compared to unmodified tRNA and suggests that the thumb loop of TruB must undergo a conformational change to accommodate this modification. We speculate that within the kinetic mechanism of TruB, the presence of $m^5U54$ could lead to a faster dissociation of tRNA compared to unmodified tRNA ($k_{-1}$ and $k_{-2}$, Fig. 5.5; (13)). However, since the catalysis of pseudouridylation is the slow and rate-limiting step for TruB (19), which is not affected by $m^5U54$, once bound, no change in the reaction velocity is observed.

As $s^4U8$ and $m^7G46$ are not in contact with TruB, their effects on TruB's activity must be indirect and communicated through the tRNA structure. Structural and functional evidence clearly shows that TruB must break interactions between the T and D arm to gain access to the target U55 (13,26). Both $s^4U8$ and $m^7G46$ stabilize the D arm in mature tRNA as a part of a strong network of tertiary interactions in tRNA in this region (27). Notably, the methylation of G46 introduces a positive charge in the variable loop near the negatively charged backbone of the base of the D arm, thereby acting like a staple closely connecting the variable loop and the D arm. Similarly, $s^4U8$ is also interacting with the D arm. By positioning and stabilizing the D arm, $m^7G46$ and $s^4U8$ may render it more difficult for TruB to disrupt the tRNA elbow structure, to displace the D arm, and to gain access to U55. Accordingly, we hypothesize that both modifications slow down the conformational change ($k_2$) thereby decreasing the affinity of TruB for tRNA (Fig. 5.5A). In the case of $m^7G46$, the initial velocity of pseudouridylation is also affected

(Table 5.1). We can envision that the constrained location of the D arm may affect correct positioning of the T arm within the active site thereby slowing down catalysis.

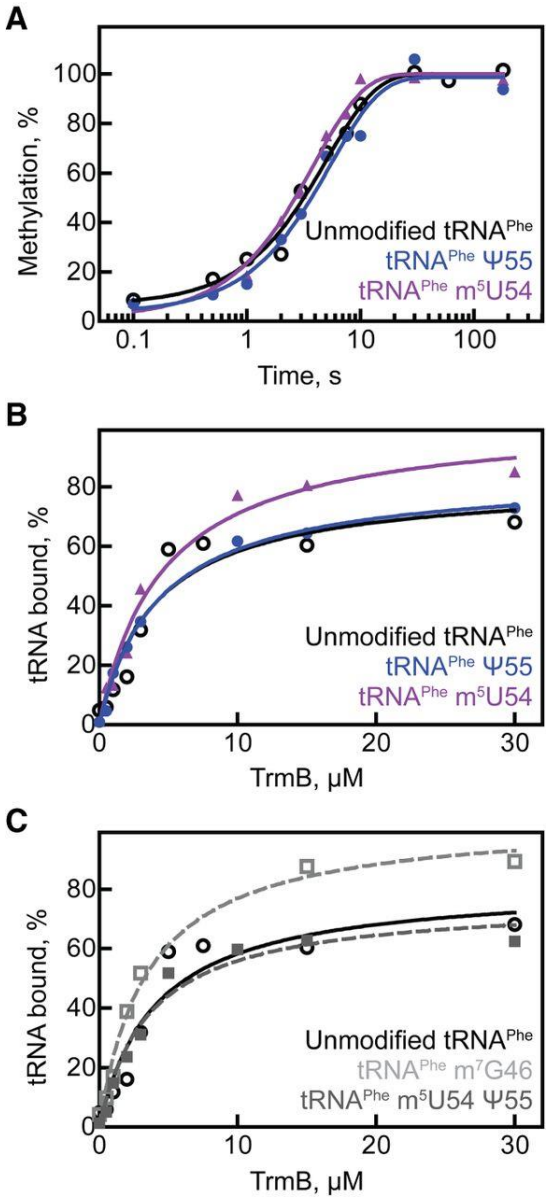
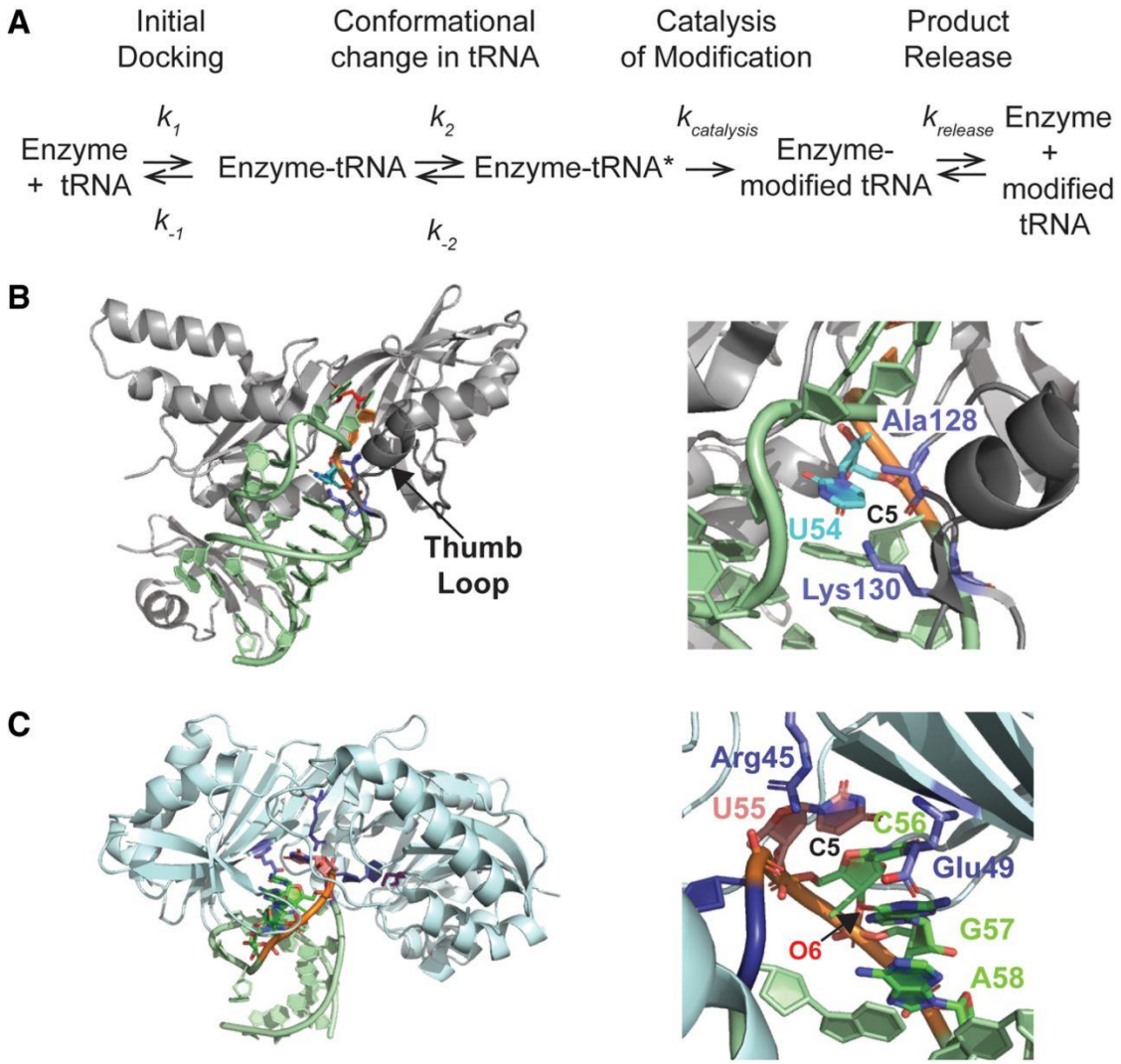



**Figure 5.4. tRNA modification and binding preferences for TrmB. (A)** Single-turnover modification by 5 μM TrmB and 50 μM [$^{3H}$]SAM mixed with 1 μM unmodified tRNA$^{Phe}$ (open circles), tRNA$^{Phe}$ m$^5$U54 (purple triangles), or tRNA$^{Phe}$ Ψ55 (blue circles). The average apparent rate of modification from three time courses (one representative each shown here) is reported in Table 5.2. *Caption continued on the next page.*

TrmB binding to 20 nM **(B)** unmodified $tRNA^{Phe}$ (open circles), $tRNA^{Phe}$ $m^5U54$ (purple triangles), $tRNA^{Phe}$ Ψ55 (blue circles), or to **(C)** unmodified $tRNA^{Phe}$ (black circles, same as shown in B), $tRNA^{Phe}$ $m^5U54$ Ψ55 (gray squares, dashed line), or $tRNA^{Phe}$ $m^7G46$ (open squares, dashed line). The experiments were repeated at least three times, and the average $K_D$ for each tRNA is summarized in Table 5.3.




*Caption on next page.*

**Figure 5.5 Kinetic mechanism and tRNA interactions of TruB and TrmA. (A)** Both TruB and TrmA have a two-step tRNA binding mechanism where the tRNA is first docked onto the enzyme ($k_1$, $k_{-1}$) followed by a conformational change that disrupts T and D arm interactions ($k_2$, $k_{-2}$) such that the enzyme gains access to its target uridine. Both binding steps are reversible and contribute to the dissociation constant. Subsequently, the tRNA is modified in one or more catalytic steps ($k_{catalysis}$), and finally the modified tRNA is released as the reaction product ($k_{release}$). All steps can contribute to the overall velocity ($v_0$) of the enzyme. **(B)** Interaction of TruB with the T arm, in particular U54. (Left) Overview of TruB bound to the T arm (PDB ID 1K8W (26)). U54 is shown in cyan, U55 in orange, and the catalytic aspartate 48 in red. The thumb loop is depicted in dark gray. (Right) Close-up view on the interaction of Lys130 and Ala128 (purple) in the thumb loop with U54. The backbone oxygen of Ala128 is 3.8 Å apart from C5 in U54 whereas the ζN of Lys130 is located 3.6 Å away from C5 of U54. The site of methylation by TrmA, C5 in U54, is indicated suggesting that the methyl group would cause a steric clash with Lys130 and Ala128. **(C)** Interaction of TrmA with the T arm, in particular U55. (Left) Overview of TrmA bound to the T arm (PDB ID: 3BT7 (17)). U54 is shown in blue, the catalytic cysteine in purple, and U55 in orange. (Right) Close-up, back view of U55 stacking with C56, G57, and A58 (green). The position of C5 of U55 which would be occupied by N1 in pseudouridine is indicated. Residues Arg45 and Glu49 (purple) are in proximity of U55, but not in direct contact with C5, that is, N1 in Ψ55 (3.1 Å between the ηN of Arg45 and C5 of U55, and 5.9 Å between the εO of Glu49 and C5 of U55). A pseudouridine would further strengthen the depicted base stacking interactions of U55–C56–G57–A58 in the T loop. Moreover, a water molecule could potentially form a hydrogen bonding bridge between N1 of pseudouridine and O6 of G57 (4.3 Å apart, labeled). All structural representations were prepared with the PyMOL software package.

The negative impact of $s^4U8$ and $m^5U54$ on TruB's affinity for tRNA (Table 5.3) is reminiscent of tRNA binding by ArcTGT, for which a negative correlation was observed between $K_M$ and tRNA melting temperature causing ArcTGT modification of unmodified tRNA to be quick in comparison to modification of tRNA containing all modifications except for its product, archaeosine (28). This similarity between ArcTGT and TruB is notable since both enzymes disrupt the tRNA tertiary structure in order to access their target base (26,29). A rigid tRNA elbow already containing one or more modifications may provide TruB and ArcTGT with a greater challenge in the accession of their target base. Likewise, *Bacillus subtilis* TrmK methylates A22 and also prefers to bind to completely unmodified tRNA compared to tRNA containing prior modifications (30).

As is the case for pseudouridylation by TruB, tRNA methylation at U54 by TrmA is affected by tRNA modifications. We found that TrmA is sensitive to the presence of $m^7G46$ and Ψ55, but not $s^4U8$. The presence of $m^7G46$ decreases methylation activity and affinity for tRNA. Again, this effect is most likely a result of the stabilization of the D arm by $m^7G46$, which hinders the disruption of T and D arm interactions by TrmA (decreased $k_2$), causing a higher dissociation constant. We also observed that the presence of Ψ55 decreases the initial velocity ($v_0$) of methylation by TrmA, but without affecting the catalytic step ($k_{methyl}$) itself (Tables 5.1, 5.2). In contrast to the negative effect on multiple-turnover methylation, we surprisingly note that Ψ55 has a positive effect on the affinity of TrmA for tRNA. As we will further explain below, these observations for Ψ55 can only be explained if a forward reaction step other than catalysis is slowed down ($k_1$, $k_2$, or $k_{release}$), reducing the multiple-turnover activity, and if a reverse reaction step (e.g., $k_{-2}$) is reduced even more, leading to tighter tRNA binding (Fig. 5.5A).

In the structure of TrmA bound to an unmodified T arm, U55 forms base stacking interactions with nucleotides 56–58, and the base of U55 is contacted by the side chain of Arginine 45 and the backbone of Glutamate 49 (Fig. 5.5C, PDB ID 3BT7; (17)). When U55 is converted to pseudouridine, N1 of pseudouridine will occupy the position of C5. None of TrmA's contacts with U55 involve this atom in the uracil ring such that it seems unlikely that TrmA

residues directly sense the presence of Ψ55. However, TrmA can only gain access to U54 by imposing a structural rearrangement in the T loop including formation of the base stack between U55, C56, G57, and A58 (17). As pseudouridine forms stronger base stacking interactions than uridine (31), the presence of Ψ55 will stabilize the T arm conformation as bound to TrmA. In addition, it is conceivable that a water molecule could possibly form a hydrogen bonding bridge between N1 of Ψ55 and O6 of G57, further rigidifying the base stacking interactions. The stabilization of the T arm conformation as bound to TrmA could reduce the dissociation of tRNA from TrmA ($k_{-1}$ or $k_{-2}$, Fig. 3.5C) explaining the lower dissociation constant of TrmA for tRNA containing Ψ55.

The initial velocity of tRNA methylation of TrmA, but not the catalytic rate, is reduced by Ψ55, despite tighter binding of TrmA to tRNA containing Ψ55. Thus surprisingly, despite tighter binding, the overall multiple-turnover reaction comprising all reaction steps is slower whereas the catalytic step is not directly affected. As mentioned, these observations can only be explained by slower tRNA binding ($k_1$ or $k_2$) or slower product release, limiting the turnover of TrmA. Slower product release could be a result of more stable binding of tRNA containing Ψ55 which should equally affect $k_{-2}$ and $k_{release}$ (Fig. 5.5A). The presence of Ψ55 itself will also stabilize tertiary interactions within the tRNA substrate, rendering it possibly more difficult for TrmA to gain access to U54 when disrupting the interaction between the D arm and the T arm (reduced $k_2$), which may further contribute to the reduced multiple turnover rate of TrmA when Ψ55 is present.

Notably, TrmB is not affected in its activity or tRNA binding by the presence of $m^5U54$ or Ψ55. Since no structure of TrmB bound to tRNA has been determined so far, we can only speculate whether and how TrmB interacts with other regions of the tRNA besides the variable loop. It may be that TrmB does not contact the T arm and that it does not affect the tertiary interaction between the D and T arm. Alternatively, TrmB may interact with the T arm, but this interaction may not be affected by T loop modifications.

*5.5.2 Preferred order of tRNA modification*

Based on our data, we hypothesize that there is a preferred order, although not a strict hierarchy, of tRNA modification in *E. coli*. Specifically, we propose that TruB and TrmA prefer to act earlier than TrmB and ThiI during tRNA maturation. Since TruB and TrmA are faster acting on tRNA lacking other modifications (Tables 5.1, 5.2, 5.3), TruB and TrmA are likely to act during the relatively early stages of tRNA maturation within the cell prior to TrmB introducing $m^7G46$ in the variable arm. This hypothesis is supported by our observations that $m^7G46$ presence negatively affects tRNA modification by TruB and TrmA, but TrmB is unaffected by $m^5U54$ and Ψ55. Accordingly, TrmB is hypothesized to preferentially act after TrmA and TruB, but this does not mean that *E. coli* TrmB is acting late during tRNA modification as also observed in yeast (24). Notably, a recent study uncovered that the presence of $m^7G46$ is highly advantageous for the formation of the adjacent aminocarboxypropyluridine ($acp^3U47$) modification in *E. coli* tRNA by the newly discovered YfiP/TapT enzyme (32). Conversely, TrmB does not depend on the prior formation of $acp^3U47$. Therefore, it is plausible that TruB and TrmA prefer to modify tRNA very early followed by TrmB, which methylates tRNA shortly after and before the likely late-acting TapT enzyme (32,33). The presence of the $s^4U8$ modification did not affect the apparent rate of modification for either TrmA or TruB (Table 5.2; Figs. 5.2B, 5.3B); however, $s^4U8$ presence did lower the affinity of TruB for $tRNA^{Phe}$ (Table 5.3; Fig. 5.2C). From these data, we cannot decisively predict temporally when ThiI is likely to act during tRNA maturation; however, it does seem probable that ThiI may act after TruB in order to provide TruB with an ideal substrate.

In accordance with our suggestion that TruB and TrmA may act prior to TrmB during tRNA modification in *E. coli*, a similar preferred modification order was reported in a recent study of in vitro transcribed $tRNA^{Phe}$ modification in *Saccharomyces cerevisiae* cell extracts monitored by time-resolved NMR. Here, Ψ55 was the first modification to be introduced within tRNA, with both $m^5U54$ and $m^7G46$ appearing second within the tRNA where the introduction of $m^7G46$ finished after the complete introduction of $m^5U54$ (24). Thus, in both *E. coli* and *S. cerevisiae*, TruB prefers to act prior to TrmB. But in yeast, it could be possible that TrmB and TrmA homologs

act in parallel during tRNA maturation, which may be a result of species-specific differences between monomeric *E. coli* TrmB (34) and the heterodimeric Trm8-Trm82 complex from yeast (35).

Regarding tRNA modification by TruB and TrmA in *E. coli*, the most likely temporal order is not obvious because $m^5U54$ hinders TruB binding and Ψ55 slows modification by TrmA (Tables 5.1, 5.2,5.3). On the other hand, Ψ55 presence increases the affinity of TrmA for $tRNA^{Phe}$ approximately five-fold (Table 5.3). The seemingly opposite effects of Ψ55 on TrmA are not without precedence as the apparent rate of U34 modification by CmoM is decreased when 1-methylguanosine 37 formed by TrmD is present within *E. coli* $tRNA^{Pro}_{UGG}$, despite 1-methylguanosine 37 lowering the affinity of CmoM for $tRNA^{Pro}_{UGG}$ (36). Interestingly, bulk tRNAs from yeast missing TruB's homolog, Pus4, have been found to lack $m^5U54$ content, suggesting, at least in yeast, introduction of $m^5U54$ actually relies on previous modification by TruB to a certain extent (24). Accordingly, the yeast tRNA methyltransferase Trm2 may be differently affected by the presence of Ψ55 than its *E. coli* homolog TrmA.

Several reasons support our suggestion that both TruB and TrmA modify tRNA relatively early within the overall hierarchy of tRNA modifications. First, with TrmA and TruB as the only enzymes to modify all species of *E. coli* tRNA, it seems unlikely for these enzymes to rely on the previous introduction of another tRNA modification as a positive determinant as no other modification can be present within each of their substrate tRNAs. In contrast, it is possible for other modification enzymes to rely on either $m^5U54$ or Ψ55 modifications as a determinant, considering that $m^5U54$ and Ψ55 conversely are present within the tRNA substrates of every *E. coli* tRNA modification enzyme. Second, evidence for early tRNA modification by TrmA and TruB during tRNA maturation is provided by the observation that both $m^5U54$ and Ψ55 appear prior to intron splicing and 5′ and 3′ end trimming when yeast $tRNA^{Tyr}$ was injected into *Xenopus* oocytes (37). Similarly, yeast $tRNA^{Phe}$ contains $m^5U54$ and Ψ55 prior to intron splicing (38,39). Third, considering that both TruB and TrmA are known tRNA chaperones (12), a scenario in which TruB and TrmA have evolved to interact with tRNA during the early stages of its maturation in order to

quickly promote correct tRNA folding may be beneficial. In particular, the correct three-dimensional structure of tRNA promoted by the action of TruB and TrmA will allow the tRNA to be modified by other tRNA modification enzymes that recognize the entire tRNA structure (40).

## 5.6 Conclusion

In summary, our proof-of-principle study describes that TruB and TrmA prefer to act on unmodified tRNA compared to tRNA already containing a single modification whereas TrmB does not appear to have a preference for the modification status of tRNA. Our results corroborate recent findings in yeast (24) and additionally provide mechanistic insight for why Ψ55 and $m^5U54$ are likely to appear early during tRNA maturation. A recent review (20) hypothesized that tRNA modification circuits, that is, a strict order of modification, are most common within the anticodon loop because modifications can serve as additional sequence or structure determinants to ensure tRNA anticodon loop modification enzymes only modify their desired substrates, which is particularly critical considering the diversity of anticodon loop modifications and their importance for translational fidelity. Taking our results together with other studies of tRNA elbow modification enzymes (23,24,28,30,32,41), it becomes clear that modifications in the tRNA elbow region do not occur in strict order where one modification is dependent on the other. However, there is still a preferred pathway of tRNA elbow modification and the modification enzymes do not act randomly on tRNA.

## 5.7 Materials and Methods

### *5.7.1 Buffers and reagents*

Experiments were performed in $TAKEM_4$ buffer (50 mM Tris-HCl, pH 7.5, 70 mM $NH_4Cl$, 30 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$). [C5-$^3$H]UTP for *in vitro* transcription of [$^3$H]$tRNA^{Phe}$ was purchased from Moraveck Biochemicals and radioactive adenosyl-L-methionine S-[methyl-$^3$H]

([$^3$H]SAM) was purchased from PerkinElmer. Nonradioactive SAM was obtained from New England Biolabs (NEB). All other chemicals were purchased from Fisher Scientific.

*5.7.2 Protein expression and purification*

To generate a plasmid encoding *E. coli trmB*, the forward primer, 5′-GCCAGAGCTAGCAAAAACGACGTCATTTCACCG-3′ and reverse primer, 5′-GCCACCGGATCCTTATTTCACCCTCTCGAACATTAAGTCCC-3′ were used to amplify the trmB open reading frame. This was cloned into the pET28a(+) vector using the NheI and BamHI restriction sites, creating pET28a-TrmB. The gene sequence was confirmed by DNA sequencing (Genewiz) and the plasmid was transformed into BL21 (DE3) cells. Cells were grown in LB supplemented with 50 μg/mL kanamycin at 37°C and protein expression was induced at an $OD_{600}$ of approximately 0.6 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 3 h, cells were harvested by centrifugation at 5000*g* for 15 min, flash frozen, and stored at −80°C.

Wild-type TruB and TruB D48N were expressed from pET28a-TruB in BL21 (DE3) cells as previously described elsewhere (19). For the expression of ThiI and IscS in BL21 (DE3) cells, plasmids pBH113 and pBH402 were obtained from Eugene Mueller (42,43). TrmA wild-type and TrmA C324A proteins were overexpressed from the pCA24N(GFP minus)-TrmA (JW3937) plasmid in AG1 (ME5305) cells (12,44). Similarly, the tRNA pseudouridine synthases TruA and RluA were expressed using the respective pCA24N(GFP minus) plasmids (19).

All proteins were purified via their amino-terminal hexahistidine tag using nickel-sepharose and Superdex 75 chromatography as described (19). Protein concentrations were determined by absorbance at 280 nm using molar extinction coefficients of 35,870 $M^{-1}$ $cm^{-1}$ for TrmA, 20,860 $M^{-1}$ $cm^{-1}$ for TruB, and 27,960 $M^{-1}$ $cm^{-1}$ for TrmB (calculated using ProtParam (45)), 63,100 $M^{-1}$ $cm^{-1}$ for ThiI and 25,400 $M^{-1}$ $cm^{-1}$ for IscS (as determined in (43)). Concentrations were confirmed using comparative SDS-PAGE.

*5.7.3 tRNA preparation*

The *E. coli* $tRNA^{Phe}$ gene was amplified from the pCF0 plasmid (46) for *in vitro* transcription. To produce internally tritium-labeled tRNA, [C5-$^3$H]UTP was included in the reaction as previously described (19). Radioactive $tRNA^{Phe}$ transcripts were purified by Nucleobond Xtra Midi anion-exchange gravity columns (Macherey-Nagel) as described elsewhere (19). Nonradioactive transcripts were purified by phenol-chloroform extraction to remove proteins followed by Superdex 75 (XK 26/100 column, GE Healthcare) size exclusion chromatography to remove unincorporated nucleotides. Subsequently, $tRNA^{Phe}$ was concentrated by isopropanol precipitation and resuspended in water. $tRNA^{Phe}$ concentration was determined by $A_{260}$ measurements using a molar extinction coefficient of 500,000 $M^{-1}$ $cm^{-1}$ (47). Scintillation counting was used to determine the specific activity of radioactive tRNA.

*5.7.4 Partial modification of $tRNA^{Phe}$*

*In vitro* transcribed $tRNA^{Phe}$ was refolded in $TAKEM_4$ buffer by heating to 65 °C for 5 min and cooled to room temperature for 10 min. To prepare singly-modified $tRNA^{Phe}$ Ψ55, $tRNA^{Phe}$ $m^5U54$, and $tRNA^{Phe}$ $m^7G46$, 1.6 μM of tritium-labeled or nonradioactive refolded $tRNA^{Phe}$ was incubated with 5 μM of purified TruB, TrmA, or TrmB enzyme for 2 h at 37 °C in a 5 mL reaction. Methylation reactions included 50 μM SAM. To prepare [$^3$H]$tRNA^{Phe}$ $s^4U8$, 4 μM of folded [$^3$H]$tRNA^{Phe}$, 2 μM of purified ThiI, and 1 μM purified IscS was incubated with 40 μM pyridoxal 5′-phosphate, 4 mM ATP, 0.5 mM L-cysteine, and 1 mM DTT in a 10 mL reaction volume in $TAKEM_4$ buffer for 2 h at 37 °C. To prepare a [$^3$H]$tRNA^{Phe}$ with five modifications, 5 μM [$^3$H]$tRNA^{Phe}$ was incubated with 7.5 μM of each RluA, TruA, TruB, TrmA, and TrmB in the presence of 50 μM SAM in a 100 μL reaction. In all cases, enzymes were subsequently removed by phenol-chloroform extraction followed by ethanol precipitation of the tRNA. After resuspending modified $tRNA^{Phe}$ in water, $tRNA^{Phe}$ concentration was determined by $A_{260}$ measurements and scintillation counting.

To quantify the level of modification for prepared [$^3$H]tRNA$^{Phe}$ m$^5$U54 and [$^3$H]tRNA$^{Phe}$ Ψ55, 600 nM of the now singly-modified tRNA was incubated with 5 μM TrmA or TruB, respectively. The end level of modification by the enzyme after 1 h at 37 °C was determined in triplicate by tritium release assays in order to determine the percentage of the tRNA that had remained unmodified. Thereby, it was determined that [$^3$H]tRNA$^{Phe}$ m$^5$U54 was 78 ± 5% modified and [$^3$H]tRNA$^{Phe}$ Ψ55 was 81 ± 4% modified. The Ψ55 content within the [$^3$H]tRNA$^{Phe}$ with five modifications was similarly quantified, and Ψ55 was found to be present in 83 ± 3% of this preparation. To assess the level of modification within [$^3$H]tRNA$^{Phe}$ m$^7$G46 and nonradioactive tRNA$^{Phe}$ m$^5$U54 and tRNA$^{Phe}$ Ψ55 preparations, small scale reactions with [$^3$H]SAM or [$^3$H]tRNA$^{Phe}$ were performed in parallel in triplicate to estimate the level of modification within each respective large-scale preparation. The respective tRNA$^{Phe}$ preparations contained 83 ± 5% of m$^7$G46, 88 ± 2% of m$^5$U54, and 89 ± 3% of Ψ55. Finally, to quantify the level of s$^4$U8 within tRNA, modified tRNA was analyzed in triplicate on an [(N-acryloylamino)phenyl]mercuric chloride (APM) gel (48). In brief, 50 pmol of RNA was analyzed on a denaturing gel containing 10% acrylamide (19:1 acrylamide:bisacrylamide), 7 M urea, and 20 μM APM in Tris/Borate/EDTA (TBE) buffer. Following SYBR Green II staining, the ratio of thiolated tRNA retarded by APM to nonthiolated, faster migrating tRNA was determined using ImageJ software and [$^3$H]tRNA$^{Phe}$ s$^4$U8 was determined to be 83 ± 2% modified.

### *5.7.5 Tritium release assay to measure Ψ55 and m$^5$U54 formation*

Tritium release assays were performed as previously described to detect pseudouridylation by TruB and C5-methylation by TrmA (12,19). First, tritium-labeled tRNA$^{Phe}$ was refolded as before. All reactions with TrmA included 50 μM SAM. For multiple-turnover reactions, 600 nM [$^3$H]tRNA$^{Phe}$ was incubated with 10 nM enzyme. The initial velocity ($v_0$) of the reaction was determined by linear fitting of the first 1.5 min of the reaction. For single-turnover reactions, enzyme and [$^3$H]tRNA$^{Phe}$ were mixed in a Kintek quench-flow apparatus with final concentrations of 5 μM of enzyme and 1 μM of tRNA. Reactions were stopped by addition of

0.1 M hydrochloric acid after 0.1, 0.5, 1, 2, 3, 5, 7.5, 10, 30, 60, and 180 s, and the level of tritium released was determined as previously described (19). To determine the apparent reaction rate ($k_{app}$), time courses were fit with a one-exponential equation:

$$Y = Y_0 + (Y_\infty - Y_0) \times \exp(-k_{app} \times t)$$

where $Y$ is the percentage of pseudouridine formation at a given time point, $Y_0$ is the initial level of apparent pseudouridylation (due to tritium release from the tRNA in absence of enzyme), $Y_\infty$ is the end level of pseudouridylation, and $t$ is the time.

For TruB and TrmA, the apparent reaction rate is concentration-independent and therefore directly reflects the rate constant of pseudouridylation ($k_\Psi$) and the rate constant of methylation ($k_{methyl}$), respectively (12,19).

*5.7.6 Methylation assay to measure $m^7G46$ formation*

To determine methylation by TrmB, TrmB and [$^3$H]SAM were rapidly mixed in a quench-flow apparatus with nonradioactive $tRNA^{Phe}$ to final concentrations 5 μM, 50 μM, and 1 μM, respectively, and reactions were stopped by addition of 0.1 M HCl at the same timepoints as described above. A constant volume of each quenched sample was precipitated on Whatman paper disks presoaked with 5% (w/v) trichloracetic acid. To remove unincorporated [$^3$H]SAM, disks were washed three times with 5% (w/v) trichloracetic acid for 5 min followed by a final wash in ethanol. After drying, paper disks were added to 4 mL EcoLite(+) scintillation cocktail (MP Biomedical), and the amount of [$^3$H]methyl incorporated was determined by scintillation counting. The apparent rate of methylation was determined by fitting to a single exponential function, as stated above. Since percent tRNA methylated cannot be determined directly with this assay, the maximum amplitude determined by fitting was set to be 100%.

*5.7.7 Nitrocellulose filtration to determine the affinity for tRNA*

Refolded, uniformly tritium-labeled $tRNA^{Phe}$ (20–40 nM) was incubated with increasing concentrations of enzyme (0, 0.5, 1.0, 2.0, 3.0, 5.0, 7.5, 10.0, 15.0, 30.0 μM for TruB D48N and wild-type TrmB, and 0, 0.15, 0.2, 0.3, 0.75, 1.0, 3.0, 5.0, 15.0, 30.0 μM for TrmA C324A) in $TAKEM_4$ buffer for 10 min at room temperature prior to filtration through a nitrocellulose membrane. The proportion of tRNA bound to enzyme was determined by scintillation counting as described in (19). The dissociation constant ($K_D$) was determined by plotting percent RNA bound (*Bound*) as a function of enzyme concentration ([*enzyme*]) and fitting to a hyperbolic equation:

$$Bound = Bound_{max} \times [enzyme]/(K_D + [enzyme])$$

## 5.8 Acknowledgements

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**Chapter 6:**
**Molecular mechanism of tRNA binding by the Escherichia coli N7 guanosine methyltransferase TrmB**

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## 6.1 Preface

A version of this chapter is a re-formatted version of a publication titled “Molecular mechanism of tRNA binding by the Escherichia coli N7 guanosine methyltransferase TrmB” that was accepted in the *Journal of Biological Chemistry (JBC)* on March 16 2023 and appears in Volume 299 Issue 5, 104612 (https://doi.org/10.1016/j.jbc.2023.104612) as part of the Research Article Collection: RNA (1). Sarah K. Schultz, Kieran Meadows, and Dr. Ute Kothe are authors. As first author, I performed and analyzed the majority of the experiments and supervised Kieran Meadows, an undergraduate student who prepared TrmB variants, performed the rapid-kinetic stopped-flow experiments with the TrmB variants, and analyzed the resulting data. Together, Dr. Ute Kothe and I conceptualized the project and planned study methodology. I wrote the first draft of the manuscript, which was edited by Dr. Ute Kothe and myself. Dr. Ute Kothe was additionally involved in supervision and funding acquisition. We thank two anonymous reviewers for their feedback on this manuscript during the revision process, resulting in the form it appears now.

## 6.2 Abstract

Among the large and diverse collection of tRNA modifications, 7-methylguanosine ($m^7G$) is frequently found in the tRNA variable loop at position 46. This modification is introduced by the TrmB enzyme, which is conserved in bacteria and eukaryotes. However, the molecular determinants and the mechanism for tRNA recognition by TrmB are not well understood. Complementing the report of various phenotypes for different organisms lacking TrmB homologs, we report here hydrogen peroxide sensitivity for the *Escherichia coli ΔtrmB* knockout strain. To gain insight into the molecular mechanism of tRNA binding by *E. coli* TrmB in real time, we developed a new assay based on introducing a 4-thiouridine modification at position 8 of *in vitro* transcribed $tRNA^{Phe}$ enabling us to fluorescently label this unmodified tRNA. Using rapid kinetic stopped-flow measurements with this fluorescent tRNA, we examined the interaction of WT and single substitution variants of TrmB with tRNA. Our results reveal the role of S-adenosylmethionine for rapid and stable tRNA binding, the rate-limiting nature of $m^7G46$ catalysis

for tRNA release, and the importance of residues R26, T127, and R155 across the entire surface of TrmB for tRNA binding.

### 6.3 Introduction

tRNAs contain the greatest density and diversity of modifications among RNA species (2). Among the assortment of chemical modifications, single methylations occur at different atoms of each of the four canonical nucleobases or the ribose sugar and are introduced by a collection of unrelated methyltransferases (3,4). Whereas some modifications are present in only certain tRNA isoacceptors in specific organisms, other modifications are highly conserved. The N7-methylguanosine ($m^7G$) modification is common in bacterial and eukaryotic tRNAs and has been found in a few archaea (5). Most often, $m^7G$ is present at position 46 in the variable loop of tRNAs, although it has additionally been found at tRNA positions 34, 36, and 49 in a handful of organisms (5). Within tRNAs, the base at position 46 is involved in a tertiary base pair with C13-G22, where it acts as a staple connecting the variable loop and the D arm. At this position vital for tRNA tertiary structure, the $m^7G46$ modification introduces a site-specific positive charge amongst the negatively charged tRNA backbone (6,7). In *Escherichia coli*, $m^7G46$ is introduced in about half of all tRNAs by TrmB, an S-adenosylhomocysteine (SAM)-dependent methyltransferase (Fig. 6.1) (8,9).

Similarly to many tRNA modification enzymes, TrmB is nonessential in bacteria and knockout of the *E. coli trmB* gene does not impair growth under ideal conditions (8). While this observation has raised questions about the functions of TrmB and other tRNA modification enzymes, newer findings in different organisms suggest a role for the $m^7G46$ modification under certain disease or stress conditions. Several recent studies have identified an importance for the human TrmB homolog METTL1 for cancer cell progression across several cancer types (reviewed in (10,11), wherein the $m^7G46$ modification prevents degradation of specific tRNAs, leading to an increase in global translation in addition to biased translation of growth-promoting genes (12-14). Thus, this tRNA methyltransferase has gained attention as a potential biomarker

for cancer prognosis and as a potential chemotherapeutic target. Moreover, human mutations resulting in the reduction of $m^7G$ methylation have been associated with a distinct form of microcephalic primordial dwarfism (15). Yeast lacking the TrmB homolog Trm8 display a specific growth defect in synthetic minimal media containing 2% glycerol at 38 °C (16). This phenotype is exacerbated upon deletion of certain additional tRNA modification enzymes due to rapid decay of specific tRNA isoacceptors (17). Within eubacteria, *Pseudomonas aeruginosa* lacking TrmB has been shown to be sensitive to hydrogen peroxide ($H_2O_2$) (18). A similar peroxide sensitivity has also been shown for the phytopathogenic fungus *Colletotrichum lagenarium* lacking the *trmB* homolog *aph1*. *C. lagenarium* cells lacking *aph1* additionally grow poorly in high-salt concentrations and are unable to infect plant cells (19). Finally, *Thermus thermophilus ΔtrmB* cells exhibit severe growth defects at high temperatures, accompanied by decreases in other tRNA modifications, tRNA stability, and protein synthesis (20).

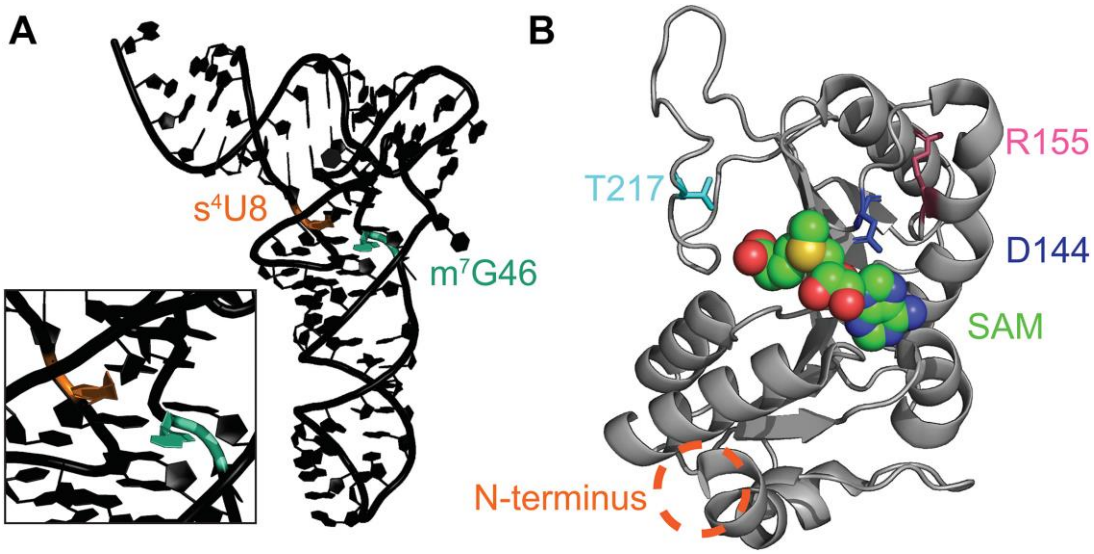


**Figure 6.1 Structures of tRNA and TrmB. A**, crystal structure of unmodified *E. coli* $tRNA^{Phe}$ (PDB 3L0U; (21)) highlighting the locations of G46, where TrmB introduces the 7-methylguanosine modification ($m^7G$, teal) and U8, which was modified by ThiI and IscS to 4-thiouridine ($s^4U$, orange) for subsequent fluorescent labeling in this study. *Caption continues on the next page.*

Panel inset is a close-up view for better visualization of the U8 and G46 nucleotides. **B**, crystal structure of *E. coli* TrmB in complex with its cofactor SAM (PDB 3DXY; (9)). The methyl donor SAM is shown as spheres and residues addressed in this study are shown as sticks, with residue D144 highlighted in blue, T217 in teal, and R155 in pink. As the first 36 amino acids of TrmB were not resolved in this structure, the location of the N terminus, which would include residue R26, is circled in orange.

Some structural information about TrmB and its homologs is available but we still do not know how exactly TrmB recognizes tRNA. Formation of $m^7G46$ in bacteria requires only the TrmB protein; however, in eukaryotes, two nonrelated enzymes form a complex to modify tRNA. Whereas *E. coli* TrmB behaves as a monomer (8), other bacterial TrmB homologs have been shown to be homodimeric in solution, including *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Aquifex aeolicus* TrmB homologs (9,22,23) . In contrast, the eukaryotic TrmB homolog, known as Trm8 in yeast and METTL1 in humans, forms a heterodimer with Trm82 (yeast) or WDR4 (humans) (24,25). Crystal structures for *E. coli*, *A. aeolicus*, *B. subtilis*, and *Saccharomyces cerevisiae* TrmB homologs have been solved alone, with substrate SAM, or product S-adenosylhomocysteine (SAH) (9,22,25-27). These structures have revealed that all TrmB homologs are class I methyltransferases deviating from the classic Rossman fold only by an insertion between two β-strands (9,22). In *E. coli* TrmB, this insertion lacks secondary structure (9), whereas an α-helix is evident in other TrmB/Trm8 structures (22,25). A structure for bacterial TrmB in complex with tRNA does not yet exist and as such, we are lacking an understanding of how TrmB binds its substrate tRNA.

A prior study of *E. coli* TrmB identified several TrmB residues necessary for TrmB methylation activity *in vitro* but did not distinguish which of these residues are directly responsible for catalysis and which are involved in tRNA binding (28). Single-residue alanine substitutions at conserved residues R26, D144, H151, R154, R155, D180, T217, and E220 reduced multiple-turnover methylation activity to less than 10% of that of the WT enzyme, with substitutions at

D144, R154, and R155 abolishing methyltransferase activity completely under these conditions (28). Steady-state kinetic analysis for several equivalent single alanine substitutions in *A. aeolicus* TrmB has suggested that TrmB D144A, R155A, and T217A variants display slower methylation than WT TrmB, whereas SAM binding is unaffected for these variants (29). The authors of this study proposed a potential catalytic mechanism wherein D180 and the adjacent T179 residue (*E. coli* TrmB numbering) interact with the N1-proton and O6 atom of the target guanosine 46 base, respectively, thus positioning the N7 atom for nucleophilic attack by the SAM methyl group (29).

Here, we investigate the function of TrmB for *E. coli* fitness and dissect its interaction with tRNA using a novel assay. Thereby, we report that the model bacterium *E. coli* is sensitive to hydrogen peroxide stress in the absence of TrmB. In order to gain a better understanding as to how this tRNA modification enzyme binds tRNA, we prepared partially modified $tRNA^{Phe}$ containing the 4-thiouridine 8 ($s^4U8$) modification using purified ThiI (also known as TtuI (30,31)) and IscS enzymes. The reactivity of the $s^4U8$ modification was then used to fluorescently label the tRNA for pre-steady-state kinetic analysis using a stopped-flow instrument. Moreover, to investigate the roles of specific TrmB residues for tRNA binding, we analyzed four TrmB variants: R26A, D144A, R155A, and T217A. The binding of these variant enzymes to tRNA was examined in real time using our stopped-flow assay, the affinity of these enzymes for tRNA was determined using nitrocellulose filtration, and the methylation activity of TrmB WT and variants was assessed in single-turnover assays. Taken together, our results suggest that tRNA binding by TrmB is a complex, multistep process aided by prior binding of SAM.

## 6.4 Results

### *6.4.1 E. coli ΔtrmB grows slowly in the presence of hydrogen peroxide*

*E. coli trmB* is nonessential for cell growth and no growth phenotypes have been reported for the *E. coli ΔtrmB* strain under ideal growth conditions (8). Since previous work has

shown *P. aeruginosa* and the parasitic fungus *C. lagenarium* lacking the *trmB* gene grow slowly in media containing hydrogen peroxide, we asked whether *E. coli* lacking *trmB* displays the same phenotype (18,19). Indeed, *E. coli* BW25113 *ΔtrmB* reproducibly grows slower than its parental strain in LB medium supplemented with $H_2O_2$. Although the exponential and stationary phases are not affected by *trmB* KO, the lag phase for the *ΔtrmB* cells is longer than that of the WT (Fig. 6.2). Thus, an increased sensitivity to hydrogen peroxide in the absence of *trmB* seems to be common between *E. coli*, *P. aeruginosa*, and the eukaryotic fungus *C. lagenarium*.

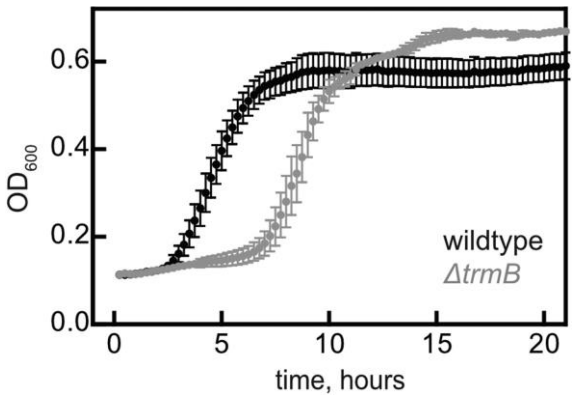


**Figure 6.2. *E. coli* BW25113 *ΔtrmB* grows slowly in LB medium-containing hydrogen peroxide.** Four biological replicates for WT *E. coli* BW25113 (black) and *E. coli* BW25113 *ΔtrmB* (gray) were seeded with a starting $A_{600}$ of 0.1 in a 96-well plate. Average $A_{600}$ values recorded every 15 min and error bars represent SEM.

*6.4.2 Partial modification and fluorescent labeling of $tRNA^{Phe}$ for rapid kinetic analysis of tRNA binding by TrmB*

In order to examine how TrmB is binding tRNA, we sought to introduce a fluorescent label into tRNA that would enable a fluorescence change that could be observed in real time using stopped-flow fluorescence spectrometry. Within the three-dimensional tRNA structure, the

$s^4U8$ residue within the acceptor stem is in close proximity to the TrmB target nucleoside G46 (Fig. 6.1A). Previous studies of tRNAs interacting with the ribosome have exploited the reactive nature of the $s^4U8$ thiol modification to introduce different fluorescent dyes attached to iodoacetamide into native tRNA isoacceptors purified from cells (32). In order to study the interaction of TrmB with its substrate tRNA lacking $m^7G46$ and other modifications, we instead *in vitro* transcribed tRNA and introduced the single $s^4U8$ modification using purified ThiI and IscS enzymes for fluorescent labeling with 5-iodoacetamidofluorescein (5-IAF) (Fig. 6.3A). Single-turnover methylation assays confirm that fluorescein-s4U-$tRNA^{Phe}$ can be rapidly methylated (Fig. A1.1A).

Using the fluorescein-$s^4U8$-$tRNA^{Phe}$, we monitored the binding of WT TrmB to tRNA in the millisecond-to-seconds range using a stopped-flow apparatus. In the presence of 50 μM SAM, mixing 15 μM TrmB with 1 μM fluorescein-s4U8-$tRNA^{Phe}$ resulted in a biphasic signal with an initial fluorescence decrease followed by a subsequent fluorescence increase (Fig. 6.3B). This curve was fit with a 2-exponential equation, yielding apparent rates of 180 $s^{-1}$ ($k_{app1}$) and 9 $s^{-1}$ ($k_{app2}$) (Fig. 4.3B). In contrast, mixing fluorescein-$s^4U8$-$tRNA^{Phe}$ with the active site variant TrmB D144A resulted in a single fluorescence decrease with an apparent rate of 77 $s^{-1}$ (Fig. 6.3C). As we will show below, this variant is deficient only in methylation but not tRNA binding. In the presence of SAM, WT TrmB forms product (28,33). Thus, the fluorescence decrease reflects an event related to tRNA binding prior to methylation. Conversely, the fluorescence increase that we observed only with the WT enzyme is likely to occur at a step after catalysis and may reflect release of the methylated tRNA.

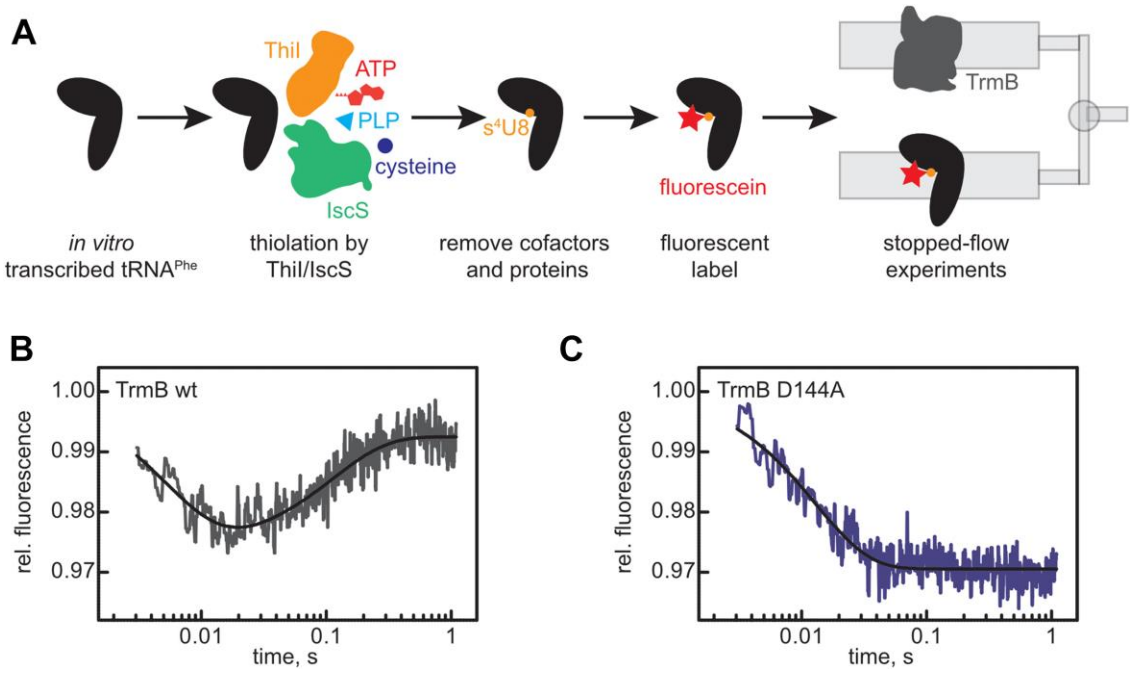


**Figure 6.3 Partial modification and fluorescent labeling of tRNA$^{Phe}$ for rapid kinetic stopped-flow experiments. A**, labeling scheme detailing the introduction of s$^4$U8 by ThiI and IscS, modified tRNA purification, and fluorescent labeling with fluorescein for use in rapid kinetic assays. **B**, rapid mixing of 15 μM WT TrmB and 50 μM SAM with 1 μM fluorescein-s$^4$U8-tRNA$^{Phe}$. The data was fit to a two-exponential equation to determine apparent rate constants: $k_{app1}$: 180 s$^{-1}$ and $k_{app2}$: 9 s$^{-1}$. **C**, rapid mixing of catalytically inactive 15 μM TrmB D144A and 50 μM SAM with 1 μM fluorescein-s$^4$U8-tRNA$^{Phe}$. Fitting the data with a one-exponential equation determined a $k_{app1}$ of 77 s$^{-1}$.

### *6.4.3 TrmB variant binding and methylating tRNA$^{Phe}$*

As previous work with *E. coli* TrmB variants has measured only overall tRNA methylation activity, but not tRNA binding, we investigated which TrmB residues are important for tRNA binding. To this end, we prepared two TrmB variants that were previously shown to be less than 10% as active in multiple-turnover methylation assays compared to WT TrmB (28) that we hypothesized would retain tRNA binding ability while being unable to methylate tRNA: TrmB

D144A and T217A (Fig. 6.1B). Here, we confirm that both residues are indeed vital for multiple-turnover methylation by TrmB (Fig. 6.4A). However, we find that at high TrmB D144A or TrmB T217A concentrations, methylation does slowly occur and reaches a similar final methylation end level as WT TrmB, with the apparent rate of methylation >150-fold slower for TrmB D144A and 35-fold slower for TrmB T217A than for WT TrmB (Fig. 6.4B and Table 6.1). Both residues are proposed to participate in methylation, wherein the side chain of the aspartate equivalent to D144 has been shown to form hydrogen bonds with N6 of the SAM adenosine moiety in *B. subtilis* TrmB (27). Residue T217 is present within the TrmB-specific insertion that is unstructured in *E. coli* TrmB (9). Within *B. subtilis* TrmB, this threonine residue is located at the bottom of the SAM-binding pocket, wherein its side-chain hydrogen bonds to the SAM methionyl moiety (22,27). However, this interaction is not observed within the *E. coli* TrmB–SAM complex (9) and thus T217 may only move in close enough proximity to interact with SAM upon structural rearrangement after tRNA binding. Alternatively, this residue may play a different role in *E. coli* TrmB.

**Table 6.1. Average apparent methylation rates for TrmB variants at 37°C.**

| | $k_{app}$, $min^{-1}$ |
|---|---|
| TrmB wildtype | 14 ± 2 * |
| TrmB D144A | 0.09 ± 0.04 |
| TrmB T217A | 0.4 ± 0.1 |
| TrmB R26A | 0.5 ± 0.1 |
| TrmB R155A | 0.7 ± 0.2 |

* apparent rate for TrmB wildtype at 37°C was previously determined using rapid kinetic quench flow (33).

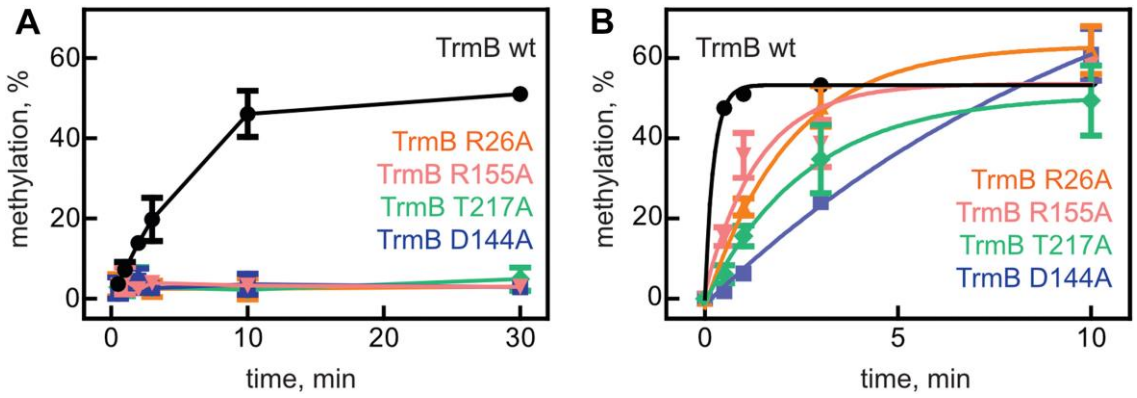


**Figure 6.4. Time courses for methylation of tRNA$^{Phe}$ by TrmB WT and variants. A**, methylation of 1 μM tRNA$^{Phe}$ under multiple turnover conditions using 50 nM TrmB variants and **(B)** methylation under single turnover conditions using 5 μM TrmB variants, both in the presence of 50 μM tritium-labeled SAM. Percent methylation was plotted against time. For the single-turnover methylation experiments, apparent rates of methylation for each TrmB variant were determined by fitting with a single exponential equation and rates are reported in Table 6.1. Note that methylation by TrmB WT shown here acts as visual comparison for variant TrmB methylation; the rate of TrmB WT methylation was previously determined using rapid kinetic quench flow (33).

**Table 6.2. Average dissociation constants ($K_D$) for TrmB wildtype and variants binding to [$^3$H]tRNA$^{Phe}$ in the presence and absence of 50 μM SAM.**

| | **+SAM, μM *** | **-SAM, μM** |
|---|---|---|
| TrmB wildtype | 2.1 ± 0.5 | 6.6 ± 1.9 |
| TrmB D144A | 0.8 ± 0.2 | 2.5 ± 0.7 |
| TrmB T217A | 2.6 ± 0.6 | 5.4 ± 1.4 |
| TrmB R26A | 5.1 ± 0.8 | 6.4 ± 2.5 |
| TrmB R155A | 5.8 ± 0.9 | 10 ± 2.8 |

* TrmB binding to tRNA in the presence of SAM for 10 min results in m$^7$G46 formation such that the affinity of TrmB to its product rather than its substrate is measured.

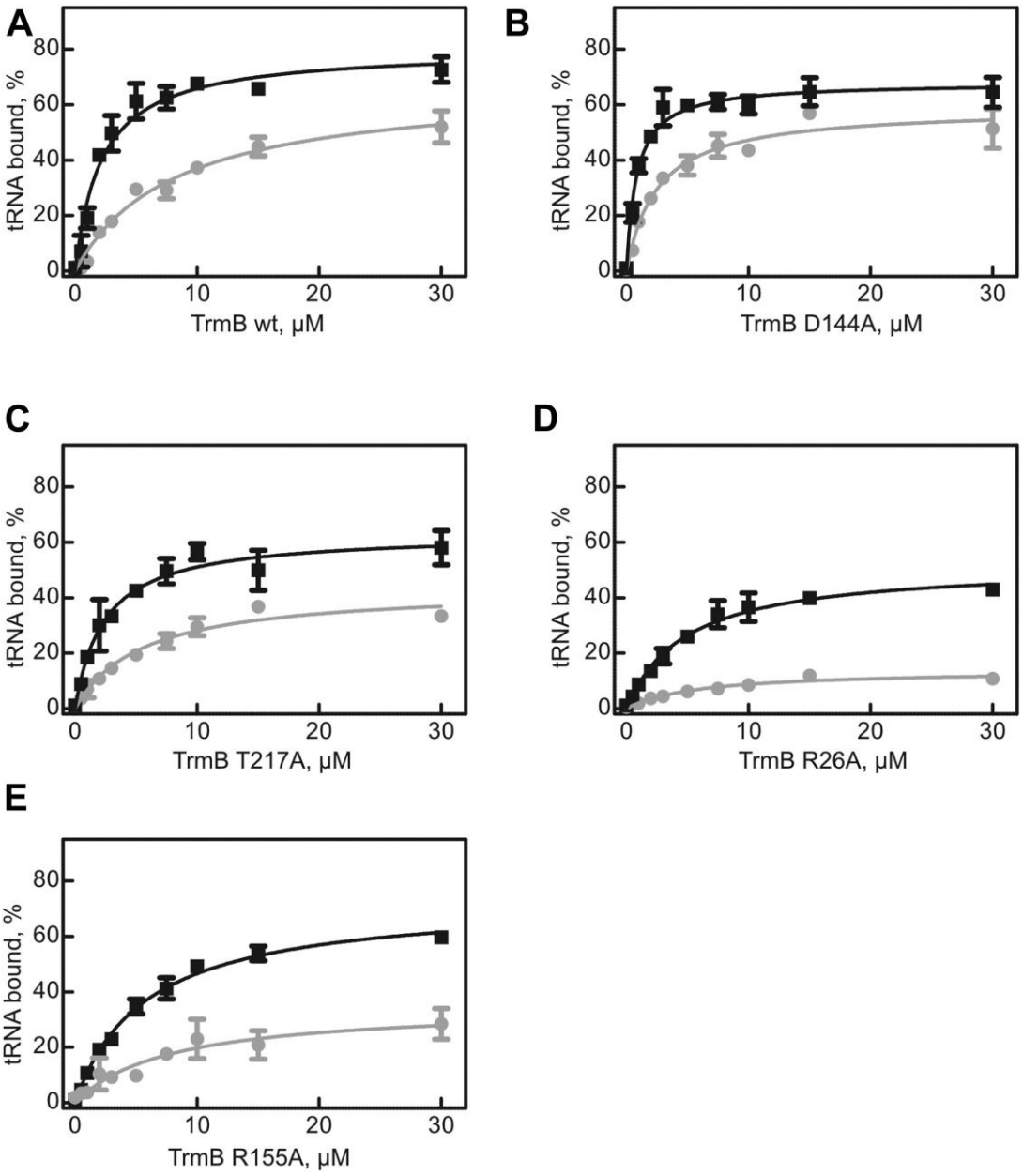


**Figure 6.5. tRNA binding by TrmB WT and variants.** To determine the affinity of TrmB in the presence (black squares) or absence (gray circles) of 50 μM SAM, 20 nM of [$^{3}$H]tRNA$^{Phe}$ was incubated for 10 min with increasing concentrations of TrmB WT **(A)**, TrmB D144A **(B)**, TrmB T217A **(C)**, TrmB R26A **(D)**, or TrmB R155A **(E)**. Percent tRNA bound was determined by nitrocellulose filtration. Averages of at least three experiments are shown with error bars representing SD. The data was fit with a hyperbolic equation to determine the dissociation constants (see Table 6.2).

To measure the affinity of each TrmB variant for $tRNA^{Phe}$, nitrocellulose filter binding using tritium-labeled tRNA was performed. Previously, we have shown that WT TrmB in the absence of SAM has a relatively low affinity for tRNA with a dissociation constant of 6.6 μM (Fig. 6.5A and Table 6.2) (33). When TrmB WT is preincubated with SAM, the affinity of TrmB for tRNA increases about 3-fold to 2.1 μM (Fig. 6.5A and Table 6.2). Thus, prior binding of SAM to TrmB has a positive allosteric effect on subsequent tRNA binding, as has previously been shown for TrmA (34).

Next, we examined the binding of TrmB D144A and T217A variants to $tRNA^{Phe}$. In both the absence and presence of SAM, TrmB D144A binds tRNA with a 2.5-fold higher affinity than the WT enzyme, with a $K_D$ of 2.5 μM and 0.8 μM, respectively (Fig. 6.5B and Table 6.2). Additionally, we observed that alanine substitution at residue T217 does not change the tRNA affinity in the presence and absence of SAM compared to TrmB WT (Fig. 6.5C and Table 6.2).

Moreover, we prepared two additional TrmB variants, that were previously shown to be less than 10% as active as WT TrmB in multiple turnover assays and we hypothesized that these TrmB variants would be deficient in tRNA binding: R26A and R155A (Fig. 6.1B). Residue R26 is present within the N-terminal extension of TrmB that is presumably flexible because this region was not resolved in the *E. coli* TrmB structure (9). As a positively charged residue, R26 may contribute to tRNA binding via electrostatic interactions. Another positively charged residue, R155, is one of two adjacent conserved arginine residues along with R154. Previous work showed that alanine substitution of either residue abolishes methylation activity under multiple-turnover conditions (28). Similarly, we find both variants are not active in multiple-turnover conditions (Fig. 6.4A), but slowly methylate tRNA in single-turnover conditions, each displaying an apparent rate that is ~30-fold slower than WT TrmB (Fig. 6.4B and Table 6.1).

In the absence of SAM, TrmB R26A has a similar, low affinity for tRNA as TrmB WT but in the presence of SAM its tRNA affinity is 2.5-fold lower (Fig. 6.5D and Table 6.2). Notably, the maximum binding amplitude for TrmB R26A in the absence of SAM is very low, with only around 20% tRNA binding, further suggesting that this TrmB variant is deficient in stable tRNA binding

(Fig. 6.5D). In the absence of SAM, TrmB R155A binds tRNA with a $K_D$ of 10 μM, which is lower than the affinity of TrmB WT for tRNA (Fig. 6.5E and Table 6.2). Similarly, in the presence of SAM, the affinity of TrmB R155A is 3-fold lower than the TrmB WT (Fig. 6.5E and Table 6.2). Like the R26A variant, the maximal level of tRNA bound by TrmB R155A is low, reaching only about 30% tRNA bound (Fig. 6.5E). Thus, substitution at residues R26 and R155 negatively affect tRNA binding.

*6.4.4 Rapid kinetic analysis of TrmB variants*

To monitor the interaction between TrmB variants and tRNA in real time, we repeated the stopped-flow assays using fluorescein-s$^4$U-tRNA$^{Phe}$ with TrmB variants. Importantly, for all TrmB variants, no significant product formation occurs even at high TrmB concentrations during the course of the stopped-flow experiments (1–100 s; Fig. 4.4B). Binding of the TrmB D144A variant to tRNA in the absence of SAM (Fig. 6.6A) displays a similar single-phase fluorescence decrease as seen when mixing this variant and fluorescein-s$^4$U8-tRNA$^{Phe}$ in the presence of SAM (Fig. 6.3B). Fitting the binding curve of TrmB D144A in the absence of SAM with an exponential equation yielded an apparent rate of about 60 $s^{-1}$ which is comparable to the apparent rate of 77 $s^{-1}$ observed in the presence of SAM.

The affinity of the TrmB T217A variant is similar to that of the WT enzyme but reduced compared to TrmB D144A (Fig. 6.5 and Table 6.2). Notably, the fluorescence change observed upon mixing TrmB T217A with fluorescein-s$^4$U8-tRNA$^{Phe}$ is different from TrmB WT and D144A. Here, we observe two subsequent fluorescence decreases, with apparent rates of 8 $s^{-1}$ ($k_{app1}$) and 0.2 $s^{-1}$ ($k_{app2}$) (Fig. 6.6B). Likewise, mixing TrmB R26A with fluorescein-s$^4$U8-tRNA$^{Phe}$ reveals two fluorescence decreases with a $k_{app1}$ of 2 $s^{-1}$ and $k_{app2}$ of 0.03 $s^{-1}$ (Fig. 6.6C). Finally, the binding of TrmB R155A with fluorescein-s$^4$U8-tRNA$^{Phe}$ in real time occurs in three phases with an initial fluorescence increase ($k_{app(increase)}$) with a rate of 176 $s^{-1}$, followed by two decreases in fluorescence with rates of 3 $s^{-1}$ ($k_{app1}$) and 0.03 $s^{-1}$ ($k_{app2}$) (Fig. 6.6D). Thus, our rapid kinetic assays reveal fast tRNA binding only in the case of the WT enzyme and the catalytically impaired

variant TrmB D144A. In accordance with the defects in tRNA binding for TrmB T217A, R26A, and R155A compared to the TrmB variant D144A observed in equilibrium binding conditions in the nitrocellulose-filtration assays (Fig. 6.5 and Table 6.2), the TrmB T217A, R26A, and R155A variants display vastly different tRNA-binding time courses, suggesting that substitutions to these residues significantly perturb how these variants bind tRNA. (Fig. 6.6 and Table A1.1).

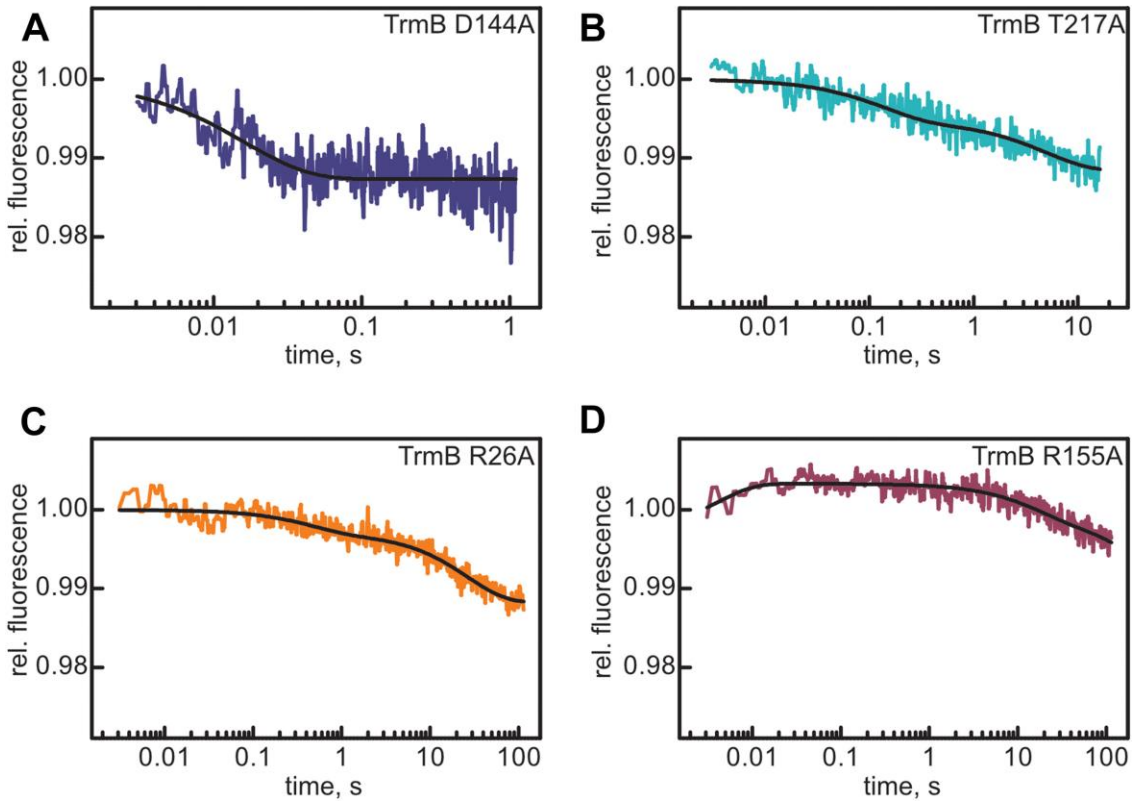


**Figure 6.6 Rapid kinetic analysis of TrmB variant binding to tRNA.** Time courses displaying rapid mixing of 15 μM TrmB variants with 1 μM fluorescein-s$^4$U8-tRNA$^{Phe}$ in a stopped-flow apparatus. Each trace is an average of at least eight independent time courses. Note the different x-axis range between panels. The time course for TrmB D144A **(A)** was fit to a one-exponential equation with a $k_{app1}$ of 62 ± 5 s$^{-1}$, whereas data for TrmB T217A **(B)** was fit to a two-exponential equation with a $k_{app1}$ of 8 ± 1 s$^{-1}$ and a $k_{app2}$ of ~0.2 s$^{-1}$. Time courses for TrmB R26A **(C)** were fit to a two-exponential equation, with a $k_{app1}$ of 2 ± 0.2 s$^{-1}$ and $k_{app2}$ of ~0.04 s$^{-1}$. Finally, data for TrmB R155A **(D)** was fit to a three-exponential equation, revealing a $k_{app1}$ of 176 ± 46 s$^{-1}$, $k_{app2}$ of 2 ± 1 s$^{-1}$, and a $k_{app3}$ of ~0.03 s$^{-1}$. Apparent rates are summarized in Table A1.1.

*6.4.5 Rapid kinetic analysis of TrmB binding and methylating tRNA$^{Phe}$*

In order to kinetically characterize the association of TrmB to tRNA$^{Phe}$ in the absence of methylation, we used the TrmB D144A variant that is highly deficient in catalysis but not tRNA binding (Figs. 4.5 and 4.6, (28)). Increasing concentrations of TrmB D144A were rapidly mixed with fluorescein-s$^4$U8-tRNA$^{Phe}$ and one-exponential equations were fit to the resulting curves. In the absence of the methyl donor SAM, the apparent rate is not dependent on enzyme concentration, with an average rate of 69 ± 5 $s^{-1}$ (Fig. 6.7A). This suggests that in the absence of SAM the observed apparent rate is indicative of a unimolecular conformational change, rather than a bimolecular binding event. In contrast, when TrmB D144A is prebound to SAM, the apparent rate of tRNA binding is concentration-dependent and therefore is indicative of a bimolecular interaction. Determination of the slope reveals an association rate constant, $k_1$, of 2.4 ± 0.5 $\mu M^{-1}$ $s^{-1}$ (Fig. 6.7B). As we will further discuss below, these results can be explained if a SAM-induced conformational change in TrmB is required for stable binding of tRNA.

To complement the kinetic analysis of tRNA binding to TrmB, we also kinetically characterized the methylation activity of TrmB WT using single-turnover quench-flow experiments and [$^3$H]SAM (Fig. S1). At 20 °C, the apparent rate for tRNA methylation is 0.04 $s^{-1}$ and this apparent rate is not concentration dependent (Fig. S1B). This observation suggests that methylation is not limited by tRNA binding.

## 6.5 Discussion

*6.5.1 Peroxide sensitivity of E. coli ΔtrmB*

In this study, we report for the first time a growth phenotype for an *E. coli ΔtrmB* strain, wherein *ΔtrmB* cells grow slowly in hydrogen peroxide-containing LB medium. This phenotype matches previous reports of phenotypes for *trmB* KOs in *P. aeruginosa* and *C. lagenarium* (18,19). During the course of our study, this phenotype was reported to be absent for the *E. coli* BW25113 *ΔtrmB* strain, even in the presence of 10 mM $H_2O_2$ (35). However, this phenotype is

highly reproducible in our experiments (Fig. 6.2) and even the BW25113 WT strain does not significantly grow in the presence of 10 mM $H_2O_2$ within the small volume of 96-well plates (data not shown). This lack of growth for the BW25113 strain at high $H_2O_2$ concentrations has recently been corroborated by others (36). Given the unstable nature of hydrogen peroxide rendering it difficult to use precise concentrations and the conserved nature of this phenotype across different organisms, we conclude that TrmB is important for fitness during oxidative stress.

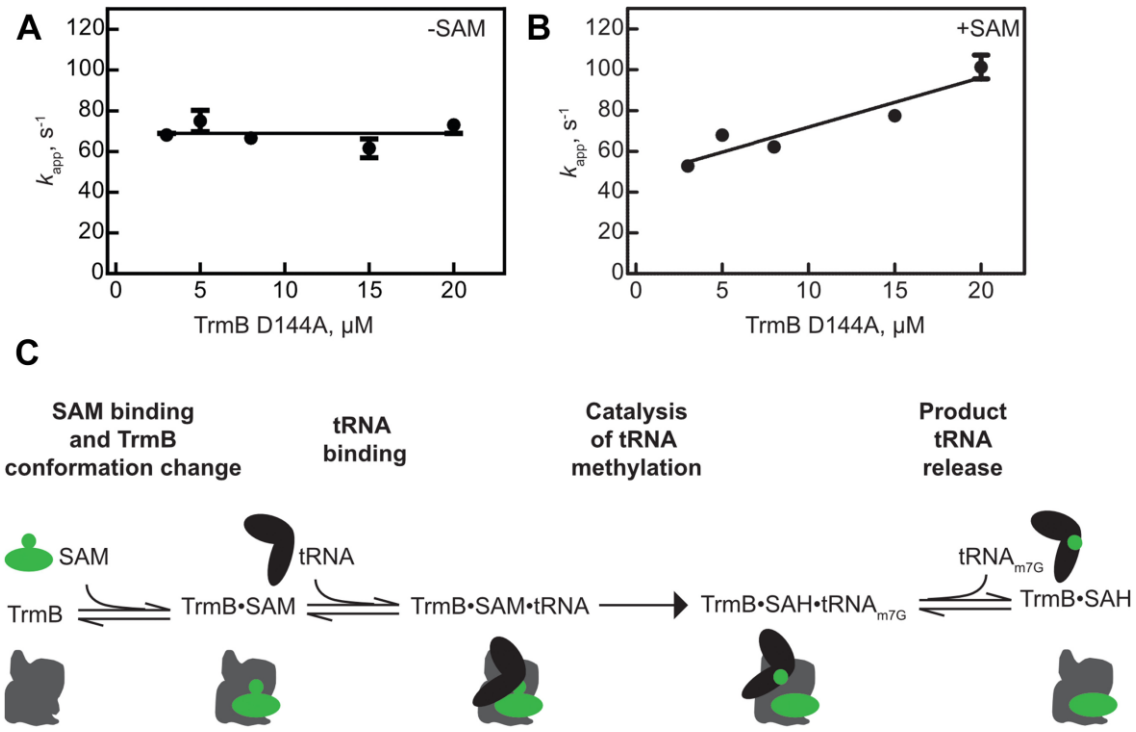


**Figure 6.7. Concentration dependence of tRNA binding by TrmB D144A.** Time courses of TrmB D144A binding to 1 μM fluorescein-$s^4$U8-$tRNA^{Phe}$ were fit to single exponential equations to determine the concentration dependence of apparent rates. In the absence of SAM **(A)**, the $k_{app}$ is not concentration-dependent and the horizontal line represents the average apparent rate of 69 ± 5 $s^{-1}$. In the presence of SAM **(B)**, the apparent rate of tRNA binding by TrmB is dependent on TrmB concentration. *Caption continues on next page.*

Thus, data was fit with a linear regression to determine the slope representing the association rate constant ($k_1$) of 2.4 ± 0.5 $\mu M^{-1}$ $s^{-1}$. (**C)**, Kinetic mechanism for tRNA methylation by TrmB. Herein, SAM binding to TrmB prompts a conformational change within TrmB, which facilitates rapid and stable tRNA binding by TrmB. tRNA methylation occurs slowly and is followed by the rapid and reversible release of product (methylated) tRNA.

It is notable that sensitivity to hydrogen peroxide stress is present in two different bacterial species, in addition to *C. lagenarium*, a eukaryote. This suggests the $m^7G46$ modification and/or TrmB plays a conserved role in mediating oxidative stress. Within *P. aeruginosa*, TrmB was found to mediate the peroxide stress response by optimizing translation of Phe and Asp codons, which are enriched within the *P. aeruginosa* catalase genes (18). Interestingly, *E. coli* catalase genes *katE* and *katG* similarly display an overabundance of Phe(UUC), Asp(GAC), and Asp(GAT) codons. The precise mechanism how TrmB enhances Phe/Asp translation remains unclear. It could be that the $m^7G46$ modification is directly important for tRNA aminoacylation or for the role of tRNA on the ribosome during translation or $m^7G46$ could act to increase the cellular stability of certain tRNAs, thereby increasing their cellular abundances and availability for translation. Considering the demonstrated importance of the $m^7G46$ modification by Trm8/Trm82 for preventing rapid decay of $tRNA^{Val}_{(AAC)}$ in yeast (17), in addition to the recent evidence that $m^7G46$ formation by METTL1/WDR4 in mammals is important for the stability of at least $tRNA^{Arg}_{(UCU)}$ (12) or $tRNA^{Lys}_{(CUU)}$ (37) and $tRNA^{Lys}_{(UUU)}$ (14), it is likely that methylation by TrmB is important for the cellular stability of certain tRNAs in bacterial cells. This possibility will be interesting to examine in the future. Moreover, given the specific importance of $m^7G46$ for only certain tRNAs across different organisms, despite the fact that TrmB modifies almost all tRNAs containing short variable loops, investigation of the mechanism underlying why TrmB is important for peroxide tolerance in *E. coli* and whether or not this is similar to the determined mechanism for *P. aeruginosa* is interesting to consider.

*6.5.2 In vitro partial modification and fluorescent tRNA labeling*

Fluorescent labeling of tRNAs at different specific internal sites by taking advantage of the reactivity of native tRNA modifications has been used to study the dynamics of tRNAs on the ribosome during protein translation (32,38). However, few studies have prepared *in vitro* transcribed tRNAs with only a select modification for internal fluorescent modification of a mostly unmodified tRNA (39,40). Using *in vitro* transcribed tRNA is advantageous for studying its interaction of tRNA-modifying enzymes because this allows for examination of the binding of a modification enzyme to its substrate (rather than product), unmodified tRNA, without the laborious process of extracting specific tRNA isoacceptors from an appropriate KO strain. Moreover, previous work has suggested that TrmB homologs tend to modify tRNA in the relatively early stages of tRNA maturation (41,42) and *E. coli* TrmB seems to not have a preference for tRNA modification status (33). This suggests that a mostly unmodified tRNA is an appropriate substrate for studying the interaction between TrmB and tRNA. As ThiI has few sequence constraints for modifying U8-containing tRNAs and instead recognizes the overall L-shaped structure (43), this strategy can be used to introduce $s^4U8$ within a wide selection of tRNAs. Thus, the preparation of fluorescently labeled, partially modified tRNA for kinetics studies described here lays the foundation for study of additional tRNA modification enzymes or other tRNA-interacting enzymes, demonstrating another use for *in vitro*-modified tRNAs (44).

*6.5.3 Molecular mechanism of E. coli TrmB interacting with tRNA*

As we have previously shown, in comparison to other tRNA modification enzymes, *E. coli* TrmB has a relatively low affinity for tRNA with a $K_D$ of about 6 μM in the absence of its cofactor SAM (Fig. 6.5 and Table 6.2) (33). Interestingly, *E. coli* TrmB binds tRNA with a lower affinity than its characterized homolog; homodimeric *B. subtilis* TrmB was shown to bind $tRNA^{Phe}$ in the absence of SAM with an affinity of about 0.1 μM using fluorescence anisotropy (27). This raises the possibility that TrmB enzymes with different quaternary structures may have distinct tRNA binding mechanisms. As such, the affinity may increase for homodimeric enzymes

compared to monomeric *E. coli* TrmB. No investigations concerning the affinity of the yeast Trm8/Trm82 heterodimer for tRNA have been conducted to date, and it is possible the heterodimeric complex again binds tRNA differently compared to monomeric bacterial TrmB. Supporting this possibility, differences in tRNA structural requirements have been identified between homodimeric *A. aeolicus* TrmB and heterodimeric yeast Trm8, wherein both enzymes require the T stem for modification activity but only yeast Trm8 additionally requires the D stem (45,46). Moreover, the R26 residue, that we demonstrate here to be important for tRNA binding (Table 6.2, Figs. 6.5 and 6.6), is not conserved in *B. subtilis*, *S. cerevisiae* or human TrmB homologs, and the N-terminal region present in mesophilic TrmB proteins is entirely absent in *A. aeolicus* TrmB. Notably, TrmB is one of several tRNA modification enzymes that utilizes only a single subunit in bacteria but requires an auxiliary protein for catalysis in eukaryotes (47,48). As such, a similar difference in tRNA affinity may exist for other bacterial tRNA modification enzymes and their eukaryotic two-subunit homologs.

Interestingly, we are demonstrating here that the affinity of TrmB WT and variants for tRNA is significantly increased in the presence of SAM (Table 6.2). Thus, prior binding of the cofactor SAM has a positive allosteric effect on the interaction of TrmB with tRNA, presumably by ordering and preorienting the active site. Previously, we have described a similar positive allostery for SAM and tRNA binding for the methyltransferase TrmA responsible for the $m^5U54$ modification in tRNAs (34). Alternatively, the presence of SAM may play a more direct role in tRNA binding by contributing interactions with the tRNA itself. Recent structures of the human TrmB homolog METTL1 in complex with its partner protein WDR4, tRNA and SAH support the hypothesis that prebinding of SAM/SAH by TrmB preorients the TrmB active site (49,50); however, as the quaternary structures of *E. coli* TrmB and human METTL1 are different, a ternary structure of TrmB in complex with SAM/SAH and tRNA would be required to fully explain the allosteric role of SAM in tRNA binding by TrmB.

In order to characterize the molecular mechanism of TrmB's interactions with tRNA, we report here a novel fluorescent assay enabling rapid kinetic studies of tRNA binding to TrmB (Fig.

4.3A). In the stopped-flow assay, we observe how fast TrmB binds to unmodified substrate tRNA giving us insight into the initial steps of TrmB's molecular mechanism. Thus, the stopped-flow assay provides different types of information (kinetics of substrate binding) about TrmB than the nitrocellulose-filtration assay (affinity of product binding). By comparing how tRNA interacts with WT TrmB compared to catalytically impaired TrmB D144A, we can gain insight into the kinetic mechanism of tRNA binding for this tRNA modifying enzyme. In these rapid kinetic stopped-flow experiments, we observe first a fluorescence decrease when either WT TrmB or TrmB D144A interact with fluorescein-s$^4$U8-tRNA in the presence of SAM. This fluorescence decrease upon tRNA binding may reflect partial unfolding of the tRNA structure as TrmB accesses its target base, G46, which is buried within the tRNA elbow forming a base pair triple with C13-G22. Notably, the apparent rate for the fluorescence decrease upon tRNA binding in the presence of SAM is 2-fold higher for WT TrmB than TrmB D144A (Fig. 6.3), suggesting that substitution of D144 for alanine slightly slows the association of TrmB with unmodified tRNA relative to WT TrmB. This is supported by the fact that tRNA methylation by TrmB D144A appears to be partially limited by tRNA binding (Fig. 6.4) as some methylation activity is observed in single-turnover methylation assays using 5 μM TrmB concentrations but not in multiple-turnover methylation assays using 50 nM TrmB.

Following the first fluorescence decrease in the rapid kinetic stopped-flow assays with WT TrmB in the presence of SAM discussed above, we then observe a second fluorescence increase with an apparent rate of ~9 $s^{-1}$. Presumably, this phase reflects the release of the modified tRNA, as this fluorescence change is absent when the catalytically impaired D144A variant is mixed with tRNA in the presence of SAM (Fig. 6.3C). We have previously reported the $k_{app}$ for tRNA methylation is 0.2 $s^{-1}$ at 37 °C (Table 6.1, (33)) and measured here that the apparent rate for tRNA methylation at 20 °C is 0.04 $s^{-1}$ and is not concentration-dependent (Fig. A1.1B), suggesting that methylation is not limited by tRNA binding. Thus, catalysis of m$^7$G46 formation may be the rate-limiting step in the molecular mechanism of TrmB which is followed by rapid product tRNA release (Fig. 4.7C). The apparent rate of the fluorescence increase observed with TrmB WT presumably reflects a combination of slow tRNA release and some product tRNA

rebinding as the affinity of TrmB WT for modified tRNA is 2.1 μM (Table 6.2). Notably, we have likewise reported that many tRNA pseudouridine synthases are characterized by a rate-limiting catalytic step (51,52).

To gain further insight into the mechanism of tRNA binding by TrmB, we performed a titration of TrmB D144A binding to tRNA in the stopped-flow experiments (Fig. 6.7). In the absence of SAM, we observed that the apparent rate, $k_{app}$, is not dependent on enzyme concentration, suggesting a unimolecular conformational change rather than a bimolecular binding event. In contrast, $k_{app}$ was found to be dependent on TrmB D144A concentration in the presence of SAM, which suggests that in this case we observe a bimolecular binding event. This different kinetic behavior in the presence and absence of SAM indicates that different mechanisms are at play (Fig. 6.7C). As prior SAM binding has a positive allosteric effect on tRNA binding (vide supra, Table 4.2), it is conceivable that SAM binding may be necessary to induce a conformational change in TrmB, which structurally prepares TrmB for stable tRNA binding. As this conformational change has already occurred when TrmB is preincubated in the presence of SAM, the initial fluorescence decrease in the stopped-flow experiments likely reflects the bimolecular interaction of TrmB and tRNA. However, in the absence of SAM, it is likely that tRNA binding is rate-limited by a preceding unimolecular conformational change in TrmB which is slow due to the absence of SAM. Thus, the observed apparent rate is not dependent on the TrmB concentration. Thereby, our rapid kinetic stopped-flow experiments provide further insight into the role of SAM for facilitating tRNA binding to TrmB. Building on the mechanism of TrmB described here, it will be interesting to analyze in the future whether and how TrmB influences tRNA folding as it likely disrupts critical tertiary interactions in the tRNA elbow region. Thus, TrmB has the potential to also act as a tRNA chaperone as previously reported *for E. coli* TruB and TrmA (34,52,53).

*6.5.4 Structural features of tRNA binding by TrmB*

In order to identify *E. coli* TrmB residues with roles in tRNA binding, we prepared four TrmB variants for characterization both by equilibrium filter-binding assays as well as our novel rapid kinetic stopped-flow assay. For the first time, this allows us to quantitatively investigate tRNA binding by TrmB independent of its catalytic activity allowing us to dissect the contribution of specific TrmB resides for these steps. Rapid kinetic stopped-flow experiments indicate that the D144A substitution may slightly impair the kinetics of tRNA association (Fig. 6.3). In comparison to TrmB D144A, the TrmB T217A variant has an approximately 3-fold lower affinity for tRNA as measured in equilibrium nitrocellulose-filtration binding experiments (Table 6.2). Interestingly, the rapid kinetic stopped-flow experiments provide further detailed insight into the mechanism of tRNA binding by TrmB variants. Here, we observed that tRNA associates to TrmB T217A much slower than to TrmB D144A (Fig. 6.6). Together, the equilibrium and rapid kinetic experiments thus demonstrate unambiguously that TrmB T217A is not only impaired in $m^7G46$ formation as previously reported but that the substitution of T217 with alanine also impairs tRNA binding to TrmB. Similarly, even stronger defects with respect to the affinity and kinetics of tRNA binding were observed for TrmB R26A and TrmB R155A (Table 6.2 and Fig. 6.5). For both variants, the tRNA affinity in the presence of SAM is at least 5-fold decreased compared to TrmB D144A and the apparent association rate is reduced from approximately 60 $s^{-1}$ to 2 $s^{-1}$. As tRNA binding is severely compromised for the TrmB R26A and R155A variants, additional steps in tRNA association are observed in the rapid kinetic stopped-flow experiments which may reflect conformational events that are typically hidden. In summary, residues R26, T217, and R155 are critically involved in tRNA binding to TrmB. Interestingly, these residues map to three different areas on the surface of TrmB (Fig. 4.1B). As TrmB is a relatively small enzyme of only 27 kDa, our results suggest that TrmB utilizes its extended surface to interact with tRNA. Such a relatively large interaction site between a modification enzyme and tRNA is not uncommon and was also reported for the human pseudouridine synthase PUS7 (54). Further structural studies will be needed to uncover the precise orientation of tRNA relative to *E. coli* TrmB.

Most recently, two publications reported structures of the human TrmB homolog, METTL1 in complex with its partner protein, WDR4, and tRNA, in the presence and absence of SAM or SAH obtained by cryo-EM (49,50). The biochemical results described here for *E. coli* TrmB corroborate several similar features and highlight important differences for tRNA binding by monomeric bacterial TrmB and its heterodimeric human counterpart METTL1. First, for both TrmB and METTL1, the entire positively charged surface of the relatively small methyltransferase is utilized for tRNA binding. However, the METTL1/WDR4 quaternary structures demonstrate for the first time that WDR4 also makes direct contacts with tRNA, thereby increasing the affinity of METTL1 for tRNA (49,50). These interactions obviously cannot take place for monomeric TrmB and may provide an explanation why the affinity of monomeric TrmB for tRNA is comparatively weak relative to that of the METTL1-WDR4 heterodimer with affinities of ~6 μM and ~90 nM respectively (Fig. 6.5A and Table 6.2, (50)). Secondly, comparison of the METTL1·WDR4·tRNA structure to that of the METTL1·WDR4·tRNA·SAH structure demonstrates that although METTL1·WDR4 can form a specific complex with tRNA, several local METTL1 conformational changes take place upon cofactor (SAH) binding to facilitate stable METTL1 binding and tRNA methylation. Particularly, in the presence of cofactor, the METTL1 N terminus becomes ordered, sandwiching itself between the tRNA and cofactor. This supports the accession of the buried G46 nucleobase by METTL1 and facilitates the overall METTL1 movement nearer to the tRNA and protrusion of the catalytic loop toward that target base (50). Although the details of methyltransferase conformation change may be distinct between the heterodimeric human methyltransferase and monomeric *E. coli* TrmB, structural rearrangements upon cofactor binding for the METTL1·WDR4·tRNA provides precedent for the suggested local methyltransferase conformational changes upon SAM binding to TrmB facilitating stable tRNA binding, consistent with our equilibrium binding experiments with WT TrmB (Fig. 6.5 and Table 6.2) and our rapid kinetic analyses of inactive TrmB (Fig. 6.7). Finally, the new METTL1·WDR4·tRNA complexes reveal cofactor and tRNA binding are necessary for organization of the flexible METTL1 N terminus which coordinates cofactor binding with structural changes within METTL1, WDR4, and the tRNA (49,50). Here, we show alanine substitution within the TrmB N terminus at R26 impairs

tRNA binding (Figs. 6.5D and 6.6C; Table 6.2), suggesting an important role for this residue for the interaction between TrmB and tRNA. Although the R26 residue is not conserved in human METTL1 and is generally a glutamine (flanked by an arginine on each side) within eukaryotic TrmB homologs, our findings suggest a conserved role for arginine residues in the flexible N terminus among TrmB/METTL1 homologs.

### 6.6 Conclusion

Taken together, our results show that TrmB is a biologically relevant enzyme that confers a fitness advantage to *E. coli* under oxidative stress. As previous reports suggest that this effect may be tRNA-specific, it is crucial to understand the interaction of TrmB with tRNA. As a first critical step in this direction, we established a new rapid kinetics assay complementing standard equilibrium binding assays such as nitrocellulose filtration. Thereby, we have dissected the molecular mechanism of TrmB. Our data suggest that binding of SAM to TrmB induces a conformational change that is required for fast and tight tRNA binding, which is corroborated by two recent METTL1·tRNA complex structures (49,50), suggesting the potential for a common mechanism for homologous N7-methylguanosine methyltransferases with distinct quaternary structures. Moreover, we show three residues located at different areas on the TrmB surface (R26, T217, and R155) are critically contributing to the stable and fast binding of tRNA to this enzyme. Following tRNA binding, the formation of $m^7G46$ is the rate-limiting step which is followed by rapid release of the modified tRNA (Fig. 4.7C). This insight into the molecular mechanism of TrmB thus lays the ground for future studies to address the role of TrmB-mediated modification of different tRNAs and for structural studies of the TrmB–tRNA complex.

## 6.7 Experimental Procedures

### *6.7.1 Buffers and reagents*

Experiments were performed in TAKEM$_4$ buffer (50 mM Tris–HCl pH 7.5, 70 mM $NH_4Cl$, 30 mM KCl, 1 mM EDTA, 4 mM $MgCl_2$). [C5-$^3$H]UTP for *in vitro* transcription of [$^3$H]tRNA$^{Phe}$ was purchased from Moravek Biochemicals. 5-IAF was from Sigma-Aldrich. Nonradioactive SAM was purchased from New England Biolabs and [methyl-$^3$H]SAM was purchased from PerkinElmer. All other chemicals were purchased from Thermo Fisher Scientific.

### *6.7.2 Hydrogen peroxide growth comparison for ΔtrmB and its parental strain*

The identity of the *ΔtrmB* strain from the Keio collection was validated by colony PCRs targeting the region upstream (5′-GCTGCAACTTCCTCAAAGG-3′) and downstream (5′-CGTCACTGAAAGTGCTGCC-3′) of the *trmB* locus and the kanamycin cassette (k1/k2) (55). For growth analysis, four biological replicates for each the *ΔtrmB* strain and its parental strain, BW25113, were grown overnight in 3 to 5 ml LB in the presence of 50 μg/ml kanamycin (*ΔtrmB*) or in the absence of antibiotic (WT). Cells were resuspended in fresh LB medium lacking antibiotic and diluted to an $A_{600}$ of 0.1 in 150 μl LB containing 5 mM $H_2O_2$. Cultures were incubated at 37 °C for 48 h with continuous shaking and the absorbance was measured every 15 min at 600 nm in an Eon BioTek 96-well plate reader. The average $A_{600}$ reading of at least three biological replicates for each strain was plotted versus time with error bars representing the SEM.

### *6.7.3 Site-directed mutagenesis to prepare TrmB variants*

Expression plasmids for TrmB variant expression were prepared from the pET28a-TrmB plasmid (33) using Quikchange site-directed mutagenesis with the following overlapping primers:

TrmB D144A: 5′-TTTTCCCTGCCCCGTGGCACAAAGCGC-3′ and

5′- GCCACGGGGCAGGGAAAAAGAGCTGC-3′.

TrmB T217A: 5′- CCGGTGGCGAAATTTGAACAACGTGG-3′ and

5′-CAAATTTCGCCACCGGACGTGATGCC-3′.

TrmB R26A: 5′- TTTGTGCGCGCCCAGGGGCGACTGAC-3′ and

5′-CCCCTGGCGGCGCACAAAACTACGG-3′.

TrmB R155A: 5′- AATAAACGCGCTATCGTTCAGGTGCCG-3′ and

5′- GAACGATAGCGCGTTTATTATGGCGCGC-3′.

The sequences of pET28a-TrmB D144A, pET28a-TrmB R26A, pET28a-TrmB R155A, pET28a-TrmB T217A were confirmed by Sanger sequencing (Genewiz).

*6.7.4 Protein expression and purification*

Plasmids encoding WT or variant TrmB were transformed into BL21 (DE3) cells. Similarly, plasmids pBH113 and pBH402 for ThiI and IscS overexpression were transformed into BL21 (DE3) for overexpression as described in (33). In brief, for overexpression, cells were grown at 37 °C in LB broth supplemented with 50 μg/ml kanamycin or 100 μg/ml ampicillin. Protein overexpression was induced at an $A_{600}$ of approximately 0.6 with 1 mM IPTG. After 3 h, cells were collected by centrifugation at 5000*g* for 15 min, flash frozen, and stored at −80 °C.

Proteins were purified via their amino-terminal hexahistidine tag using nickel-sepharose followed by Superdex 75 (XK 26/100) chromatography as described elsewhere (51). TrmB protein concentrations were determined by absorbance at 280 nm using an extinction coefficient of 27,960 $M^{-1}$ $cm^{-1}$ (calculated using ProtParam (56)). Similarly, ThiI and IscS concentrations were determined by $A_{280}$ using experimentally determined extinction coefficients 63,100 and 25,400 $M^{-1}$ $cm^{-1}$, respectively (57). Protein concentrations were validated using comparative SDS-PAGE and Bradford assays.

### *6.7.5 tRNA$^{Phe}$ preparation*

*E. coli* tRNA$^{Phe}$ was prepared as described elsewhere (51) by amplification from the pCF0 plasmid (58) followed by in vitro transcription. The resulting tRNA$^{Phe}$ was purified by phenol extraction and Superdex 75 (XK 26/100) chromatography. For uniformly tritium-labeled tRNA$^{Phe}$, [5-$^{3}$H]UTP was included in the reaction and [5-$^{3}$H]tRNA$^{Phe}$ was purified by Nucleobond Xtra Midi anion exchange gravity columns (Machery-Nagel) (51).

### *6.7.6 tRNA$^{Phe}$ modification and fluorescent labeling*

In order to introduce a specific fluorescent label at position 8 of the tRNA structure, purified ThiI and IscS enzymes were used to prepare s$^{4}$U8-tRNA$^{Phe}$. For this reaction, tRNA$^{Phe}$ was first folded in TAKEM$_4$ buffer by heating to 65 °C for 5 min and cooling to room temperature for at least 10 min. Folded tRNA$^{Phe}$ at a final concentration of 5 µM was incubated with 3 µM IscS and 3 µM ThiI in the presence of 40 µM pyridoxal 5'-phosphate, 4 mM ATP, 500 µM L-cysteine, 0.07 U/µl RiboLock RNase inhibitor (Thermo Fisher Scientific), and 1 mM dithiothreitol in TAKEM$_4$ buffer for at least 2 h at 37 °C in a 20 ml total volume. The reaction was stopped by enzyme denaturation via heating at 80 °C for 15 min and enzymes were then removed by phenol/chloroform extraction. Following isopropanol precipitation to reduce the volume, reaction cofactors were removed by Superdex 75 (10/300 GL) chromatography. tRNA thiolation was validated to be at least 80% by ImageJ analysis of 5 to 100 pmol of tRNA on a 20 µM [(N-acryloylamino)phenyl]mercuric chloride, 7 M urea, 10% polyacrylamide gel (59).

Fluorescent labeling of the s$^{4}$U8 residue was achieved by incubating 60 µM s4U8-tRNA$^{Phe}$ with 3.2 mM 5-IAF in 12 mM HEPES-KOH pH 8.2 containing 80% (v/v) dimethyl sulfoxide at 65 °C in the dark for 4 h, similarly to the procedure in (38). To remove unincorporated dye, at least eight successive phenol extractions were performed until the organic layer was no longer yellow. To remove phenol, two chloroform extractions were performed followed by ethanol precipitation. The final fluorescein-s$^{4}$U8-tRNA$^{Phe}$ was resuspended in water and the tRNA

concentration was determined spectrophotometrically at 260 nm. The labeling efficiency was determined by absorbance at 492 nm and was typically ~2 to 15%.

### *6.7.7 Stopped-flow to monitor TrmB–tRNA$^{Phe}$ binding*

Following tRNA folding, fluorescein-s$^4$U8-tRNA$^{Phe}$ (final concentration: 1 μM) was rapidly mixed with TrmB at a final concentration between 3 to 20 μM in TAKEM$_4$ buffer in a KinTek SF-2004 stopped-flow apparatus at 20 °C. Fluorescein was excited at 480 nm and emission monitored from 505 nm onward. Relative fluorescence ($Y$) was plotted against time ($t$) and traces were fit to a one-, two-, or three-exponential function to determine apparent rates ($k_{app}$) using TableCurve 2D:

$$Y = Y_\infty + Amp \times \exp(-k_{app} \times t)$$

$$Y = Y_\infty + Amp_1 \times \exp\left(-k_{app1} \times t\right) + Amp_2 \times \exp(-k_{app2} \times t)$$

$$Y = Y_\infty + Amp_1 \times \exp\left(-k_{app1} \times t\right) + Amp_2 \times \exp\left(-k_{app2} \times t\right) + Amp_3 \times \exp(-k_{app3} \times t)$$

Data shown are averages of at least eight independent time courses.

For TrmB D144A, apparent rates were plotted against enzyme concentration and fit with a linear equation to determine the association rate constant $k_1$:

$$k_{app} = k_1 \times [enzyme] + k_{-1}$$

### *6.7.8 Nitrocellulose filtration to determine affinity of TrmB for tRNA*

Prior to the reaction, [$^3$H]tRNA$^{Phe}$ was refolded in TAKEM$_4$ buffer by heating and cooling as described above. Refolded [$^3$H]tRNA$^{Phe}$ (40 nM) was incubated with increasing concentrations of TrmB WT or variant enzyme (0–30 μM) in TAKEM$_4$ buffer in the presence or absence of 50 μM SAM for 10 min at room temperature.

As described in (49), the enzyme–tRNA mixture was filtered through a nitrocellulose membrane under vacuum and the proportion of bound tRNA was determined by scintillation counting. To determine the dissociation constant ($K_D$), percent tRNA bound (Bound) was plotted as a function of enzyme concentration ([enzyme]) and fit with a hyperbolic equation using GraphPad Prism software (https://www.graphpad.com/):

$$Bound = Bound_{max} \times [enzyme]/(K_D + [enzyme])$$

*6.7.9 Methylation assay to monitor tRNA modification by TrmB*

Refolded $tRNA^{Phe}$ (1 μM) was incubated with TrmB (5 μM for single-turnover assays or 50 nM for multiple-turnover experiments) in the presence of 50 μM [$^3$H]SAM (PerkinElmer; diluted with nonradioactive SAM to achieve a final specific activity of 800 dpm/pmol) in $TAKEM_4$ buffer at the temperature indicated (37 °C for Fig. 4 and 20 °C for Fig. S1). Reaction samples (190 μl for reactions mixed in a KinTek Quench Flow or 20 μl for reactions not performed in quench flow) were quenched at indicated times by spotting onto Whatman filter papers presoaked with 5% (w/v) trichloroacetic acid. After letting filters dry for at least 10 min, filters were washed three times with 5% (w/v) trichloroacetic acid followed by a final wash in anhydrous ethanol. After drying for ~30 min at 60 °C, the radioactivity remaining on filters was quantified by scintillation counting in 4 ml EcoLite (+) scintillation cocktail (MP Biomedicals). Percent methylation or raw decays per minute were plotted against time. Single-turnover methylation time courses were fit with a one-exponential equation to determine the apparent rate, $k_{app}$:

$$Y = Y_\infty + Amp \times \exp(-k_{app} \times t)$$

### 6.8 Acknowledgements

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# Chapter 7:
# Modifications in the T arm of tRNA globally determine tRNA maturation, function and cellular fitness

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## 7.1 Preface

This chapter is a manuscript intended for submission that has been re-formatted in accordance with the University of Lethbridge thesis requirements. Dr. Ute Kothe and I together conceptualized this project and together wrote the manuscript, with input from Dr. Richard Fahlman at the University of Alberta and Dr. Tao Pan at the University of Chicago. MSR-seq experiments were performed and analyzed by Dr. Christopher Katanski, Mateusz Halucha, Noah Pena, and Dr. Tao Pan with total RNA samples I had prepared, and mass spectrometry experiments were conducted and analyzed by Dr. Richard Fahlman from cellular extract I prepared. All other experiments were conducted by myself, and analyzed by myself and Dr. Ute Kothe. Dr. Ute Kothe, Dr. Tao Pan, and Dr. Richard Fahlman were responsible for supervision and funding acquisition.

## 7.2 Abstract

All elongator tRNAs harbor 5-methyluridine 54 and pseudouridine 55 in the T arm, which are generated by the enzymes TrmA and TruB, respectively. *Escherichia coli* TrmA and TruB both act as tRNA chaperones, and strains lacking *trmA* or *truB* are outcompeted by wildtype. Here, we investigate how TrmA and TruB contribute to cellular fitness. Deletion of *trmA* and *truB* in *E. coli* causes a global decrease in aminoacylation and alters other tRNA modification such as $acp^3U47$. Whereas overall protein synthesis is not affected in *ΔtrmA* and *ΔtruB* strains, the translation of a specific subset of codons is significantly impaired, and the expression of many specific proteins is translationally changed. In conclusion, we demonstrate that universal modifications of the tRNA T arm are critical for global tRNA function by enhancing tRNA maturation, tRNA aminoacylation, and translation, thereby improving cellular fitness and explaining *trmA* and *truB* conservation.

### 7.3 Introduction

Transfer RNAs (tRNAs) are the most densely and most diversely modified class of RNA, and all organisms invest significant energy into synthesizing large numbers of tRNA modifying enzymes (1). In humans, defects in tRNA modification enzymes are frequently associated with diseases including cancer, neurological disorders, and mitochondrial diseases (2). Within tRNAs, two general clusters of modifications are apparent. Modifications in the anticodon stem-loop generally play direct and essential roles during translation; however, functional roles for modifications within the tRNA elbow oftentimes remain elusive (3,4). Two of the most common and conserved tRNA modifications are 5-methyluridine ($m^5U$) 54 and pseudouridine (Ψ) 55 (Figure 7.1). These modifications are found at adjacent positions within the T arm of nearly every elongator tRNA throughout all domains of life. In *Escherichia coli*, $m^5U54$ is formed by the methyltransferase TrmA (5), whereas Ψ55 is generated by the pseudouridine synthase TruB (6,7). Like their modifications, the TrmA and TruB enzymes are highly conserved throughout all domains of life, although some archaea and diverse bacteria utilize alternative enzymatic strategies to introduce $m^5U54$ and Ψ55 (4).

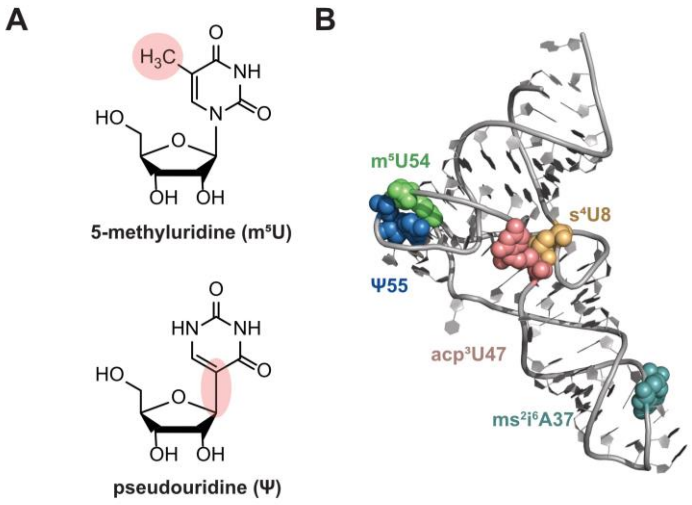



**Figure 7.1. Structures and location of $m^5U54$ and Ψ55. (A)** Chemical structures of $m^5U$ (top) and Ψ (bottom) nucleosides. The region of the modification is highlighted in red. *Caption continued on next page.*

**(B)** Location of $m^5U54$ (green spheres), Ψ55 (blue spheres), $s^4U8$ (yellow spheres), $acp^3U47$ (pink spheres), and $ms^2i^6A37$ (teal spheres) within the crystal structure of unmodified *E. coli* $tRNA^{Phe}$ (PDB 3L0U) (8).

Highlighting the importance of TrmA and TruB and their respective modifications, $m^5U54$ and Ψ55 are the only two modifications found within every *E. coli* tRNA (9). In this model organism, the *trmA* and *truB* genes are non-essential under ideal conditions but are important for bacterial fitness in co-culture experiments (10-13). In addition to modifying tRNAs, TrmA and TruB act as tRNA chaperones, folding tRNA independently of their modification activity (11,12,14). The tRNA chaperone activity of TruB is responsible for optimizing fitness in *E. coli* (10,11), whereas TruB pseudouridylation is important for growth at cold temperatures in *Thermus thermophilus* (15), heat shock recovery in *E. coli* (16), and *Shigella flexneri* virulence (17). Both the chaperone activity and modification activities of TrmA play roles in *E. coli* fitness (11), but the methylation activity seems dispensable in yeast (13,18). Finally, the $m^5U54$ and Ψ55 modifications themselves stabilize tRNA (3). Taken together, the conserved enzymes TrmA and TruB are bifunctional enzymes, playing two distinct roles during tRNA maturation by folding tRNA and introducing tRNA-stabilizing modifications. Supporting the importance for TrmA and TruB homologs in humans, the TrmA homolog TRMT2A is a potential biomarker for the recurrence of certain cancers and is a potential target for polyQ diseases (19,20). Moreover, the TruB homologs, TRUB1/2, are linked to maternally inherited diabetes through a mitochondrial $tRNA^{Glu}$ mutation that prevents tRNA pseudouridylation (21,22). Contrasting the conservation of TrmA and TruB enzymes, their implications in human diseases, and abundance of $m^5U54$ and Ψ55 modifications, only a handful of subtle growth phenotypes have been identified for model organisms lacking these enzymes (16,17).

Here we address the paradox of almost all cells investing energy into modifying and folding the majority of tRNAs by TrmA and TruB, although these enzymes seem to be unimportant for growth during ideal and various stress conditions. We examined which functional

tRNA processes are affected in the absence of *trmA* and *truB* genes by measuring tRNA abundance, tRNA modification, tRNA aminoacylation, as well as codon-specific and global protein translation within *E. coli ΔtrmA* and *ΔtruB* knockout strains. Thereby, this work highlights that absence of TrmA or TruB results in several defects during tRNA maturation, aminoacylation and protein translation, explaining why cells lacking TrmA or TruB exhibit reduced fitness.

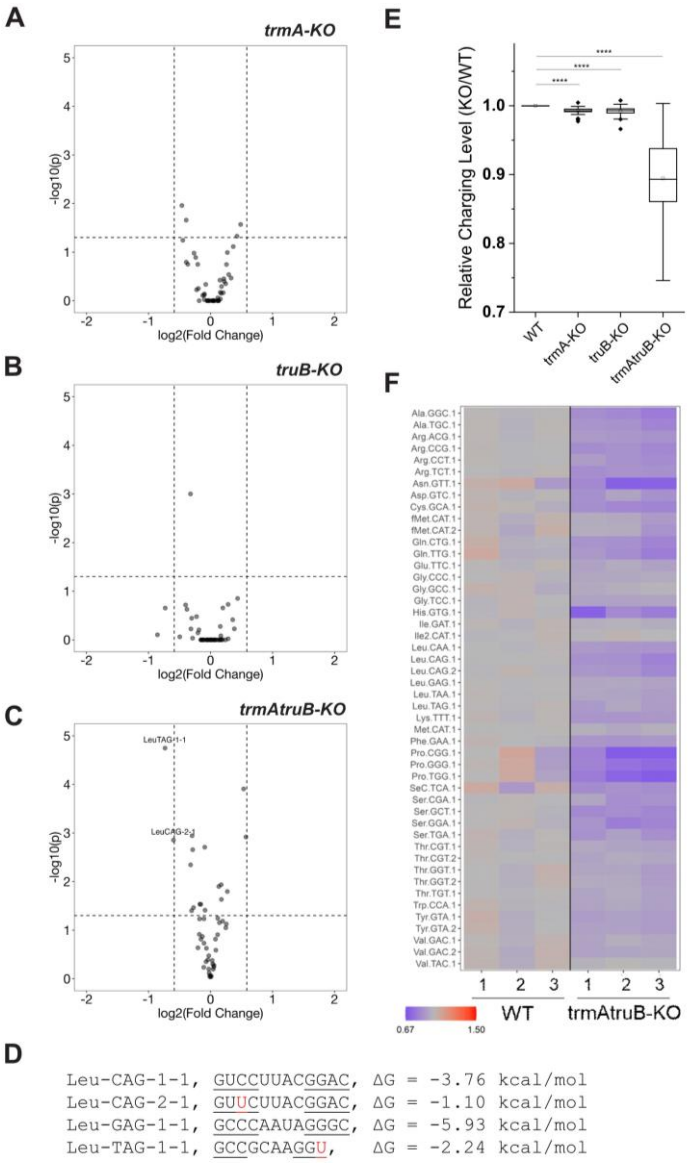
## 7.4 Results

### *7.4.1 tRNA T arm modification globally enhances aminoacylation*

To dissect the roles of TruB and TrmA for tRNA function and protein synthesis, we used *E. coli ΔtruB* and *ΔtrmA* strains from the Keio Collection (23) in addition to preparing a double-knockout strain lacking both *truB* and *trmA* genes. Previously, TrmA and TruB chaperone activity has been assessed *in vitro* by detecting tRNA aminoacylation (charging) because only correctly folded tRNA is efficiently charged (11,12,24). Therefore, we hypothesized that the deletion of *trmA* and/or *truB* increases the fraction of misfolded tRNA and in turn decreases the aminoacylation levels of some or all tRNAs *in vivo*. To quantitatively compare both the abundance and the aminoacylation levels for all tRNAs, we used multiplex small RNA sequencing (MSR-seq) which simultaneously measures tRNA abundance, modification, and charging (25). Herein, the 3′ ribose of uncharged tRNA is oxidized leading to β-elimination of the 3′ adenine of the tRNA whereas the terminal 3′ adenine is protected in aminoacylated tRNA. After Illumina sequencing, comparison of sequences ending in 3′ CC (deacylated) to 3′ CCA (aminoacylated) allows for quantification of the aminoacylation level for individual tRNA isoacceptors while also providing information about relative tRNA abundance and modification content (25,26).

First, we asked whether TrmA and/or TruB and their resulting modifications $m^5U54$ and Ψ55 promote the cellular stability and thereby steady-state abundance of tRNAs *in vivo* as observed for other tRNA modifying enzymes (1,27,28). Within the single *ΔtrmA* and *ΔtruB*

strains, no tRNA is decreased or increased more than 1.5-fold upon modification enzyme knockout (Fig. 7.2A, Fig. 7.2B, Fig. A2.1). In the double-knockout strain, only two tRNAs, $tRNA^{Leu}_{TAG}$ and $tRNA^{Leu}_{CAG-2-1}$ are downregulated by ~1.5-fold compared to the wildtype strain (Fig. 7.2C). The decreased abundances for these two tRNAs may be associated with lower stability of their stem-loop in the variable region compared to closely related $tRNA^{Leu}_{GAG}$ and $tRNA^{Leu}_{CAG-1-1}$ (Fig. 7.2D). These results indicate that the lack of TrmA and TruB has only minimal effects on the overall cellular stability of tRNAs in *E. coli*.




*Caption on next page.*

**Figure 7.2. tRNA aminoacylation is reduced globally in *trmA* and *truB* knockout strains.** tRNA abundance change in single and double knockout strains depicted as volcano plots. Vertical dashed lines indicate threshold change of 1.5-fold, horizontal dashed lines indicate $p$ = 0.05. **(A)** *ΔtrmA*; **(B)** *ΔtruB*; **(C)** *ΔtrmAΔtruB*. **(D)** Variable region stem-loop sequences and free energy calculations of $tRNA^{Leu}$(xAG). U of G-U pair is shown in red. **(E)** Charging level change of all tRNAs in wild-type, *ΔtrmA*; *ΔtruB*; and *ΔtrmAΔtruB* strains. Box and whisker plot, **** $p < 10^{-4}$. **(F)** Heatmap of charging level change for each tRNA in *ΔtrmAΔtruB* over *E. coli* BW25113 wildtype (WT) showing each biological replicate.

Next, we examined changes in aminoacylation upon *trmA* or *truB* knockout. Comparison of aminoacylation levels revealed a significant global decrease in tRNA charging in the *ΔtruB* and *ΔtrmA* strains, with more pronounced effects in the double *ΔtrmAΔtruB* strain (Fig. 7.2E-F). In the double knockout strain, tRNA aminoacylation is on average 10% lower than in the wildtype strain, suggesting additive effects of removing both TrmA and TruB (Fig. 7.2E). Almost all individual tRNAs are less charged, but changes in charging level are variable (Fig. 7.2F). This data supports our hypothesis that TrmA and TruB affect tRNA charging of almost all tRNAs *in vivo*, and the effect is much more pronounced upon losing both modifications.

### *7.4.2 $m^5U54$ and Ψ55 influence the modification of several other tRNA nucleotides*

Since certain tRNA modifications, such as 3-(3-amino-3-carboxypropyl)uridine at position 47 in the tRNA variable loop ($acp^3U47$, Fig. 7.3A), cause mutations or deletions during reverse transcription when a thermostable reverse transcriptase (Superscript IV) is used for MSR-seq, the tRNA sequencing dataset also enables detecting changes in mutation and/or deletion frequencies at specific nucleotides that reflect tRNA modifications (25). Comparing the *ΔtrmA* strain to wild-type *E. coli*, we observe reduced mutation and deletion frequencies at position 47 within the majority of tRNAs that carry the $acp^3U47$ modification, suggesting that the $acp^3U47$

modification is less abundant in the absence of TrmA/$m^5$U54 (Fig. 7.3B, Fig. 7.3C). Similarly, further reduced mutation and deletion frequencies at U47 are observed for the *ΔtrmAΔtruB* strain; however, single knockout of *truB* does not affect $acp^3$U47 content in most tRNAs with the exception of $tRNA^{Phe}$ (Fig 7.3C). To validate the $acp^3$U47 content in the absence of TrmA and TruB, we conducted primer extension analysis using a reverse transcriptase with low processivity (AMV RT) for tRNA extracted from *E. coli* observing the reaction stop at the bulky modification $acp^3$U47 (Fig. 7.3D); in the absence of this modification, the primer is extended either to the 5′ end of the tRNA or to the next bulky tRNA modification ($ms^2i^6$A37 in the case of $tRNA^{Phe}_{GAA}$). Determining the ratio of band intensities for primer extension stops at position 47 to 37 confirms a reduction of $acp^3$U47 in $tRNA^{Phe}_{GAA}$ upon deletion of *trmA*, *truB*, or both (Fig. 7.3D, Fig. A2.2A-C). Again, TrmA seems to play a larger role in $acp^3$U47 formation than TruB (Fig. 7.3C, Fig. 7.3D).

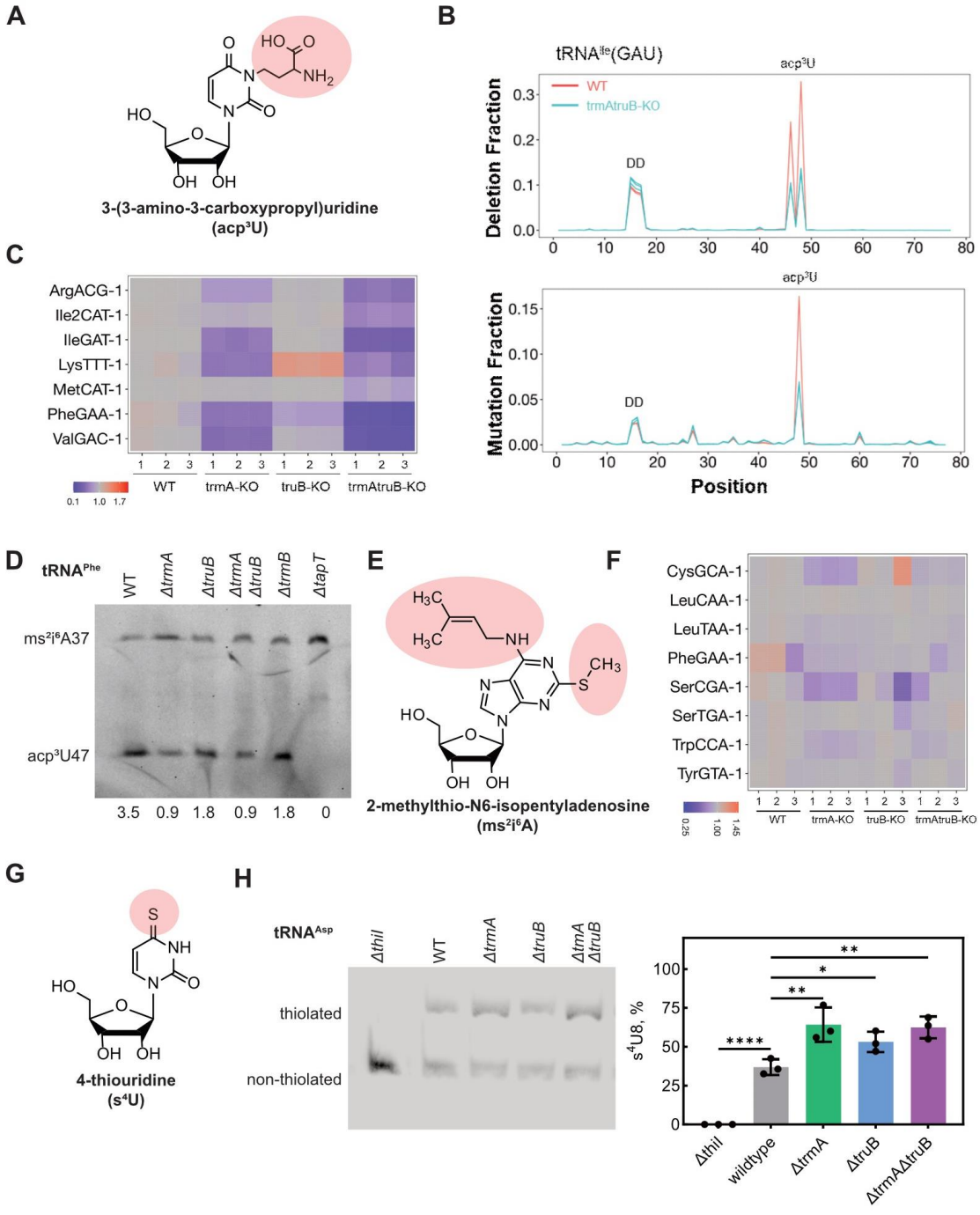



**Figure. 7.3. Deletion of *trmA* and/or *truB* changes abundance of additional tRNA modifications. (A)** Chemical structure of acp³U with modified portions highlighted in red. **(B)** Mutation and deletion fraction of $tRNA^{Ile}_{(GAU)}$ reads in WT and *ΔtrmAΔtruB* strains *(caption continued on the next page).*

Known modifications for this tRNA include acp$^3$U47 which generates strong mutation and deletion signatures, and consecutive dihydrouridines **(D)** D19D20 which also generate mutation and deletion signatures. *ΔtrmAΔtruB* reduces the mutation and deletion fraction of acp$^3$U47, but not those of D19D20. **(C)** Heatmap of acp$^3$U47 level change of *ΔtrmA* and/or *ΔtruB* over WT showing each biological replicate. All 7 *E. coli* tRNAs with known acp$^3$U47 modifications are shown. Data shown are the sum of mutation+deletion fraction at corresponding U47 position relative to WT. Blue indicates a decrease of acp$^3$U in the double-KO strain. **(D)** Validation of a decrease in acp$^3$U47 content upon deletion of either *trmA* or *truB* by tRNA$^{Phe}$ primer extension and reverse transcription (RT). Ratios of acp$^3$U47/ms$^2$i$^6$A37 intensities are indicated at the bottom of the gel. Gel displayed is representative of two biological replicates. All uncropped gels and quantifications are displayed in Figure A2.2. **(E)** Chemical structure of ms$^2$i$^6$A37 with modified portions highlighted in red. **(F)** Heatmap of mutation and deletion frequency changes at position 37 for all *E. coli* tRNAs harboring a ms$^2$i$^6$A37 modification. **(G)** Chemical structures of s$^4$U with modified portions highlighted in red. **(H)** The presence of s$^4$U8 in knockout strains grown in LB medium containing 2% formate was detected by APM-PAGE followed by Northern blotting for tRNA$^{Asp}$ (left) and quantified based on the band intensities of the thiolated and non-thiolated form (right). **** indicates $p < 10^{-4}$, ** indicates $p < 0.01$, and * indicates $p < 0.05$. Three biological replicates were performed, and all replicates are shown in Figure A2.3.

In addition to this change in the tRNA variable loop, the tRNA sequencing data also indicate potential changes in the anticodon arm. Among these, we noticed several changes at the conserved purine 37 adjacent to the anticodon, which is modified to ms$^2$i$^6$A in several tRNAs (Fig. 7.3E). Particularly in the absence of *trmA*, the deletion/mutation signature at A37 is changed relative to wildtype within several tRNAs known to contain ms$^2$i$^6$A (tRNA$^{Leu}_{TAA}$, tRNA$^{Phe}_{GAA}$, tRNA$^{Trp}_{CCA}$, tRNA$^{Cys}_{GCA}$, and tRNA$^{Ser}_{CGA}$); however, other ms$^2$i$^6$A37-containing tRNAs may not be affected (tRNA$^{Tyr}_{GTA}$, tRNA$^{Leu}_{CAA}$, Fig. 7.3F). A smaller effect on ms$^2$i$^6$A37 is observed in the *ΔtruB* and double knockout strains. Only *E. coli* tRNA$^{Arg}_{ACG}$ contains the edited base inosine 34, which

generates a mutation during reverse transcription. The absence of *trmA* and/or *truB* does not affect tRNA deamination, as all strains had a high level of mutation (>97%) at A34 of $tRNA^{Arg}_{ACG}$ (Fig.A2.2D).

Finally, we also wondered if other common modifications closer to $m^5U54$ and Ψ55 within the tRNA tertiary structure are affected by the loss of TrmA and/or TruB. In particular, we examined whether $s^4U8$ (Fig. 7.3G) abundance is dependent on presence of TrmA or TruB by using urea-PAGE containing a phenylmercury compound that decreases the migration velocity of sulfur-containing tRNAs, wherein we detected specific tRNAs by Northern blotting (29). For many tRNAs, a high level (near 100%) of tRNA thiolation is observed in the absence and presence of TrmA and TruB enzymes (Fig. A2.3). In contrast, $tRNA^{Asp}$ was only ~30-40% thiolated in the wildtype strain. Interestingly, the proportion of thiolated $tRNA^{Asp}$ increased to ~50% in the *ΔtruB* strain and to ~60% in the *ΔtrmA* and *ΔtrmAΔtruB* strains (Fig. 7.3H), suggesting that the absence of TrmA and TruB promotes $s^4U8$ formation by ThiI. In summary, TruB and TrmA affect other tRNA modifications at several positions including U8, A37 and U47 (and possibly more that do not provide strong reverse transcription signatures in sequencing) thereby influencing several steps during tRNA maturation.

### *7.4.3 TruB and TrmA affect translation of specific codons*

Since tRNA aminoacylation and modification are affected in the absence of TruB and TrmA, we next investigated how cellular protein synthesis is impacted. To assess potential effects on global translation, we utilized biorthogonal noncanonical amino acid tagging (BONCAT), wherein L-azidohomoalanine (L-AHA) is incorporated into newly synthesized proteins instead of L-methionine allowing for labelling with a rhodamine dye (30). Overall quantification of new proteins does not reveal statistically significant changes between *E. coli* wild-type and the *ΔtrmA/ΔtruB* strains (Fig. 7.4A, Fig. A2.4). However, upon careful inspection of the SDS-PAGE, we noticed that selected proteins seem to be expressed differently in the deletion strains, suggesting that trmA and truB affect the synthesis of specific proteins.

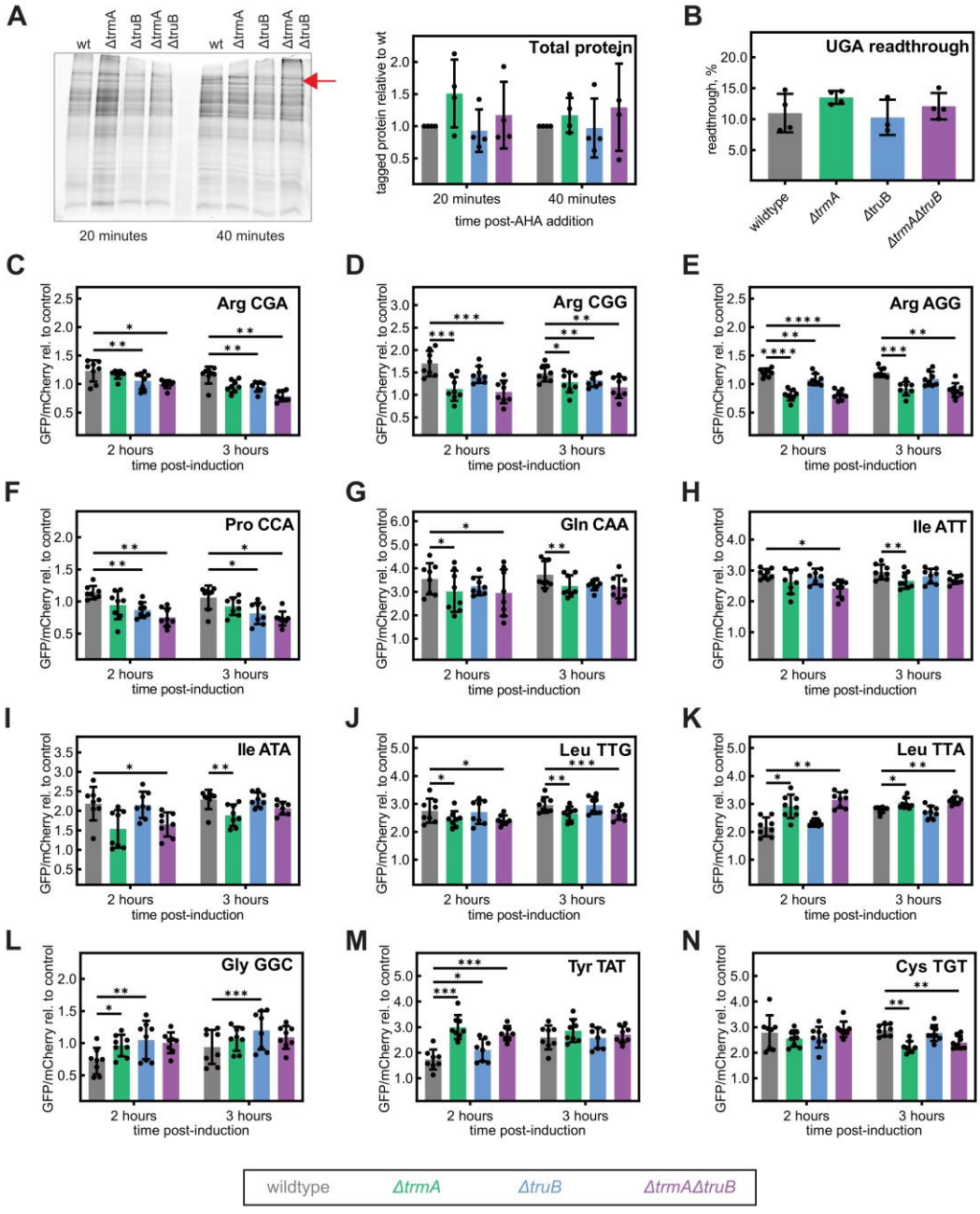



**Figure 7.4. Absence of TrmA and/or TruB does not affect global protein translation, but differentially affects translation of specific codons. (A)** Determination of global translation by pulse-labeling BONCAT experiments. SDS PAGE shown (left side of panel) is representative of four biological replicates. *(Caption continued on next page)*

All replicates are present in Figure A2.4. Red arrow features potential specific protein differences between wildtype and knockout strains. Densitometry of each total lane (right side of panel) was used to estimate global translation in each strain. **(B)** Proportion of UGA readthrough by Sec-tRNA$^{Sec}$ in the context of the *fdhF* SECIS element in a dual luciferase construct. **(C – N)** Measurements of codon-specific translation using GFP reporters. The GFP gene is preceded by four consecutive codons as indicated in the top right for each panel, and GFP expression is recorded 2 and 3 hours after induction relative to mCherry expression encoded on the same plasmid. Codon-specific GFP translation is compared for the *ΔtrmA* (green), *ΔtruB* (blue), *ΔtrmAΔtruB* (purple) and wild-type (grey) *E. coli* strains for indicated codons. Relative translation measurements for the remaining 49 sense codons are present in Figure A2.4 – 6. Eight biological replicates were performed for each strain; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

To understand how *trmA* and *truB* affect specific protein synthesis, we analyzed variations in translation for all 61 sense codons in addition to changes in UGA stop codon recoding by the selenoprotein machinery. First, we measured UGA stop codon readthrough by tRNA$^{Sec}$ in response to a selenocysteine insertion sequence (SECIS) element using a luciferase reporter where the UGA codon and the SECIS element of the *E. coli fdhF* gene are placed between a Firefly luciferase and Renilla luciferase gene (31). No significant differences in selonocysteine incorporation are noted between *E. coli* wild-type and the *ΔtrmA* and/or *ΔtruB* deletion strains (Fig. 7.4B). Subsequently, we dissected how changes in tRNA maturation and charging affect protein synthesis in a sense-codon-specific manner by utilizing a series of reporter constructs where green fluorescent protein (GFP) is preceded by four identical codons (32). As an internal reference, mCherry is expressed from the same plasmid. We monitored the ratio of GFP and mCherry fluorescence to determine codon-specific changes in translation in the *ΔtrmA* and *ΔtruB* single and double-knockout strains (Fig. 7.5.4C-N, Fig.A2.4-6). For most codon repeats, we did not observe significant differences between the *E. coli* wild-type and the deletion

strains, in accordance with the observation that global translation is unaffected. However, a specific set of codon repeats leads to altered expression of GFP in the *ΔtrmA* and/or *ΔtruB* deletion strains.

Previously, it was reported that deletion of *truB* (but not *trmA*) results in decreased translation of a reporter containing nine consecutive Arg CGA codons; interestingly, no effect was observed for translation of consecutive CGU codons, which are read by the same tRNA ($tRNA^{Arg}_{ACG}$) (17). We detect a similar decrease in translation for only four consecutive CGA codons in strains lacking *truB* (Fig. 7.4C) and similarly no effect for consecutive Arg CGU codons (Fig. A2.4D). Additionally, we detect translational decreases for other arginine codons, CGG and AGG, which are read by different tRNAs, $tRNA^{Arg}_{CGG}$ and $tRNA^{Arg}_{CCT}$, respectively (Fig. 7.4D, E).

In the *ΔtruB* and *ΔtrmAΔtruB* knockouts, repeats of the proline CCA codon (read by $tRNA^{Pro}_{TGG}$) displays significantly lower GFP production (Fig. 7.4F). Less pronounced, but still significant decreases in translation are noted in the absence of the *trmA* gene for Gln CAA codon repeats (read by $tRNA^{Gln}_{TTG}$) as well as for repeats of the Ile ATT and ATA codons, which are read by $tRNA^{Ile}_{GAT}$ and $tRNA^{Ile2}_{CAT}$, respectively (Fig. 7.4G-I). Interestingly, we observe opposing effects of *trmA* deletion for Leu codon reading: GFP expression is decreased in the presence of consecutive Leu TTG codons (read by $tRNA^{Leu}_{CAA}$), but increased for repeated Leu TTA codons (read by $tRNA^{Leu}_{TAA}$; Fig. 7.4J, K). For the Gly GGC codon, significantly increased GFP expression is noted in the absence of *truB* (Fig. 7.4L), and deletion of *trmA* and/or *truB* results in increased translation in the presence of consecutive Tyr TAT codons (Fig. 7.4M). Knockout of *trmA* decreases translation in the presence of consecutive Cys TGT codons (Fig. 7.4N). In conclusion, although the translation of many codons is unaffected by *trmA* and/or *truB* deletion, certain codons are translated poorly in the absence of *trmA* and/or *truB*, and for other codons translation is enhanced.

*7.4.4 TrmA and TruB impact the E. coli transcriptome and proteome*

Since we observed no overall change in protein synthesis, but a change in translation efficiency for individual codons in the absence of *trmA* and/or *truB*, we hypothesized that a subset of proteins, possibly enriched in the codons with altered translation efficiencies, may be differentially synthesized in the deletion strains. To test this hypothesis, we examined RNA abundance in the single- and double-deletion strains by total RNA-seq, and quantitated protein abundance by mass spectrometry, allowing us to correlate the relative abundances of transcripts and proteins in each knockout compared to wildtype (Fig. 7.5, Fig. A2.7). Overall, these experiments reveal several changes between wild-type and deletion strains on both the transcriptome and proteome level. Particularly, the transcript levels of several genes are increased, but the protein levels are decreased, suggesting deficiencies in translation for the *trmA* and *truB* single and double knockout strains (Fig. 7.5). This observation is particularly pronounced in the *ΔtrmAΔtruB* double-knockout strain. Only a few proteins increase in abundance despite decreased transcript levels. In general, a larger number of proteins are found to be decreased (rather than increased) upon knockout of *trmA* and/or *truB* compared to wildtype (Fig. A2.7).

Analyzing for gene ontology (GO) terms among the down-regulated proteins in the knockout strains (Fig. A2.8) shows that in particular metabolic processes are impacted in the single knockout strains. In the double-knockout strain, both metabolic processes and gene expression processes are affected. While some proteins are decreased in two or three of the knockout strains, other proteins are only decreased in one of the strains (Fig. A2.8) in accordance with strain-specific features observed in the codon-specific GFP translation assays. Overall, these findings are in accordance with our observation that global translation is not affected upon deleting *trmA* or *truB*, but that individual codons are impaired in translation, resulting in specific proteome changes that likely cause the previously reported decrease in cellular fitness (11,12).

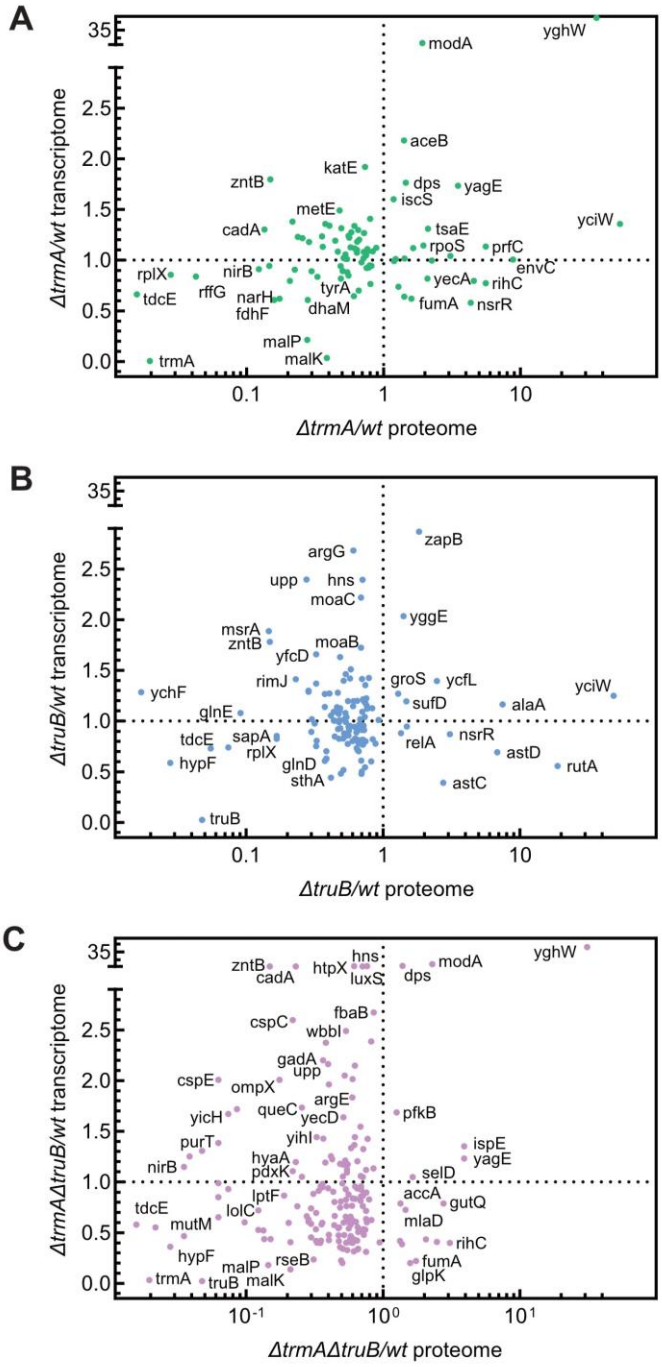



**Figure 7.5. Gene expression is altered upon *trmA* and/or *truB* deletion.** Transcriptome and proteome changes were recorded in *ΔtrmA* **(A)**, *ΔtruB* **(B)**, and *ΔtrmAΔtruB* **(C)** knockout strains. Each data point represents a protein with a significantly ($p < 0.05$) altered abundance within the knockout compared to wildtype, with fold protein change (knockout/wildtype) indicated by position on the x-axis. *(Caption continued on next page*)

To address proteins that were not identified in any wildtype replicates (fold change = division by zero) or not identified in a knockout (fold change = 0), a small value (0.1) was added to each value. For each significantly altered protein, the respective mRNA fold change (knockout/wildtype) is indicated by the value on the y-axis. Proteomics and transcriptomics experiments are the average of four and three biological replicates, respectively.

### 7.5 Discussion

Here, we provide comprehensive insight into the cellular functions of the universally conserved tRNA modifying enzymes TrmA and TruB that introduce $m^5U54$ and Ψ55 in all elongator tRNAs (Fig. 5.6). Thereby, we answer the decades-old question why these enzymes are conserved, but do not display phenotypes upon deletion in many organisms despite their disease relevance in humans and their contributions to cellular fitness. We previously demonstrated that TrmA and TruB fulfill two molecular functions by both modifying and folding tRNAs (11,12). To identify their overall function for cellular fitness, we analyzed here the effect of deleting these enzymes in *E. coli* without separating the tRNA modification and folding activities, since our previous data indicate that both are likely contributing to cellular fitness. Specifically, we demonstrate that TrmA and TruB lead to globally increased tRNA aminoacylation without significantly affecting tRNA abundance. Moreover, we uncover functional connections between $m^5U54$ and Ψ55 and several other modifications in tRNA (e.g., $acp^3U47$, $ms^2i^6A37$, and $s^4U8$). In accordance with the absence of major growth phenotypes in *ΔtrmA* and/or *ΔtruB E. coli* strains, overall translation is unaffected. However, translation for several codons is decreased, most notably the Proline CCA codon and three Arginine codons (CGA, CGG, and AGG). Interestingly, the abundance of several proteins is changed in the *ΔtrmA* and/or *ΔtruB* strains, which is not correlated with transcript abundance, revealing that the synthesis of specific proteins is impaired. These depleted proteins are often implicated in metabolism or gene expression, which explains the fitness defect of *ΔtrmA* and/or *ΔtruB* strains. In conclusion, TrmA and TruB enhance

aminoacylation and modification of all tRNAs in *E. coli*, thereby fine-tuning their functionality and ensuring efficient protein synthesis of specific codons.

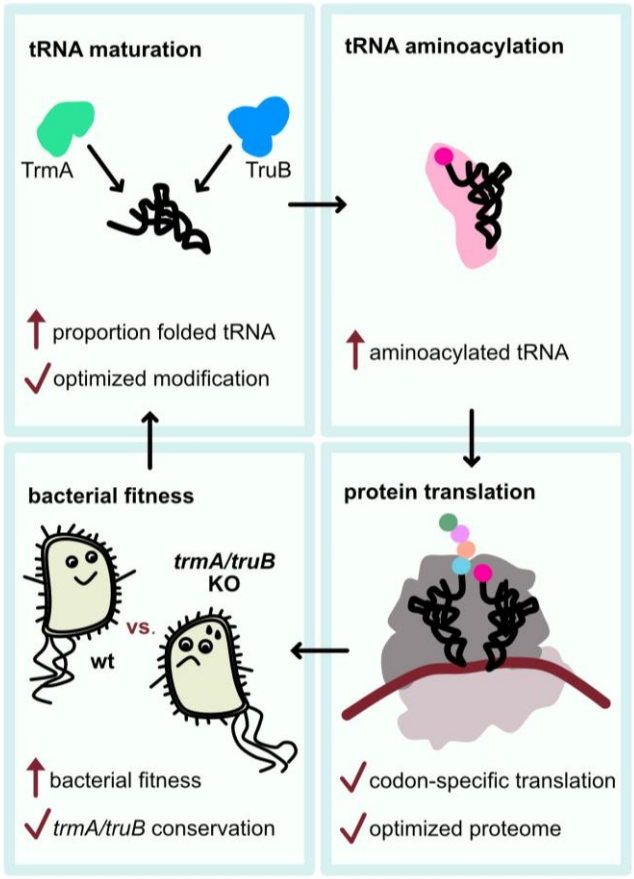



**Figure 7.6. Molecular and cellular function of the tRNA modification enzymes TrmA and TruB.** By interacting with the T arm of all tRNA, these enzymes enhance tRNA maturation by introducing $m^5U54$ and Ψ55, respectively, promoting tRNA folding, and modulating the formation of several other tRNA modifications. Thereby, they globally increase the levels of tRNA aminoacylation. Whereas these enzymes do not affect overall protein synthesis, translation of several codons is optimized by the presence of TrmA and TruB resulting in a proteome that promotes bacterial fitness in competition assays. The effects of TrmA and TruB on tRNA maturation, aminoacylation, codon-specific translation, the proteome and cellular fitness explain the conservation of these enzymes from bacteria to humans where they are implicated in several diseases.

The data presented here uncover a critical role of TrmA and TruB for tRNA maturation and aminoacylation. Specifically, we reveal global defects in aminoacylation in *ΔtrmA* and/or *ΔtruB E. coli* strains, wherein presence of TrmA and TruB enzymes collectively increase global aminoacylation by about 10% compared to the double knockout strain (Fig. 7.5.2E-F). Hence, TrmA and TruB affect charging of most or all tRNAs *in vivo*, most likely by enhancing the correct folding of all tRNAs (11,12). This decrease in aminoacylation in the knockout strains compared to the wild-type cells is small enough to explain why deletion strains do not display growth defects, but large enough to affect cellular fitness in competition assays over several days (11,12). Clearly, it is beneficial for the cell to rescue a small percentage of potentially misfolded tRNAs through the action of the tRNA chaperones TruB and TrmA.

TrmA and TruB and their homologs act early during tRNA maturation, thereby preparing all tRNAs for subsequent maturation events (33-35). In both yeast and Thermus thermophilus, deletion of *trmA* or *truB* homologs leads to altered abundances of other tRNA modifications at various positions, including $m^1A$, Gm, and $m^5U$ (15,34). Here, we reveal that the action of *E. coli* TrmA and TruB modulates the levels of other tRNA modifications including $s^4U8$, $ms^2i^6A37$, and $acp^3U47$ (Fig. 7.3). These results agree with recent LC/MS data from the Koutmou lab reporting decreased levels of $ms^2i^6A37$ and $acp^3U47$ in $tRNA^{Phe}$ from a *ΔtrmA E. coli* strain (*Jones et al*., *submitted*). Together, these findings indicate that many other tRNA modifying enzymes act on tRNA lacking $m^5U54$ and/or Ψ55 with reduced efficiency. This effect might be mediated by the chaperone activity of TrmA and TruB, as certain tRNA modification enzymes require correctly folded tRNA, or it may be due to lack of the $m^5U54$ and Ψ55 modifications. Further, it is conceivable that the lower levels of other tRNA modifications in the *ΔtrmA* and/or *ΔtruB* strains contribute to low aminoacylation, as several modifications at positions 34 and 37 act as determinants for aminoacyl-tRNA synthetases (36).

Given that $m^5U54$ and Ψ55 affect tRNA structural stability (3), it is notable that few effects on tRNA abundance were detected in the *ΔtrmA* and/or *ΔtruB* strains. Only two $tRNA^{Leu}$ isoacceptors are significantly downregulated in the *ΔtrmAΔtruB* strain (Fig. 7.2C); however, the

translation of the Leu CTG and CTA codons read by these tRNAs is not affected by *trmA* and *truB* deletion (Fig. A2.4). This contrasts studies of yeast tRNA modification enzymes, wherein certain enzymes are vital for the stability of a few tRNAs, but dispensable for other tRNAs (1,27,37,38). Thus, TruB and TrmA act differently from other tRNA modification enzymes as they do not impair the abundance of selected tRNAs but enhance the maturation of all tRNAs.

Despite the global effect of TrmA and TruB on the maturation and aminoacylation of all tRNAs, there are no global translation changes. However, we observed significant codon-specific defects in translation. In particular, translation of Pro CCA, Arg CGA, CGG and AGG, Gln CAA, Ile ATT and ATA, Cys TGT, and Leu codons TTG were negatively affected by the deletion of the *trmA* and/or *truB* gene. These codons are all used at much lower frequencies in *E. coli* than the other codons for the same amino acid. Accordingly, the tRNAs decoding these codons are often at a lower abundance compared to their isoacceptors; however, the decrease in their activity cannot be attributed to a reduction in their abundance compared to the knockout strains (Fig. 7.2A-C). Instead, these tRNAs are particularly susceptible to defects in codon-specific decoding abilities in the absence of TrmA and TruB, whether that be due to the loss TrmA/TruB chaperone activity and/or the loss of the $m^5U54$ and Ψ55 modifications. Alternatively, defects in these tRNAs may be caused by changes in the activity of other tRNA modifying enzymes, including those that form $ms^2i^6A37$ or $acp^3U47$, as these modifications tend to be decreased within the tRNAs affected in our codon-specific translation assay.

Of the seven tRNAs that contain $acp^3U47$, six have decreased $acp^3U47$ levels in the *ΔtrmAΔtruB* knockout strain. Remarkedly, these six tRNAs decode at least one codon differently in the absence of *trmA* (and *truB*) compared to the wildtype strain (Fig. 7.3C). Translation of CGA by $tRNA^{Arg}_{ACG}$, ATT and ATA by $tRNA^{Ile}_{GAT}$, ATA by $tRNA^{Ile2}_{CAT}$, AAA by $tRNA^{Lys}_{TTT}$, and GTT by $tRNA^{Val}_{GAC}$ are significantly lower in the *ΔtrmA* and/or *ΔtruB* strain, whereas translation of Phe TTC codons is improved in the *ΔtrmA* strain. The $ms^2i^6A37$ modification also has a lower abundance in several tRNAs within the *ΔtrmA* strain (Fig 7.3F), and half (4/8) of the $ms^2i^6A37$-containing tRNAs display different translation of certain codons in the absence of *trmA*. In the

absence of *trmA* (and *truB*), translation is decreased for TGT and TTG, read by $tRNA^{Cys}_{GCA}$ or $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{TAA}$, respectively. In contrast, translation is increased for TAT read by $tRNA^{Tyr}_{GTA}$ and TTC read by $tRNA^{Phe}_{GAA}$. Notably, several hypomodified tRNAs discussed above also decode additional codons whose translation is not altered in the absence of *trmA*/*truB*, supporting the notion that TrmA and TruB modulate codon-specific tRNA interactions. Interestingly, recent in vitro translation assays by the Koutmou group suggest translocation in the presence of the antibiotic hygromycin B is improved in the absence of $m^5U54$ (*Jones et al*., *submitted*), providing further evidence for tRNA elbow modifications altering translation. The observed tRNA- and codon-specific effects in the absence of $m^5U54$ and Ψ55 could be explained by changes in the tRNA dynamics which are critical when the tRNAs translocated through the ribosome.

In accordance with codon-specific translational impairments in the knockout strains, we noted a decreased abundance of specific proteins in the *ΔtrmA* and/or *ΔtruB* strains although their transcripts levels were unchanged or even increased, demonstrating translation-specific defects in gene expression. Indeed, analysis of the most decreased proteins in the *ΔtrmAΔtruB* strain shows that these are often enriched in the most affected codons such as the CGA and CGG Arg codons (ZntB) and the CCA Pro codon (YicH, PurT, NirB). There are 20 proteins whose abundance is decreased in all three single- and double-knockout strains (Fig. A2.8); nine genes encoding these proteins are enriched in Pro CCA and Arg CGA and CGG codons (*adhP, hrpA, hyaA, rbsK, upp, ybiS, ycaO, ygaM, and zntB*). Evidently, the codon-specific defects in translation upon *trmA* and/or *truB* deletion affect the overall proteome underlining the importance of these two tRNA modifying enzymes for protein synthesis. Moreover, 9 of the 20 translationally downregulated proteins are large *E. coli* proteins with more than 500 residues (*rpoC, fusA, hrpA, hyaB, metE, nrdA, pheT, tdcE, ycaO*). For these large proteins, subtle effects on translation in the *ΔtrmA* and/or *ΔtruB* strains likely accumulate leading to lower protein abundance. The codon-specific translation effects and decreases in selected proteins explain the reduced cellular fitness of the *ΔtrmA* and/or *ΔtruB* strains.

As TrmA and TruB are conserved across organisms from bacteria to humans, the link between their molecular function during tRNA maturation and aminoacylation and their cellular role for protein synthesis is likely similar in eukaryotes. In yeast, studies similarly suggest a role for the TrmA and TruB homologs, Trm2 and Pus4, in translation. Global ribosome profiling has revealed both Trm2 and Pus4 have subtle effects on ribosome occupancy at different codons, suggesting codon-specific translation defects in yeast knockout strains, similar to our observations in bacteria (39). Moreover, yeast Pus4 can act as a prion, and protein synthesis is altered in these prion strains likely due to a different translation of rare codons (40). The human homologs, TRMT2A and TRUB1 have also been implicated in modulating protein synthesis. Although human TRMT2A does not affect synthesis of polyQ proteins, the aggregation of polyQ is reduced (20) and translation fidelity is decreased in the absence of TRMT2A (41). Moreover, human TRUB1 affects mitochondrial tRNA conformation and mitochondrial translation thus decreasing the assembly and activity of oxidative phosphorylation complexes, without altering tRNA abundance (22). In conclusion, our findings regarding the molecular and cellular function of *E. coli* TrmA and TruB, together with reports of the importance of their yeast and human homologs for protein synthesis indicates a conserved mechanism for these tRNA modifying enzymes. Herein, TrmA and TruB homologs promote tRNA maturation and aminoacylation and affect codon-specific translation thus providing an explanation for their implication in human diseases, including diabetes, deafness and cancer (19-22).

In summary, we comprehensively identify the functions of the conserved tRNA modifying enzymes, TrmA and TruB, which introduce $m^5U54$ and Ψ55 in the TΨC arm of all tRNAs (Fig. 7.6). Thereby, these enzymes promote correct tRNA folding and enable efficient modification by other tRNA modifying enzymes. These positive effects during maturation lead to significant improvements in aminoacylation for all tRNAs. Thus, TruB and TrmA stand out as enzymes that globally affect all tRNAs, contrasting other tRNA modifying enzymes which only act on a subset of tRNAs (27). Other tRNA modifying enzymes targeting the elbow region of many tRNAs such as the dihydrouridine synthases may have similar global roles. The positive impact of TrmA and TruB on the cell is particularly evident in the decoding of codons by tRNAs harboring

$ms^2i^6A37$ and $acp^3U47$ modifications and the translation of large proteins leading to significant changes in protein expression in *ΔtrmA* and/or *ΔtruB* knockout strains. These codon- and protein-specific translation defects reduce the cellular fitness under competition conditions. Similarly, the human homologs of *E. coli* TrmA and TruB are likely to impact tRNA maturation and aminoacylation and to modulate protein synthesis explaining their implication in human diseases. Unlike other tRNA modification enzymes that have tRNA- and stress-specific functions, we therefore demonstrate that TrmA and TruB act as global enhancers of protein synthesis and cellular fitness.

## 7.6 Methods

### *7.6.1 Chemicals and reagents*

Unless otherwise stated, all reagents were purchased from ThermoFisher Scientific. All oligonucleotides were purchased from Integrated DNA Technologies (IDT) and are listed in Table A2.1.

### *7.6.2 E. coli strains*

All *E. coli* wildtype and single knockout strains are BW25113 derivatives from the Keio Collection (23). To construct the *ΔtrmAΔtruB* double knockout, the FLP recognition target (FRT)-flanked kanamycin resistance cassette was removed from the Keio *ΔtrmA* strain, and the *truB* gene was subsequently replaced by a FRT-flanked kanamycin resistance cassette using λ Red recombinase (42). All gene deletions were confirmed by colony PCR using primers specific for the kanamycin resistance cassette (k1/k2) and primers targeting the locus upstream and downstream of the deleted gene (Table A2.1).

### *7.6.3 Total RNA extractions for tRNAseq, Northern blotting, and primer extension*

Cells were grown overnight in LB medium (wildtype) including 50 µg/mL kanamycin (knockout strains) from single colonies. Cultures were centrifuged and resuspended in fresh LB media or LB including 2% (w/v) sodium formate as indicated. Cultures (50 mL) were started at an $OD_{600}$ of 0.1 and grown until reaching an $OD_{600}$ of 0.3-0.4, when 15 mL culture was harvested and shock frozen. RNA extractions were accomplished under acidic conditions (pH <5) to preserve the aminoacyl bond using TRIzol (Invitrogen), following the manufacturer's instructions. In brief, cells were resuspended in 3 mL TRIzol, followed by the addition of 0.6 mL chloroform. After centrifugation (30 minutes, 6000*g*), the aqueous layer was removed and ethanol precipitated several times. Purified RNA was resuspended in 10 mM NaOAc (pH 4.8) and stored at -80 °C. Concentrations were determined using UV spectrometry (NanoDrop 2000c).

### *7.6.4 MSR-tRNA-seq for tRNA abundance, aminoacylation, and certain modifications*

Library preparation, MSR-tRNA-seq and data analysis were conducted as previously published (25). Three biological replicates were sequenced for each strain and condition. The *E. coli* tRNA reference was obtained from the Genomic tRNA database (43), Modification was identified by mutation and deletion signatures that were generated by Superscript IV read-through. Source codes for all custom scripts are available on GitHub (https://github.com/ckatanski/CHRIS-seq).

### *7.6.5 Northern blotting for tRNA thiolation*

To compare the fraction of thiolated tRNAs to non-thiolated tRNAs, 5 µg total RNA was separated on a 20 µM [(N-acryloylamino)phenyl]mercuric chloride (APM, Toronto Research Chemicals) 7 M urea 12% polyacrylamide gel 28. To reduce APM-thiol linkages, APM gels were soaked in 0.2 M β-mercaptoethanol for one hour prior to transfer. RNA was transferred to a nylon membrane (Amersham Hybond-XL) at 23 V for 7 minutes using a Trans-Blot Turbo Transfer System (Bio-Rad). Following transfer, blots were crosslinked twice at 1200 mJ. Northern blotting

was performed similarly as described in (44) using biotinylated probes (Table A2.1), which were detected after incubation with 120 ng/mL streptavidin-HRP (Genscript) in hybridization buffer for one hour. To detect chemiluminescence, a solution containing 375 ng/mL luminol, 55 ng/mL p-coumaric acid, and 0.1% (v/v) hydrogen peroxide was applied to the blot immediately prior to imaging with a FluorChem Q imager (Cell Biosciences). Densitometry was performed using ImageJ.

### *7.6.6 Primer extension to detect acp$^3$U47 modification*

Reverse transcriptions were performed by first annealing 1700 ng of total RNA to 3 pmol Cy5-labeled tRNA$^{Phe}$ reverse transcription primer by heating to 65 °C and cooling to 47 °C. The RNA-primer mixture was then added to a reaction mixture containing final concentrations of 136 ng/µL total RNA, 240 nM Cy5-labeled reverse transcription primer, 0.4 mM dNTPs, and 8 mM DTT, in 1 X AMV reverse transcription buffer. After pre-heating to 47 °C, AMV reverse transcriptase (New England Biolabs) was added to a final concentration of 0.4 U/µL. and incubated at 47 °C for 45 minutes. The reaction was ended by heating to 70 °C for 15 minutes. Samples (7 µL of the reverse transcription reaction) were analyzed on an 8 M urea 8% polyacrylamide sequencing gel in TBE which was preheated to 50 °C at 50 W until the bromophenol blue dye front was halfway down the gel. Gels were imaged on a Typhoon 5 scanner.

### *7.6.7 Total RNA sequencing*

As TRIzol extractions from *E. coli* cells are known to be biased towards the extraction of small RNAs (45), and to promote proportionate presence of mRNAs of all sizes, *E. coli* cells were first resuspended in 300 µL of a cell opening buffer (100 mM Tris, 10 mM EDTA, 0.5% SDS, 286 mM β-mercaptoethanol) containing 10 mg/mL lysozyme, 0.1 U/µL DNase I, and 0.1 U/µL RiboLock RNase inhibitor (ThermoFisher) and incubated at 37 °C for 5 minutes. Subsequently,

Proteinase K (ThermoFisher) was added to a final concentration of 0.5 mg/mL, and lysates were incubated at 50 °C for 10 minutes. RNA extractions were performed as described above. Further DNase treatment, RNA quality analysis by TapeStation and DNA/RNA Qubit, library preparation, strand-specific Illumina sequencing with a HiSeq400, and data analysis were performed by Azenta. In brief, reads were trimmed with Trimmomatic v.0.36 and trimmed reads were mapped to the *E. coli* BW25113 genome using Bowtie2 v.2.2.6. Unique gene hit counts were calculated by using featureCounts from the Subread package v.1.5.2 and gene expression was compared between *E. coli* strains using DESeq2.

*7.6.8 Bioorthogonal noncanonical amino acid tagging (BONCAT)*

*E. coli* pre-cultures were grown overnight in the appropriate antibiotic in 5 mL LB medium. Stationary-phase cells were harvested, washed, and resuspended in M9 minimal media containing 1.75% sodium formate, and 50 mL cultures were grown at 37 °C starting at an $OD_{600}$ of 0.1. When cultures reached an $OD_{600}$ of ~0.3, L-azidohomoalanine (L-AHA, Sigma) was added to a final concentration of 1 mM. Twenty minutes and 40 minutes post-L-AHA addition, 0.5 $OD_{600}$ units of cells were harvested, fixed with 3% (v/v) formaldehyde, washed into PBS, and stored at -20 °C. Cell opening and fluorescent labeling of L-AHA-containing proteins with DBCO-PEG4-Carboxyrhodamine 110 (Click Chemistry Tools) were performed as described elsewhere (30). Proteins were purified using chloroform-methanol extraction and resuspended in 6.4 M urea and 200 mM DTT; total protein concentration was determined by UV spectrometry:

$$Concentration\ (mg/mL)\ = (1.55 \times A_{280}) - (0.76\ \times\ A_{260})$$

Protein samples were denatured at 65 °C prior to analyzing 5 µg total protein on a 12% SDS-PAGE. Labeled proteins were detected using the 488 nm laser on a Typhoon 5 Imager (Cytiva). To confirm equal loading, gels were stained with Coomassie. Relative global translation for each strain was estimated by densitometry (ImageJ).

*7.6.9 Proteome comparison by mass spectrometry*

Four biological replicates of each strain were grown at 37 °C in LB media containing 2% (w/v) sodium formate until reaching an $OD_{600}$ between 0.3 and 0.4. Following harvesting, cells were resuspended in 8 M urea containing SDS loading buffer lacking any colored dyes and 0.3 $OD_{600}$ units of each sample were analyzed on a 12% SDS PAGE and stained with Coomassie.

Each lane was excised and subsequently cut into 14 equal bands, each gel fraction was subjected to in-gel tryptic digestion as previously described (46) and the resulting peptides were dried and resuspended in 60 L of 0.2% formic acid in 5% acetonitrile (ACN). Digested peptides were analyzed by LC-MS/MS using a ThermoScientific Easy nLC-1000 in tandem with a Q-Exactive Orbitrap mass spectrometer. Each sample (5 µL) was resolved using a 120 min gradient [0%–45% Buffer B; Buffer A (0.2% formic acid in 5% ACN); Buffer B (0.2% formic acid in 100% ACN)] on a 2 cm Acclaim 100 PepMap Nanoviper C18 trapping column in tandem with a Thermo EASY-Spray column (PepMap® RSLC, C18, 3 µm, 100 Å, 75 m × 150 mm). For data-dependent analysis, full scans were acquired at 35000 resolution at a range of 400–200 m/z while 17500 resolution was used for MS/MS scans. Only the top 15 ions with +2 and +3 charges were selected for MS/MS with 10 s dynamic exclusion applied to prevent continuous reanalysis of abundant peptides. Following data acquisition, raw data files were compiled for each gel lane and searched with Proteome Discoverer 1.4's SEQUEST search algorithm using the reviewed, non-redundant *E. coli* complete proteome retrieved from UniProtKB. The search parameters and quantification were as previously described (47). Proteins were considered to be significantly different in each knockout compared to the wildtype for p values $<0.05$ determined by a two-tailed paired Student's t test. Significantly enriched GO terms in each dataset were determined using the PANTHER GO biological complete annotation set and *E. coli* reference list by Fisher's Exact test, correcting for False Discovery Rate (48).

*7.6.10 Translation reporter assays to examine codon-specific translation*

BW25113 wildtype, *ΔtrmA*, *ΔtruB*, and *ΔtrmAΔtruB* strains were transformed with reporter plasmids carrying an arabinose-inducible fluorescent transcriptional cassette encoding superfolder green fluorescent protein (sfGFP) followed by mCherry fluorescent protein (kind gift of Assaf Katz, Universidad de Chile, Santiago, Chile) (32). Each reporter contains a set of four tandem repeats of a codon following the third sfGFP codon, such that sfGFP expression relies on translation of the four repeated codons. To account for small differences in translation between strains, plasmid S1 was used as a control, which lacks any additional codons in sfGFP.

sfGFP and mCherry fluorescence was determined similarly as previously described (32). In short, overnight cultures were diluted 1:20 to an $OD_{600}$ of ~0.1 and grown in LB medium supplemented with 100 µg/mL ampicillin until reaching an $OD_{600}$ of 0.4-0.6. At this point, cells were diluted 1:4 into fresh LB medium containing a final concentration of 100 µg/mL ampicillin and 0.4% (w/v) arabinose in black, optical bottom 96 well plates and grown at 37 °C with shaking at 100 rpm. Two- and three-hours post-induction, $OD_{600}$, sfGFP fluorescence (excitation: 480 nm, emission: 515 nm), and mCherry fluorescence (excitation: 587 nm, emission: 610 nm) were measured using a FlexStation 3 plate reader (Molecular Devices). sfGFP/mCherry ratios for each test codon were normalized to the sfGFP/mCherry ratio of plasmid S1 for the respective strain:

$$sfGFP/mCherry\ ratio\ relative\ to\ control\ = \frac{sfGFP_{test\ codon}/mCherry_{test\ codon}}{sfGFP_{S1}/mCherry_{S1}}$$

For each codon, translation between strains was compared using two-way ANOVA. Significant differences ($p < 0.05$) between strains are indicated.

*7.6.11 Sec-tRNA$^{Sec}$ mediated UGA readthrough reporter assay*

To prepare a dual luciferase reporter for measuring selenocysteine insertion sequence-directed UGA readthrough that is suitable for expression within kanamycin-resistant and DE3-lacking BW25113 knockout strains, pTrc99a-fluc-fdhF130-179UGA-rluc was prepared by

PCR amplification and ligation of the DNA sequence encoding the Fluc-FdhF-Rluc fusion protein within pET24-fluc-fdhF130-179UGA-rluc (31) (kind gift from Marina Rodnina, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany). To account for subtle differences in canonical translation between strains, site-directed mutagenesis was used to construct the control plasmid, pTrc99a-fluc-fdhF130-179UUC-rluc, which encodes a UUC Phe codon in place of the UGA Sec codon. Plasmids were transformed into BW25113 wildtype, *ΔtrmA*, *ΔtruB*, and *ΔtrmAΔtruB* strains.

To determine the proportion of UGA readthrough in each strain, cultures were grown at 37 °C with shaking, starting at an $OD_{600}$ of ~0.1 in LB media supplemented with 50 μM sodium selenite. At an $OD_{600}$ between 0.5-0.7, fusion protein expression was induced with 1 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG). Following 30 minutes of growth, 0.1 $OD_{600}$ of cells were harvested, opened by lysozyme, and firefly and Renilla luciferase were measured using the Dual-Luciferase® Reporter Assay System (Promega) as per the manufacturer's instructions with a Spectramax i3 plate reader (Molecular Devices). Recoding efficiency for each strain was calculated using the following formula:

$$\%\, readthrough = \frac{Rluc_{UGA}/Fluc_{UGA}}{Rluc_{UUC}/Fluc_{UUC}} \times 100\%$$

### 7.7 Submission numbers

The data discussed in his publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE233667 for MSR-tRNA-seq data and GSE237609 for total RNA sequencing data.

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# Chapter 8:
# Conclusions and future directions

## 8.1 Summary

In this thesis, I have examined the mechanisms and cellular functions of three highly conserved tRNA modifying enzymes, TrmA, TruB, and TrmB. In order to determine if these enzymes have a preference for the modification status of their substrate tRNAs, I first established a system to prepare partially modified tRNAs *in vitro*, for which the methods are explained in detail in Chapter 3. By preparing tRNAs that contained only a single modification (one of $s^4U8$, $m^7G46$, $m^5U54$, or Ψ55) and comparing the activity and affinity of TrmB, TrmA, and TruB for singly-modified and unmodified tRNA, the results shown in Chapter 5 suggest that TruB prefers to bind and modify unmodified tRNA. TrmA also prefers to modify unmodified tRNA, but interestingly binds Ψ55 tRNA more tightly than unmodified tRNA. In contrast, TrmB does not have a binding or modification preference for the modification status of tRNA. Therefore, these results suggest that TrmA and TruB are likely to act in the early stages of tRNA maturation, whereas TrmB is likely to act later.

To further our understanding of the mechanism through which TrmB binds tRNAs, I then examined how TrmB binds tRNA by preparing a tRNA containing only the $s^4U8$ modification and labeling the thiol residue with a fluorescein derivative. In Chapter 4, I explain this method to prepare tRNA with an internal fluorophore in detail and in Chapter 6, I present the results of the TrmB•tRNA binding mechanism. Using the fluorescent tRNA, pre-steady-state stopped-flow studies were used to monitor tRNA binding to TrmB in real time. Taking the results of these rapid kinetic experiments together with equilibrium binding assays and tRNA methylation assays, this study identified that TrmB requires the presence of the methyl donor SAM for rapid and stable binding of tRNA. By preparing single-residue substitution variants of TrmB, the residue D144 was found to be primarily involved in catalysis, whereas R26, R155A, and T217 are all important for tRNA binding. Thus, the small TrmB enzyme binds tRNA using the entirety of its surface. Finally, in this study, I identified that the *E. coli trmB* gene is necessary for robust growth in hydrogen peroxide stress conditions.

In Chapter 7, I examine the roles of the early-acting TrmA and TruB enzymes within *E. coli* cells. In addition to modifying tRNAs early, thereby setting the stage for the remainder of tRNA maturation, the Kothe lab has previously demonstrated that both enzymes act as tRNA chaperones (1,2), and it has been long-known that these enzymes are highly conserved in all domains of life and target the majority of all tRNAs for $m^5U54$ and Ψ55 formation. In contrast to the apparent importance and abundance of TrmA and TruB enzymes, they are non-essential in bacteria and their deletion results in only subtle phenotypes. To understand what roles tRNA folding and modification play within cells, I examined tRNA abundance, aminoacylation, and modification, in addition to protein translation within *E. coli* lacking one or both of these genes. Interestingly knockout of *trmA* and/or *truB* was found to globally decrease cellular tRNA aminoacylation, while not disturbing tRNA abundance. Moreover, the stoichiometry of additional tRNA modifications (including $s^4U8$, $ms^2i^6A37$, and $acp^3U47$) within tRNAs is altered in the absence of TrmA and TruB. These disruptions to cellular tRNA modification were not found to alter global translation; however, the translation of specific codons and specific mRNAs was impacted by the loss of TrmA and TruB. Thus, TrmA and TruB play important roles in fine-tuning cellular tRNA maturation and aminoacylation, and thereby affect the expression of proteins within the cell and finally explain why TrmA and TruB are so highly conserved throughout all domains of life.

Therefore, the results from the three studies I have presented here further our information on the mechanisms by which tRNA modifications act and what roles highly conserved tRNA modifying enzymes play within cells. Taking these results together, in the sections below I will briefly discuss how my thesis lays the groundwork for future studies of tRNAs and RNA modifications.

### 8.2 Preferred order of tRNA modification

When I was preparing the manuscript for the results shown in Chapter 5 for submission and in the time since its publication, tRNA modification order within the tRNA T arm has gained

more attention. Two studies examining the temporal order of $tRNA^{Phe}$ modification in yeast cellular extract and human cells were published (3,4). The results of both studies point towards very fast pseudouridylation of newly transcribed tRNA by TruB homologs, but differ from each other in the relative order for $m^5U54$ and $m^7G46$ introduction. In yeast cellular extract, $m^5U54$ and $m^7G46$ were the next modifications to appear following Ψ55, with $m^7G46$ being quickly incorporated and $m^5U54$ appearing more slowly in the tRNA population over time (4). In human cells, $m^5U54$ is incorporated quickly, followed by a slow incorporation of $m^7G46$ (3). Interestingly, knockout of *pus4* in yeast results in a lower abundance of $m^5U54$ in tRNA (4,5), suggesting that, at least in yeast, C5-methylation of U54 may somewhat rely on previous pseudouridylation. Supporting this idea, purified yeast Trm2 has a six-fold faster activity when acting on Ψ55 tRNA compared to unmodified tRNA (6). In contrast, my results show that although the affinity of *E. coli* TrmA is five-fold tighter for Ψ55 tRNA compared to unmodified tRNA, the activity of TrmA is similar between both unmodified and Ψ55 tRNA (7). Despite these potential species-specific differences, it seems that early introduction of Ψ55 is a common feature during the maturation of tRNA throughout *E. coli*, yeast, and humans.

The mechanisms underlying several tRNA modification circuits remains to be studied. Whereas Chapter 5 identifies early-acting tRNA modifying enzymes, which enzymes are likely to act later in maturation and why remains unknown. For example, *in vivo* knockout studies have revealed that $acp^3U47$ content in several tRNAs is decreased in the absence of the *trmB* gene (8), suggesting dependence of the TapT enzyme on TrmB; however, the mechanistic basis for this crosstalk has not yet been addressed. Similarly, results presented in Chapter 7 of this thesis show that $acp^3U47$ and $ms^2i^6A$ content is decreased in several tRNAs when *trmA* and/or *truB* is deleted. By preparing partially-modified tRNA, the binding and modification preferences of TapT (responsible for $acp^3U47$) and MiaA/MiaB (responsible for $ms^2i^6A37$) can be examined to determine which prior modification(s) are necessary for efficient enzyme activity, and why. Moreover, the yeast dihydrouridine synthase Dus2 has been shown to require one or modifications for activity, but which modification(s) are needed remains unknown (9). Thus, the method presented in Chapter 3 for preparing partially modified tRNAs will be powerful for

studying the mechanistic basis of the hierarchical order of tRNA modification. Studies examining the relative order of tRNA modification and identifying early-acting modifying enzymes are critical to gain an understanding of the overall tRNA modification state when a single modification enzyme is lost or mutated. Thus, we can explain the downstream effects resulting from mutations to early-acting modification enzymes implicated in diseases (for example, the likely "intermediate" tRNA modifying enzyme, WDR4 involved in $m^7G46$ formation, as will be discussed below). Moreover, using these studies of partially-modified tRNAs as a model, modification interdependencies can be examined in additional types of RNAs containing multiple modifications, including rRNA, small nucleolar RNA (snoRNA), microRNA (miRNA), long non-coding RNA (lncRNA), and small nuclear (snRNA).

### 8.3 tRNA binding by TrmB

During the preparation of the manuscript presented in Chapter 6, two independent groups determined cryo-electron microscopy and X-ray diffraction structures of the human TrmB homolog (METTL1) in complex with its partner protein (WDR4, absent in bacteria) and tRNA (10,11). These structures corroborated several of our findings, including that METTL1 (or TrmB) only becomes ordered upon cofactor binding (11). However, as METTL1 requires WDR4 for efficient tRNA binding and modification since WDR4 contributes critical interactions with tRNA, the details of tRNA binding compared to bacterial TrmB are likely to be different. Thus, to fully understand how bacterial TrmB binds tRNA, structural studies will be necessary. Furthermore, *Bacillus subtilis* TrmB is a homodimer, seemingly acting as an evolutionary intermediate between the monomeric *E. coli* TrmB and the heterodimeric eukaryotic TrmB. By repeating the rapid-kinetic and equilibrium binding experiments with homodimeric *B. subtilis* TrmB and heterodimeric eukaryotic METTL1/WDR4, the kinetics and molecular mechanisms for tRNA binding can be compared between the homologous enzymes. This will provide insights into the evolution of this enzyme as well as potentially identify differences between human and bacterial TrmB enzymes.

Since TrmB is important for bacterial fitness, specific targeting of the bacterial TrmB enzyme may be used as an adjunctive antibiotic therapy.

Moreover, mutations in the human *WDR4* gene are known to cause a distinct form of primordial dwarfism (12). Using an *in vivo* yeast model system, previous research has shown levels of $m^7G46$ in tRNA are reduced for yeast with corresponding mutations, suggesting that a lack of tRNA methylation may be implicated in this genetic disease (12). Interestingly, it seems that an abnormal $m^5C$ is present in the absence of $m^7G46$ - at least in certain tRNAs; however, the reasoning for and any possible implications of this extra modification remain unstudied (12). As WDR4 is the partner protein to the catalytic subunit METTL1, why do *WDR4* mutations result in decreased tRNA methylation? The SAM binding and the catalytic pockets are located in METTL1 (not WDR4), but structures of the METTL1•WDR4•tRNA complex do indicate that WDR4 is implicated in tRNA binding, and thus substitutions may alter tRNA binding. Alternatively, since these single amino acid substitutions are at the interface of the METTL1•WDR4 binding surface, perhaps WDR4 binding to METTL1 impaired, thus also impairing tRNA binding. This possibility can be tested using techniques such as size exclusion chromatography or isothermal calorimetry. Does decreased tRNA methylation directly contribute to this disease, or do alternative functions of this enzyme (including, potential tRNA chaperone activity)? Since different substitutions in WDR4 have been observed for this disease, do WDR4 variants have similar defects in tRNA modification to each other, or do these affect tRNA binding and/or modification differently? To begin to answer these questions, the binding of purified human METTL1•WDR4 variants to tRNA can be determined first under equilibrium conditions to determine if the variants have a similar affinity for tRNA as the wildtype enzyme. The details of the binding interaction can then be studied using fluorescent $s^4U8$-tRNA using rapid-kinetics stopped-flow experiments.

### 8.4 Fluorescent labeling of partially modified tRNA to study tRNA interactions

In addition to studying the binding of TrmB homologs, using internal native tRNA modifications to fluorescently label tRNA enables studies of additional tRNA modification enzymes and other tRNA interacting factors. As shown in Chapter 4 the interaction of the U20-modifying enzyme DusA can also be monitored using $s^4$U8-fluorescein-$tRNA^{Phe}$ (13). Bacterial DusA modifies U20 within many tRNAs to dihydrouridine (D), and no *in vitro* studies of this enzyme have yet been accomplished. Interestingly, the yeast enzyme that targets this position (Dus2) seems to require one or more modifications for activity, but it is not yet known which modification(s) are necessary (9). In contrast, no modifications seem to be necessary for modification by *Thermus thermophilus* Dus targeting U20 of $tRNA^{Phe}$ *in vitro* (14). From ongoing stopped-flow experiments in the Kothe lab, it is known that DusA can bind mostly unmodified tRNA (unmodified except for the $s^4$U8 residue to which fluorescein is attached) (13), but whether it binds unmodified tRNA more efficiently than modified tRNA remains unknown. Additionally, the human Dus2 homolog is overexpressed in different cancers, leading to an increase in dihydrouridine in tRNA (15,16). Finally, the proof-of-principle study presented in Chapter 6 presents more opportunities to similarly use the unique chemistry of tRNA modifications to fluorescently label tRNAs at different positions in tRNAs using thiol (e.g. $s^2$U34) and other reactive modifications (including dihydrouridine and wybutosine) (13,17). This will enable mechanistic binding studies of additional tRNA modifying enzymes and other tRNA binding factors for mostly unmodified tRNA. Additionally, alternative tRNA isoacceptors can be used, and different tRNA variants can be produced, allowing for investigation of tRNA determinants necessary for enzyme binding.

### 8.5 tRNA modifying enzymes and chaperones, TrmA and TruB, enhance cellular protein synthesis

Results from Chapter 7 suggest that TrmA and TruB optimize protein translation in *E. coli* by fine-tuning tRNA maturation and aminoacylation and addresses the long-standing question of why these non-essential enzymes are so highly conserved. These results raise two important

questions: why and how does the loss of TrmA and TruB affect translation of these codons? First, the observed effect on translation *in vivo* could be directly due to the loss of $m^5U54$ and Ψ55, but this defect in translation could also be caused by the subtly decreased cellular levels of the corresponding aminoacyl-tRNAs or due to the loss of additional modifications within the tRNA, including $ms^2i^6A37$ and $acp^3U47$. Moreover, the tRNA folding activity of TrmA and TruB may be responsible for these processes rather than formation of $m^5U54$ and Ψ55.

To untangle effects of tRNA folding from tRNA modification, codon reporter assays can be repeated for *ΔtrmA* and *ΔtruB* cells carrying the appropriate catalytically-inactive enzyme for affected codons to determine if the tRNA folding function of TrmA and TruB can rescue translation for these codons (1,2). If the tRNA folding role of TrmA and TruB cannot rescue translation, then the roles of $m^5U54$ and Ψ55, in addition to the other affected modifications, can be examined *in vitro* to determine which modifications affect translation, and which step of translation elongation they affect (18,19). For such experiments, it may be interesting to use the Arg CGA codon or the Pro CCA codon, as these were among the most affected in the *in vivo* reporter assays in Chapter 7.

### 8.6 Concluding remarks

Mutations within tRNA modification enzyme genes or tRNA genes that result in aberrant modification are implicated in genetic diseases, including several mitochondrial diseases and neurological disorders, in addition to Type 2 diabetes (20). For example, defective G46 methylation results in primordial dwarfism and mitochondrial tRNA mutations that result in the loss of TRUB1 activity results in deafness in humans (21). Furthermore, the expression and activity of several tRNA modifying enzymes is dysregulated in several cancers and may contribute to cancer progression (22,23). Notably, TRM2 is overexpressed in cancer, and METTL1 has gained much recent attention as a driver of cancer progression and thus a potential chemotherapeutic target for several cancers (20). In order to fully understand these diseases, the

precise cellular roles for tRNA modifying enzymes must be uncovered in addition to the molecular mechanisms through which modification enzymes act.

The work presented in my thesis provides a benchmark for the importance of investigating the cellular effects of tRNA elbow modification enzymes and also provides a framework for using partially modified tRNAs to examine mechanisms of tRNA modification enzymes. Further studies of additional tRNA elbow modifying enzymes (for example, dihydrouridine synthases) should examine the impacts of these enzymes for global tRNA abundance, aminoacylation, modification, and global and codon-specific translation. Importantly, similar experiments should be performed in human cells to examine cellular roles of tRNA elbow modification enzymes in humans, in particular, for those enzymes with disease relevance. Thus, the broad implications and far-future objectives of the work presented here sets the stage for a complete understanding of the physiological relevance of tRNA modifying enzymes and methods to target those relevant for disease.

### 8.7 References


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**Appendix 1 – Supplemental Information for Chapter 6:**

**Molecular mechanism of tRNA binding by the *Escherichia coli* N7 guanosine methyltransferase TrmB**

**Table A1.1. Average apparent rates ($k_{app}$) for the association of TrmB variants and fluorescein-s$^4$U8-tRNA$^{Phe}$.** Rates were determined by fitting data shown in Figure 6.6 with 1-, 2-, or 3-exponential equations. $k_{app}$ increase refers to an event wherein fluorescence is increased, whereas $k_{app1}$ and $k_{app2}$ correspond to decreases in fluorescence.

| | $k_{app}$ increase ($s^{-1}$) | $k_{app1}$ ($s^{-1}$) | $k_{app2}$ ($s^{-1}$) |
|---|---|---|---|
| TrmB D144A | N/A | 62 ± 5 | N/A |
| TrmB T217A | N/A | 8 ± 1 | 0.2 ± 0.02 |
| TrmB R26A | N/A | 2 ± 0.2 | 0.04 ± 0.002 |
| TrmB R155A | 176 ± 46 | 2 ± 1 | 0.03 ± 0.002 |

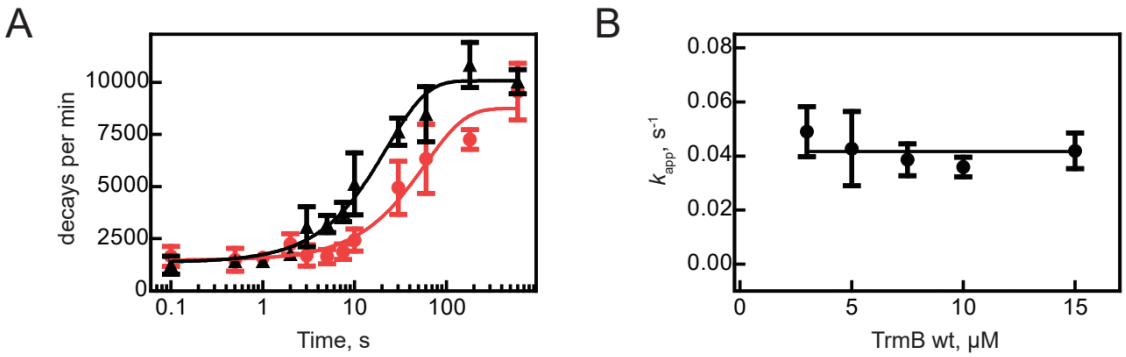



**Figure A1.1. Rapid tRNA methylation by TrmB at 20°C. (A)** Time courses of tRNA methylation are recorded using a quench-flow apparatus. TrmB (5 μM) in the presence of 50 μM SAM is rapidly mixed with 1 μM of unmodified *in vitro* transcribed $tRNA^{Phe}$ (black triangles) or fluorescein-$s^4U8$-$tRNA^{Phe}$ (red circles) at 20°C. Error bars display the average of three replicates. Both time courses were fit with a 1-exponential equation to determine the apparent rate of methylation ($k_{app}$), which was determined to be 0.04 ± 0.01 $s^{-1}$ for unmodified $tRNA^{Phe}$ and 0.02 ± 0.01 $s^{-1}$ for fluorescein-$s^4U8$-$tRNA^{Phe}$. **(B)** Concentration dependence of tRNA methylation by TrmB. Time courses of unmodified tRNA methylation by TrmB were fit with single-exponential equations, and the resulting $k_{app}$ was plotted against TrmB concentration. Apparent rates are not dependent on TrmB concentration, and the horizontal line represents an average $k_{app}$ of 0.04 ± 0.01 $s^{-1}$.

## Appendix 2 – Extended Data for Chapter 7:

## Modifications in the T arm of tRNA globally determine tRNA maturation, function and cellular fitness

**Table A2.1. Oligonucleotides used in this study (primers and Northern blotting probes)**

| Name | Sequence |
|---|---|
| *trmA* left | 5'-GAGCAATCCCTACAATCGCCGCGTACTTTAATTTTTCAGGATACATCATGATTCCGGGGATCCGTCGACC-3' |
| *trmA* right | 5'-GCCGGATGCTGGCGCATCCGGCATGGGTTTTACTTCGCGGTCAGTAATACTGTAGGCTGGAGCTGCTTCG-3' |
| *truB* left | 5'-CATGACGAAGAACGTCGTGTTAACCCGGACGACAGCAAGGAGGACTAATGATTCCGGGGATCCTTCGACC-3' |
| *truB* right | 5'-AGCGACCTGTTATCGCAAGACGGTTAACATTACGCCGGGTATTCAACCACTGTAGGCTGGAGCTGCTTCG-3' |
| Upstream *trmA* | 5'-GGCTGTTCAAAACGGTTAGC-3' |
| Downstream *trmA* | 5'-GCTGTTTAGCTCCATTGTGCC-3' |
| Upstream *truB* | 5'-ATCAGGTCAACGTGAACAGC-3' |
| Downstream *truB* | 5'-CGAGCAGTTTACGACGCTGAG-3' |
| Upstream *thiI* | 5'-TAACGCAGGCTTCGAGTTGC-3' |
| Downstream *thiI* | 5'-GCACTCTGCTGGTTGAAACC-3' |
| Upstream *tapT* | 5'-CAGGTTGTCGACATGCTTAATGG-3' |
| Downstream *tapT* | 5'-AGTACCGGTCCGTTAAAGC-3' |
| k1 (kan internal forward) | 5'-CAGTCATAGCCGAATAGCCT-3' |
| k2 (kan internal reverse) | 5'-CGGTGCCCTGAATGAACTGC-3' |
| fluc-fdhF-rluc sense | 5'-GTACTCCATGGATGGAAGACGCCAAAAACATAAAGAAAGGC-3' |
| fluc-fdhF-rluc antisense | 5'-CATATGGATCCTTATTGTTCATTTTTGAGAACTCGCTCAACG-3' |
| fdhF130-179UUC sense | 5'-GCTCGTGTCTTCCACGGCCCATCGGTTGC-3' |
| fdhF130-179UUC antisense | 5'-GCCGTGGAAGACACGAGCGCAGCAGTCAACG-3' |
| Cy5 tRNAPhe RT primer | 5'-/Cy5/TGGTGCCCGGACTCGG-3' |
| tRNAPhe biotin | 5'-/5Biosg/TGGTGCCCGGACTCGGAATCGAAC-3' |
| tRNAAsp biotin | 5'-/5Biosg/TGGCGGAACGGACGGGACTCG-3' |
| tRNAIle(CAT) biotin | 5'-/5Biosg/TGGTGGCCCCTGCTGGACTTGAACC-3' |
| tRNAArg(CCT) biotin | 5'-/5Biosg/TGGTGTCCCCTGCAGGAATCG-3' |

| | |
|---|---|
| tRNAArg(ACG) biotin | 5'-/5Biosg/TGGTGCATCCGGGAGGATTCG-3' |
| tRNALeu(CAG) biotin | 5'-/5Biosg/TGGTGCGAGGGGGGGGACTTG-3' |
| tRNALeu(TAG) biotin | 5'-/5Biosg/TGGTGCGGGAGGCGAGACTTG-3' |
| tRNAGln(TTG) biotin | 5'-/5Biosg/TGGCTGGGGTACCTGGATTCG-3' |
| tRNAPro(GGG) biotin | 5'-/5Biosg/TGGTCGGCACGAGAGGATTTG-3' |
| tRNAPro(CGG) biotin | 5'-/5Biosg/TGGTCGGTGATAGAGGATTCGAACCTC-3' |
| tRNAPro(UGG) biotin | 5'-/5Biosg/TGGTCGGCGAGAGAGGATTCGAAC-3' |
| 5S rRNA biotin | 5'-/5Biosg/ATGCCTGGCAGTTCCCTACTCTCGCATGG-3' |

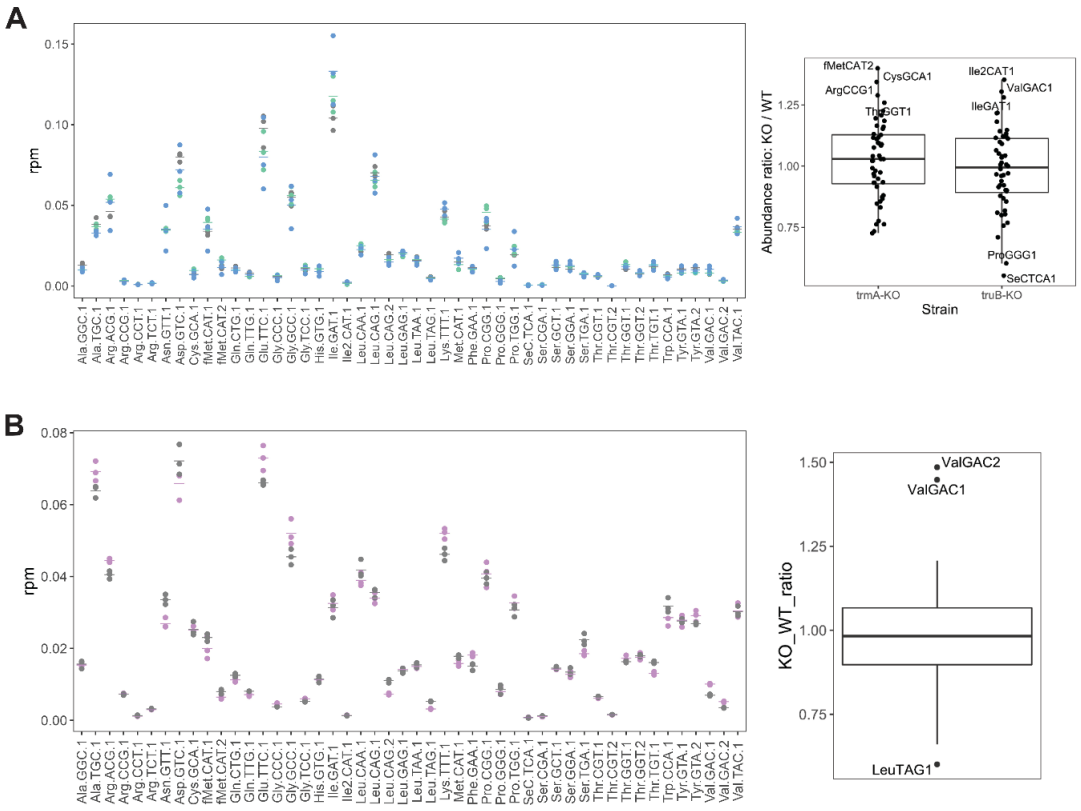



**Figure A2.1. Global tRNA abundance does not change in the absence of *trmA* and/or *truB* observed by RNAseq.** Reads per million (RPM) counted for each tRNA species in wildtype and single *ΔtrmA* and *ΔtruB* knockout (KO) strains **(A)** or wildtype and the double *ΔtrmAΔtruB* strain **(B)**.

**A** Mutation rate at position 34 of $tRNA^{Asp}$(ACG):

| Strain | Mutation rate (avg) | stdev |
|---|---|---|
| WT | 0.979 | 0.002 |
| trmA-KO | 0.987 | 0.001 |
| truB-KO | 0.989 | 0.002 |
| trmAtruB-KO | 0.975 | 0.002 |

**D** Ratio of stops at position 47 vs. position 37 during reverse transcription of $tRNA^{Phe}$:

| Strain | replicate 1 | replicate 2 |
|---|---|---|
| WT | 3.5 | 2.9 |
| *ΔtrmA* | 0.9 | 1.8 |
| *ΔtruB* | 1.8 | 2.0 |
| *ΔtrmAΔtruB* | 0.9 | 1.2 |
| *ΔtrmB* | 1.8 | 3.5 |
| *ΔtapT* | 0 | 0 |

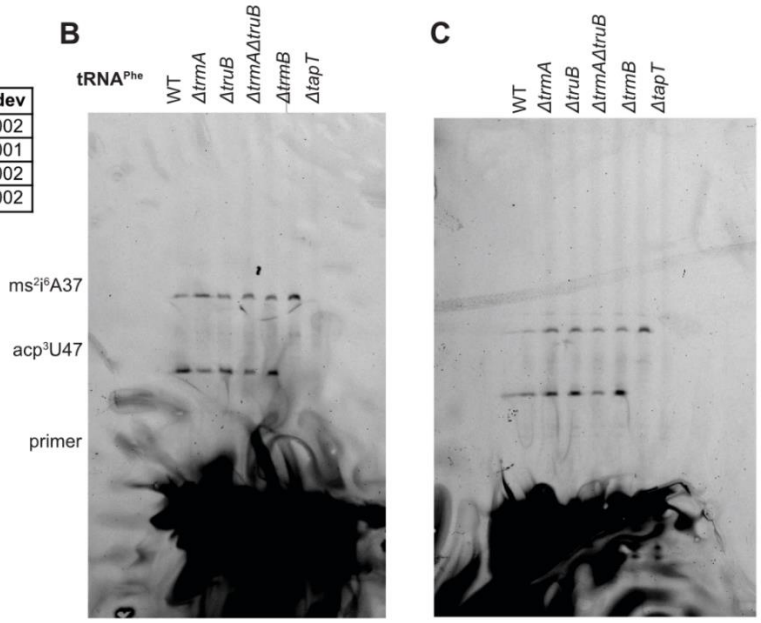



**Figure A2.2. tRNA modifications in the absence of *trmA* and/or *truB*. (A)** Mutation rate observed for position 34 of $tRNA^{Arg}_{ACG}$, which indicates presence of the inosine modification. **(B, C)** Full, unedited gel images for two biological replicates (replicate 1, B; replicate 2, C) for $tRNA^{Phe}$ primer extension to detect $acp^3U47$ modification. To determine the relative $acp^3U47$ content between strains, the reverse transcription stop corresponding to $acp^3U47$ was compared to that for $ms^2i^6A37$ **(D).**

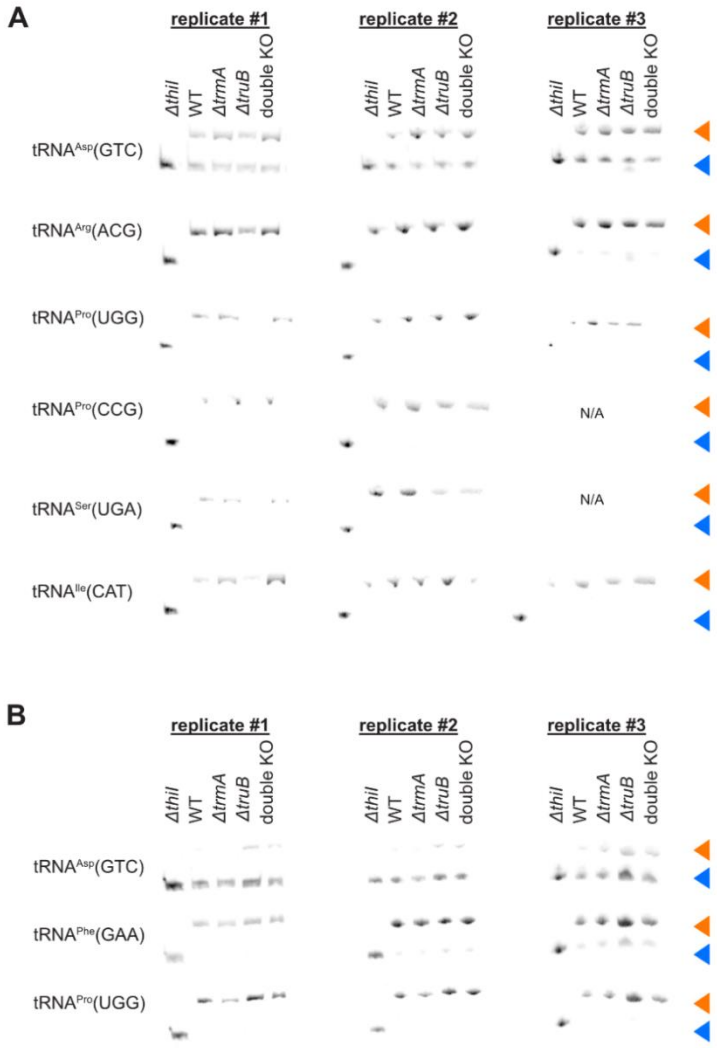
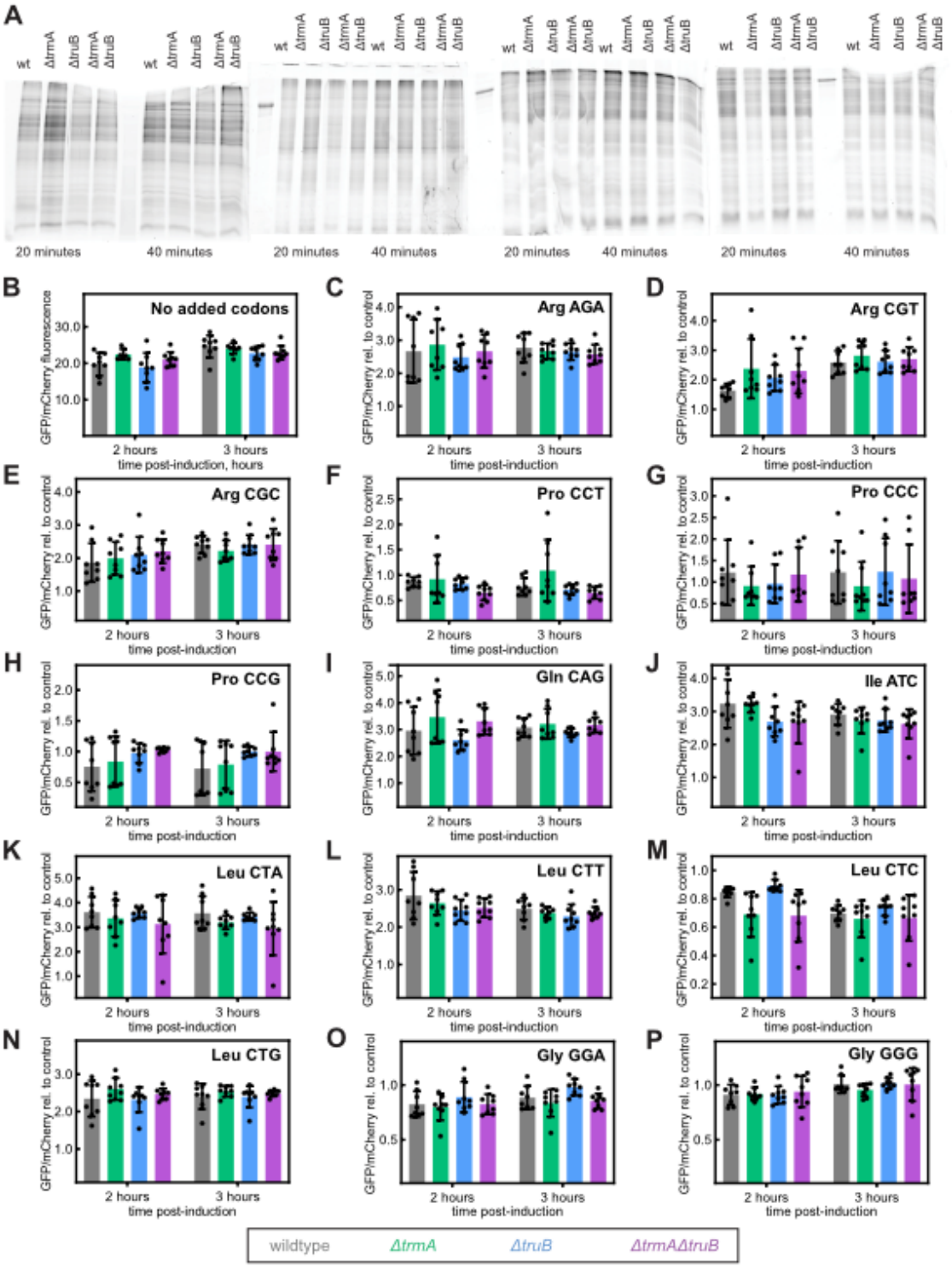
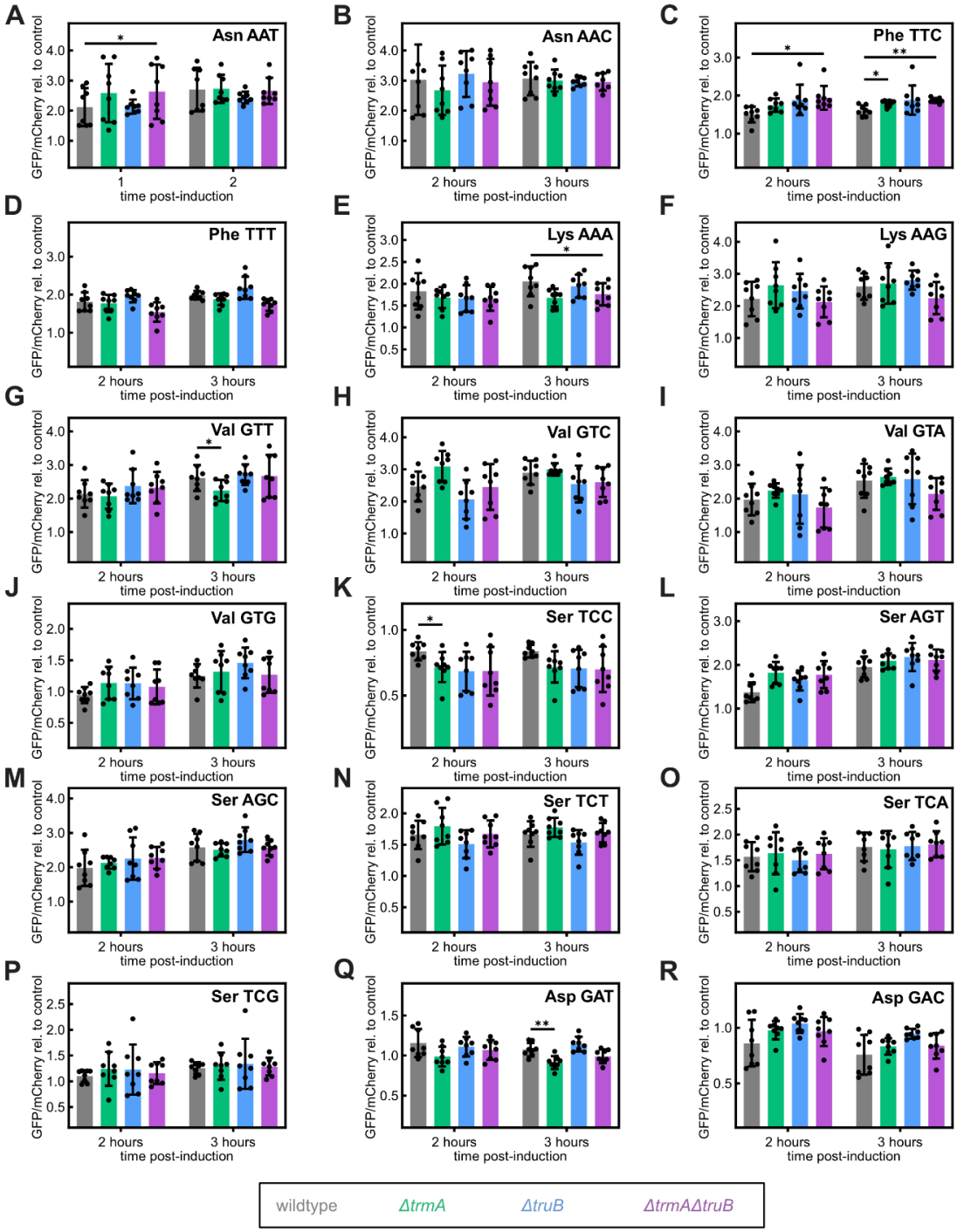



**Figure A2.3. APM Northern blotting to determine the levels of $s^4U8$ in different tRNAs. (A)** *E. coli* strains were grown in rich LB media, total tRNA was extracted and thiolation levels of specific tRNAs were determined by APM-Urea PAGE followed by detection using northern blotting. No significant differences between tRNA thiolation for these tRNAs were identified in this growth condition. **(B)** Thiolation levels of specific tRNAs were determined as in (A) after growing *E. coli* under stress conditions in LB media containing 2% sodium formate. The thiolated tRNA migrates slower and is indicated with an orange arrow in contrast to the non-thiolated form (blue triangle). Thiolation levels were found to be different in *trmA* and *truB* knockout strains compared to wildtype for $tRNA^{Asp}_{GTC}$ (see Figure 7.3).



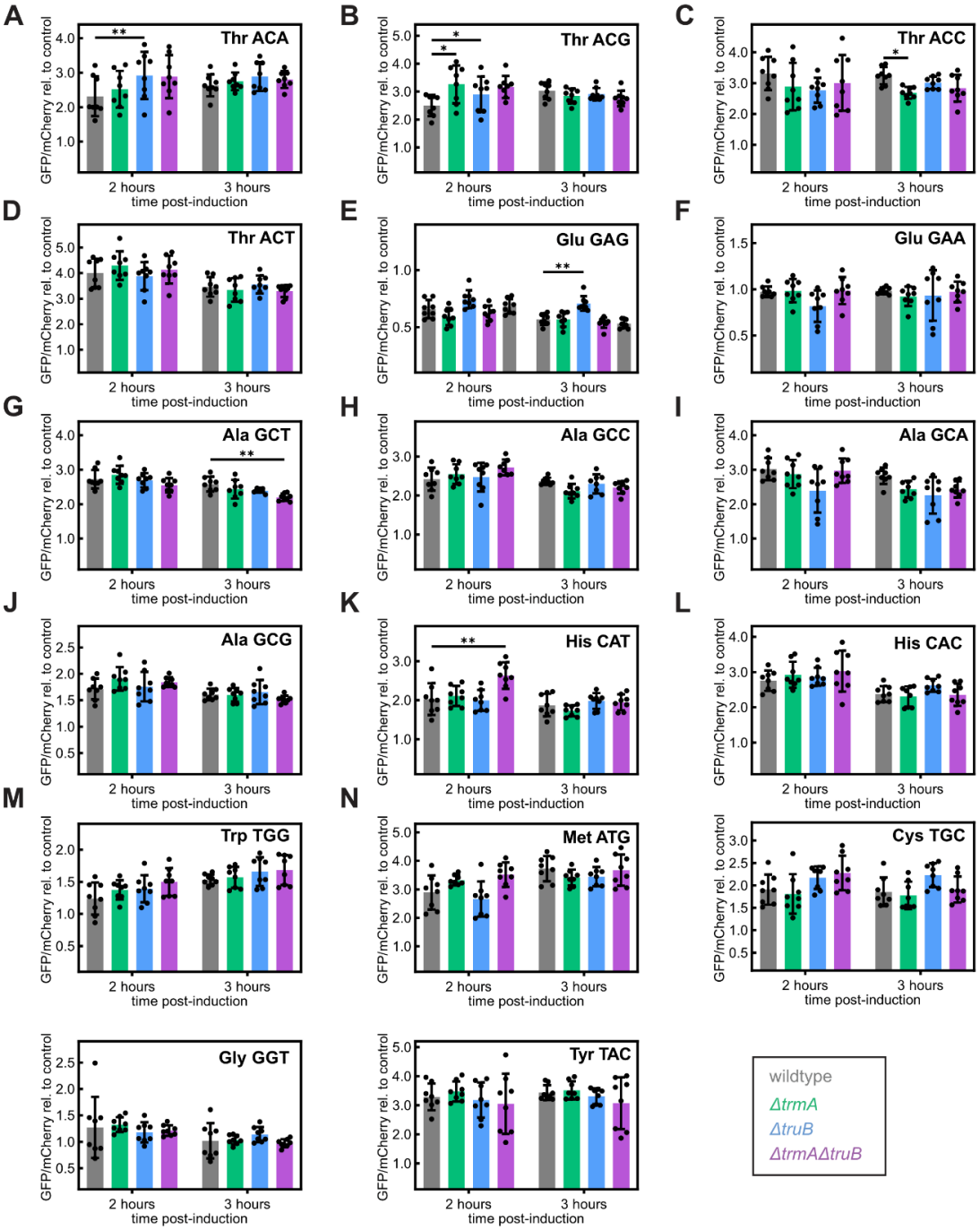

*Caption on next page.*

**Figure A2.4. Absence of TrmA and/or TruB disproportionately affects translation of specific codons (set 1). (A)** Original SDS-PAGEs detecting newly synthesized proteins in the *ΔtrmA*, *ΔtruB*, *ΔtrmAΔtruB* and wild-type *E. coli* strains. **(B to P)** The GFP reporter gene is preceded by four consecutive codons as indicated in the top right for each panel, and GFP expression is compared 2 and 3 hours after induction relative to mCherry expression encoded on the same plasmid. Codon-specific GFP translation is reported for the *ΔtrmA* (green), *ΔtruB* (blue), *ΔtrmAΔtruB* (purple) and wild-type (grey) *E. coli* strains. Significant changes ($p < 0.05$) are marked with an asterisk. In panel (B), a control is shown where no consecutive codons precede the GFP gene showing that general translation is not affected in the knockout strains.




*Caption on next page.*

**Figure A2.5. Absence of TrmA and/or TruB disproportionately affects translation of specific codons (set 2).** The GFP reporter gene is preceded by four consecutive codons as indicated in the top right for each panel, and GFP expression is compared 2 and 3 hours after induction relative to mCherry expression encoded on the same plasmid. Codon-specific GFP translation is reported for the *ΔtrmA* (green), *ΔtruB* (blue), *ΔtrmAΔtruB* (purple) and wild-type (grey) *E. coli* strains. Significant changes ($p < 0.05$) are marked with an asterisk.




*Caption on next page.*

**Figure A2.6. Absence of TrmA and/or TruB disproportionately affects translation of specific codons (set 3).** The GFP reporter gene is preceded by four consecutive codons as indicated in the top right for each panel, and GFP expression is compared 2 and 3 hours after induction relative to mCherry expression encoded on the same plasmid. Codon-specific GFP translation is reported for the *ΔtrmA* (green), *ΔtruB* (blue), *ΔtrmAΔtruB* (purple) and wild-type (grey) *E. coli* strains. Significant changes ($p < 0.05$) are marked with an asterisk.

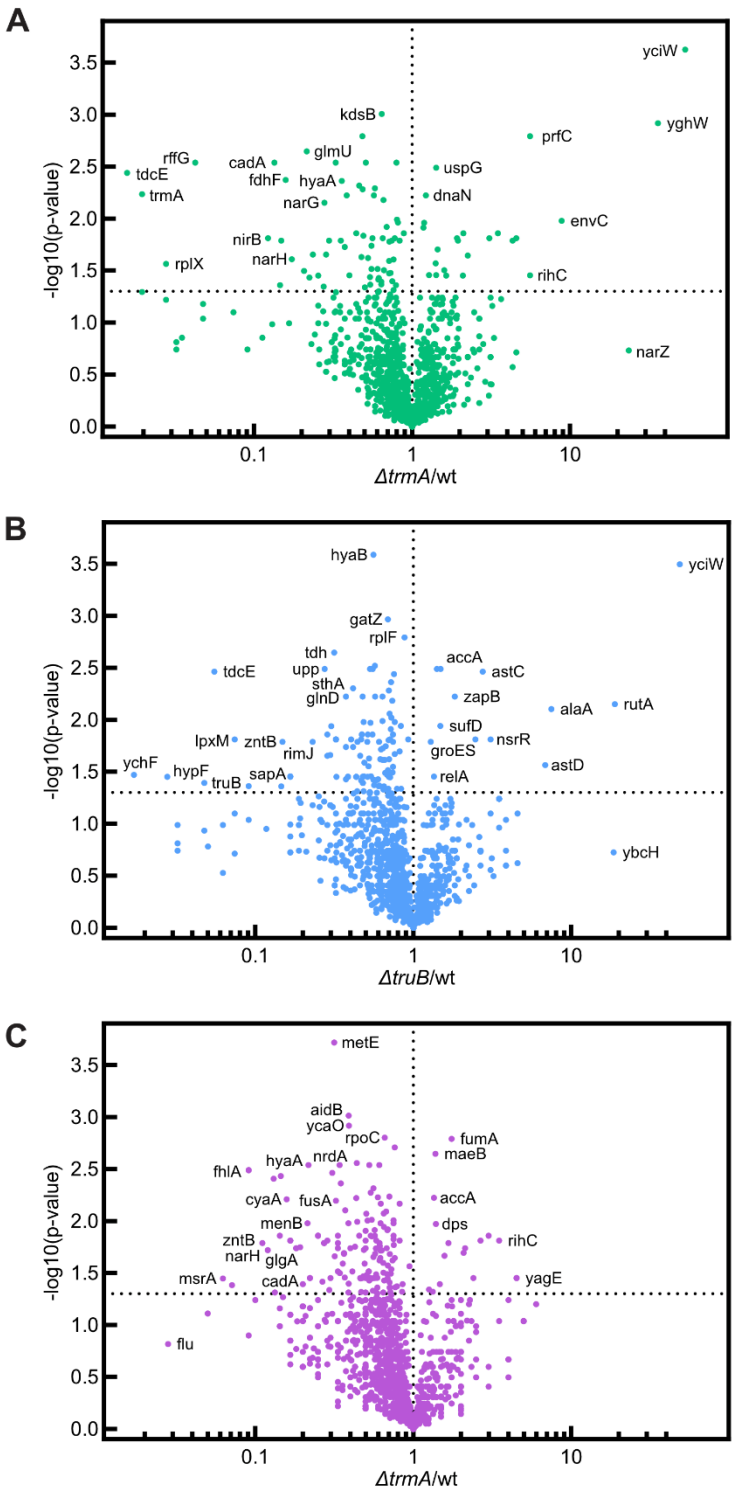



**Figure A2.7. Volcano plots showing the change in relative protein abundance between *E. coli* wildtype and *ΔtrmA* (A), *ΔtruB* (B), and *ΔtrmAΔtruB* (C).**

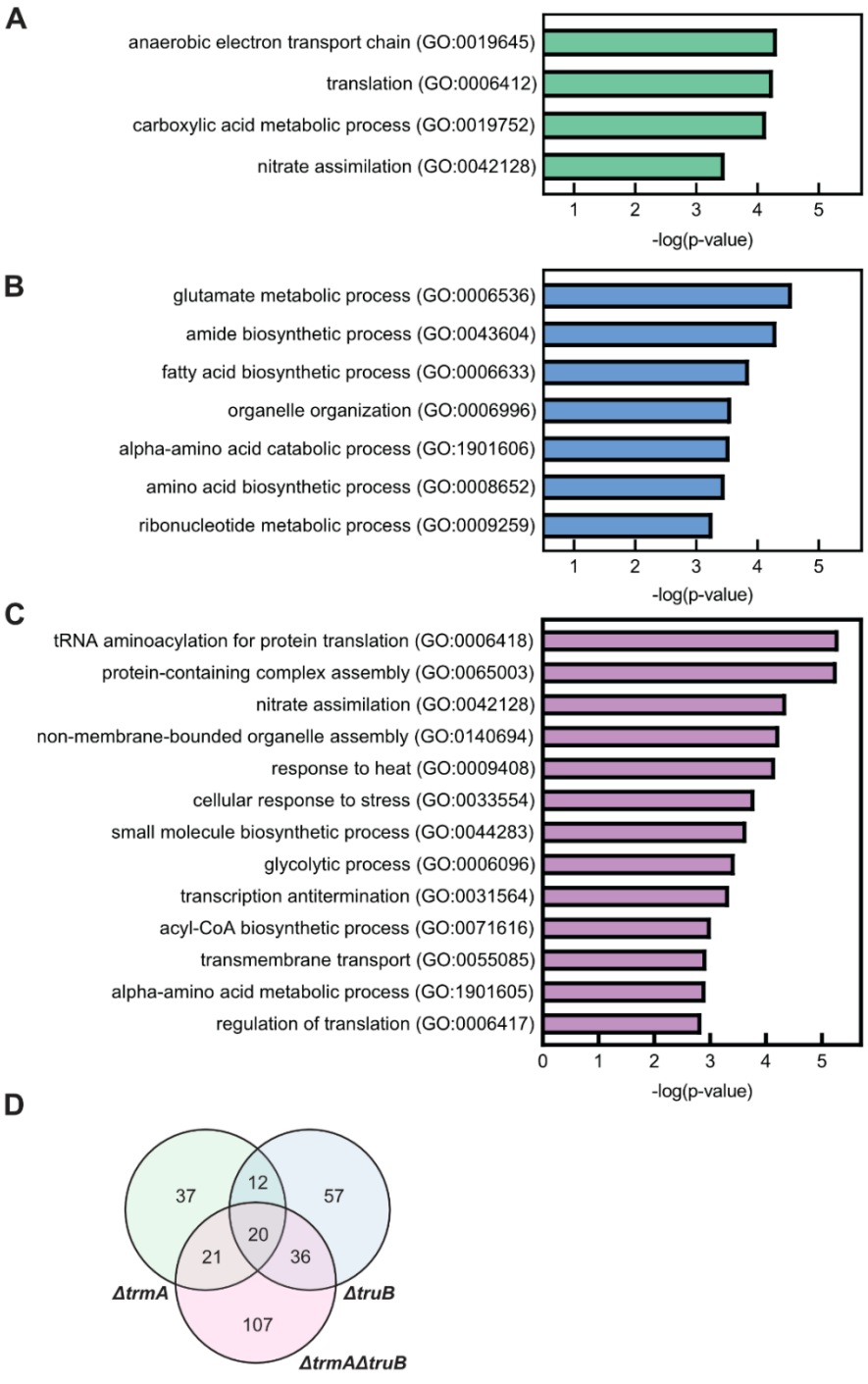



**Figure A2.8. Gene ontology analysis for up/downregulated proteins in *ΔtrmA* (A), *ΔtruB* (B), and *ΔtrmAΔtruB* (C) strains and overlap between dysregulated proteins for each strain (D).**