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

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Almost all elongator tRNAs (Transfer RNAs) harbor 5-methyluridine 54 and pseudouridine 55 in the T arm, generated by the enzymes TrmA and TruB, respectively, in *Escherichia coli*. TrmA and TruB both act as tRNA chaperones, and strains lacking *trmA* or *truB* are outcompeted by wild type. 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 modifications such as acp^{3}U47. While overall protein synthesis is not affected in Δ*trmA* and Δ*truB* strains, the translation of a subset of codons is significantly impaired. As a consequence, we observe translationally reduced expression of many specific proteins, that are either encoded with a high frequency of these codons or that are large proteins. The resulting proteome changes are not related to a specific growth phenotype, but overall cellular fitness is impaired upon deleting *trmA* and *truB* in accordance with a general protein synthesis impact. 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 irrespective of the growth conditions which explains the conservation of *trmA* and *truB*.

tRNA modification | pseudouridine | methylation | aminoacylation | protein synthesis

Significance

tRNAs (Transfer RNAs) are the most highly and diversely modified RNAs across all domains of life, and cells invest significantly in synthesizing numerous tRNA-modifying enzymes. Nevertheless, decades-long research has not resolved the paradox that deletion of many tRNA-modifying enzymes causes no obvious phenotype. Here, we identify the biological function of TrmA and TruB generating the most conserved modifications, 5-methyluridine 54 and pseudouridine 55 in the T arm of almost all tRNAs. Unlike other studied modification enzymes, TrmA and TruB globally enhance modification, folding, and aminoacylation of all tRNAs. Additionally, they promote codon- and protein-specific translation explaining their importance for cellular fitness across many growth conditions. The impact on gene expression of TrmA and TruB clarifies their role in human diseases.

Transfer RNAs (tRNAs) are the most densely and most diversely modified class of RNA, and all organisms invest significant energy into synthesizing many 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 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^{5}U) 54 and pseudouridine (Ψ) 55 (*SI Appendix*, Fig. S1). 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^{5}U54 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 in almost all bacteria and eukaryotes (8, 9), although some archaea and diverse bacteria utilize alternative enzymatic strategies to introduce m^{5}U54 and Ψ55, for example, with the enzymes TrmFO and Pus10, respectively (4, 10).

Highlighting the importance of TrmA and TruB and their respective modifications, m^{5}U54 and Ψ55 are the only two modifications found within every *E. coli* tRNA (9, 11). In this model organism, the *trmA* and *truB* genes are nonessential under ideal and several stress conditions but are important for bacterial fitness in coculture experiments (12–15). In addition to introducing stabilizing modifications within tRNAs (3), TrmA and TruB act as tRNA chaperones, folding tRNA independently of their modification activity (13, 14, 16). The tRNA chaperone activity of TruB is responsible for optimizing fitness in *E. coli* (12, 14), whereas TruB pseudouridylation is important for growth at cold temperatures in *Thermus thermophilus* (17), heat shock recovery in *E. coli* (18), and *Shigella flexneri* virulence (19). Both the chaperone and modification activities of TrmA play roles in *E. coli* fitness (13), but the methylation activity seems dispensable in yeast (15, 20). Taken together, the conserved enzymes TrmA and TruB are bifunctional enzymes that play 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 biomarker for the recurrence of certain cancers and a potential target for polyQ diseases (21, 22). Moreover, the TruB homologs, TRUB1/2,

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are linked to maternally inherited diabetes through a mitochondrial $tRNA^{Glu}$ mutation that prevents tRNA pseudouridylation (23, 24). 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 (18, 19).

To identify the molecular and cellular function of TrmA and TruB contributing to cellular fitness, 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* Δ*trmA* and Δ*truB* knockout strains. Thereby, this work highlights that the 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.

Results

tRNA T arm Modification Globally Enhances Aminoacylation. To dissect the roles of TruB and TrmA for tRNA function, we used *E. coli* Δ*truB* and Δ*trmA* strains from the Keio Collection (25) and prepared a double-knockout strain lacking both genes. We confirmed that the double Δ*trmA*Δ*truB* knockout strain is outcompeted by *E. coli* wild-type (WT) similar to the single deletion strains (*SI Appendix*, Fig. S2*A*). Next, we analyzed growth under stress conditions to uncover a potential, previously overlooked phenotype using Biolog phenotypic screen assessing hundreds of conditions (Dataset S1). In agreement with previous reports (12, 18, 19), the knockout strains grew similar to WT in most cases. For specific growth conditions, we checked the Biolog results both in liquid culture and on spot plates. However, we did not identify a phenotype, where the WT strain grows better than the deletion strains (*SI Appendix*, Fig. S2 *B* and *C*). Accordingly, we conclude that *trmA* and *truB* are generally important for cellular fitness irrespective of the cellular growth conditions.

Previously, TrmA and TruB chaperone activity was assessed in vitro by detecting tRNA aminoacylation, since only correctly folded tRNA is efficiently charged (13, 14, 26). Therefore, we hypothesized that the deletion of *trmA* and/or *truB* increases the fraction of misfolded tRNA and decreases the aminoacylation levels of some or all tRNAs in vivo. To quantitatively compare 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 (27).

First, we asked whether TrmA and/or TruB and their resulting modifications promote the cellular stability and thereby steady-state abundance of tRNAs in vivo as observed for other tRNA-modifying enzymes (1, 28, 29). Within the single Δ*trmA* and Δ*truB* strains, no tRNA is decreased or increased more than 1.5-fold relative to WT and on a global level, tRNA abundance is unchanged compared to WT (Fig. 1 *A* and *B*, *SI Appendix*, Fig. S3, and Dataset S2). In the double-knockout strain, only two tRNAs, $tRNA^{Leu}_{TAG}$ and $tRNA^{Leu}_{CAG\text{-}2\text{-}1}$, are downregulated by ~1.5-fold compared to the WT strain (Fig. 1*C*). 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 $tRNA^{Leu}_{CAG\text{-}1\text{-}1}$ (Fig. 1*D*). Thus, TrmA and TruB have only minimal effects on the overall cellular stability of tRNAs in *E. coli*.

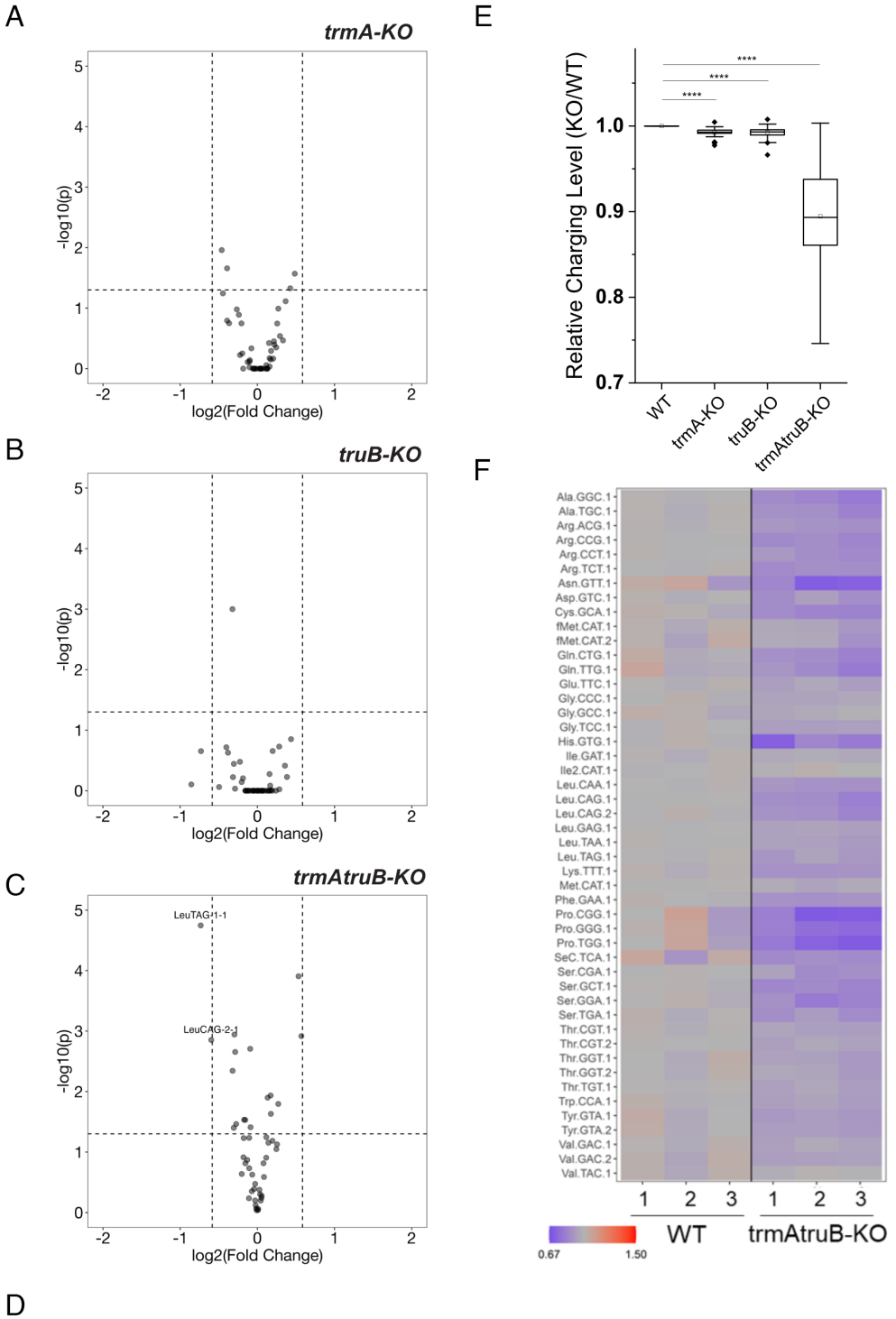
In contrast, a comparison of aminoacylation levels in the absence of *trmA* or *truB* reveals a significant global decrease in tRNA charging in the Δ*truB* and Δ*trmA* strains, with more pronounced effects in the double-knockout strain (Fig. 1 *E* and *F* and Dataset S3). In the Δ*trmA*Δ*truB* strain, tRNA aminoacylation is on average 10% lower than in the WT strain, suggesting additive effects of removing both TrmA and TruB (Fig. 1*E*). Almost all individual tRNAs are less charged, but changes in charging level are variable (Fig. 1*F*), supporting our hypothesis that TrmA and TruB affect tRNA charging of almost all tRNAs in vivo.

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. 2*A*) cause mutations or deletions during RT when a thermostable reverse transcriptase (Superscript IV) is used, the tRNA sequencing dataset detects changes in the relative modification level in the knockout strains compared to WT (27). Comparing the Δ*trmA* strain to WT *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^5U54 (Fig. 2 *B* and *C*). Similar, further reduced mutation and deletion frequencies at U47 are observed for the Δ*trmA*Δ*truB* strain; however, single knockout of *truB* does not affect acp^3U47 content in most tRNAs with the exception of $tRNA^{Phe}$ (Fig. 2*C*), which we validated by primer extension using a reverse transcriptase with low processivity that stops at the bulky acp^3U47 modification (Fig. 2*D* and *SI Appendix*, Fig. S4 *B*–*D*).

Additionally, the tRNA sequencing data indicate potential changes in anticodon loop modifications. Particularly in the absence of *trmA,* there are several changes at the conserved purine 37 adjacent to the anticodon within several tRNAs known to contain ms^2i^6A (Fig. 2 *E* and *F*). A smaller effect on ms^2i^6A37 is observed in the Δ*truB* and double-knockout strains. Only *E. coli* $tRNA^{Arg}_{ACG}$ contains the edited base inosine 34, which generates a mutation during RT. This mutation is detected in all strains, suggesting the absence of *trmA* and/or *truB* does not affect tRNA deamination (*SI Appendix*, Fig. S4*A*).

Finally, we also wondered whether other common modifications near 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. 2*G*) abundance is dependent on TrmA or TruB by using an APM-urea-PAGE and Northern blotting (30). For many tRNAs, a nearly stoichiometric level of tRNA thiolation is observed in the absence and presence of TrmA and TruB (*SI Appendix*, Fig. S5). In contrast, $tRNA^{Asp}$ was only ~30 to 40% thiolated in the WT strain; however, tRNA thiolation was found to increase to ~50% in the Δ*truB* strain and to ~60% in the Δ*trmA* and Δ*trmA*Δ*truB* strains (Fig. 3*H*), suggesting the absence of TrmA and TruB promotes s^4U8 formation in $tRNA^{Asp}$ 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.

TruB and TrmA Affect Translation of Specific Codons. Since tRNA aminoacylation and modification are affected in the absence of TrmA and TruB, we next investigated how cellular protein synthesis is impacted. To assess potential effects on global translation, we utilized biorthogonal noncanonical amino acid tagging (31). Quantification of new proteins does not reveal statistically significant changes in overall translation between *E. coli* WT and the Δ*trmA*/Δ*truB* strains (*SI Appendix*, Fig. S6 *A* and *B*). To understand whether *trmA* and *truB* affect synthesis of specific proteins, we analyzed variations in



D

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Leu-CAG-1-1, GUCCUUACGGAC, ΔG = -3.76 kcal/mol
Leu-CAG-2-1, GUUCUUACGGAC, ΔG = -1.10 kcal/mol
Leu-GAG-1-1, GCCCAAUAGGGC, ΔG = -5.93 kcal/mol
Leu-TAG-1-1, GCCGCAAGGU,   ΔG = -2.24 kcal/mol
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Fig. 1. tRNA aminoacylation is reduced globally in *trmA* and *truB* knockout strains. tRNA abundance change in single- and double-knockout strains depicted as volcano plots (n = 3 for each strain). Vertical dashed lines indicate threshold change of 1.5-fold, horizontal dashed lines indicate $P = 0.05$. (*A*) *ΔtrmA*; (*B*) *ΔtruB*; (*C*) *ΔtrmAΔtruB*. Abundance comparisons for each tRNA isoacceptor and for global tRNA abundance are present in *SI Appendix*, Fig. S3. (*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 WT, *ΔtrmA*; *ΔtruB*; and *ΔtrmAΔtruB* strains. Box and whisker plot, $^{****}P < 10^{-4}$ (n = 3 for each strain). (*F*) Heatmap of charging level change for each tRNA in *ΔtrmAΔtruB* over *E. coli* BW25113 WT showing each biological replicate. The data for tRNA abundance and charging levels can be found in Datasets S2 and S3, respectively.

translation for all 61 sense codons in addition to UGA stop codon recoding by the selenoprotein machinery. To measure UGA stop codon read-through by $tRNA^{Sec}$ in response to a selenocysteine insertion sequence (SECIS) element, we used a luciferase reporter where the UGA codon and the SECIS element of the *E. coli fdhF* gene are placed between Firefly and *Renilla* luciferase genes (32); however,

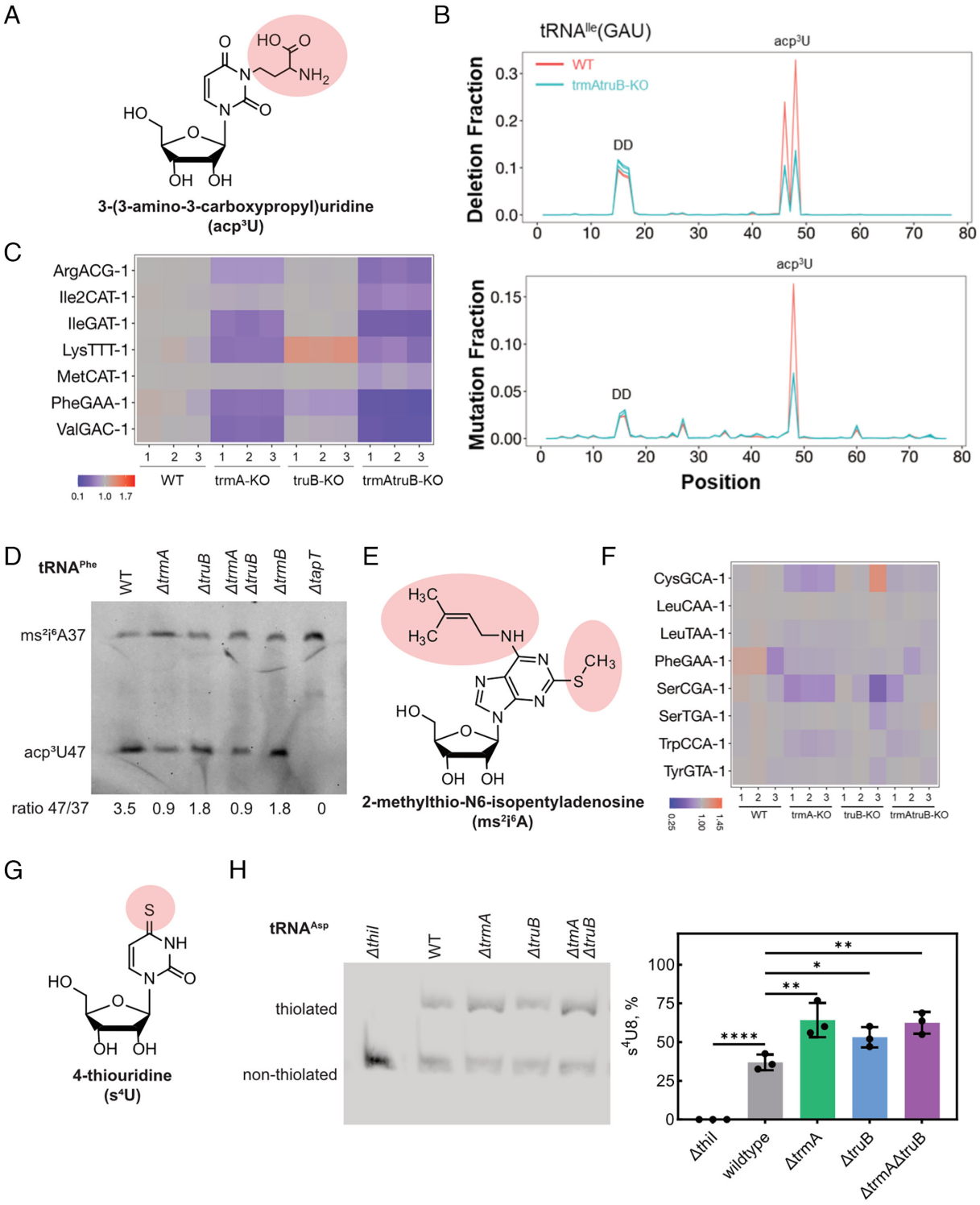

Fig. 2. Deletion of *trmA* and/or *truB* changes abundance of additional tRNA modifications. (*A*) Chemical structure of acp^{3}U with modified portions highlighted in red. (*B*) Mutation and deletion fractions of tRNA^{Ile}(GAU) reads in WT and *ΔtrmAΔtruB* strains. Deletion of *trmA* and *truB* (cyan) lowers the deletion (*Top*) and mutation (*Bottom*) fraction at position 47 compared to the WT (pink). (*C*) Heatmap showing changes in the sum of the deletion and mutation fraction at position 47 relative to WT for all *E. coli* tRNAs known to contain acp^{3}U47. (*D*) Primer extension of tRNA^{Phe} validates the decrease in acp^{3}U47 content in the absence of *trmA/truB*. Numbers underneath each lane denote the intensity ratio for the bands corresponding to reverse transcription stops at position 47 vs. 37. Full gel and replicate are in *SI Appendix*, Fig. S4 *B–D*. (*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 Lysogeny Broth (LB) medium containing 2% sodium formate was detected by [(N-acryloylamino) phenyl]mercuric chloride (APM) urea-PAGE followed by Northern Blotting for tRNA^{Asp} (*Left*) and quantified based on the band intensities of the thiolated and nonthioated form from three biological replicates (*Right*). **** indicates $P < 10^{-4}$, ** indicates $P < 0.01$, and * indicates $P < 0.05$. No changes in s^{4}U8 content were found for other tested tRNAs (*SI Appendix*, Fig. S5).

no significant differences in selenocysteine incorporation were noticed in the absence of TrmA or TruB (*SI Appendix*, Fig. S6*C*). To dissect whether and how changes in tRNA maturation and charging affect protein synthesis in a sense codon-specific manner, we utilized a series of reporter constructs where green fluorescent protein (GFP) is preceded by four identical codons (33). As an internal reference,

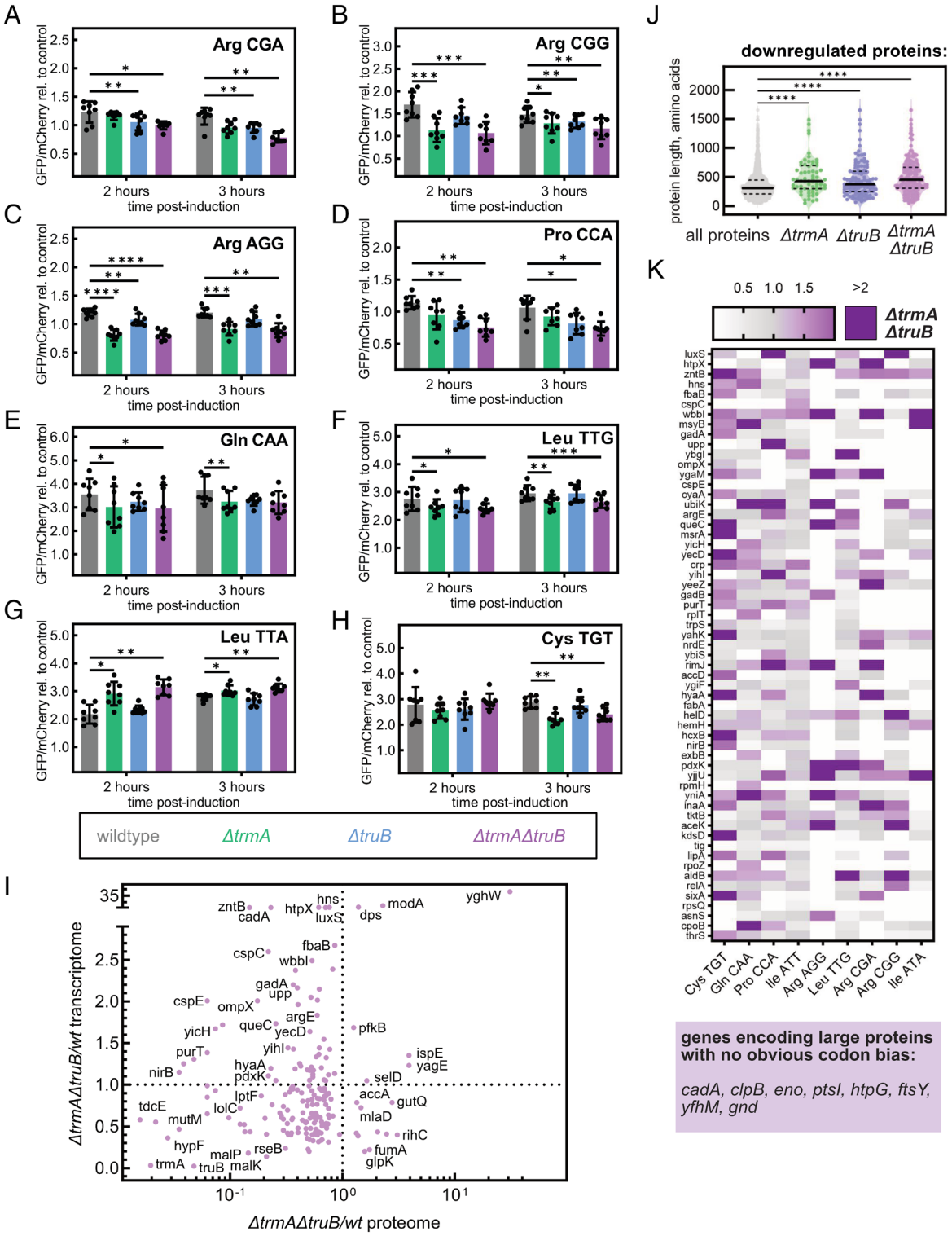

Fig. 3. TrmA and/or TruB differentially affect translation of specific codons and proteins. (*A*–*H*) Measurements of codon-specific translation using green fluorescent protein (GFP) reporters containing four tandem repeats of the indicated codon (*SI Appendix, SI Methods*). Relative translation measurements for the remaining 53 sense codons are present in *SI Appendix*, Figs. S6–S9. Eight biological replicates were performed for each strain; * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$. (*I*) Changes in gene expression in the double *ΔtrmAΔtruB* deletion strain. Each data point represents a protein with a significantly ($P < 0.05$) altered abundance within the knockout compared to WT, with fold protein change (knockout/WT) indicated on the *x*-axis and the respective mRNA fold change (knockout/WT) on the *y*-axis. Proteomics and transcriptomics experiments are the average of four and three biological replicates, respectively. Changes in gene expression for *trmA* and *truB* single-knockout strains are present in *SI Appendix*, Fig. S12. (*J*) Protein lengths in amino acids for significantly ($P < 0.05$) down-regulated proteins compared to WT within *ΔtrmA* (green, n = 65, median = 428 amino acids), *ΔtruB* (blue, n = 111, median = 375 amino acids), and *ΔtrmAΔtruB* (purple, n = 164, median = 453 amino acids) strains. The median protein size of downregulated proteins within each strain is significantly ($P < 0.001$) larger compared to the median size for all proteins detected by our mass spectrometry experiments (gray, n = 1,951, median = 309 amino acids). Solid lines represent median and dashed lines represent quartiles. No increase in median protein size is observed for proteins upregulated within the knockout strains (*SI Appendix*, Fig. S14). (*K*) Heat map showing the relative codon frequencies (codon frequency per 1,000 for given gene/codon frequency per 1,000 within *E. coli* genome; see *SI Appendix, SI Methods*) for down-regulated proteins with up-regulated mRNAs (*Top Left Quadrant* of panel *I*) for codons found to be poorly translated in the *ΔtrmAΔtruB* knockout strain. Genes listed in the box below heat map are large proteins (>447 amino acids, 75th percentile or above for proteins detected in our mass spectrometry experiments) with no increases >1.5-fold for any poorly translated codons. Similar analysis for downregulated proteins in *trmA* and *truB* single deletion strains is present in *SI Appendix*, Fig. S14. Full codon analyses can be found in Dataset S5.

mCherry is expressed from the same plasmid. To determine codon-specific changes in translation, we monitored the ratio of GFP and mCherry fluorescence in strains lacking *trmA* and/or *truB* (Fig. 3 *A–H* and *SI Appendix*, Figs. S6–S9). No significant differences between the *E. coli* WT and the deletion strains were observed for most codons, in accordance with the observation that global translation is unaffected. However, repeats of specific codons lead to altered expression of GFP in the knockout 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 CGT codons, which are read by the same tRNA ($tRNA^{Arg}_{ACG}$) (19). We detect a similar decrease in translation for only four consecutive CGA codons in strains lacking *truB* (Fig. 3*A*) and similarly no effect for consecutive Arg CGT codons (*SI Appendix*, Fig. S6*G*). 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. 3 *B* and *C*).

In the Δ*truB* and Δ*trmA*Δ*truB* knockouts, the proline CCA codon (read by $tRNA^{Pro}_{TGG}$) displays significantly lower GFP production (Fig. 4*D*). Less pronounced, but still significant decreases in translation are noted in the absence of the *trmA* gene for the Gln CAA codon (read by $tRNA^{Gln}_{TTG}$) (Fig. 3*E*). 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 $tRNA^{Leu}_{CAA}$) in the absence of *trmA*, but increased for Leu TTA codons (read by $tRNA^{Leu}_{TAA}$; Fig. 4 *F* and *G*). For the Cys TGT codon, significantly decreased GFP expression is noted in the strains lacking *trmA* after 3 h (Fig. 4*H*). Altered translation was additionally found for several other codons (*SI Appendix*, Figs. S6–S9). In conclusion, although translation of many codons is not affected in the absence of *trmA* and/or *truB,* translation of certain codons is enhanced or decreased.

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 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 WT (Fig. 3*I*, *SI Appendix*, Figs. S10–S13, and Dataset S4). These experiments reveal several changes between WT and deletion strains in the transcriptome and proteome. At the transcriptome level, approximately the same number of genes are increased and decreased within each knockout strain compared to the WT strain (*SI Appendix*, Fig. S10). In contrast, at the proteome level, many proteins have a decreased abundance within the deletion strains compared to the WT strain, and a relatively fewer number of proteins are found to be increased in the knockout strains (*SI Appendix*, Fig. S11). For many genes with decreased protein abundance, the respective transcript levels are increased, suggesting deficiencies in translation in the absence of *trmA* and/or *truB;* in contrast, only few proteins increase in abundance despite decreased transcript levels (Fig. 3*I* and *SI Appendix*, Fig S12).

To shed light on the cellular impact of these transcriptome and proteome changes, we analyzed gene ontology (GO) terms (*SI Appendix*, Fig. S13). For the mRNA changes, we detect effects on several processes (*SI Appendix*, Fig. S13 *A–C*). For the down-regulated protein, no significant GO terms were found for the Δ*truB* strain, and in the Δ*trmA* strain, only the anaerobic electron transport chain was significantly affected. Several processes displayed reduced proteins in the Δ*trmA*Δ*truB* double-knockout strain, in particular, processes related to nitrogen and nitrate metabolism although we did not detect an associated phenotype (*SI Appendix*, Figs. S13*D* and S2). Similar to the strain-specific features observed in the codon-specific GFP translation assays, instances of strain-specific changes in mRNA and protein abundances are evident (*SI Appendix*, Fig. S13 *E–H*). Overall, these findings are in line with our observation that global translation is not affected upon deleting *trmA* or *truB* but that translation of individual codons is impaired, resulting in specific proteome changes that cause the previously reported decrease in cellular fitness (13, 14).

We tested several hypotheses to explain why specific proteins are down-regulated upon *trmA* and/or *truB* deletion. As the observed translational effects are subtle, we first tested whether these effects are most prominent on the synthesis of large proteins leading to their decrease in the *E. coli* proteome. Indeed, the median protein length among proteins that are down-regulated in each of the three deletion strains is significantly larger than that of the median length for all proteins detected in our mass spectrometry experiments (Fig. 3*J*), suggesting that translation of large proteins is impaired. Second, we analyzed all down-regulated proteins for their codon content to test whether less efficiently translated codons such as Cys TGT, Gln CAA, Pro CAA, and Arg CGA and CGG are more frequent among these

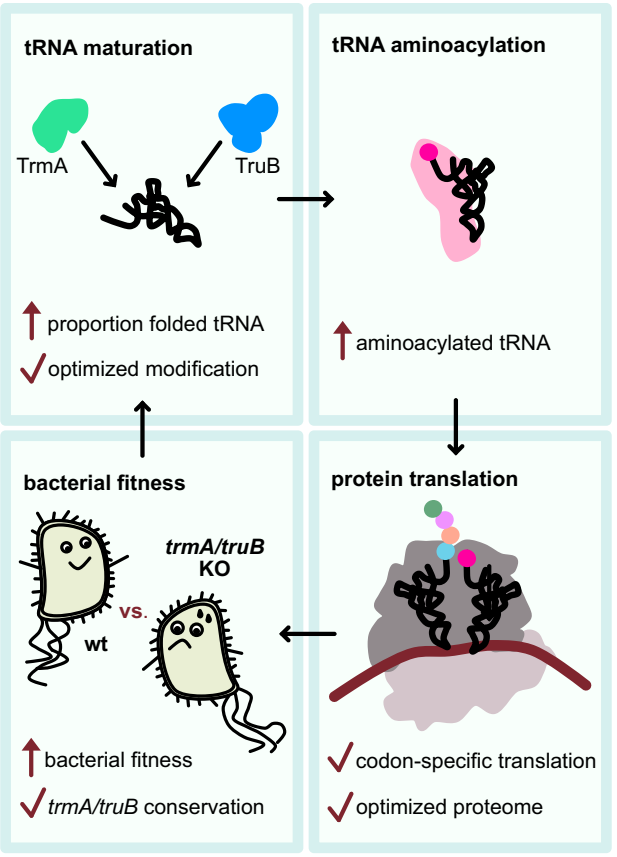

Fig. 4. Molecular and cellular function of the tRNA modification enzymes TrmA and TruB. By interacting with the T arm of all tRNAs, these enzymes enhance tRNA maturation by introducing m^5U54 and Ψ55, respectively, promoting tRNA folding, and modulating the formation of several other tRNA modifications. Thereby, they globally increase the levels of tRNA aminoacylation. While 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.

proteins (Fig. 3*K*, *SI Appendix*, Fig. S14, and Dataset S5). This analysis revealed that almost all down-regulated proteins with up-regulated mRNAs are either large or have coding sequences where these less efficiently translated codons are more frequent than usual. Third, we assessed whether the decreased aminoacylation of tRNAs in the deletion strains triggers the bacterial stringent response pathway (*SI Appendix*, Fig. S15), which has been previously observed for bacterial strains lacking the tRNA modification enzyme TrmD (34). Compared to overexpression of RelA, similar yet smaller changes in mRNA and protein changes were observed in the absence of *trmA* or *truB*, in particular in the *ΔtrmAΔtruB* double-knockout strain. In conclusion, the observed effects on codon-specific translation by *trmA* and *truB* explain the proteome changes and the reduced overall fitness of the deletion strains.

Discussion

Here, we provide comprehensive insight into the cellular functions of the universally conserved TrmA and TruB enzymes that introduce m^5U54 and Ψ55 in elongator tRNAs (Fig. 4). 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 (13, 14). 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 both likely contribute 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 other modifications in tRNA including 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 (*SI Appendix*, Fig. S2), 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). The abundance of several proteins is changed in the *ΔtrmA* and/or *ΔtruB* strains and is often not correlated with transcript abundance, revealing that the translation of specific proteins is impaired explaining the fitness defect of *ΔtrmA* and/or *ΔtruB* strains. In particular, TrmA and TruB promote the synthesis of large proteins and proteins with mRNAs containing a high frequency of certain codons (Fig. 3). In conclusion, by enhancing the aminoacylation and modification of all tRNAs in *E. coli,* TrmA and TruB fine-tune the functionality of all tRNAs and ensure efficient protein synthesis.

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 the presence of TrmA and TruB enzymes collectively increase global aminoacylation by about 10% (Fig. 1 *E* and *F*). Hence, TrmA and TruB affect charging of most or all tRNAs in vivo, most likely by enhancing the correct folding of tRNAs (13, 14). This decrease in aminoacylation in the knockout strains compared to the WT 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 (13, 14). 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, preparing all tRNAs for subsequent maturation events (35–37). In both yeast and *T. thermophilus,* deletion of *trmA* or *truB* homologs leads to altered abundances of other tRNA modifications at various positions (17, 36). Here, we reveal that the action of *E. coli* TrmA and TruB modulates the levels of at least s^4U8, ms^2i^6A37, and acp^3U47 (Fig. 2). 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 *ΔtrmA E. coli* (38). Together, these findings indicate that several other tRNA-modifying enzymes act with reduced efficiency on tRNA lacking m^5U54 and/or Ψ55. 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. Additionally, hypomodification of tRNAs may contribute to the low aminoacylation levels observed in strains lacking *trmA* and/or *truB* (39).

Given that m^5U54 and Ψ55 affect tRNA 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 down-regulated in the *ΔtrmAΔtruB* strain (Fig. 1*C*); however, the translation of the Leu CTG and CTA codons read by these tRNAs is not affected by *trmA* and *truB* deletion (*SI Appendix*, Fig. S6). This contrasts studies of several yeast tRNA modification enzymes, which demonstrate certain enzymes are vital for the stability of a few tRNAs, but dispensable for others (1, 28, 40, 41). Thus, TruB and TrmA act differently from other tRNA modification enzymes as they enhance the maturation of all tRNAs rather than increase the stability of selected 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 (Fig. 3). In particular, translation of Pro CCA, Arg CGA, CGG and AGG, Gln CAA, Cys TGT, and Leu TTG codons were negatively affected by the deletion of the *trmA* and/or *truB* gene. Many of these codons are each used at lower frequencies in *E. coli* compared to other codons encoding 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 (Fig. 1 *A*–*C*). Instead, these tRNAs seem to be particularly susceptible to defects in codon-specific decoding abilities in the absence of TrmA and TruB, due to an increase in misfolded tRNAs, loss of m^5U54 and/or Ψ55, decrease in another affected modification (discussed below), or by a combination of these effects.

Of the seven tRNAs that contain acp^3U47, six have decreased acp^3U47 levels in the *ΔtrmAΔtruB* strain. Remarkedly, these six tRNAs decode at least one codon differently in the absence of *trmA* (and *truB*) compared to the WT strain (Fig. 2*C*, CGA by $tRNA^{Arg}_{ACG}$, ATT and ATA by $tRNA^{Ile}_{GAT}$, ATA by $tRNA^{Ile2}_{CAT}$, AAA by $tRNA^{Lys}_{TTT}$, GTT by $tRNA^{Val}_{GAC}$, TTC by $tRNA^{Phe}_{GAA}$). The ms^2i^6A37 modification also has a lower abundance in several tRNAs within the *ΔtrmA* strain (Fig. 2*F*), and half of the ms^2i^6A37-containing tRNAs display different translation of certain codons in the absence of *trmA* (TGT by $tRNA^{Cys}_{GCA}$, TTG by or $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{TAA}$, TAT by $tRNA^{Tyr}_{GTA}$, TTC 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, 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 (38), 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 for tRNA translocation 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 with unchanged or even increased transcripts levels demonstrating translation-specific defects in gene expression. Codon usage analysis of the down-regulated proteins in the *ΔtrmA* and *ΔtruB* single- and double-deletion strains shows that the majority of these genes are enriched in codons that are less efficiently translated in these strains such as the CGA and CGG Arg codons and the CCA Pro codon (Fig. 3*K* and *SI Appendix*, Fig. S14). Evidently, the codon-specific defects in translation upon *trmA* and/or *truB* deletion affect the overall proteome underlining the importance of TrmA and TruB for protein synthesis. Moreover, many of the translationally down-regulated proteins are large *E. coli* proteins (Fig. 3*J*). 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. Notably, these translational changes in the knockout strains do not relate to a phenotype under specific growth conditions despite our extensive search (*SI Appendix*, Fig. S2 and Dataset S1). Rather, our data suggest that *trmA* and *truB* optimize protein synthesis in general and irrespective of the growth conditions. This interpretation is further supported by data from the Koutmou lab that deletion of *trmA* subtly changes *E. coli* growth in the presence of hygromycin indicating an alteration in tRNA translocation through the ribosome which is in agreement with a general impact on protein synthesis (38).

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. Global ribosome profiling has revealed yeast 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 (42). 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 (43). 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 (22) and translation fidelity is decreased in the absence of TRMT2A (44). Moreover, human TRUB1 affects mitochondrial tRNA conformation and mitochondrial translation thus decreasing the assembly and activity of oxidative phosphorylation complexes, without altering tRNA abundance (24). 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: TrmA and TruB homologs promote tRNA maturation and aminoacylation and affect codon-specific translation as well as synthesis of large proteins. Understanding these cellular roles of TrmA and TruB homologs provides insight into their implication in human diseases, including diabetes, deafness, and cancer (21–24).

In summary, we comprehensively identify the functions of the conserved tRNA-modifying enzymes, TrmA and TruB (Fig. 4). which promote correct tRNA folding and enable efficient modification by other tRNA-modifying enzymes. These positive effects during maturation lead to improved aminoacylation for all tRNAs. Thus, TruB and TrmA stand out as enzymes that globally affect all tRNAs, contrasting several tRNA-modifying enzymes that seem to benefit only a subset of tRNAs (28). The positive impact of TrmA and TruB on the cell is evident in the decoding of specific codons, in particular codons read by tRNAs harboring ms^2i^6A37 and acp^3U47 modifications, and in 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 cellular fitness of the deletion strains under competition conditions. The human homologs of *E. coli* TrmA and TruB likely impact tRNA maturation and aminoacylation and modulate protein synthesis similarly 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. Other tRNA-modifying enzymes targeting the elbow region of many tRNAs such as the dihydrouridine synthases may have similar global roles.

Methods

For details, see *SI Appendix, SI Methods*. In brief, single-knockout strains were obtained from the Keio collection and the *ΔtrmAΔtruB* strain was constructed as reported by Datsenko and Wanner (25, 45). MSR-seq was performed as previously reported (27). Primer extension to detect acp^3U47 was carried out using avian myeloblast virus reverse transcriptase and a Cy5-labeled primer complementary to the $tRNA^{Phe}$ 3′ end (*SI Appendix*, Table S1). Thiolated tRNA was separated using APM-urea-PAGE as previously reported (30) and specific tRNAs were detected by northern blotting with biotinylated probes. Tandem codon translation reporter assays were performed similarly as in ref. 33, and mass spectrometry to examine the cellular proteome was performed similarly to ref. 46.

Data, Materials, and Software Availability. The data discussed in his publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE233667 (47) for MSR-tRNA-seq data and GSE237609 (48) for total RNA sequencing data.

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