Supplementary Figures

tRNA pseudouridine synthase D (TruD) from *Thermus thermophilus* modifies U13 in $tRNA^{Asp}$, $tRNA^{Glu}$, and $tRNA^{Gln}$ and U35 in $tRNA^{Tyr}$

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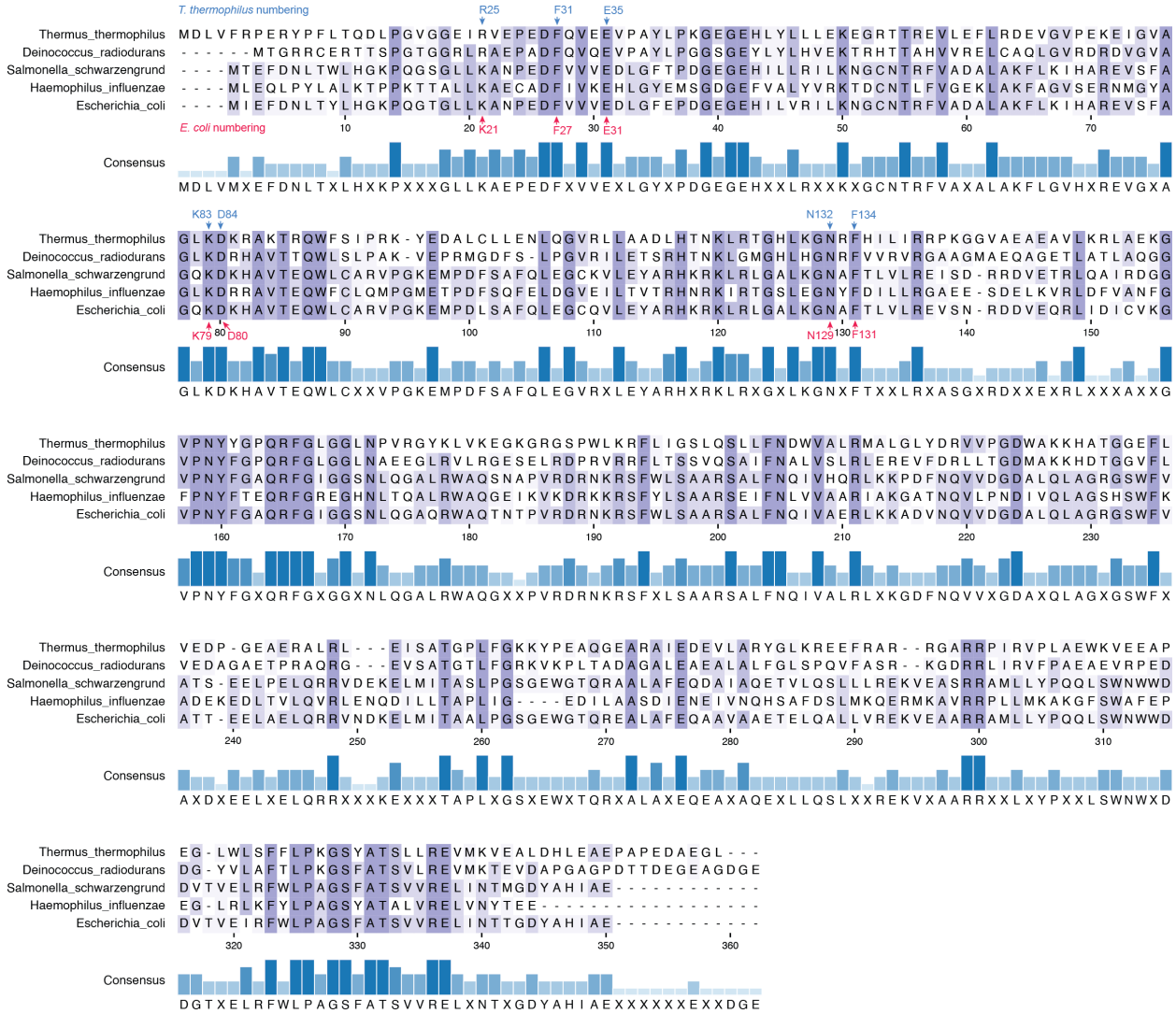
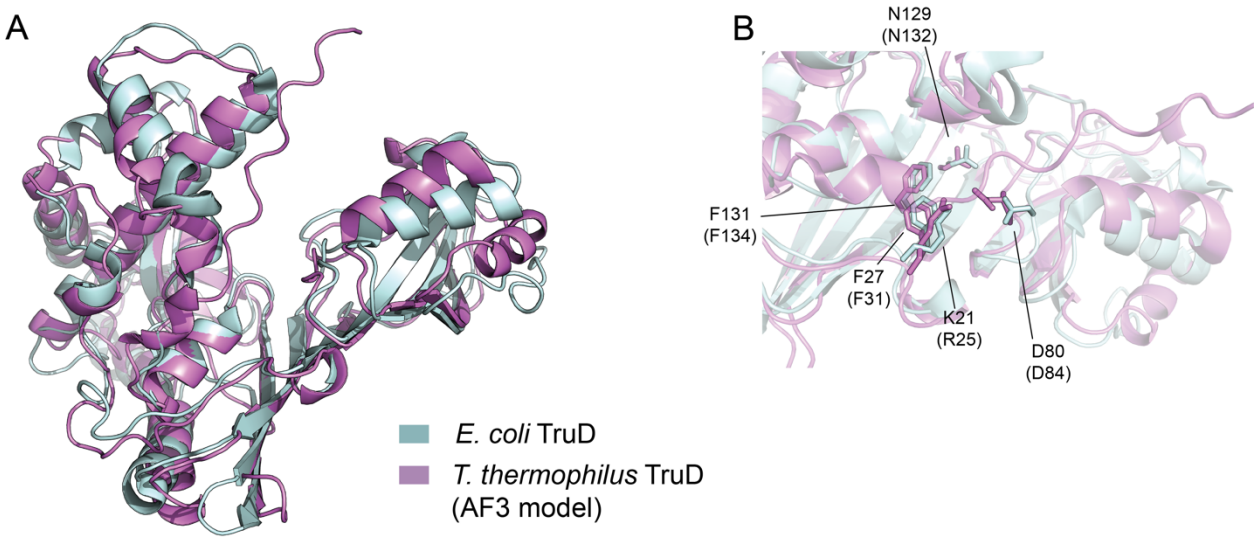
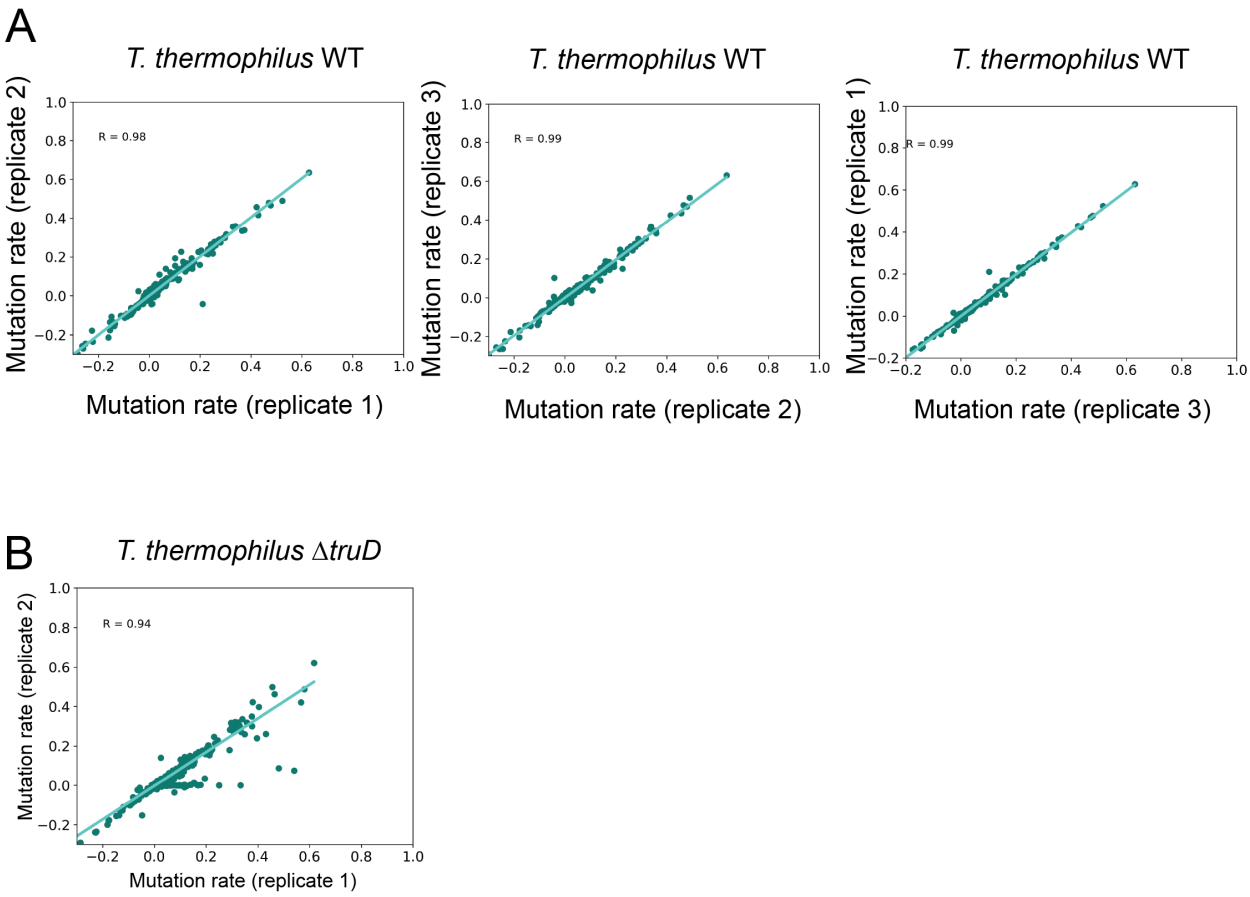


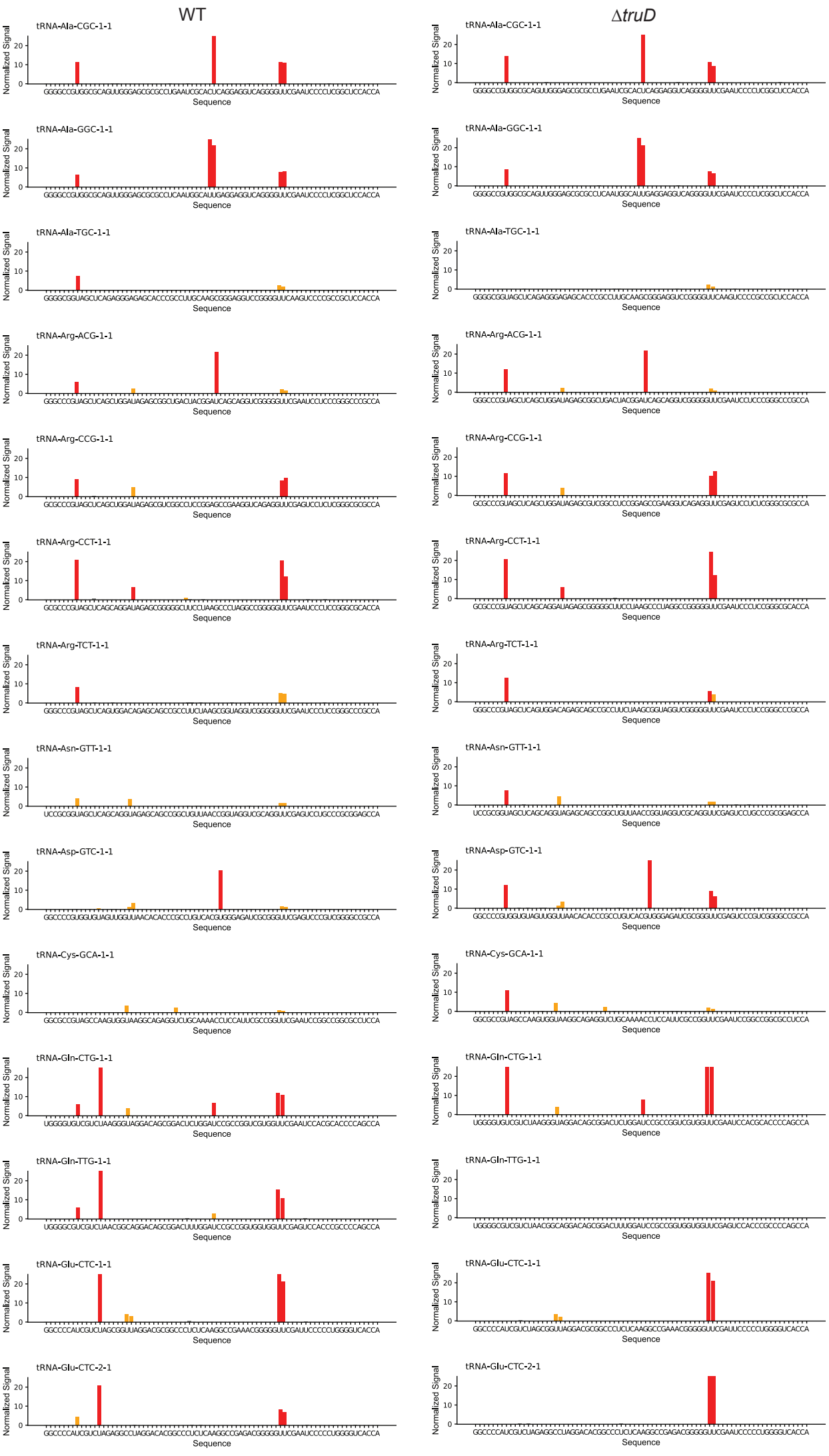
Supplementary Figure 1. Multiple sequence alignment analysis of TruD from *T. thermophilus*, *D. radiodurans*, *E. coli*, *S. schwarzengrund*, and *H. influenzae*. The amino acid sequences were retrieved from UniProt (https://www.uniprot.org/) and aligned by CLUSTALW (https://www.genome.jp/tools-bin/clustalw) with default parameters. Catalytically important residues in *E. coli* TruD and *T. thermophilus* TruD are highlighted in red and blue, respectively. Numbering of the amino acid sequence is based on the *E. coli* TruD sequence.



Supplementary Figure 2. (A) *T. thermophilus* TruD was modeled by AlphaFold3 (https://alphafoldserver.com) and visualized by PyMol. The *T. thermophilus* TruD model (Magenta) was superimposed on the crystal structure of *E. coli* TruD (PDB id: 1SB7) (White cyan). (B) The catalytically important residues *in T. thermophilus* TruD are compared with the corresponding residues in *E. coli* TruD.



Supplementary Figure 3. (A and B) The three or two biological replicates of the mutation profiling data from PRAISE experiments of (A) *T. thermophilus* WT and (B) the Δ*truD* strain, are plotted to see the correlation between each replicate. The plots were fitted with a linear regression. The Pearson correlation coefficient (R value) is shown in each graph.


WT
ΔtruD
tRNA-Ala-CGC-1-1
tRNA-Ala-GGC-1-1
tRNA-Ala-TGC-1-1
tRNA-Arg-ACG-1-1
tRNA-Arg-CCG-1-1
tRNA-Arg-CCT-1-1
tRNA-Arg-TCT-1-1
tRNA-Asn-GTT-1-1
tRNA-Asp-GTC-1-1
tRNA-Cys-GCA-1-1
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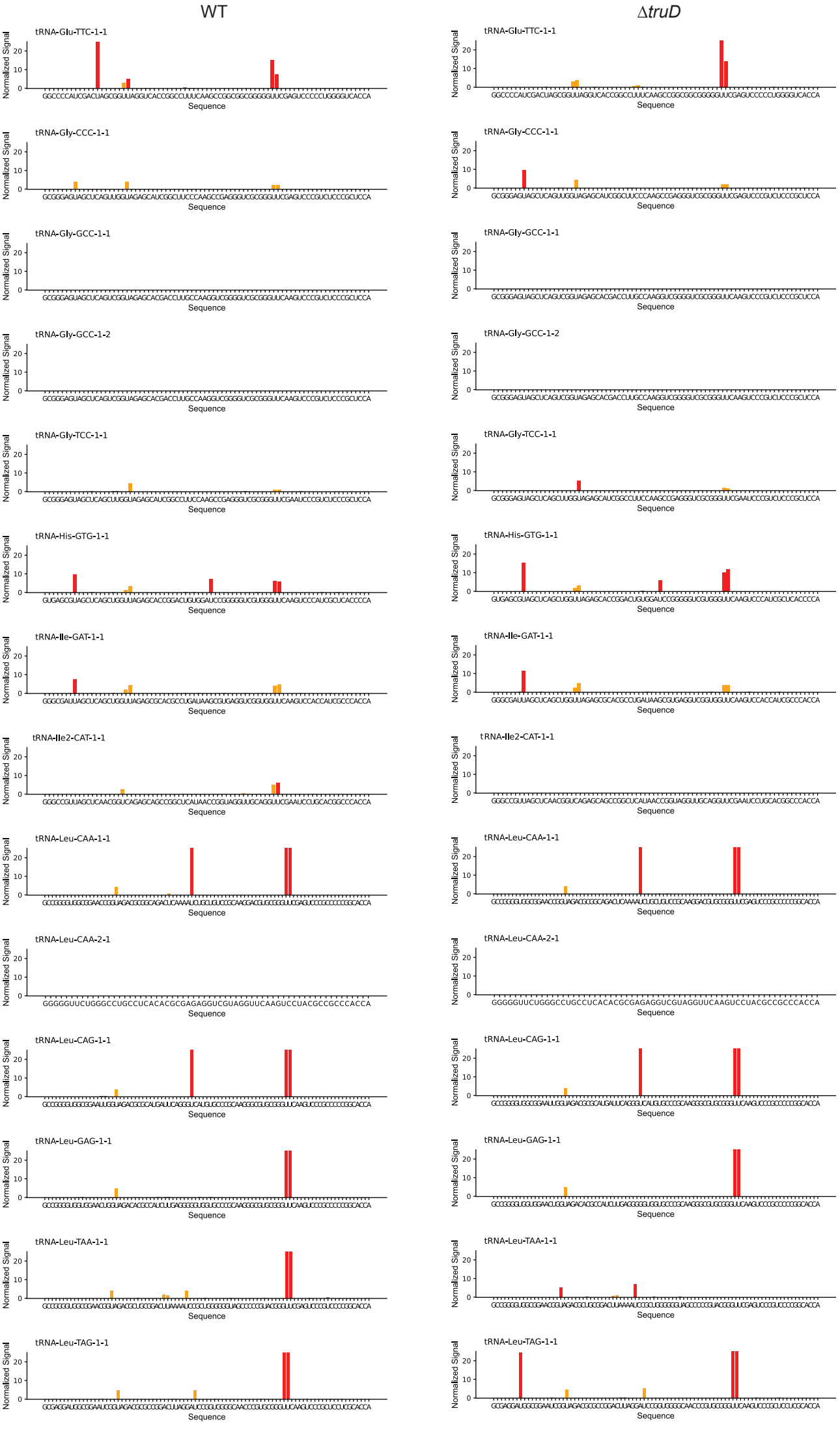
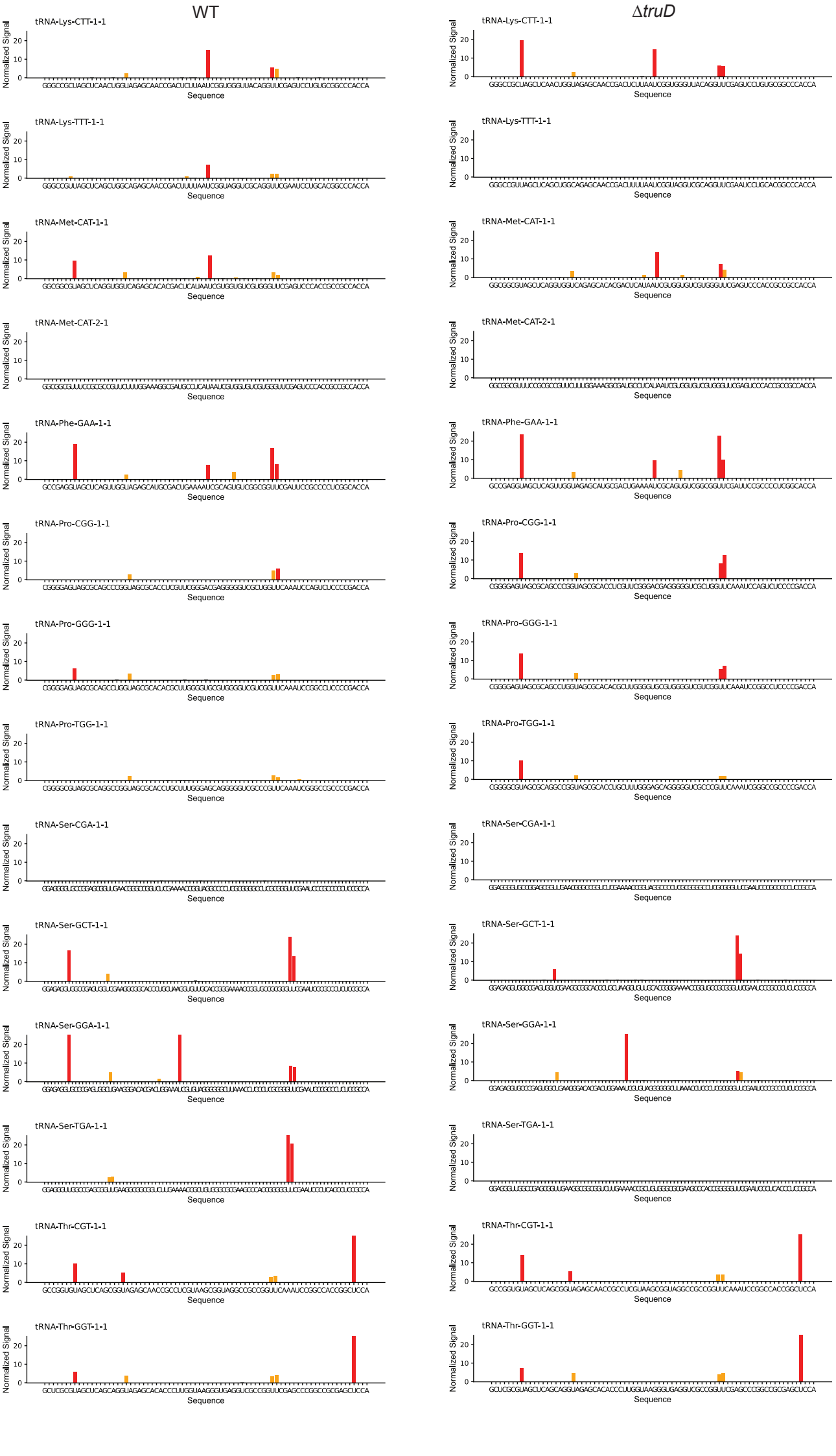
tRNA-Glu-CTC-1-1
tRNA-Glu-CTC-2-1
Normalized Signal
Sequence


WT
ΔtruD
tRNA-Glu-TTC-1-1
tRNA-Gly-CCC-1-1
tRNA-Gly-GCC-1-1
tRNA-Gly-GCC-1-2
tRNA-Gly-TCC-1-1
tRNA-His-GTG-1-1
tRNA-Ile-GAT-1-1
tRNA-Ile2-CAT-1-1
tRNA-Leu-CAA-1-1
tRNA-Leu-CAA-2-1
tRNA-Leu-CAG-1-1
tRNA-Leu-GAG-1-1
tRNA-Leu-TAA-1-1
tRNA-Leu-TAG-1-1
Normalized Signal
Sequence


WT
ΔtruD
tRNA-Lys-CTT-1-1
tRNA-Lys-TTT-1-1
tRNA-Met-CAT-1-1
tRNA-Met-CAT-2-1
tRNA-Phe-GAA-1-1
tRNA-Pro-CGG-1-1
tRNA-Pro-GGG-1-1
tRNA-Pro-TGG-1-1
tRNA-Ser-CGA-1-1
tRNA-Ser-GCT-1-1
tRNA-Ser-GGA-1-1
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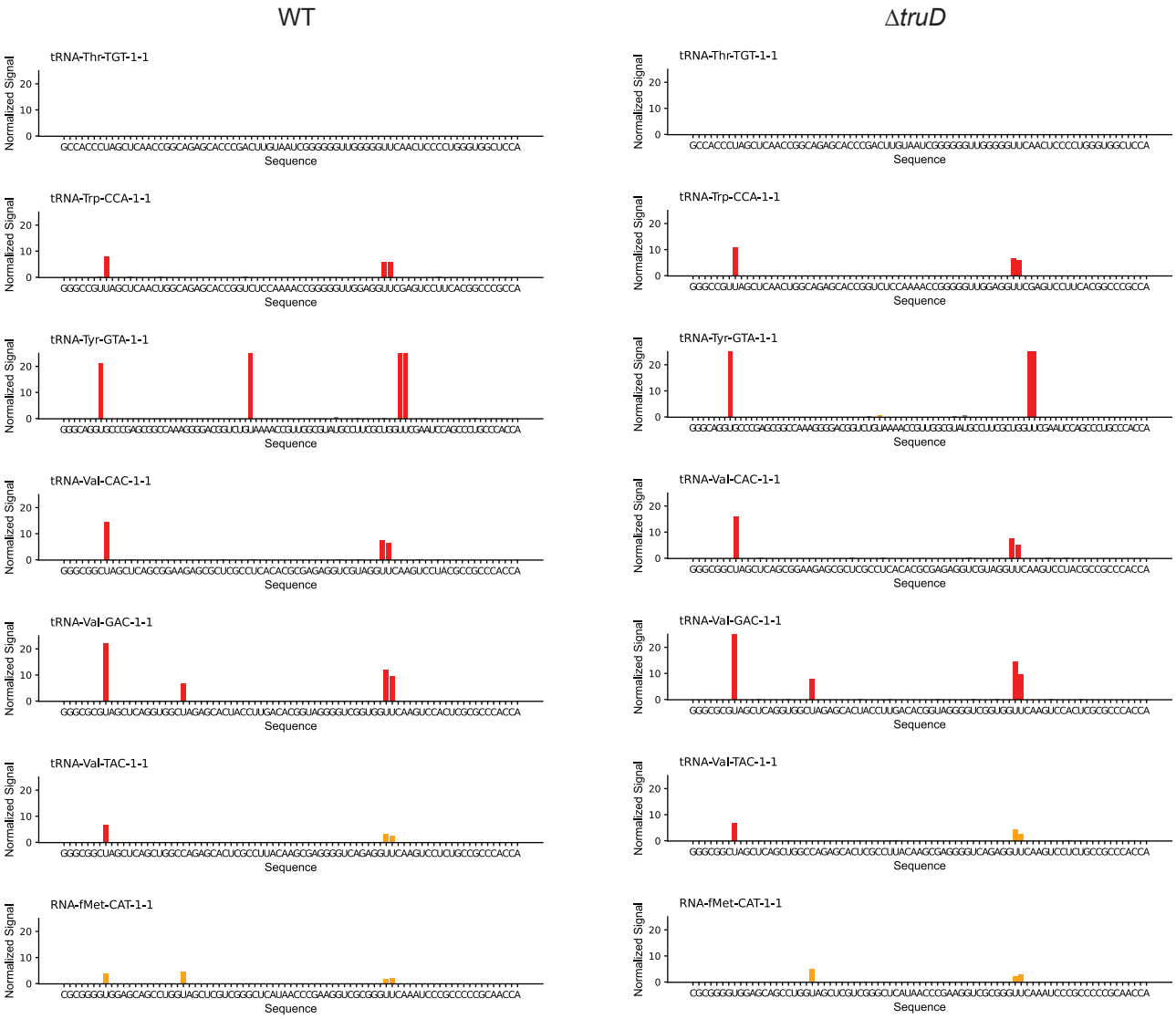
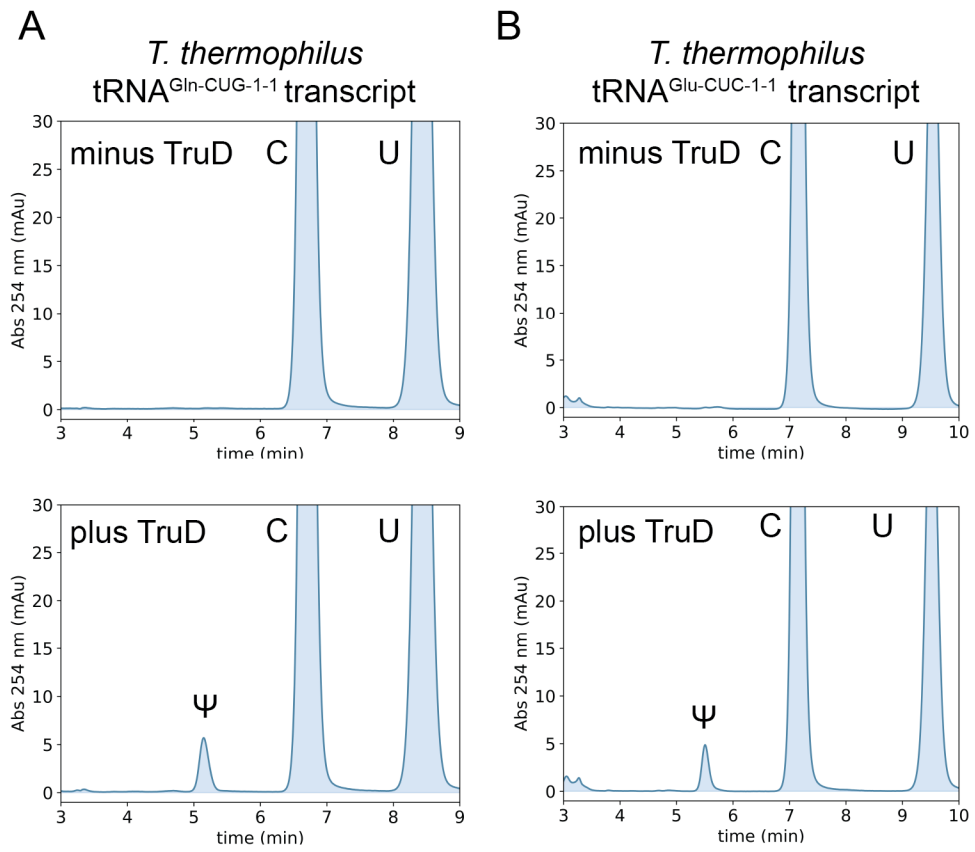
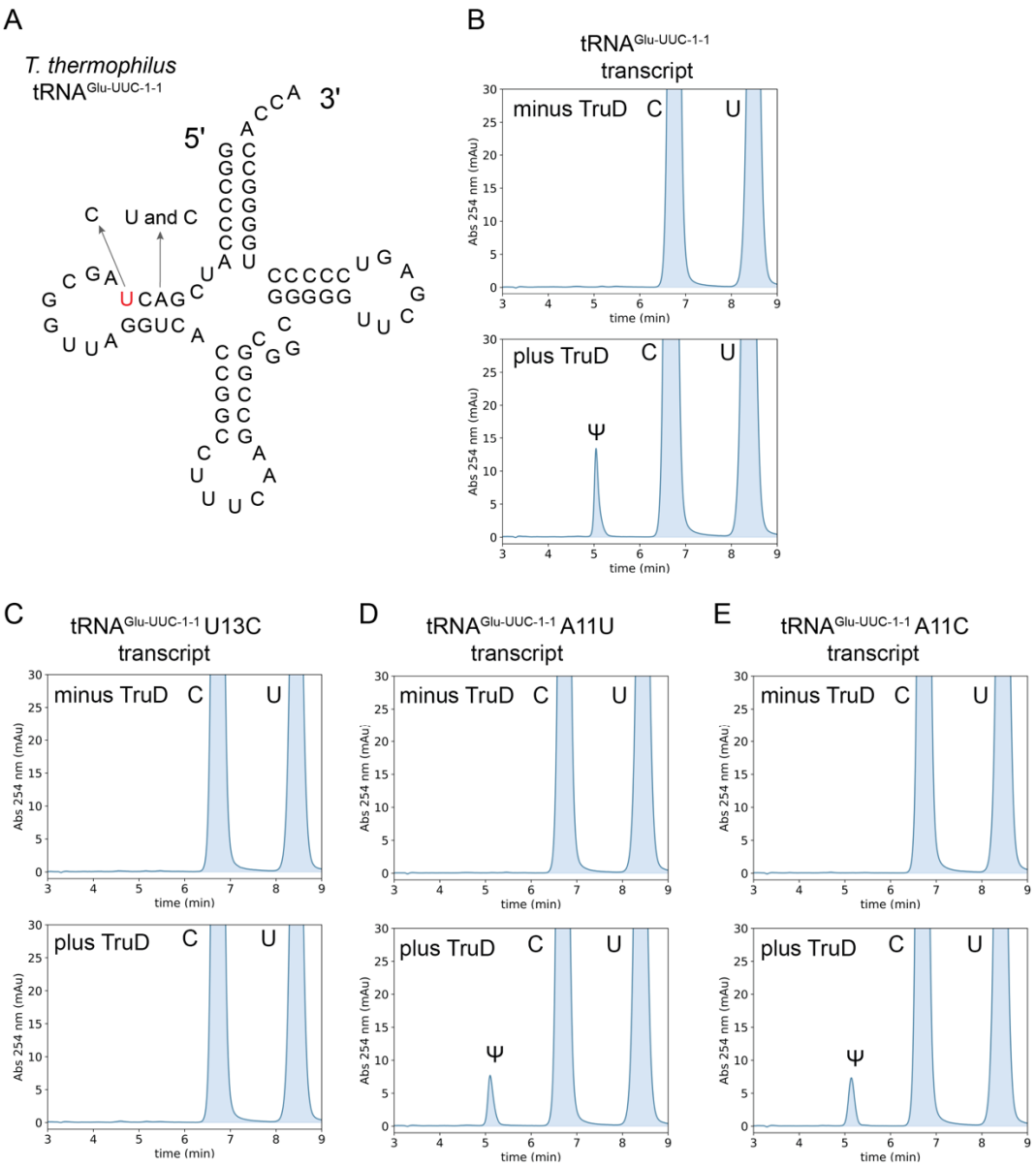
tRNA-Thr-GGT-1-1
Normalized Signal
Sequence


WT
ΔtruD
tRNA-Thr-TGT-1-1
tRNA-Trp-CCA-1-1
tRNA-Tyr-GTA-1-1
tRNA-Val-CAC-1-1
tRNA-Val-GAC-1-1
tRNA-Val-TAC-1-1
RNA-fMet-CAT-1-1
Normalized Signal
Sequence

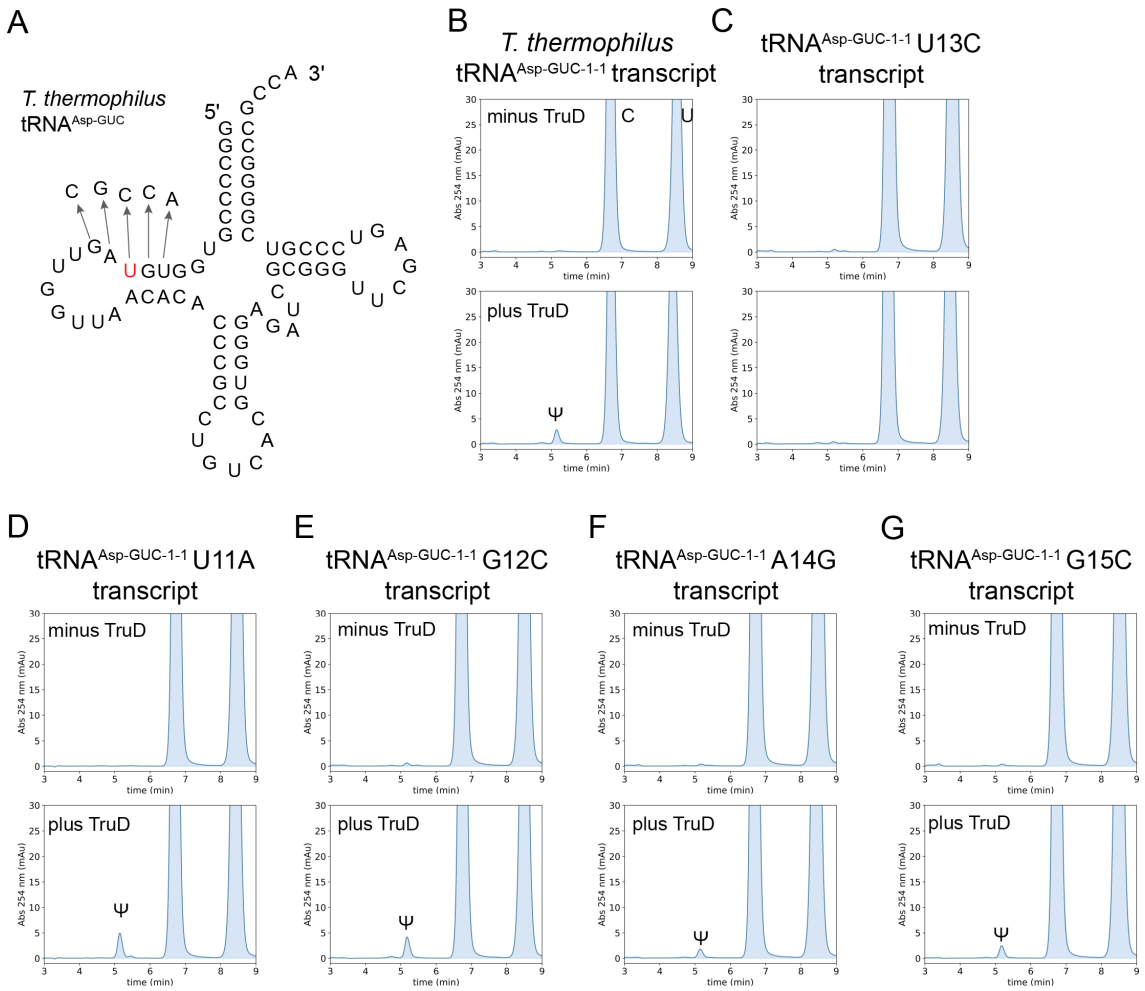
Supplementary Figure 4. PRAISE data on tRNAs from WT and Δ*truD* strains were provided.



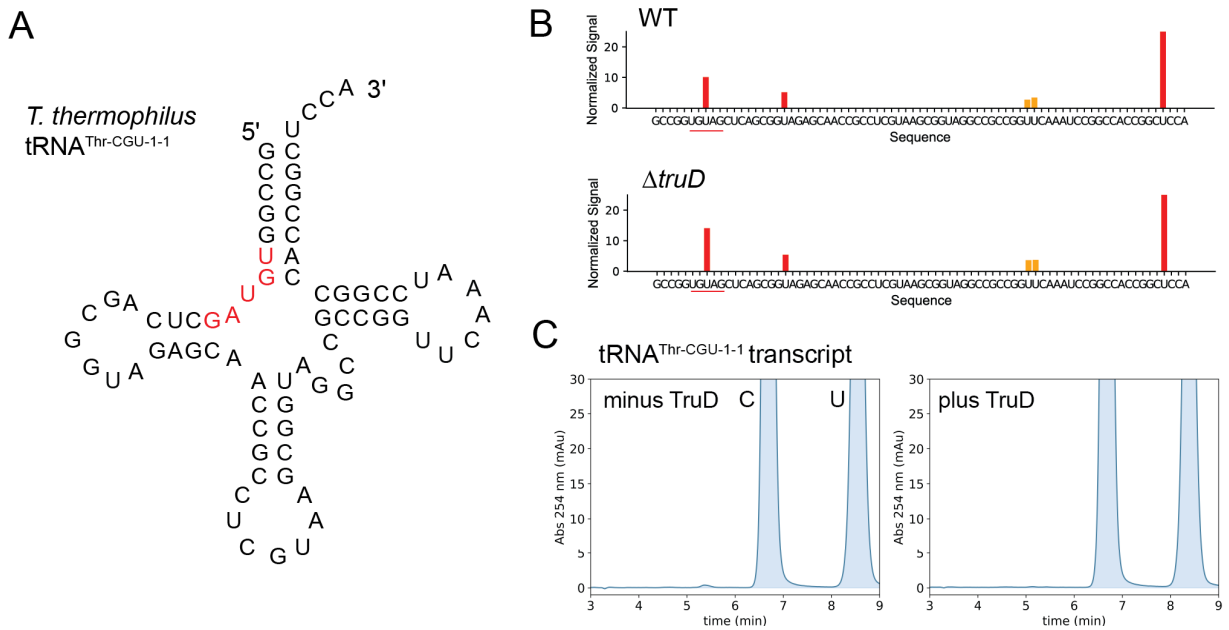
Supplementary Figure 5. (A–B) In vitro pseudouridylation assays with (plus TruD) or without (minus TruD) recombinant TruD using in vitro synthesized (A) $tRNA^{Gln\text{-}CUG\text{-}1\text{-}1}$ WT transcript, and (B) $tRNA^{Glu\text{-}CUC\text{-}1\text{-}1}$ WT transcript. Pseudouridine formation was detected by nucleoside analysis using HPLC where pseudouridine eluted around 5 min. These experiments were performed independently three times (n=3) and gave the same results; one representative chromatogram is shown.



Supplementary Figure 6. (A) Secondary structure of *T. thermophilus* tRNA$^{Glu\text{-}UUC\text{-}1\text{-}1}$. The point mutation was introduced at 2 nucleotide positions in the TruD recognition site. The target uridine is highlighted in red. (B–E) In vitro pseudouridylation assays with (plus TruD) or without (minus TruD) recombinant TruD using in vitro synthesized (B) tRNA$^{Glu\text{-}UUC\text{-}1\text{-}1}$ WT transcript, (C) U13C mutant transcript, (D) A11U mutant transcript, and (E) A11C mutant transcript. Pseudouridine formation was detected by nucleoside analysis using HPLC where pseudouridine eluted around 5 min. These experiments were performed independently three times (n=3) and gave the same results; one representative chromatogram is shown.



Supplementary Figure 7. (A) Secondary structure of *T. thermophilus* tRNA$^{Asp\text{-}GUC\text{-}1\text{-}1}$. The point mutation was introduced at 5 nucleotide positions in the TruD recognition site. The target uridine is highlighted in red. (B–G) In vitro pseudouridylation assays with (plus TruD) or without (minus TruD) recombinant TruD using in vitro synthesized (B) tRNA$^{Asp\text{-}GUC\text{-}1\text{-}1}$ WT transcript (Fig. 5C was replotted), (C) U13C mutant transcript (Fig. 5D was replotted), (D) U11A mutant transcript, (E) G12C mutant transcript, (F) A14G mutant transcript, and (G) G15C mutant transcript. Pseudouridine formation was detected by nucleoside analysis using HPLC where pseudouridine eluted around 5 min. These experiments were performed independently three times (n=3) and gave the same results; one representative chromatogram is shown.



Supplementary Figure 8. (A) Secondary structure of *T. thermophilus* tRNA$^{Thr\text{-}CGU\text{-}1\text{-}1}$. The TruD recognition sequence is highlighted in red. (B) PRAISE data for tRNA$^{Thr\text{-}CGU\text{-}1\text{-}1}$ in WT and Δ*truD* strains. The TruD recognition sequence is highlighted in red. (C) In vitro pseudouridylation assays with (plus TruD) or without (minus TruD) recombinant TruD using tRNA$^{Thr\text{-}CGU\text{-}1\text{-}1}$ transcript was performed. Pseudouridine formation was detected by nucleoside analysis using HPLC where pseudouridine eluted around 5 min. These experiments were performed independently three times (n=3) and gave the same results; one representative chromatogram is shown.