

Figure S1. Additional results for Fig. 1.

- (a) Statistics of sequencing replicates.
- (b) Mapping fraction of tRNA and other RNA. DMS0: no DMS; DMS2: 2%DMS; DMS5: 5%DMS. SnoRNA read fractions are <0.5%.
- (c) Mutation rate of each nucleotide on tRNA^{Met} with and without DMS or demethylase (DM) treatment.
- (d) Box-whisker plot showing the native m¹A mutation rate distribution in 5% DMS treated in vitro and in vivo samples without (minus) and with (plus) demethylase (DM) treatment.
- (e) Nucleotide identity of DM_{diff} samples with 5% DMS treatment.
- (f) DMS signals for cytosolic and mitochondrial tRNAs showing higher reactivity of mito-tRNAs.
- (g) Median mutation rate for A residues in tRNAs and mRNAs *in vivo*. The positional read filter to calculate the mutation rate is ≥100 for mRNA1 and ≥500 for mRNA2.
- (h) Gini-plot of DMS signal *in vitro* (left) and *in vivo* (right) samples excluding the native m¹A/m³C/m¹G modified positions.

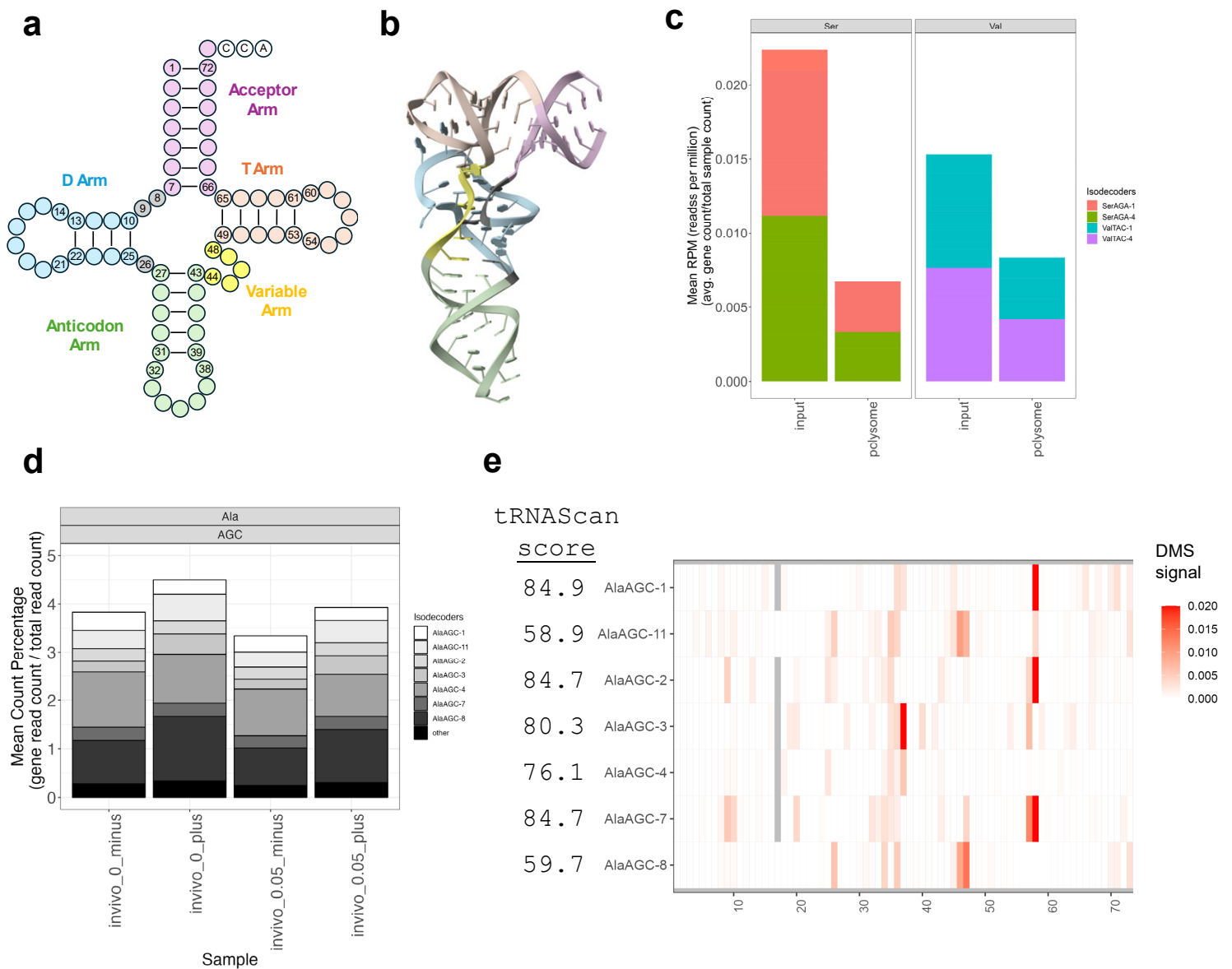


Figure S2. tRNA secondary and tertiary structure and residue nomenclature, and additional results for Fig. 2.

- (a) Cloverleaf secondary structure, also showing standard tRNA nomenclature and canonical positions used in this work.
- (b) Tertiary structure modified from a tRNA^{Val} structure (PDB: 8CTI), different stem-loop regions are color coded like in (a).
- (c) Mean RPM values of SerAGA-1/-4 and ValUAC-1/-4 isodecoders from input (total RNA) and polysome-enriched samples, sequencing data from NCBI GSE198441.
- (d) Mean read count percentage of all tRNA^{Ala}(AGC) isodecoders under different *in vivo* conditions, 0_minus/0.05_minus, no DMS (0) or 5% DMS (0.05), no demethylase (DM) treatment, 0_plus/0.05_plus, no DMS (0) or 5% DMS (0.05), +DM treatment.
- (e) Heatmap showing the *in vivo* positional DMS signals for the tRNA^{Ala}(AGC) isodecoders expressed in our samples.

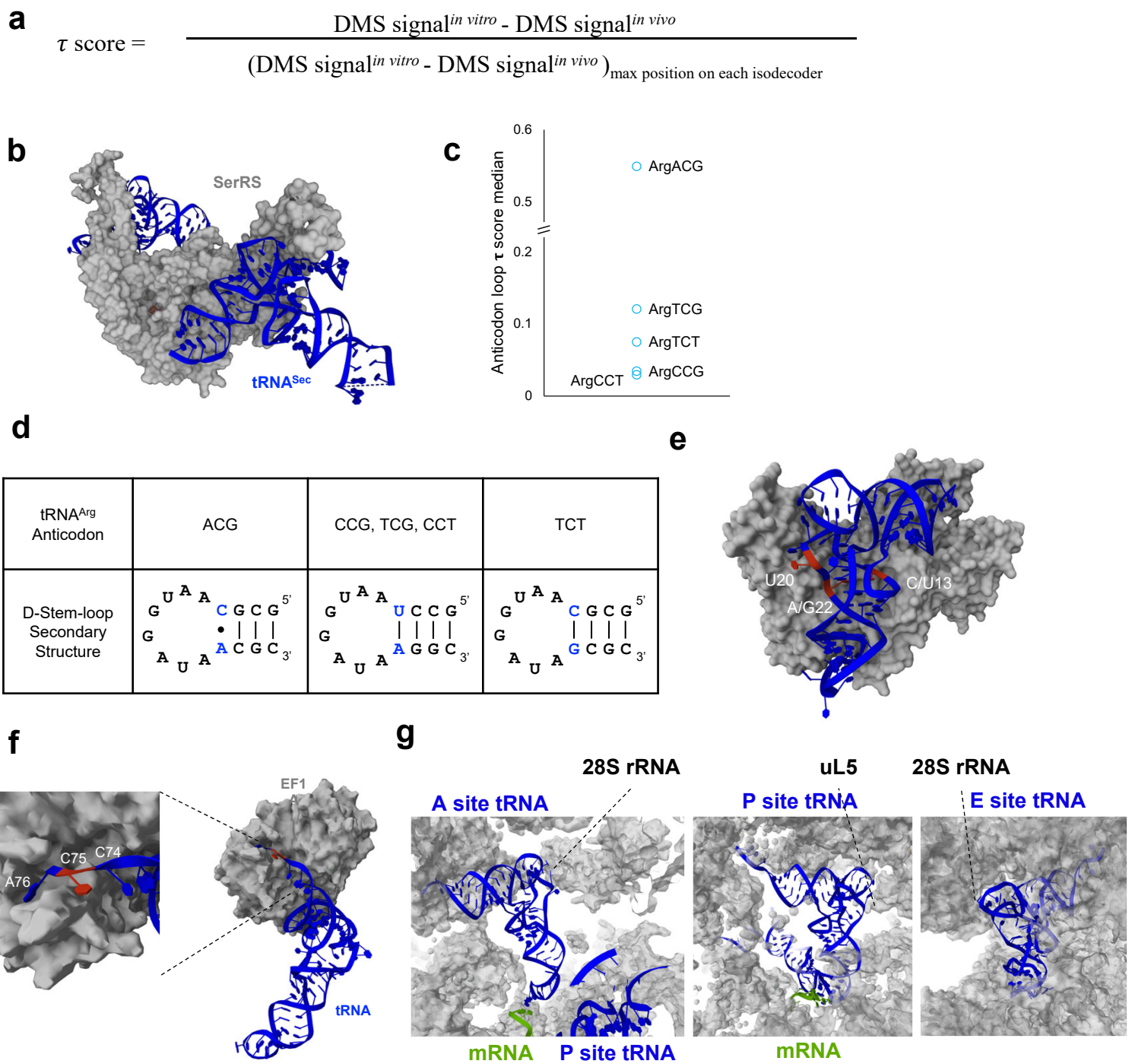


Figure S3. Additional results for Fig. 3.

- (a) Definition of τ score.
- (b) Human SerRS-tRNA^{Sec} structure showing the anticodon loop of the substrate tRNA is not bound by the SerRS (PDB: 4RQE).
- (c) Box-whisker plot showing anticodon loop τ score medians of 5 tRNA^{Arg} isoacceptors ($n = 3$ biological replicates).
- (d) D-stem loop sequence and secondary structures of 5 tRNA^{Arg} isoacceptors. Base pair differences are shown in blue.
- (e) Structure of yeast ArgRS interacting with tRNA^{Arg} (PDB: 1F7V).
- (f) EF-1A-tRNA structure showing protection of C75 residue (PDB: 8G60).
- (g) Structures of A, P, and E site tRNAs in the human ribosome showing protection of the tRNA elbow region (PDB: 8G60 (A and P site), PDB: 6QZP (E site)).

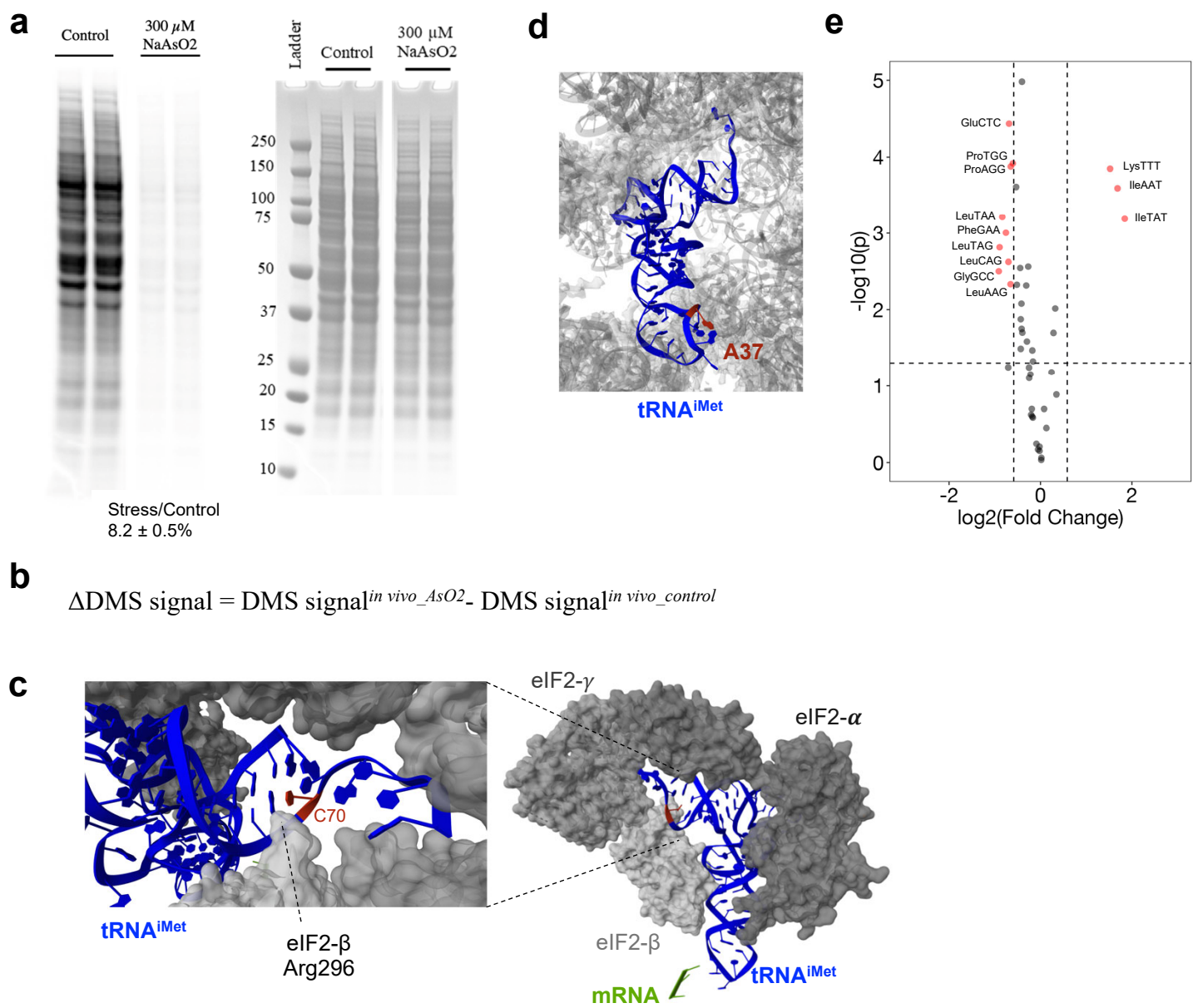
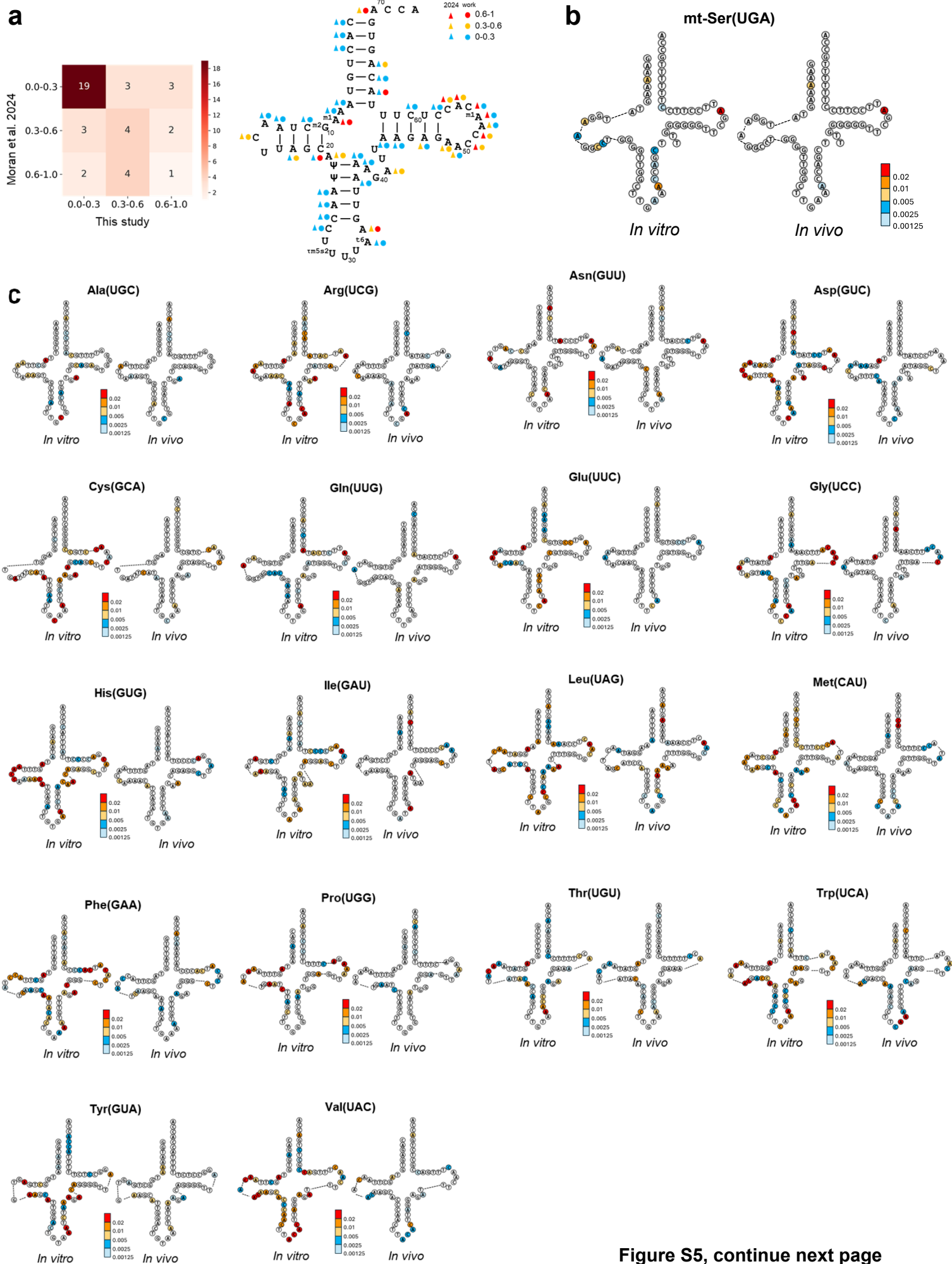


Figure S4. Additional results for Fig. 4.

- (a) ³⁵S-Met pulse-chase showing a strong reduction of translation upon arsenite treatment. Coomassie stained loading controls are on the right.
- (b) Formula of the Δ DMS signal.
- (c) eIF2B-tRNA^{iMet}-tRNA ternary complex structure showing eIF2- β Arg296 residue in proximity to C70 (C71 in tRNA nomenclature) of tRNA^{iMet} (PDB: 6YBV).
- (d) tRNA^{iMet} at the A site of the ribosome with A37 highlighted in red (PDB: 4UG0).
- (e) Cytosolic tRNA changes on the polysome under stress (AsO₂ polysome/control polysome). Vertical lines indicate a 1.5-fold change; the horizontal line indicates $p = 0.05$. Data from reference 24 and NCBI GSE198441.



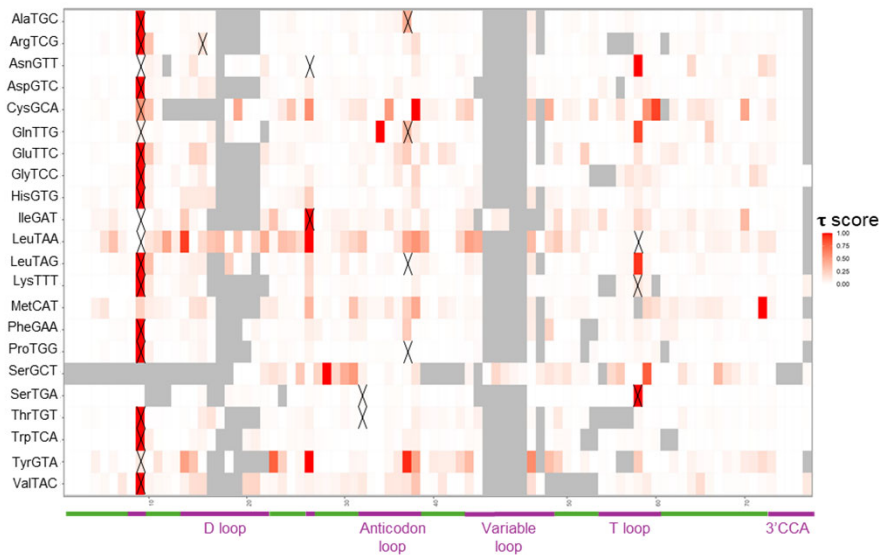
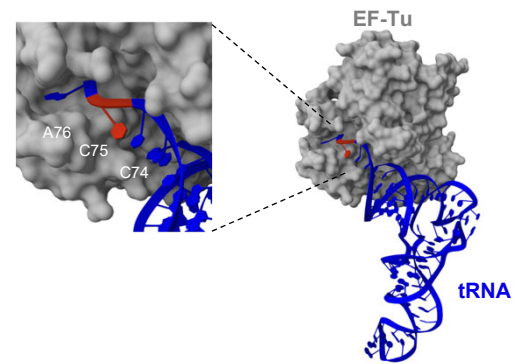
d**e**

Figure S5. Additional results for Fig. 5.

- Comparison of positional DMS reactivity of mt-tRNA^{Lys} between data obtained in this study and reference 23, presented as heatmap (left) and in secondary structure (right).
- Secondary structure of mt-tRNA^{Ser}(UGA) overlaid with *in vitro* and *in vivo* DMS signals according to the colored scale bar. The dashed lines connects the residues in this schematic for mt-tRNAs. Mt-tRNA positions are according to reference 28.
- Secondary structures of 18 type II mt-tRNAs overlaid with *in vitro* and *in vivo* DMS signals according to the colored scale bar. The dashed lines connects the residues in this schematic for mt-tRNAs. Mt-tRNA positions are according to reference 28.
- Heatmap showing the positional τ scores on each mt-tRNA. Native modifications (reference 30) that are incompletely removed by demethylases are marked with "X".
- EF-Tu-mt-tRNA structure showing C75 interaction (PDB: 1TTT).

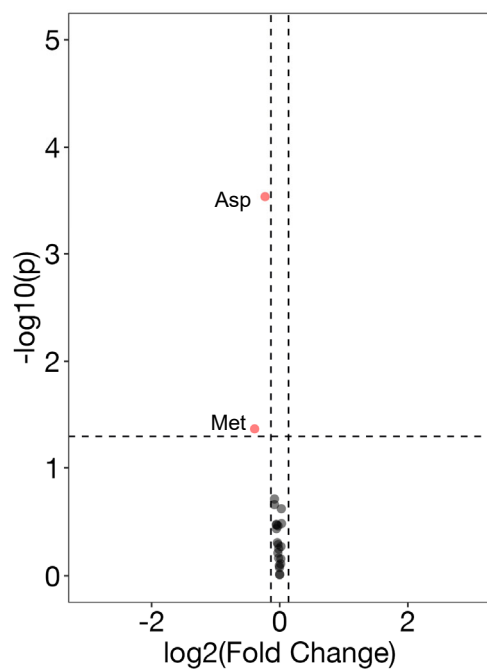
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Figure S6. Additional results for Fig. 6.

(a) mt-tRNA charging fold changes in polysomes under arsenite treatment. The dotted vertical lines indicate a 1.1-fold change (FC), and the dotted horizontal line indicates $p = 0.05$. Data from reference 24 and NCBI GSE198441.

Supplemental Table 1: Sequencing library sample description and alignment rates

Sample	Demethylase (DM)	DMS (%)	bowtie_total_reads	bowtie_alignment_rate	mimseq_total_reads	mimseq_alignment_rate
invivo rep 1	minus	0	15715439	50.75%	12344688	65%
invivo rep 1	minus	2	9722879	51.93%	7904772	55%
invivo rep 1	minus	5	16120250	34.92%	9609525	59%
invivo rep 2	minus	0	19892094	53.73%	16818898	68%
invivo rep 2	minus	2	11151715	51.78%	9427936	67%
invivo rep 2	minus	5	16013586	35.88%	10369044	61%
invivo rep 3	minus	0	22142765	68.55%	19667269	71%
invivo rep 3	minus	2	11800992	67.33%	10487449	69%
invivo rep 3	minus	5	12734766	40.63%	8290601	56%
invivo rep 1	plus	0	12147387	62.14%	9287760	67%
invivo rep 1	plus	2	7351527	64.16%	5792604	67%
invivo rep 1	plus	5	12663814	39.97%	6777626	61%
invivo rep 2	plus	0	18785916	63.98%	14830160	68%
invivo rep 2	plus	2	10125640	62.21%	7958329	66%
invivo rep 2	plus	5	16951181	35.33%	8441927	60%
invivo rep 3	plus	0	27552423	79.88%	24156280	74%
invivo rep 3	plus	2	14034843	77.87%	12190014	72%
invivo rep 3	plus	5	15057889	44.90%	9036441	60%
in vitro ctrl rep 1	minus	5	51418879	55.08%	45434448	39%
in vitro ctrl rep 2	minus	5	37675709	54.38%	33862685	37%
in vitro ctrl rep 3	minus	5	46871163	47.08%	38442226	35%
in vitro ctrl rep 1	plus	5	55892951	70.55%	48040194	49%
in vitro ctrl rep 2	plus	5	41578868	70.90%	36426561	42%
in vitro ctrl rep 3	plus	5	58474628	58.40%	44447629	39%
AsO2 stress rep 1	minus	0	16493481	51.27%	13478845	64%
AsO2 stress rep 1	minus	2	8395121	44.94%	6076516	62%
AsO2 stress rep 1	minus	5	8583125	54.49%	7141546	66%
AsO2 stress rep 2	minus	0	14869160	52.40%	12543129	68%
AsO2 stress rep 2	minus	2	7896793	46.50%	6200229	65%
AsO2 stress rep 2	minus	5	6424637	49.07%	5344485	65%
AsO2 stress rep 3	minus	0	14361049	64.91%	12524400	68%
AsO2 stress rep 3	minus	2	11080964	63.44%	9466772	68%
AsO2 stress rep 3	minus	5	7988812	66.82%	7127207	68%
AsO2 stress rep 1	plus	0	12369389	64.52%	9958992	66%
AsO2 stress rep 1	plus	2	6511664	53.60%	4388751	64%
AsO2 stress rep 1	plus	5	6609581	67.04%	5312129	68%
AsO2 stress rep 2	plus	0	13505123	63.59%	10629544	68%
AsO2 stress rep 2	plus	2	7610307	50.89%	5103341	64%
AsO2 stress rep 2	plus	5	6136073	57.92%	4624615	64%
AsO2 stress rep 3	plus	0	17275086	76.76%	14935113	71%
AsO2 stress rep 3	plus	2	12965077	72.87%	10668580	71%
AsO2 stress rep 3	plus	5	9428126	77.33%	8150711	72%