Supporting Information

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SI Materials and Methods

Synthesis of tRNA Analogs. Terminal A_{76} in *Mycoplasma mobile* $tRNA^{Leu}$ was replaced with 2′-deoxyadenosine (2′dA) or dideoxyadenosine (ddA) using an adapted protocol (1). Transcribed *M. mobile* $tRNA^{Leu}$ (~400 A_{260} units) was incubated in 1 mL 50 mM NaOAc (pH 5.2) containing 2.5 mM $NaIO_4$ at 37 °C for 2 h in the dark. Excess $NaIO_4$ was reduced at 37 °C for 30 min by incubation with 2 mM glucose. Periodate-oxidized tRNA was ethanol-precipitated and resuspended in 563 μL of water. The terminal adenosine was β-eliminated by incubating periodate-oxidized tRNA with 187 μL of 1 M lysine (pH 9.0) at 25 °C for 2.5 h, followed by desalting using a CHROMA SPIN-10 column (Clontech). The tRNA residual phosphate group was removed by incubation in 1 mL of 100 mM Tris (pH 8.0), 10 mM $MgSO_4$, and 10 U of calf intestinal alkaline phosphatase for 1 h at 37 °C. The solution was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (125:24:1, pH 5.2). The organic phase-containing protein was removed and one-half volume of 4.6 M NH_4OAc (pH 5.0) was added. The tRNA was recovered by ethanol precipitation.

Modified tRNA was incubated with 100 mM Tris (pH 9.0), 100 mM KCl, 2 mM ATP, 2′-dATP or ddATP, 10 mM $MgCl_2$, 0.5 mM DTT, and 16 μM *Escherichia coli* CCA-adding enzyme at 32 °C for 3 h. Protein was extracted and tRNA recovered as described above. The tRNA concentration was measured optically at A_{260} (ε = 840,700 $M^{-1}{\cdot}cm^{-1}$).

Biological Sample Preparation. The *Mycoplasma synoviae* leucyl-tRNA synthetase (LeuRS) gene was synthesized and cloned into pET14b plasmid with TGA codons mutated into TGG (Geneart). A SpeI restriction site was introduced into the plasmid p14LiMmoLeuRS (2) at the site encoding Glu229 and Gly232 within the *M. mobile* LeuRS gene. The resulting plasmid p14bMBMmLeuRSSpeI was linearized and ligated with PCR-amplified DNA sequence of the *M. synoviae* LeuRS connective polypeptide 1 (CP1) domain using In-Fusion Advantage PCR cloning kit (Clontech), yielding plasmid p14bMBMmLeuRS/MsLeuCP1. Protein was expressed in *E. coli* BL21 (DE3) and isolated using Affinity Gel His-Select Nickel resin (Sigma) followed by a HiTrap 16/60 size-exclusion Superdex 75 purification column (GE Healthcare) (3).

M. mobile LeuRS expression was induced overnight at 25 °C using 1 mM isopropyl β-D-1-thiogalactopyranoside using *E. coli* BL21(pLysS)Plus transformed with plasmid p14LiMmoLeuRS (2). Cells were harvested by centrifugation and resuspended in 10 mM Tris (pH 8.0), 200 mM NaCl, 5 mM NaH_2PO_4, and 5% (vol/vol) glycerol, followed by flash-freezing in liquid nitrogen and storage at −80 °C. The cells were lysed by sonication on ice for 2 min with 2-s intervals at 35% sonicator capacity (Vibra Cell; Midwest Scientific), followed by centrifugation at 23,000 × *g* for 30 min. The supernatant was applied to a HiTrap Chelating HP column and washed with 10 mM Tris-base (pH 8.0), 1 M NaCl, 5 mM NaH_2PO_4, and 5% glycerol. A gradient of 10–75 mM imidazole in this buffer was applied before eluting LeuRS with 250 mM imidazole. Subsequent purification on a Superdex 200 column was carried out in 10 mM *bis*-Tris (pH 6.5), 500 mM NaCl, 2 mM NaH_2PO_4, 20 μM $ZnCl_2$, and 1% glycerol. Protein was concentrated to 30 mg/mL and flash-frozen in liquid nitrogen for storage at −80 °C.

Aminoacylation Assays. Leucylation assays with *M. mobile*, *M. synoviae*, or hybrid LeuRS enzymes were carried out at 25 °C in a 36-μL reaction mixture containing 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 21 μM [^{3}H]leucine (318 Ci/mmol), 4 μM tRNA, and 100 nM LeuRS enzyme as described (4). To test for isoleucine mischarging, 40 μM [^{3}H]isoleucine (166 Ci/mmol) was used and 1 μM enzyme introduced. LeuRS hydrolytic activity was measured using a reaction mixture consisting of 60 mM Tris (pH 7.5), 10 mM $MgCl_2$, and about 6.5 μM [^{3}H]Ile-$tRNA^{Leu}$ that was prepared as previously described (2).

Manual Model Building of *M. mobile* LeuRS X-Ray Structure. Manual model rebuilding was carried out using COOT (5) and was interspersed with a round of structure refinement using both PHENIX (6) and REFMAC (7). Cross validation, using 5% of the data for the calculation of the free R factor, was used throughout the model-building process to monitor building bias (8).

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4. Karkhanis VA, Boniecki MT, Poruri K, Martinis SA (2006) A viable amino acid editing activity in the leucyl-tRNA synthetase CP1-splicing domain is not required in the yeast mitochondria. *J Biol Chem* 281(44):33217–33225.
5. Adams PD, et al. (2010) PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):213–221.
6. Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):486–501.
7. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53(Pt 3):240–255.
8. Kleywegt GJ, Brünger AT (1996) Checking your imagination: Applications of the free R value. *Structure* 4(8):897–904.

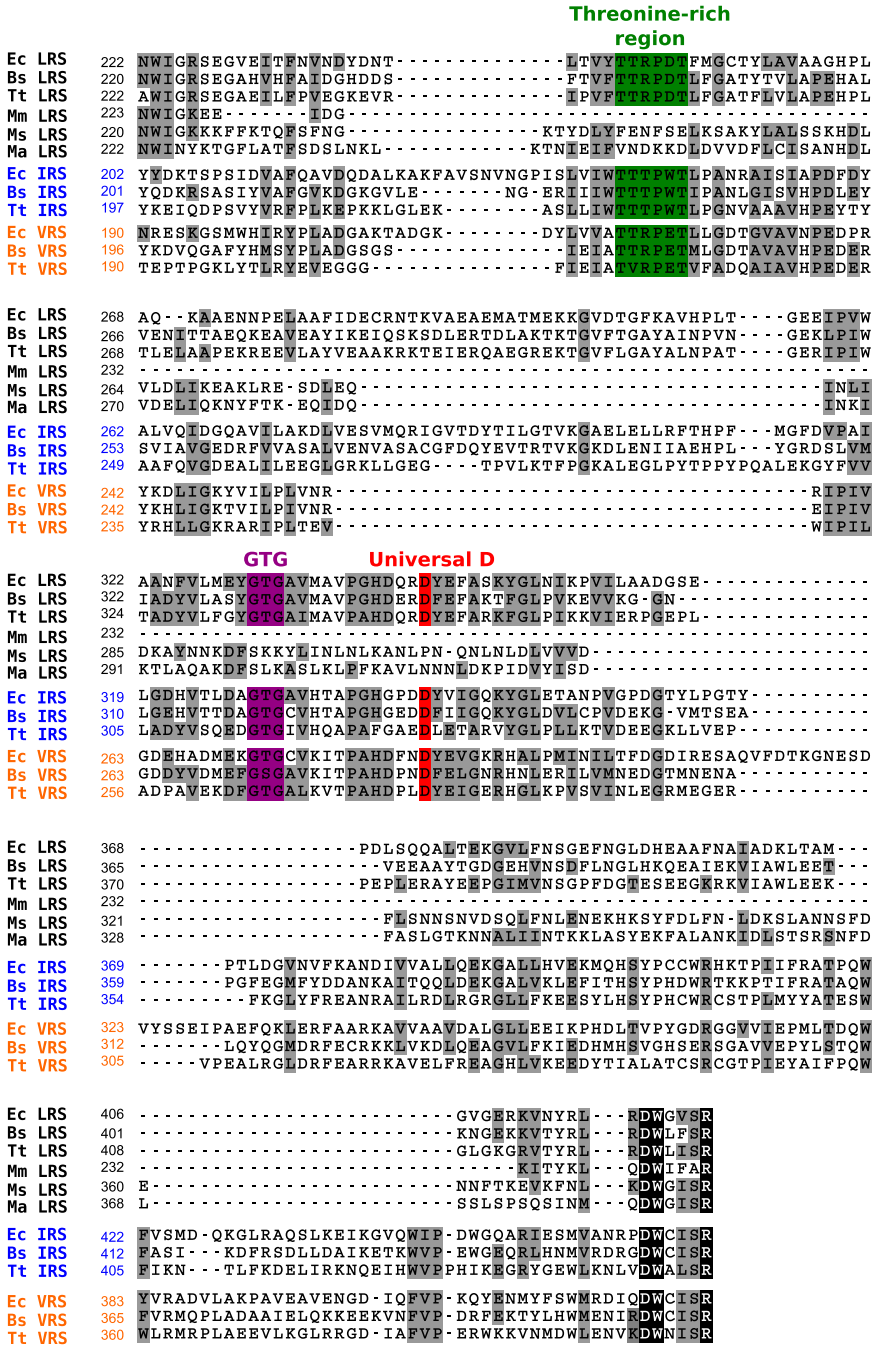

Fig. S1. Sequence alignment of CP1 domains. The CP1 domains from representative LeuRS (black), IleRS (blue), and ValRS (orange) were aligned using ClustalW, manually curated, and shaded using Biology Workbench (http://workbench.sdsc.edu). Shaded and black boxes denote homologous and conserved residues, respectively. Key motifs and residues of editing are highlighted: threonine-rich region (green), GTG motif (purple), and the universally conserved aspartic acid (red). Organism abbreviations are as follows: Bs, *Bacillus subtilus*; Ec, *Escherichia coli*, Ma, *Mycoplasma agalactiae*; Mm, *Mycoplasma mobile*; Ms, *Mycoplasma synoviae*; Tt, *Thermus thermophilus*.

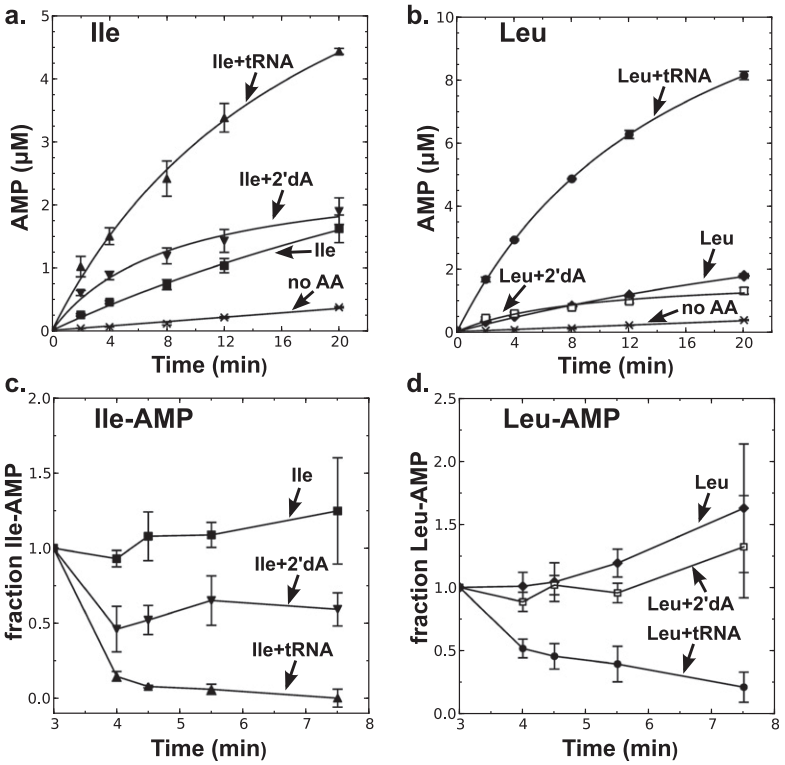

Fig. S2. *M. synoviae* LeuRS exhibits pretransfer editing activity. Reaction mixtures contained 10 μM *M. mobile* $tRNA^{Leu}$ or a tRNA analog with 2′-deoxyadenosine substituted for the A_{76} nucleoside (2′dA-tRNA), 1 μM *M. synoviae* LeuRS, and 0.5 U/mL inorganic pyrophosphatase. Amino acid-dependent AMP formation is measured by TLC of reaction aliquots from ATPase reactions with 5 mM isoleucine (*A*) or 5 mM leucine (*B*). Aminoacyl-adenylate was measured with 5 mM isoleucine (*C*) or 5 mM leucine (*D*). Abbreviations are as follows: ■, isoleucine only (Ile); ▲, isoleucine and tRNA (Ile+tRNA); ▼, isoleucine and 2′dA-tRNA (Ile+2′dA); ◆, leucine only (Leu); ●, leucine and tRNA (Leu+tRNA); □, leucine and 2′dA-tRNA (Leu+2′dA); and ×, no amino acid control (No AA). Error bars represent SDs derived from triplicated reactions.

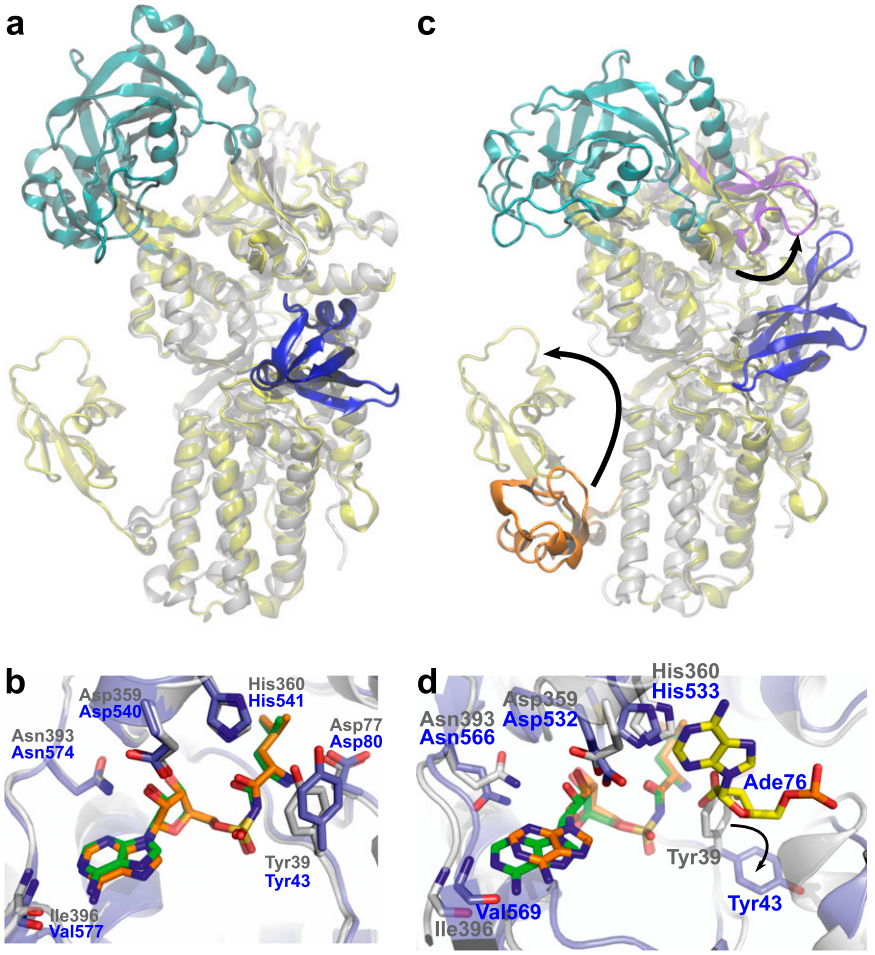

Fig. S3. Structural comparison indicates conformational changes for tRNA binding. (*A*) Structural alignment of *M. mobile* LeuRS (MmLeuRS, yellow) with *T. thermophilus apo* enzyme (TtLeuRS, PDB ID code 1H3N). The main body of TtLeuRS is colored in gray, the CP1 domain in cyan, and the leucine-specific domain in blue. The C-terminal domain of TtLeuRS was not resolved. (*B*) The aminoacylation site of MmLeuRS (gray) is structurally aligned with TtLeuRS (blue). The bound leucyl adenylate analog, Leu-AMS, is shown in green for MmLeuRS and orange for TtLeuRS. Key residues interacting with Leu-AMS are shown in licorice and labeled. (*C*) Structural alignment of MmLeuRS (yellow) with *E. coli* LeuRS aminoacylation complex (EcLeuRS, PDB ID code 4AQ7). The EcLeuRS is colored similarly to the TtLeuRS, except its C-terminal domain is shown in orange and its Zn-1 domain in purple. The major domain movement to accommodate tRNA binding is indicated by black arrows. (*D*) Structural alignment of MmLeuRS (gray) with EcLeuRS (blue). The binding of A_{76} of tRNA (yellow) induces the side chain of Tyr43 in EcLeuRS to rotate about 120° (indicated by a black arrow). The leucyl adenylate analog, Leu-AMS, is shown in green for MmLeuRS and orange for EcLeuRS. Key residues interacting with Leu-AMS are shown in stick model and labeled.

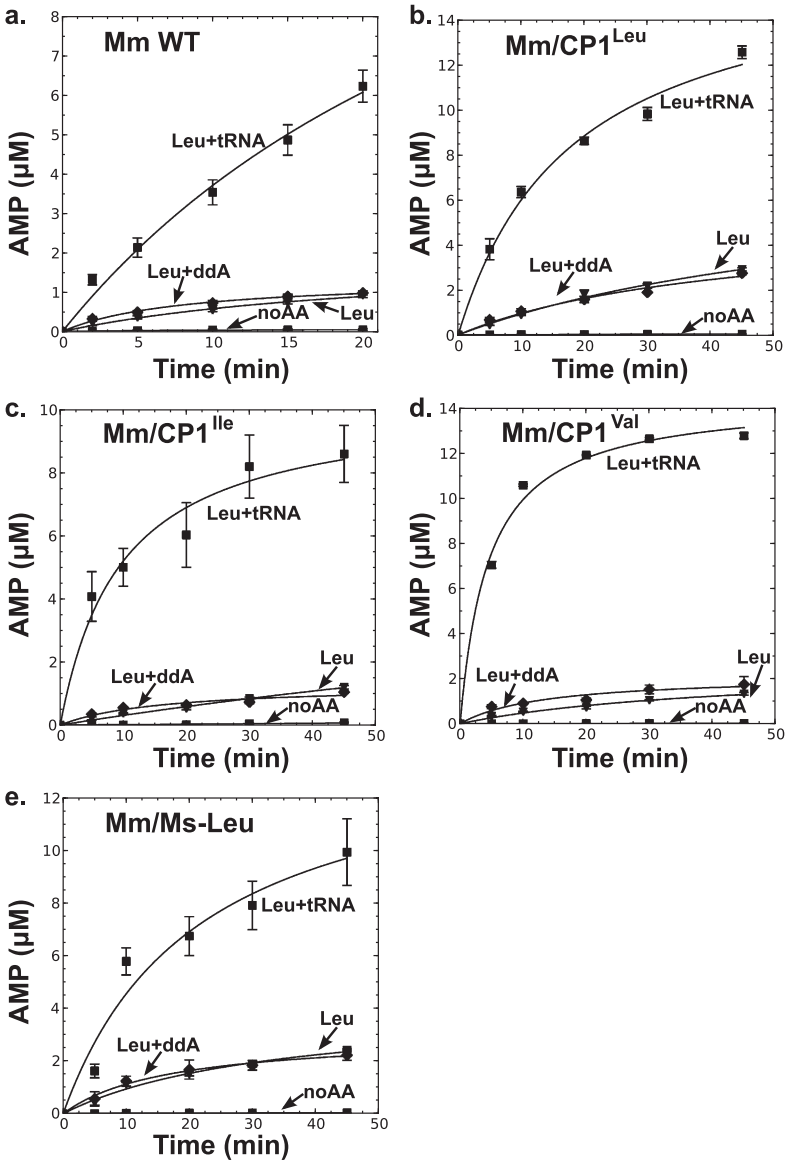

Fig. S4. tRNA-dependent pretransfer editing for *M. mobile* LeuRS is specific for nonleucine amino acids. Reaction mixture contained 1 μM enzymes, 10 μM $tRNA^{Leu}$, or $tRNA^{Leu}$ with A_{76} replaced by dideoxyadenosine analog (ddA-tRNA), 18.1 μM [α-^{32}P]ATP (40 μCi/mL), and 2.5 mM leucine. The AMP formation activities are measured for *M. mobile* LeuRS (*A*), MmLeuRS/$CP1^{Leu}$ (*B*), MmLeuRS/$CP1^{Ile}$ (*C*), MmLeuRS/$CP1^{Val}$ (*D*), and MmLeuRS/Ms$CP1^{Leu}$ (*E*). Symbols used are as follows: ◆, reaction without amino acid present (no AA); ▲, leucine (Leu); ▼, leucine with tRNA (Leu+tRNA); and ■, leucine with ddA-tRNA (Leu+ddA). Error bars represent the SD values based on three separate experiments.

Table S1. Data collection and refinement statistics

	M. mobile LeuRS + Leu-AMS*
Data collection	
Space group	*R*32
Cell dimensions	
a, *b*, *c* (Å)	202.9, 202.9, 213.9
α, β, γ (°)	90, 90, 120
Resolution (Å)	12–2.07 (2.07–2.12)
R_{merge}	0.227 (0.993)
$I/\sigma I$	18.45 (2.56)
Completeness (%)	99.95 (99.20)
Redundancy	12.4 (11.8)
Refinement	
Resolution (Å)	2.07
No. reflections (work/free)	96,445/5,079
R_{work}/R_{free}	0.159/0.199
No. of atoms	
Protein	5,164 [A]† + 4,844 [B]
Ligand	31 [E] + 31 [F] (Leu-AMS)
Water/other	1,022/10 glycerol
B-factors average	
Protein	28.4 [A]†/22.7 [B]
Ligand	12.6 [E]/12.7 [F]
Water	33.2
Rms deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.509

*Values in parentheses are for highest-resolution shell.
†Values are for each molecule in the asymmetric unit, with chain indicator given in square brackets.