

Supporting Information

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SI Materials and Methods

DNA Constructs. DNA sequences of the *ileS* gene and tRNA^{Ile} were derived from the *M. smegmatis* strain MC2 155 genome (NC 008596.1). Positions 121–169 in *M. smegmatis ileS* were replaced by 5'-CAGGGTGGTACCGCGATAATCAATCGTCCCTG-3' to shorten the antisequestator structure and to facilitate RNA folding. For the SHAPE experiments, the *M. smegmatis ileS* sequence was incorporated into a “structure cassette” (17, 39). For analysis of cross-linking by capillary electrophoresis experiments, 5'-GGAGT-3' was added 5' of the *ileS* sequence, and the tRNA^{Ile} sequence was incorporated into the “structure cassette.” The DNA sequence for noncognate tRNA^{Gly} was derived from *B. subtilis* strain BR151 (*lys3 metB10 tpC2*).

DNA templates for *B. subtilis* tRNA^{Gly} and *M. smegmatis* tRNA^{Ile} 3' and 5' fragments were generated by ligating complementary pairs of oligonucleotides (43), followed by PCR. Other DNA templates were generated by ligation of complementary pairs of oligonucleotides and insertion into plasmid pGEM4 using XbaI and PvuII restriction endonucleases, followed by PCR. Mutations were introduced by using primers containing the mutation in PCR.

RNA Synthesis. RNAs were transcribed using a T7-MEGAshortscript high-yield transcription kit (Life Technologies) and purified using 8 M urea 6% wt/vol denaturing PAGE (44), followed by electroelution, phenol-chloroform extraction, and ethanol precipitation (45). RNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).

tRNA Binding Assays. Uniformly radioactively labeled tRNA was generated by including [α -³²P]-UTP (800 Ci/mmol; 1 Ci = 37 GBq) at a final concentration of 0.85 μ M in the T7 RNAP transcription reaction. Radioactivity determined in a Packard Tri-Carb 2100TR liquid scintillation counter was used to calculate the RNA concentrations utilizing measurements of the radioactivity of [α -³²P]-UTP of known concentration and the level of [α -³²P]-UTP incorporation in the transcription reaction. Labeled tRNA (0.05 μ M) was mixed with *ileS* RNA in 1 \times transcription buffer [20 mM Tris-HCl (pH 7.9), 20 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA]. The reactions were heated to 65 °C for 5 min, slow-cooled to 40 °C, and passed through a Nanosep 10K Omega filter microconcentrator (Life Sciences), which allowed the unbound tRNA to pass through the filter. The fluid retained on the filter was combined with Packard BioScience Ultima Gold scintillation fluid and counted in a Packard Tri-Carb 2100TR liquid scintillation counter. The data were fit to the standard quadratic binding isotherm using GraphPad Prism 7 software to determine approximate K_d values and SEs.

SHAPE. The *ileS* RNA (150 nM) was folded by snap-cooling (17) in 100 mM Hepes (pH 8.0), 10 mM MgCl₂, and 20 mM NaCl. Tenfold excess of tRNA or 0.5 \times Tris-EDTA buffer was added; then, the samples were cofolded as in tRNA binding assays, modified with *N*-methylisatoic anhydride (NMIA; 6.5 mM; Sigma-Aldrich), and precipitated as described (17). A fluorescently labeled oligonucleotide (5'-GAACCGGACCGAAGCCCG, 45 nM; Applied Biosystems, ThermoFisher Scientific) was used in primer extension inhibition, and parallel RNA sequencing reactions were performed as in ref. 39, except that RNAs were denatured before primer binding and reaction conditions were changed to 20 min at 52 °C, followed by 5 min at 65 °C. Samples were analyzed on a 3730 DNA Analyzer (Applied Biosystems, Inc.), and raw SHAPE reactivity was derived

using QuShape software (46). For each nucleotide, statistical outliers were removed, and average SHAPE reactivity (the difference between frequency of RT stops at each nucleotide in the presence and absence of NMIA) was calculated. The data were normalized using the 2–8% rule (47). Relative reactivity was calculated by subtracting normalized SHAPE reactivity in the absence of tRNA from reactivity in the presence of tRNA^{Ile} or tRNA^{Gly}.

UV Cross-Linking. The zero-length cross-linking analog 4-thio-UTP (TriLink BioTechnologies) was used to identify RNA contacts. A bipartite *ileS* leader RNA was constructed to allow incorporation of 4-thio-UTP into the Stem II and IIA/B domains. The 5' fragment of the bipartite leader RNA included nucleotides 1–49, while the 3' fragment contained nucleotides 50–98; 4-thio-UTP (final concentration 7.5 mM) was substituted for UTP in the T7 RNAP transcription reactions for the 3' fragment.

Two bipartite tRNA^{Ile} constructs (A and B) were used to validate RNA–RNA interaction. For tRNA A, the 5' fragment included nucleotides 1–44 and the 3' fragment included nucleotides 45–76. For the tRNA B, the 5' fragment included nucleotides 1–39 and the 3' fragment included nucleotides 40–76. The 3' fragment of tRNA A with 4-thio-UTP specifically incorporated at nucleotide 63 and the 5' fragment of tRNA B with 4-thio-UTP specifically incorporated at nucleotide 16 were purchased from Dharmacon (GE Healthcare Life Science).

Uniformly or 5'-labeled tRNAs were used to visualize the cross-linked RNA products. The 5'-labeling was carried out with [γ -³²P]-ATP (6,000 Ci/mmol, 1 Ci = 37 GBq) using a KinaseMax 5'-end labeling kit (Life Technologies) per the manufacturer's instructions. The concentration of radiolabeled RNA was determined by analysis in a Packard Tri-Carb 2100TR liquid scintillation counter as described above.

The *ileS* RNA (1.6 μ M) was combined with radioactively labeled tRNA (0.05 μ M) in 1 \times transcription buffer, heated to 65 °C for 5 min, and slow-cooled to 40 °C. RNAs were exposed to a 365-nm UV lamp for 10 min at 4 °C, and then ethanol precipitated (18). The RNA was resolved using 6% wt/vol denaturing PAGE, visualized by PhosphorImager analysis (Molecular Dynamics), and quantified using ImageQuant 5.2 or 8.1 software.

Primer Extension Inhibition Reactions. The cross-linked RNA was purified using 6% PAGE, electroeluted, and ethanol precipitated. In the experiments with the *ileS* sequence containing 4-thio-UTP to map cross-linked nucleotides in the *ileS* sequence, a DNA oligonucleotide (5'-CGCTCAACTCATGGG-3') complementary to *ileS* nucleotides 83–98 was 5'-labeled with [γ -³²P]-ATP (6,000 Ci/mmol, 1 Ci = 37 GBq), as previously described (44), and annealed to the RNA in 1 \times extension buffer [10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 60 mM NH₄Cl, 6 mM 2-mercaptoethanol] by heating to 65 °C for 5 min and slow-cooling to 40 °C. Reactions were incubated for 10 min at 37 °C and 10 min at 45 °C, followed by the addition of avian myeloblastosis virus RT (1 unit/reaction; ThermoScript RT-PCR; Invitrogen) and dNTPs at 40 μ M and incubation at 45 °C for 15 min. Primer extension reactions were terminated by the addition of precipitation/inactivation buffer [1 M guanidinium thiocyanate, 0.167% (wt/vol) *N*-lauryl sarcosine, 10 mM DTT, 83% isopropanol], followed by 1 h incubation at –20 °C. The products were resolved using denaturing 12% PAGE and visualized by PhosphorImager analysis (Molecular Dynamics), and quantification was performed using ImageQuant 5.2 software. A primer extension reaction with non-cross-linked *ileS*

leader RNA 5' fragment was carried out as a control. DNA-sequencing ladders were generated using a DNA Sequenase 2.0 kit (USB Corporation), a pGEM4 plasmid construct containing the *ileS* leader sequence, and the oligonucleotide used in the primer extension inhibition reactions.

In the cross-linking experiments analyzed by capillary electrophoresis, primer extension inhibition and sequencing reactions were carried out as in the SHAPE experiments except for the use

of fluorescently labeled 5'-GAACCGGACCGAAGCCCG-3' or 5'-GCGCGCACGATGTGCACGC-3' primers (Applied Biosystems, ThermoFisher Scientific), respectively. For each nucleotide, statistical outliers were removed and an average RT stop (difference between the frequency of RT stops at each nucleotide in the cross-linked vs. non-cross-linked samples) value was calculated. The data were normalized to the average of the 5% highest average RT stops values.

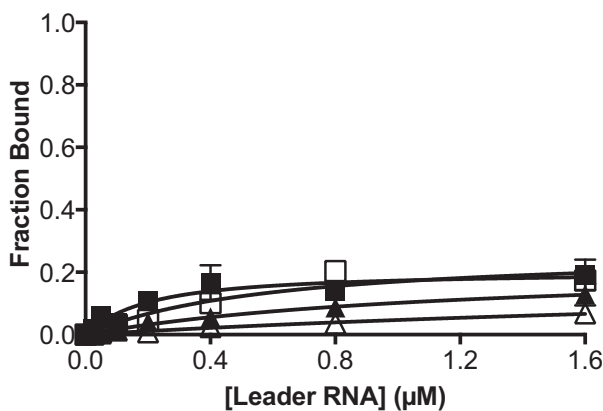


Fig. S1. tRNA^{Gly} binding to the *ileS* leader RNA variants. Labeled tRNA was incubated with increasing concentrations of the *ileS* leader RNA and passed through a size-exclusion filter, followed by quantification of the retained tRNA. Leader RNA variants correspond to those shown in Fig. 2. Variant 2, open triangles ($R^2 = 0.85$); variant 3, filled triangles ($R^2 = 0.66$); variant 4, open squares ($R^2 = 0.85$); variant 5, filled squares ($R^2 = 0.67$). Error bars denote SEM, $n = 3$.

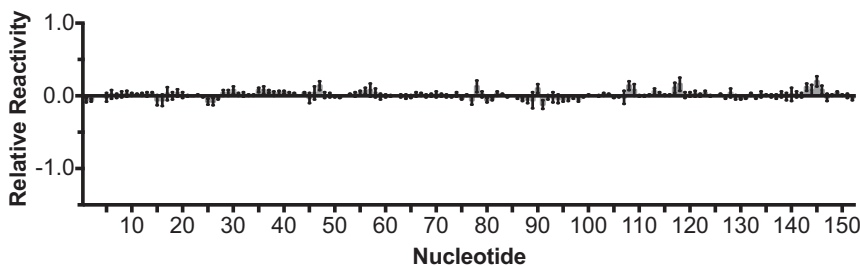


Fig. S2. Relative reactivity of *M. smegmatis* *ileS* leader RNA in the presence of tRNA^{Gly}. The *ileS* leader RNA was cofolded with 10-fold excess of noncognate tRNA^{Gly} before SHAPE experiments. Relative reactivity is the difference in SHAPE reactivity in the presence of tRNA^{Gly} vs. the absence of tRNA. Error bars denote SEM, $n = 9$.

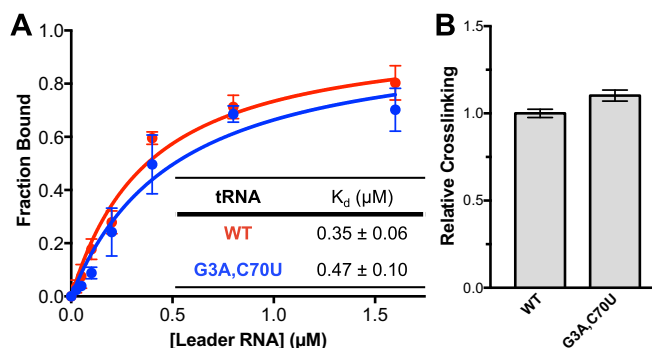


Fig. S3. (A) G3A, C70U tRNA^{le} binding to the bipartite *ileS* leader RNA variant 3. Labeled tRNA was incubated with increasing concentrations of the *ileS* leader RNA and passed through a size-exclusion filter, followed by quantification of the retained tRNA. WT, red ($R^2 = 0.97$); G3A,C70U, blue ($R^2 = 0.93$). Error bars denote SEM, $n \geq 3$. The insert shows approximate K_d values. (B) Cross-linking efficiency of mutant tRNAs relative to the WT leader RNA. Error bars denote SEM, $n \geq 3$.