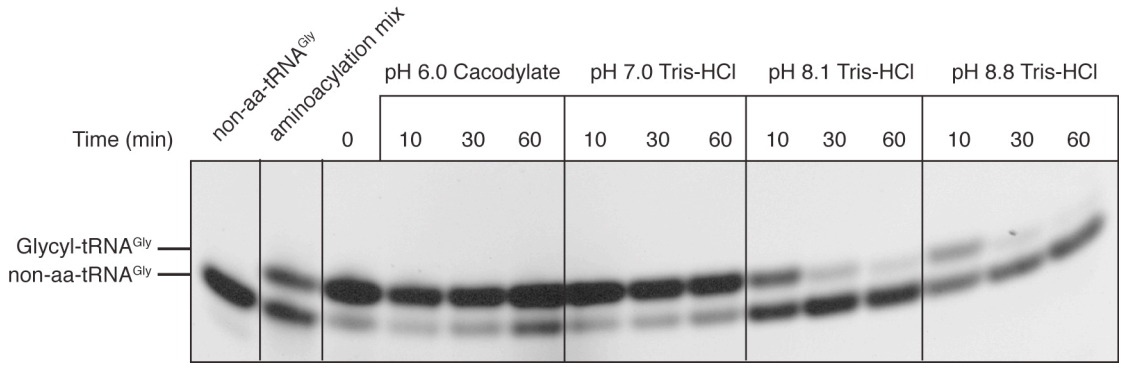
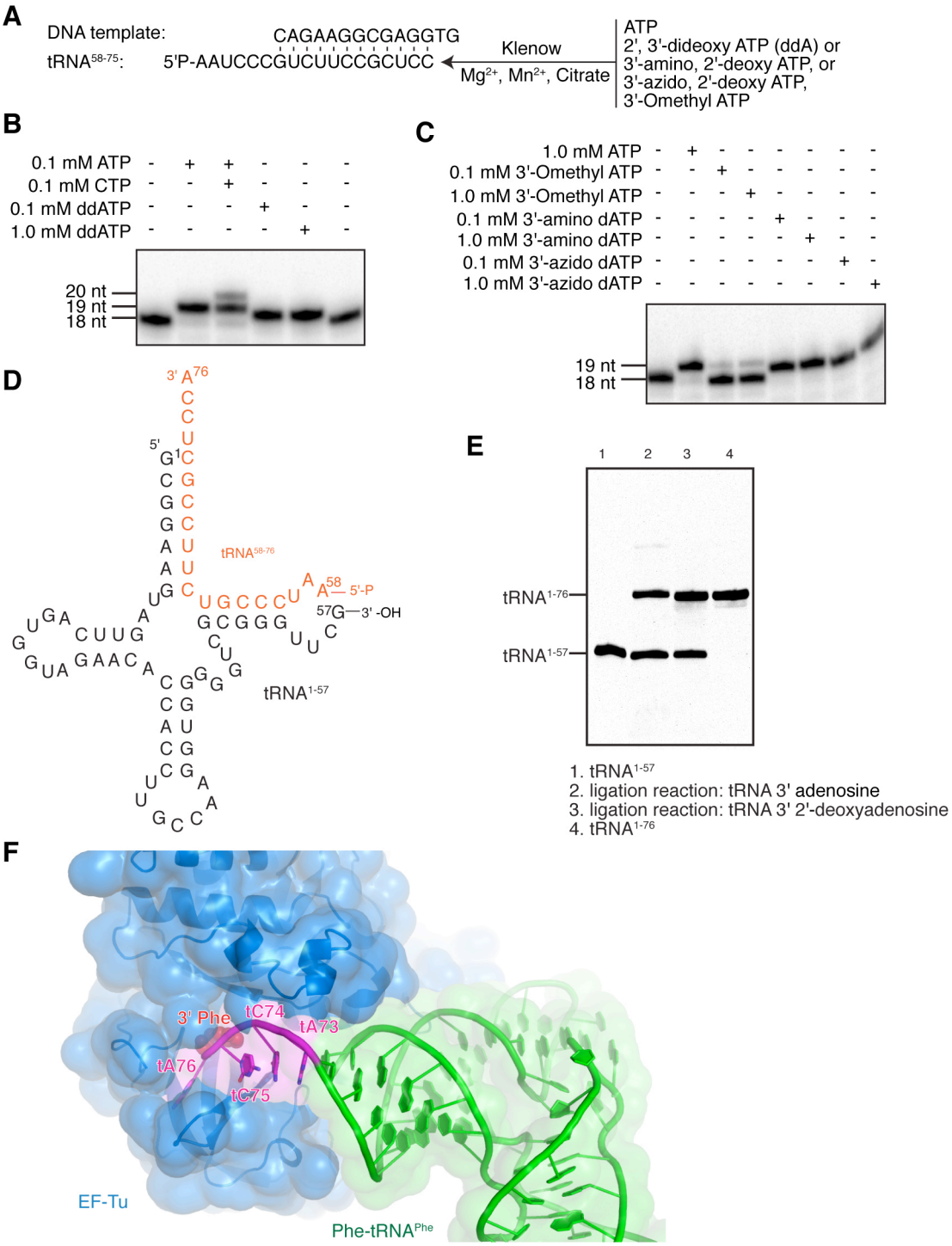



**Supplemental Figure 1. Stability of the Aminoacyl Bond in Glycyl-tRNA$^{Gly}$ at Different pH, related to Figure 1**

Acid-PAGE analysis shows that the aminoacyl bond is prone to rapid hydrolysis even at mildly alkaline pH.




**Supplemental Figure 2. Preparation of tRNA Variants that Carry a Range of Chemical Modifications on their 3' Ends, related to Figure 2**

(A) Scheme for efficient incorporation of modified 3'-terminal adenosines and analogs using the Klenow fragment of DNA polymerase I (adapted from Huang and Szostak, 1996). The combined presence of 10 mM $Mg^{2+}$ and 5 μM $Mn^{2+}$ broadens substrate selectivity while retaining robust polymerization activity of several DNA polymerases such as the Klenow fragment. The presence of a 5' overhanging nucleotide (G in this example) in the DNA template facilitates incorporation of a nucleotide in the adjacent neighboring position.

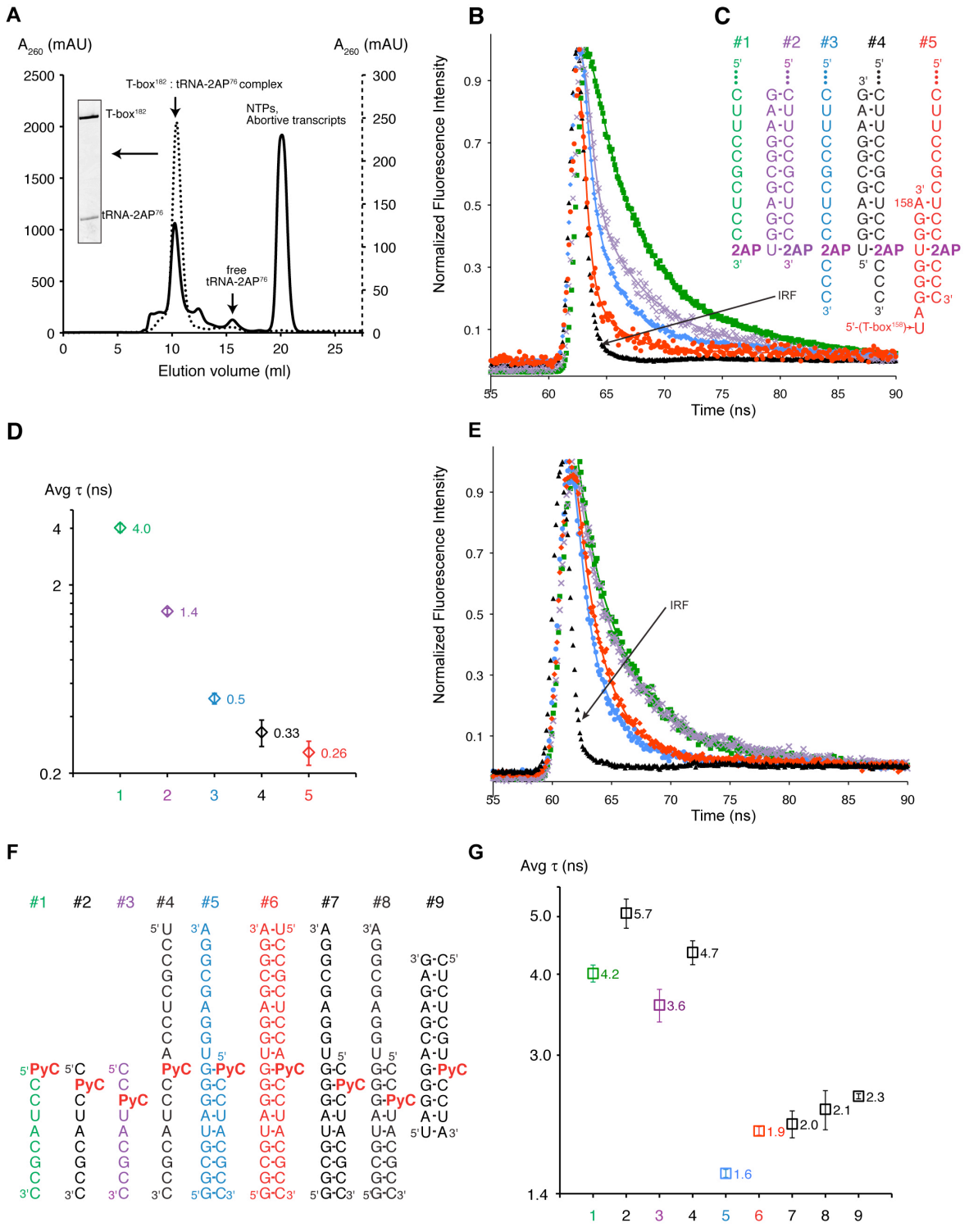
(B) Under conditions described in (A), the Klenow fragment efficiently incorporates ATP and ddATP.

(C) Under conditions in (A), the Klenow fragment efficiently incorporates 3'-amino dATP and 3'-azido dATP, but not 3'-methoxy ATP.

(D) Secondary structure schematic illustrating the reconstruction of full-length tRNA from two fragments through enzymatic RNA ligation.

(E) Urea PAGE analysis of tRNA ligation schematized in (D).

(F) EF-Tu•GTP (blue) binding to aa-tRNA (green) occludes its 3'-NCCA terminal region (magenta), preventing its base-pairing with the T-box antiterminator bulge (Nissen et al., 1995).




**Supplemental Figure 3. Benchmarking of 2AP and PyC Fluorescence Lifetimes, related to Figure 3**

(A) Chromatograms of initial fractionation (solid line, before lifetime measurements) and re-fractionation (dotted line, after lifetime measurements) of native tRNA-2AP$^{76}$-T-box$^{182}$ complex, and denaturing PAGE analysis of the RNAs in the tRNA-2AP$^{76}$-T-box$^{182}$ fraction.

(B) Normalized traces of 2AP fluorescence decay and best fits for RNAs in (C). Instrument response function (IRF) is also shown.

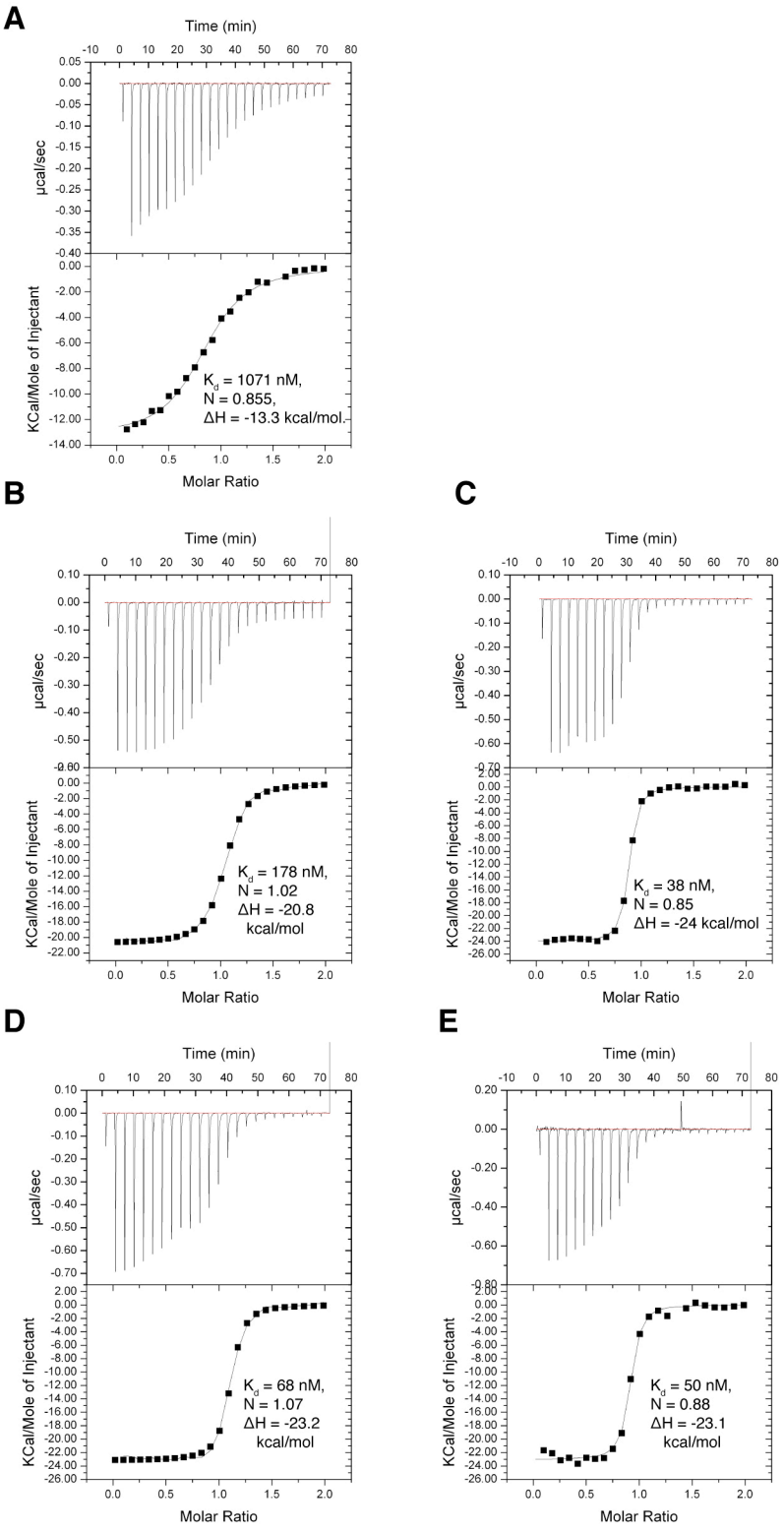
(C) Secondary structures of RNA and RNA complexes characterized in (B), with the same color code.

(D) Average 2AP lifetimes of the RNAs shown in (C), color-coded as in (B) and (C).

(E) Normalized traces of PyC fluorescence decay against time and best fits for RNAs in (F)

(F) Secondary structure schematics of RNA and RNA complexes measured in (E)

(G) Average PyC lifetimes of the RNAs shown in (F), as colored in (E) and (F).




**Supplemental Figure 4. Representative ITC Titrations, related to Figure 4**

(A) Titration of T-box$^{178\text{-}182}$ into T-box$^{158}$. $K_d$ = 1071 nM, N = 0.855, $\Delta H$ = -13.3 kcal/mol.

(B) Titration of wt tRNA into T-box$^{158}$. $K_d$ = 178 nM, N = 1.02, $\Delta H$ = -20.8 kcal/mol.

(C) Titration of wt tRNA into T-box$^{158}$ prebound with T-box$^{178\text{-}182}$. $K_d$ = 38 nM, N = 0.85, $\Delta H$ = -24 kcal/mol.

(D) Titration of tRNA$^{EX1C}$ into T-box$^{158}$. $K_d$ = 68 nM, N = 1.07, $\Delta H$ = -23.2 kcal/mol.

(E) Titration of tRNA$^{EX1C}$ into T-box$^{158}$ prebound with T-box$^{178\text{-}182}$. $K_d$ = 50 nM, N = 0.88, $\Delta H$ = -23.1 kcal/mol.

**Supplemental Table 1. Representative Fluorescence Lifetimes, related to Figures 3 & 4**

| Fluorophore | RNA in Figure | Construct number | $\chi^2$ | $\tau_1$ (ns) | Fraction ($\tau_1$) | $\tau_2$ (ns) | Fraction ($\tau_2$) | $\tau_3$ (ns) | Fraction ($\tau_3$) | Average $\tau_{Avg}$ (ns) | S.d. (ns)* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2AP | 2AP-TP | | 0.97 | 9.8 | 1.00 | | | | | 9.8 | 0.21 |
| 2AP | S3C | #1 | 1.07 | 5.9 | 0.59 | 1.34 | 0.408 | | | 4.03 | 0.21 |
| 2AP | S3C | #2 | 0.91 | 6.0 | 0.18 | 0.44 | 0.818 | | | 1.45 | 0.06 |
| 2AP | S3C | #3 | 2.08 | 4.8 | 0.09 | 1.32 | 0.160 | 0.18 | 0.88 | 0.50 | 0.03 |
| 2AP | S3C | #4 | 1.09 | 3.9 | 0.05 | 0.13 | 0.950 | | | 0.33 | 0.05 |
| 2AP | S3C | #5 | 1.04 | 3.4 | 0.05 | 0.11 | 0.955 | | | 0.26 | 0.04 |
| 2AP | 3B | #1 | 1.12 | 4.9 | 0.32 | 0.28 | 0.68 | | | 1.76 | 0.09 |
| 2AP | 3B | #2 | 0.98 | 5.1 | 0.12 | 0.21 | 0.88 | | | 0.80 | 0.06 |
| 2AP | 3B | #3 | 1.23 | 4.6 | 0.04 | 0.13 | 0.96 | | | 0.31 | 0.02 |
| 2AP | 3B | #4 | 1.08 | 4.7 | 0.06 | 0.13 | 0.94 | | | 0.40 | 0.03 |
| PyC | S3F | #1 | 1.41 | 4.2 | 1.00 | | | | | 4.2 | 0.18 |
| PyC | S3F | #2 | 1.49 | 7.3 | 0.65 | 2.24 | 0.35 | | | 5.70 | 0.41 |
| PyC | S3F | #3 | 0.97 | 5.9 | 0.45 | 1.72 | 0.55 | | | 3.60 | 0.29 |
| PyC | S3F | #4 | 1.11 | 7.1 | 0.60 | 1.34 | 0.40 | | | 4.68 | 0.28 |
| PyC | S3F | #5 | 1.05 | 3.8 | 0.20 | 0.98 | 0.80 | | | 1.55 | 0.03 |
| PyC | S3F | #6 | 1.24 | 2.0 | 1.00 | | | | | 1.92 | 0.04 |
| PyC | S3F | #7 | 1.05 | 2.9 | 0.56 | 0.82 | 0.44 | | | 1.99 | 0.13 |
| PyC | S3F | #8 | 1.13 | 3.1 | 0.44 | 1.21 | 0.56 | | | 2.14 | 0.21 |
| PyC | S3F | #9 | 1.61 | 2.3 | 1.00 | | | | | 2.30 | 0.03 |
| PyC | 4B | #1 | 1.14 | 5.1 | 0.68 | 0.90 | 0.32 | | | 3.74 | 0.23 |
| PyC | 4B | #2 | 1.55 | 2.6 | 0.68 | 0.71 | 0.32 | | | 2.00 | 0.10 |

(*) S.d. was calculated from three independent replicate experiments.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Chemical Synthesis, RNA Production, and Other Materials

All materials were purchased from Sigma-Aldrich unless otherwise indicated. Chemical syntheses of 4-pentenoyl succinamide and dinitrobenzyl glycine were performed essentially as described (Lodder et al., 1998; Lodder et al., 2005; Goto et al., 2011) and verified by mass spectrometry and NMR (for dinitrobenzyl glycine). Longer RNAs used in this study (tRNAs, T-boxes, flexizyme, etc) were transcribed in vitro, purified by electrophoresis on 8% polyacrylamide, 8 M urea TBE gels (29:1 acrylamide:bisacrylamide), electroeluted, washed once with 1 M KCl, desalted by ultrafiltration, and stored at 4°C prior to use. DNA and RNA oligonucleotides were purchased from IDT-DNA or Trilink Biotechnologies.

### Preparation of Homogeneous Aminoacylated-tRNA (aa-tRNA)

tRNAs were produced by in vitro transcription with T7 RNAP as described (Milligan et al., 1987), with the modification that 20 mM GMP and 2 mM GTP were used instead of 5 mM GTP. This GMP "priming" (Puglisi and Wyatt, 1995) produces ~90% 5'-monophosphorylated and ~10% 5'-triphosphorylated tRNA. tRNA aminoacylation using dinitro-Flexizyme (dFx, 46 nts) was performed essentially as described (Goto et al., 2011). Efficiency of aminoacylation (typically 50-60%) was evaluated using acid gel electrophoresis (6.5% polyacrylamide; 29:1 acrylamide:bisacrylamide; the gels were cast and run in 100 mM NaOAc pH 5.5; Varshney et al., 1991). Aminoacylation reactions containing non-aa-tRNA, aa-tRNA, and dFx were precipitated with 70% ethanol, washed, and dried in a centrifugal vacuum concentrator before being resuspended in 10 mM NaOAc pH 5.5. For N-protection of the aa-tRNA, this aminoacylation

mixture containing 100 μM tRNA was mixed 1:1 (v/v) with 100 mM N-pentenoyl succinamide previously dissolved in 100% dioxane. Next, 1/10 volume of 1M $NaHCO_3$ was added to raise the pH to ~ 8.5 to initiate the reaction, which was allowed to proceed for 16 hours at room temperature with gentle mixing. The reaction was quenched by adding NaOAc pH 5.5 to a final concentration of 100 mM, precipitated by adjusting to 70% (v/v) ethanol, dried, and stored at -80 ºC. For RP-HPLC, the N-protection mixture was dissolved in RP-HPLC Buffer A (20 mM $NH_4OAc$ pH 5.5, 10 mM $MgOAc_2$, 400 mM NaCl, and 5% (v/v) methanol), and fractionated on a C18 column (Waters, 4.6 x 150 mm) using a linear gradient over 10 column volumes to Buffer B [Buffer A with 60% (v/v) methanol]. Fractions containing N-protected aa-tRNA were pooled, precipitated with 70% ethanol, dried, and resuspended in 10 mM $NH_4OAc$ pH 5.5 before deprotection. For deprotection, N-protected aa-tRNA was mixed with 1/4 volume of 50 mM iodine previously dissolved in 1:1 (v/v) tetrahydrofuran:$H_2O$ and allowed to react for 0.5 h at room temperature (Lodder et al., 1998; Lodder et al., 2005). Deprotected aa-tRNA was brought up to 0.3 M NaOAc pH 5.5, precipitated by adjusting to 70% (v/v) ethanol, washed, dried, and stored at -80 ºC. Typical final purity of glycyl-$tRNA^{Gly}$ is better than 95% based on acid gel PAGE and RP-HPLC analysis.

**Preparation of tRNA Variants Carrying 5' or 3' Modifications**

A tRNA fragment (residues 1-57) was produced by in vitro transcription with GMP priming. It was then ligated with RNA oligonucleotides corresponding to tRNA residues 58-76 bearing the desired modifications. Briefly, the 100 μM tRNA 1-57 was incubated with 150 μM 5' monophosphated tRNA 58-76 in 5 mM HEPES-KOH pH 7.5 at 60 ºC for 5 min, before $MgCl_2$ was added to 10 mM. The mixture was allowed to cool to room temperature over 8 min. 1000

U/ml T4 RNA Ligase I (ssRNA ligase, New England Biolabs) and 1X T4 RNA Ligase Buffer were added and ligation was allowed to proceed for 16 hours at 16 ºC. Typical ligation yield was ~50% (Fig. S2E).

To prepare tRNA 58-76 fragment with 3' terminal 2',3'-dideoxy adenosine (ddA), 3'-amino adenosine and 3'-azido adenosine modifications, which were not commercially available, a synthetic oligonucleotide corresponding to residues 58-75 (i.e., missing the terminal A76) was extended using the Klenow fragment (New England Biolabs) and a complementary DNA template (Huang and Szostak, 1996; Fig. S2A-C) in Incorporation Buffer (40 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM dithiothreitol, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 15 mM $Na_3$Citrate pH 5.5). The $Mn^{2+}$-chelating citrate provides ~5 μM free $Mn^{2+}$ which serves to broaden the substrate specificity of DNA polymerases such as the Klenow fragment while avoiding enzymatic inhibition due to higher $Mn^{2+}$ (Tabor and Richardson, 1989). tRNA with 5'-OH was produced by co-transcriptional self-cleavage of a hammerhead ribozyme fused 5' of the tRNA sequence (Ferré-D'Amaré and Doudna, 1996).

**In Vitro Transcription Antitermination Assay**

In vitro transcription antitermination assays were performed essentially as described (Grundy et al., 2002; Grundy et al., 2005), using σ-factor-saturated *E. coli* RNA polymerase holoenzyme (Epicenter), which was previously shown to operate *B. subtilis* T-boxes to an extent comparable to *B. subtilis* RNA polymerase (Grundy et al., 2002; Grundy et al., 2005). Saturating concentrations of tRNA (3 μM) were used (Yousef et al., 2005); increasing the concentration to 10 μM did not alter the efficiency of antitermination. Instead of using heparin or rifampicin to

prevent re-initiation of transcription, elongation complexes (EC16) were purified from nucleotide triphosphates using G-25 spin columns (GE Healthcare). To preserve the aminoacyl bond, the experiments involving unprotected glycyl-$tRNA^{Gly}$ were performed in pH 7.0 instead of pH 8.1. At pH 7.0, glycyl-$tRNA^{Gly}$ was not appreciably hydrolyzed in 60 min, which was longer than the duration of the experiments (Figure S1).

**Assembly and Isolation of Native T-box-tRNA Complexes by SEC**

Wild-type and 2AP-labeled $tRNA^{Gly}$ were pre-folded by snap-cooling to suppress dimerization, as described (Zhang and Ferré-D'Amaré, 2013). T-box fragments were transcribed by T7 RNA polymerase as previously described (Klein and Ferré-D'Amaré, 2006), in the presence of pre-folded tRNA. The transcription mixture was treated with Proteinase K (Roche; final concentration in the reaction 0.033 U/mL) to remove T7 RNA polymerase, and DNase I (New England Biolabs; final concentration in the reaction 50 U/mL) to remove the DNA template, and was fractionated on a Superdex-200 10/300 GL column (GE Healthcare) previously equilibrated with SEC buffer (100 mM KCl, 50 mM HEPES-KOH pH 7.0, 10 mM $MgCl_2$). Relevant fractions were pooled and concentrated using Amicon spin concentrators (Millipore) before measurement of fluorescence lifetime. After measurements, samples were re-analyzed on the same column to verify the intactness of the tRNA-T-box complex (Fig. S3A).

**Fluorescence Lifetime Measurements for 2-aminopurine (2AP) and Pyrrolo-cytosine (PyC)**

Fluorescence lifetime measurements were performed at room temperature on a EasyLife-LS (Photon Technology International) system with diode light sources at 280 nm (for 2AP) and 340 nm (for PyC) and bandpass emission filters 376/20 nm (for 2AP) and 448/20 nm (for PyC). For

benchmarking experiments using annealed RNA oligonucleotides, 10 μM of fluorescent oligonucleotide was mixed with 50 μM of non-fluorescent RNA in SEC buffer and annealed by cooling over 8 min from 60 ºC to 4 ºC before use. Collected traces were background subtracted, normalized, and fit to minimal number of exponential decay components that produced acceptable $\chi^2$ (Table S1) and random residuals using Felix32 spectroscopy software (Photon Technology International).

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