## Supplementary Information

### TLS Refinement

Refinement using the program CNS (Brunger et al., 1998) resulted in the R factors ($R_{free}/R_{work}$) of 33.4/29.1 for crystal I, 35.0/29.8 for crystal II and 41.7/29.7 for crystal III. To examine the possible anisotropic motions of sub-domains in the complexes, rigid body refinement of sub domains (using TLS) underlayed further restrained refinement using REFMAC (Winn et al., 2001) on all three crystal structures. After many trials using different sub domains, the optimal B-factor refinement was obtained for crystal I and III as follows: for crystal I each stem loop of tRNA was treated as a separate TLS group, and for crystal III each individual protein and tRNA molecule was treated as a separate TLS group. R factors were improved to 27.9/24.6 and 35.7/25.9 for crystal I and III, respectively. No improvement was observed for crystal II.

### $^{3}$H release assay

From a 150 μl reaction containing 5.5 nM enzyme and 0.1-2 μM substrate tRNA incubated at room temperature, aliquots of 24 μl were removed at time intervals of 0.5 min up to 3 min and quenched with 0.5 mL of 5 % Norit A in 0.1 N HCl. For slow reactions ($k_{cat}/K_M < 0.1\text{x}10^{-3}$ $s^{-1}\mu M^{-1}$), 55 nM enzyme was used with the time-course of 10 min and the quenching interval of 2 min. The quenched mixture was centrifuged and filtered through Ultrafree-MC filter device (Amicon) to remove Norit A (which binds tRNA). The filtrate was counted twice in 5 ml of Aquasol-2 (New England Nuclear).

**Figure S1. Interaction between TruA and tRNA.**

(A) Surface representation of the part of TruA interacting with tRNA (within 5 Å from tRNAleu1). Colors on surface represent the electrostatic characteristics of the atoms; hydrophobic (white), positive (blue), negative (red), polar N (lightblue), polar O (salmon) and sulfur (yellow).

(B) Schemetic description of the interaction. Interacting residues are connected by solid lines. Unlike many nucleic acid binding proteins, the TruA moieties interacting with the tRNA elbow and D-stem display hydrophobic characters. A set of aliphatic residues (V46, A81, A82, L85, G86, A89) are clustered and interact with the exposed hydrophobic surface of the tRNA elbow. In addition, Y140 stacks on the base or the ribose of U16, and R78 stacks onto the C56 base, forming a pi-cation interaction. Near the D-stem backbone are also several aliphatic residues (V167, T234, A238, T239) lining the D-stem backbone and interacting with the ribose ring. The hydrophobic interaction is complemented by few hydrophilic residues such as K241 and Q168 interacting with the phosphate or ribose hydroxyl groups. The interaction of the ASL with the loop1 (L1) and loop8 (L8) are described in the main text and Fig. 2.

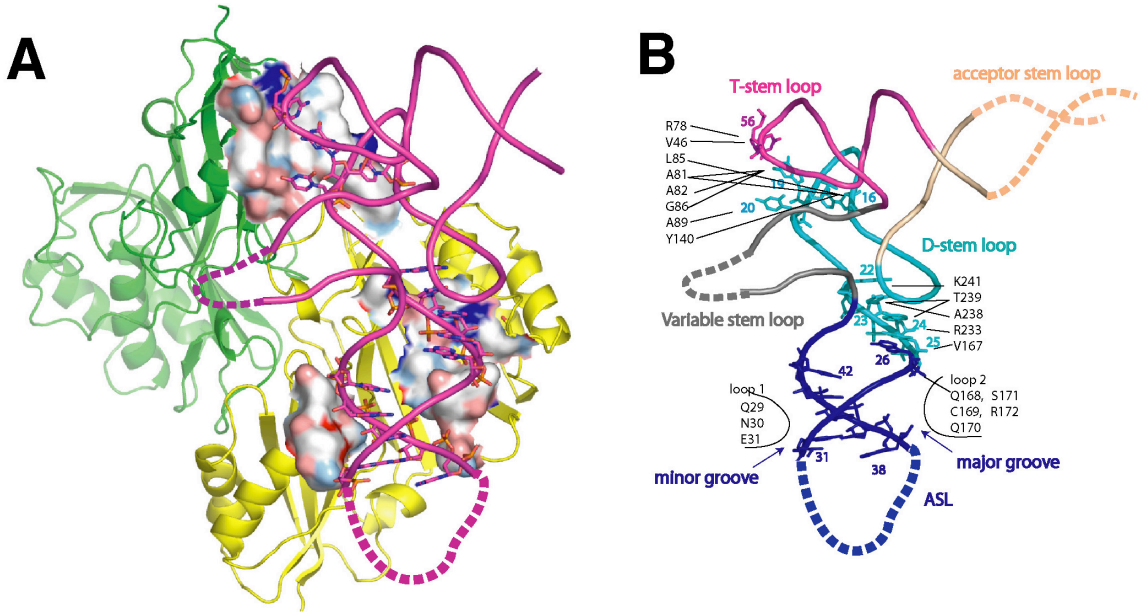


**Figure S2. Simulated Annealing Omit Map of *flipped-out* G39.**

The simulated annealing omit map (pink) is compared with the difference (Fo-Fc; blue, also shown in Fig. 3C). Both are phased with stacked conformation (green) of tRNA bound to TruA. Both maps support that the active site bound molecule is a guanidine nucleotide rather than a liberated uracil.

The possibility of the density being caused by a liberated uracil released during the chemical reaction is unlikely since it has been shown by chemical probes that TruA does not produce abasic tRNAs during the enzymatic reaction, and thus does not release *free* uracil (Zhao and Horne, 1997). In addition, the density matches the shape and size of a purine nucleotide with an explicit feature of the 5'-phosphate too close for any non-covalent interaction with a freed base. Thus, we conclude that the density is not caused by a liberated base, but by the flipped-out G39.

Zhao, X., and Horne, D. A. (1997). The role of cystein residues in the rearrangement of uridine to pseudouridine catalyzed by pseudouridine synthase I. J Bio Chem *272*, 1950-1955.

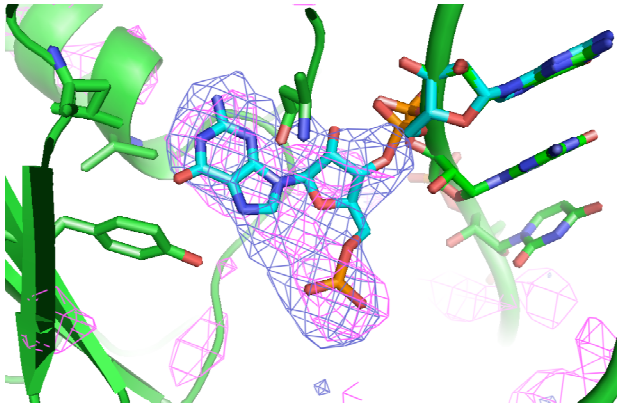
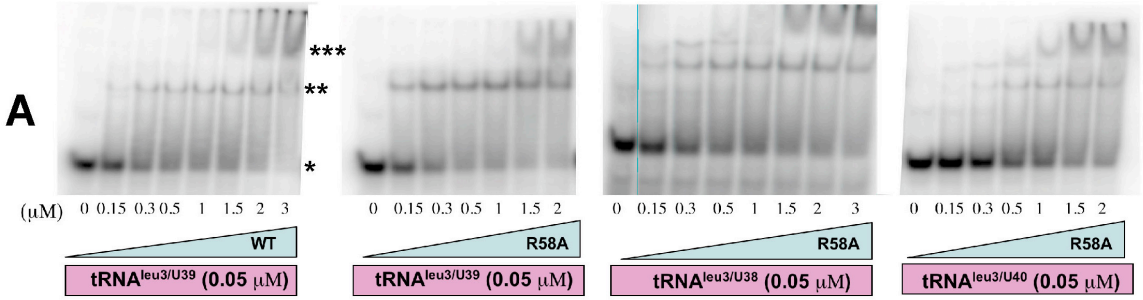


**Figure S3. Binding affinity of tRNA to wt-TruA and R58A.**

(A) Gel shift analysis using wt-TruA and [$^{32}$P]tRNA$^{leu3/U39}$; R58A and [$^{32}$P]tRNA$^{leu3/U39}$; R58A and [$^{32}$P]tRNA$^{leu3/U38}$; and R58A and [$^{32}$P]tRNA$^{leu3/U40}$. Free tRNA (*) and tRNA in complex with TruA (**). Bands with low electrophoretic mobility (***) occuring only at the protein concentration greater than 1 mM likely represent various multimeric forms of the protein interacting with the TruA•tRNA $^{leu3/U39}$complex. Curves for Kd determination are shown in Fig. 4D.

(B) To assess the specificity of the interactions shown in bands ** and ***, the complex formation of R58A•[$^{32}$P]tRNA$^{leu3/U39}$ were competed with either cold tRNA$^{leu3/U39}$ or mix of single stranded RNAs with size varying from 0.5 to 10kb, at 0.5, 1.0 and 1.5 mM of R58A. 1 mM tRNA is equivalent to 26 mg/l of RNA mix in RNA mass. Both ** and *** bands become weaker upon incubation with cold tRNA$^{leu3/U39}$, but not with the RNA mix, indicating that both bands are due to specific interaction between R58A and tRNA$^{leu3/U39}$.



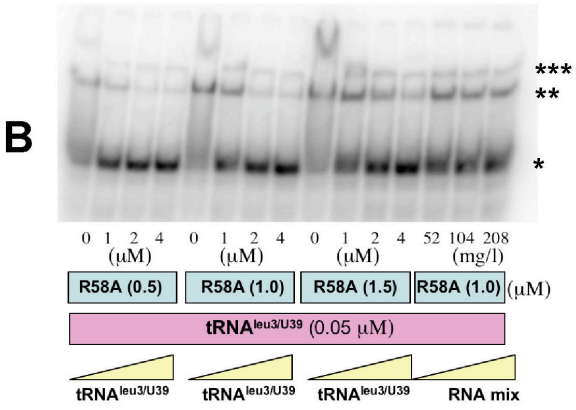



**Figure S4. Binding affinity of tRNA to wt-TruA and D60A.**

(A) In the previous studies, the dissociation constant of $tRNA^{phe}$ and D60A was measured by filter binding assay. To compare with our measurement, we performed the gel shift assay using D60A and [$^{32}$P]$tRNA^{leu3/U39}$. The binding affinity (44 nM) based on gel shift is very similar to the reported value (72 nM). The specificity of the interaction was tested by a competition experiment as described above.

(B) Curve for Kd determination.

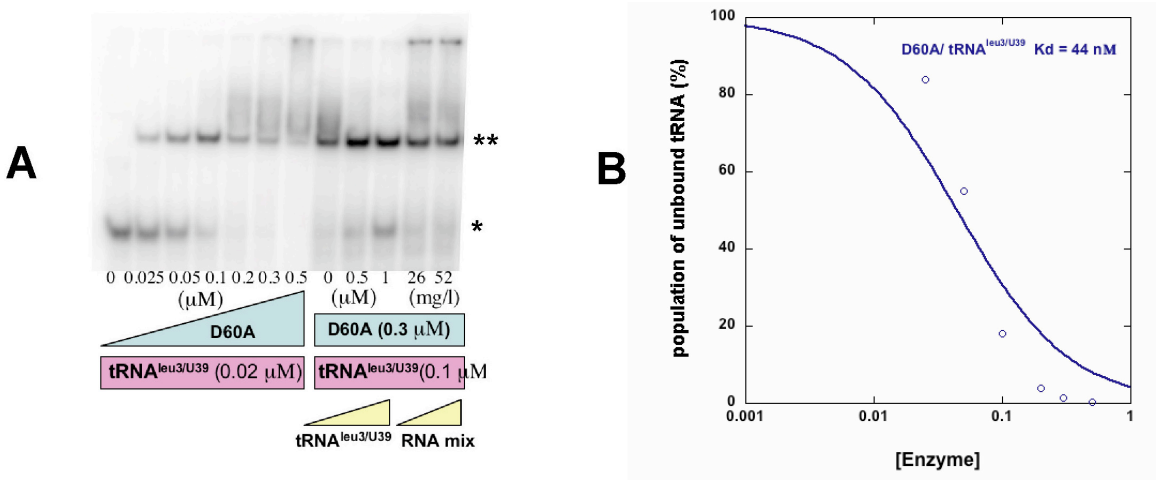
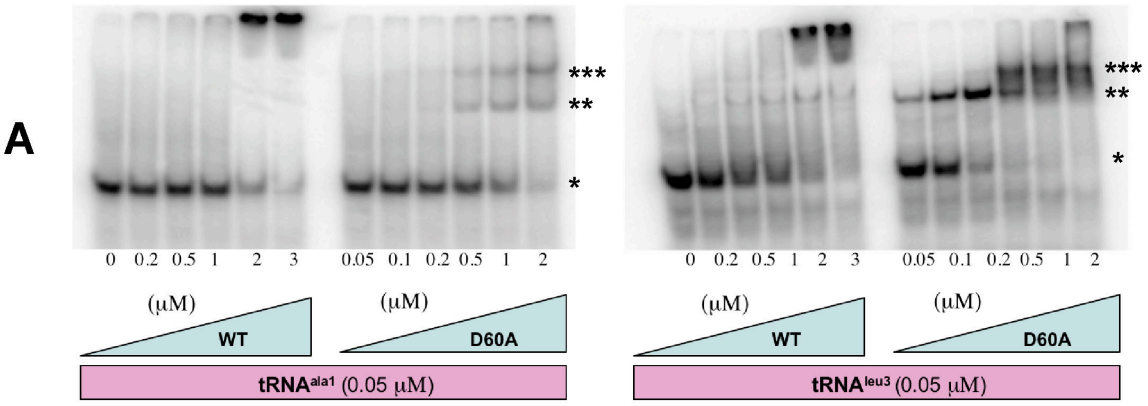


**Figure S5. Binding affinities of $tRNA^{ala1}$ and $tRNA^{leu3}$ to wt-TruA and D60A.**

(A) Gel shift analysis using Left: wt-TruA or D60A with [$^{32}$P]$tRNA^{ala1}$, and Right: wt-TruA or D60A with [$^{32}$P]$tRNA^{leu3}$.

(B) Curves for Kd determination.



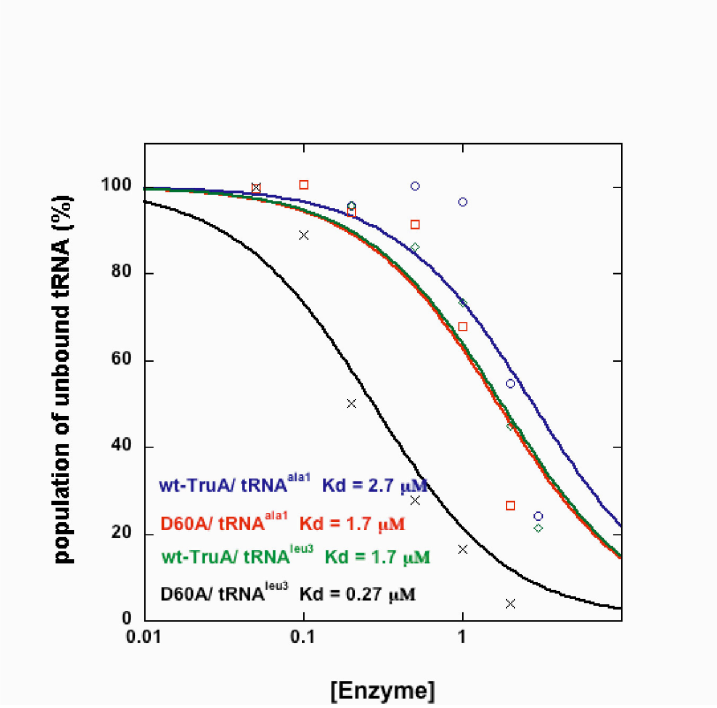



**Figure S6. Rigid-body vs. Atomic B-factors.**

Comparison of B-factors calculated by treating each stem loop of tRNA as a separate rigid body (green) or by allowing individual atomic variations (red). The lager amplitude fluctuation in B-factors from individual atomic refinement may reflect the limitation of the rigid-body assumption in describing the tRNA motion.