Supplementary Information for

Structures of the ribosome bound to EF-Tu–isoleucine tRNA elucidate the mechanism of AUG avoidance

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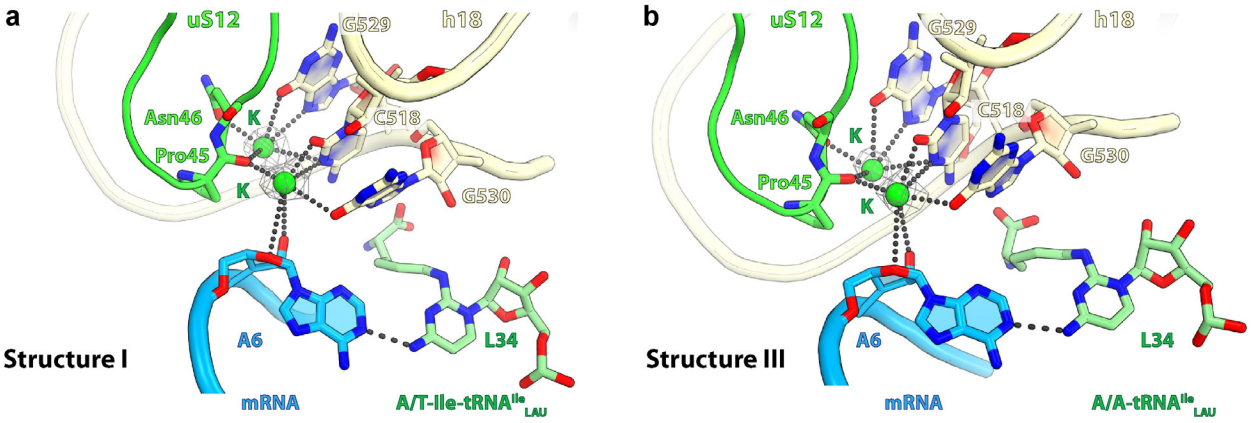
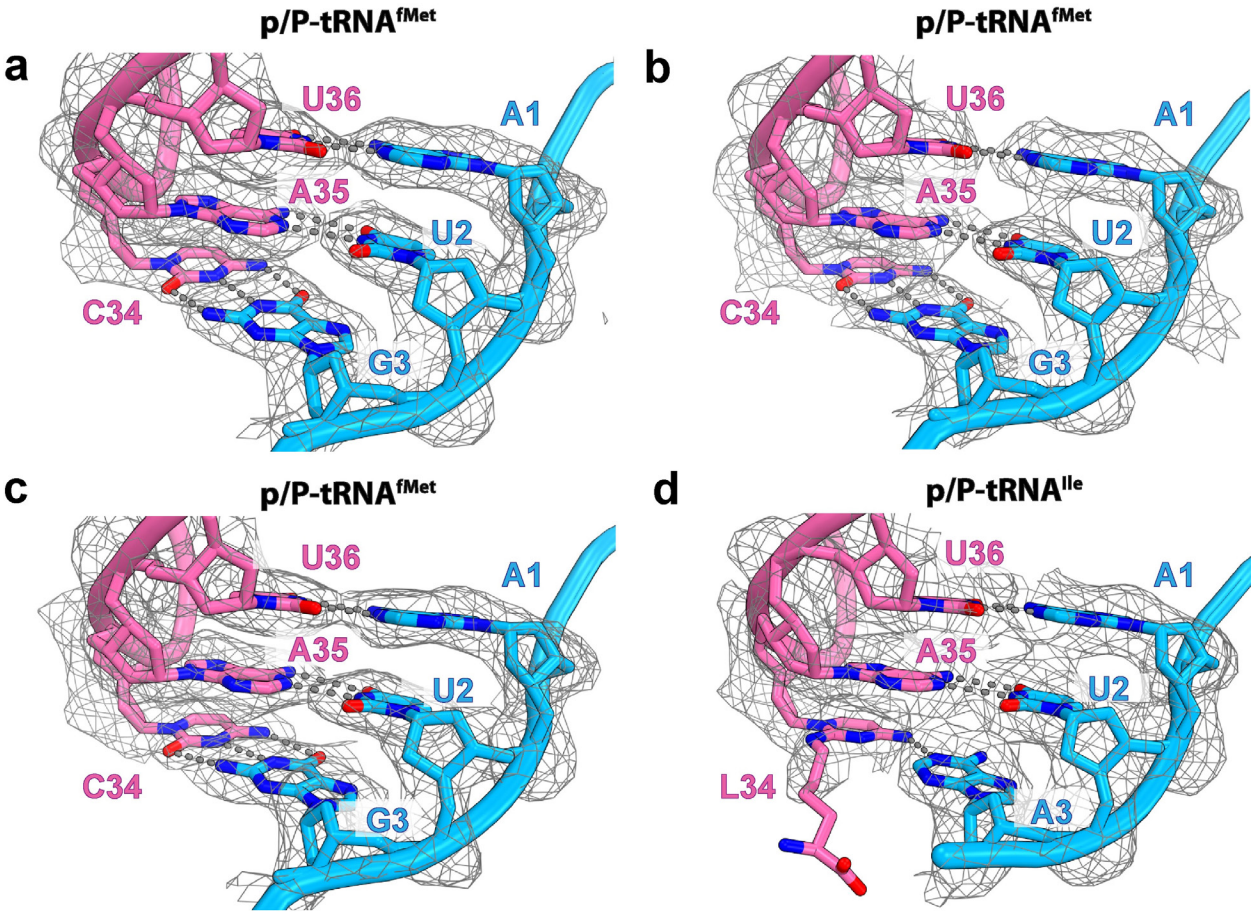
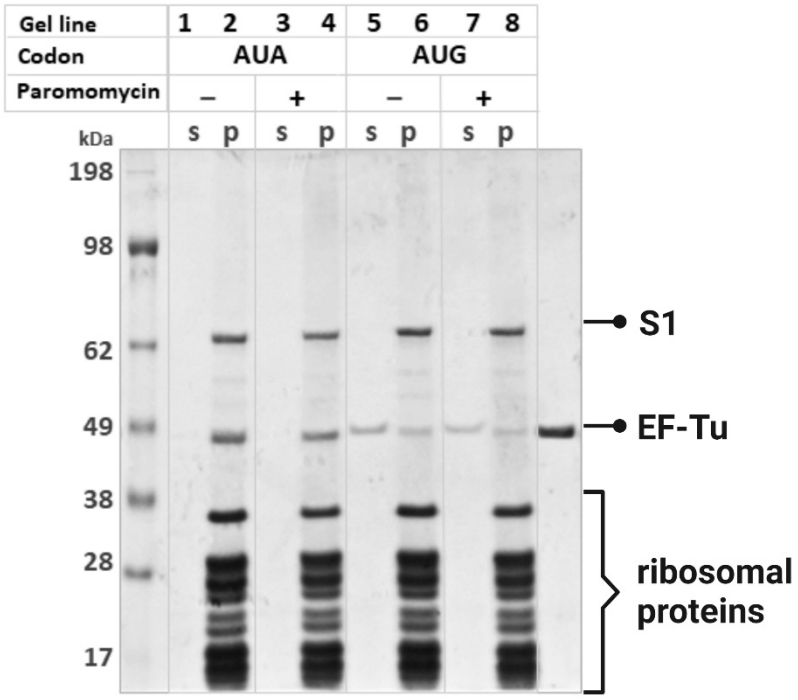


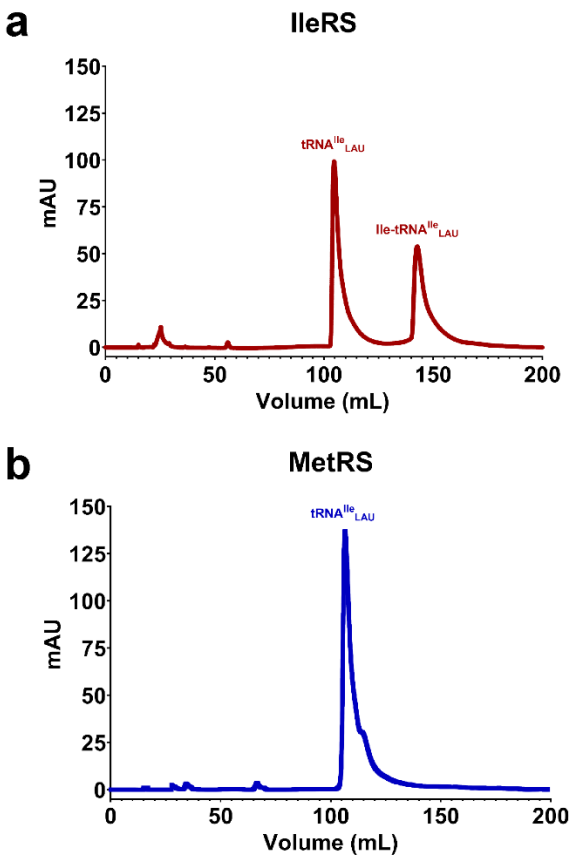
Supplementary Fig. 1. Potassium ions in the ribosome decoding center. Stabilization of the decoding center by potassium ions (green spheres) upon binding of the A-site $tRNA^{Ile}_{LAU}$ in structures I (**a**) and III (**b**), as previously observed in an elongation ribosome complex[1]. The stabilization of the third nucleotide of the A-site codon (A6) by potassium in both structures suggests that the ASL of the $tRNA^{Ile}_{LAU}$ is fully accommodated into the A site. The Coulomb potential density of the K^+ ions (gray mesh) is contoured at 2.9σ. The gray dashed lines indicate putative hydrogen bonds.



Supplementary Fig. 2. Interactions between the P-site tRNA and mRNA in structures I-IV. In structures I (**a**), II (**b**) and III (**c**), initiator $tRNA^{fMet}$ is bound to the P-site AUG codon, while in structure IV (**d**), $tRNA^{Ile}_{LAU}$ is bound to the P-site AUA codon. The Coulomb potential density (gray mesh) is contoured at 2.9σ. Putative hydrogens bonds are indicated by the gray dashed lines.



Supplementary Fig. 3. Co-sedimentation assay assessing the binding of EF-Tu•Ile-tRNA$^{Ile}_{LAU}$ to A-site AUA or AUG programmed 70S ribosome complexes. Binding of the *E. coli* ternary complex of EF-Tu, guanosine 5′-β,γ-methylenetriphosphate, and Ile-tRNA$^{Ile}_{LAU}$ to the 70S ribosome programmed with the cognate AUA (lanes 1-4) or near-cognate AUG (lanes 5-8) codon in the A site, analyzed by SDS-PAGE after centrifugation through a sucrose cushion. Gel lanes labeled (s) and (p) are proteins in the supernatant and pellet fractions, respectively. EF-Tu•GDPCP•Ile-tRNA$^{Ile}_{LAU}$ binds to the 70S ribosome with the AUA codon programmed in the A site similarly in the absence (lane 2) or presence (lane 4) of paromomycin. EF-Tu•GDPCP•Ile-tRNA$^{Ile}_{LAU}$ binds to the 70S ribosome programmed with the near-cognate AUG codon in A site with low apparent affinity with or without paromomycin (lanes 6 and 8). The results are representative of three independent experiments.



Supplementary Fig. 4. Aminoacylation reactions of tRNA$^{Ile}_{LAU}$ analyzed by reverse-phase HPLC. Aminoacylation of tRNA$^{Ile}_{LAU}$ by *E. coli* IleRS (**a**) or *E. coli* MetRS (**b**). The purified tRNA$^{Ile}_{LAU}$ is only aminoacylated by IleRS and does not show any peak for aminoacylated-tRNA^{Ile} following incubation with MetRS, confirming that lysidinylated-tRNA^{Ile} is recognized exclusively by IleRS, as reported[2].

Supplementary References

1. Rozov, A. et al. Importance of potassium ions for ribosome structure and function revealed by long-wavelength X-ray diffraction. *Nat. Commun.* **10**, 2519 (2019).
2. Nakanishi, K. et al. Structural basis for translational fidelity ensured by transfer RNA lysidine synthetase. *Nature* **461**, 1144-1148 (2009).