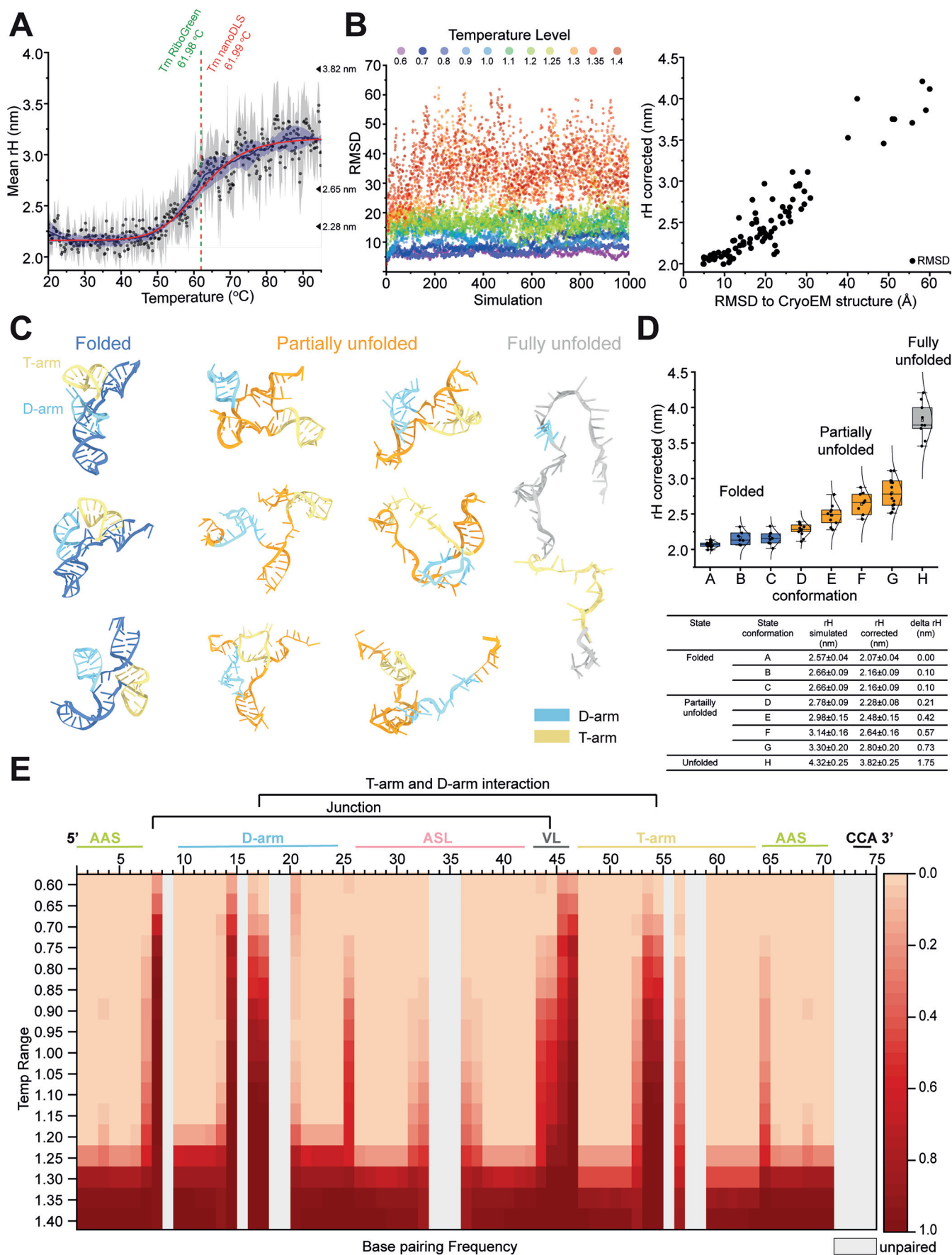
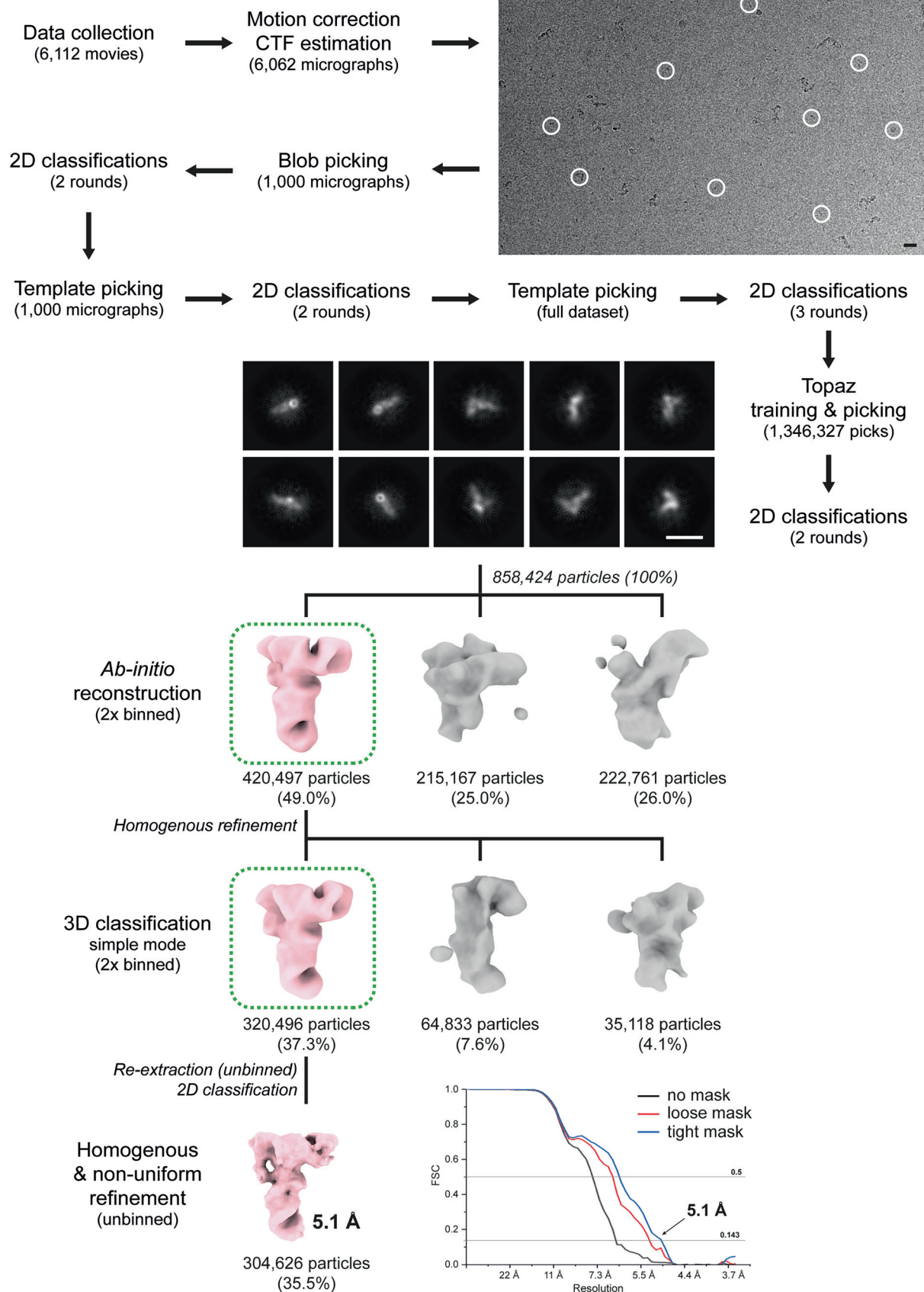


Expanded View Figures

Figure EV1. Molecular simulations of tRNA unfolding.

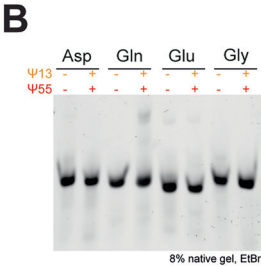
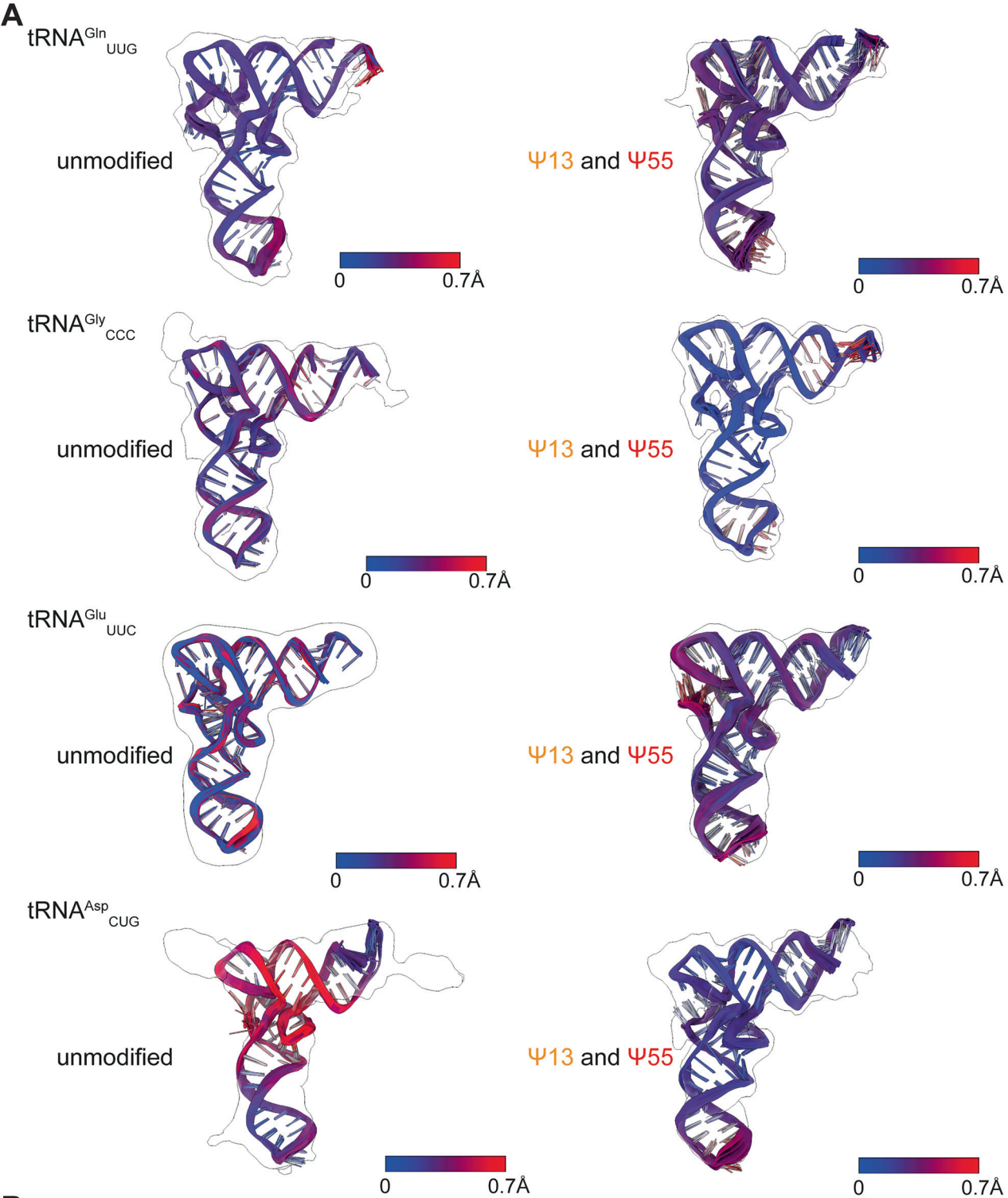
(A) NanoDLS analysis of tRNA^{Gln}_{UUG} size changes in the thermal gradient. 2.28 nm rH corresponds to the simulated start of the unfolding process, 2.65 nm rH corresponds to the experimentally calculated inflection point, 3.82 nm rH corresponds to the average value of simulated fully unfolded tRNA^{Gln}_{UUG}. (B) Conformational space sampled by SimRNA at the different values of the T (temperature) parameter, from very "cold" (0.6) sampling of near-native structures to very "hot" (1.4) sampling of unfolded conformations (left). Relationship between the deviation of the progressively unfolded RNA 3D conformation from the folded structure and the rH value (right). (C) Selected models representing each of tRNA unfolding states. Light blue indicates the D-arm, yellow indicate T-arm. (D) Calculated hydrodynamic radii of simulated different unfolding states of the tRNA^{Gln}_{UUG} (top). Summary table of mean hydrodynamic radii calculated from the simulated unfolding states of tRNA^{Gln}_{UUG} and their recalibrated values (bottom). (E) A heatmap representing the frequency of base-pairing retention in SimRNA simulations at the T parameter ranging from 0.6 to 1.4. Source data are available online for this figure.



tRNA^{Gln}_{UUG} unmodified

**Figure EV2. Cryo-EM reconstruction pipeline for unmodified tRNA^{Gln}_{UUG}.**

A representative micrograph is shown (top right) together with indicated positions of the finally selected particles (white circles); scalebar = 100 Å. Representative 2D classes, ab initio classes and further steps of 3D refinement are shown in the bottom part of the figure. Absolute numbers and percentages of particles are listed and the Fourier Shell Correlation blot (FSC) blot of the final reconstruction, highlighting the nominal resolution at FSC = 0.143.



**Figure EV3. Comparison of the obtained models within each ensemble.**

(A) From top to bottom. Cryo-EM reconstructions of unmodified (left) and Ψ_{13} - and Ψ_{55} -modified (right) human tRNA^{Gln}_{UUG}, tRNA^{Gly}_{CCC}, tRNA^{Glu}_{UUC} and tRNA^{Asp}_{GUC} with an ensemble of 10 atomic models fitted into the density colored by RMSD between each atom. (B) A native gel showing the mobility of unmodified and Ψ_{13} - and Ψ_{55} -modified tRNAs.

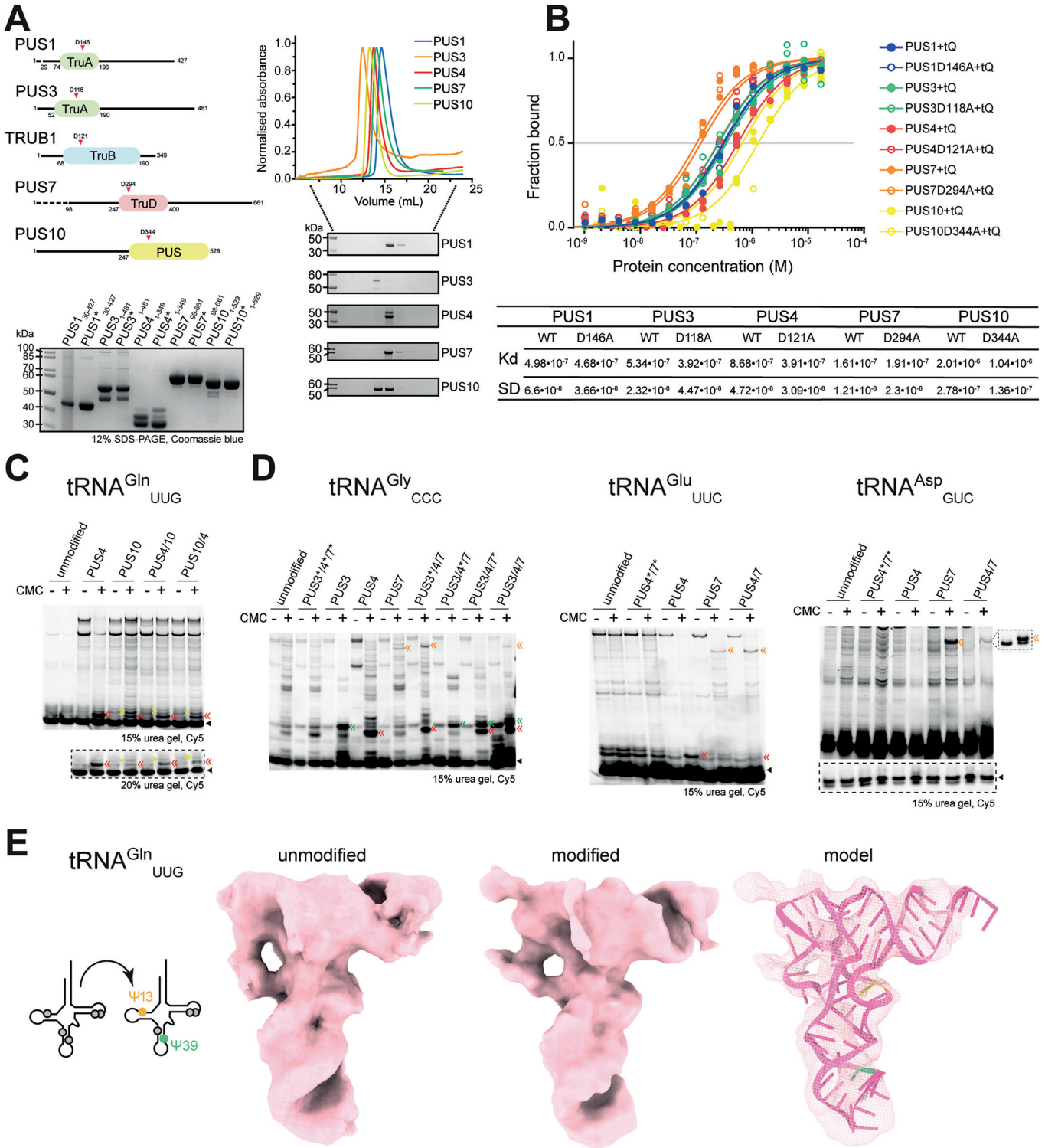




Figure EV4. In vitro pseudouridylation of human tRNAs.

(A) Cartoon presentation of domains of each PUS (upper-left). The length is labeled, and domain is highlighted where the catalytic residue is indicated by a triangle. The truncated regions of PUS1 and PUS7 are shown as dashed lines. An SDS-PAGE gel of recombinant HsPUS enzymes (lower-left). Catalytic mutants marked with stars. SEC profiles of purified human PUS enzymes (upper-right) and the collected fraction for each purification were resolved in SDS-PAGE gels (lower-right). (B) MST analyses of PUS, including wild type and inactive form (DA), binding to tRNA^{Gln}_{UUG}. The calculated K_ds of wild-type PUS and inactive forms (DA), binding to tRNA^{Gln}_{UUG}. (C, D) Detection of PUS-dependent Ψ formation on tRNA^{Gln}_{UUG}, tRNA^{Gly}_{CCC}, tRNA^{Glu}_{UUC} and tRNA^{Asp}_{GUC}. The reverse-transcribed cDNA products were resolved in a 15% or 18% urea gel and the CMC-Ψ mediated short cDNAs are indicated by double-arrows (Ψ₁₃ orange, Ψ_{27/28} blue, Ψ₃₉ green, Ψ₅₄ yellow Ψ₅₅ red). Each tRNA primer (labeled with Cy5) is indicated by a triangle. In the case of tRNA^{Asp}_{GUC}, the signal for primer is obtained from a short exposure shown in the dash lined box while the Ψ₁₃-dependent cDNA is obtained using a site-specific primer (shown in a dash lined box on the side). (E) Cryo-EM reconstructions of unmodified and Ψ₁₃- and Ψ₃₉-modified tRNA^{Gln}_{UUG}. The modified sites are highlighted by color code in the 2D cartoon and the model (Ψ₁₃ orange and Ψ₃₉ green). Source data are available online for this figure.

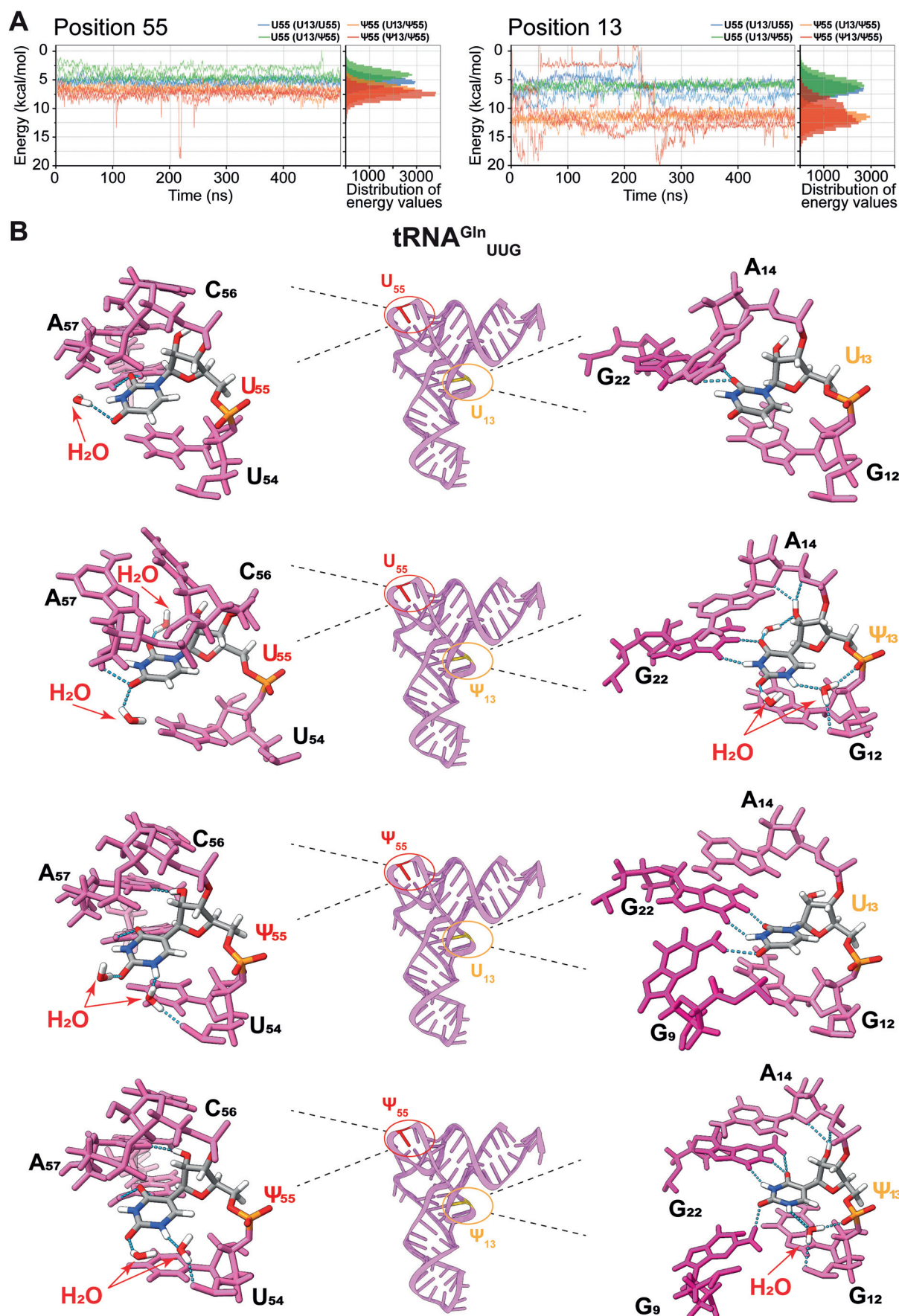



Figure EV5. MD Simulations of tRNA with and without Ψ_{13} and Ψ_{55} .

(A) Distribution of interaction energies between uridine (U) and pseudouridine (Ψ) with neighboring residues throughout the simulation and a histogram of the energy distribution for U_{55}/Ψ_{55} (left) and U_{13}/Ψ_{13} (right). The graphs emphasize the varying stability and dynamics of interactions across different simulation variants. Notably, variants containing Ψ (depicted in orange and red) exhibit greater stability compared to those with U, represented in green and blue. (B) Snapshots illustrating the local environments at positions 55 (left) and 13 (right) for all simulation variants. In the simulations where position 13 is occupied by Ψ (specifically in Ψ_{13}/U_{55} and Ψ_{13}/Ψ_{55}), a water molecule (red arrow) forms a triad of hydrogen bonds involving the imino hydrogen of Ψ_{13} and the phosphate groups of both Ψ_{13} and G_{12} . At position 55, when Ψ_{55} is present (in U_{13}/Ψ_{55} and Ψ_{13}/Ψ_{55}), a water molecule (red arrow) bridges between the imino hydrogen of Ψ_{55} and the OP of U_{54} . Notably, these specific interactions are absent when U occupies both the position 13 and 55.