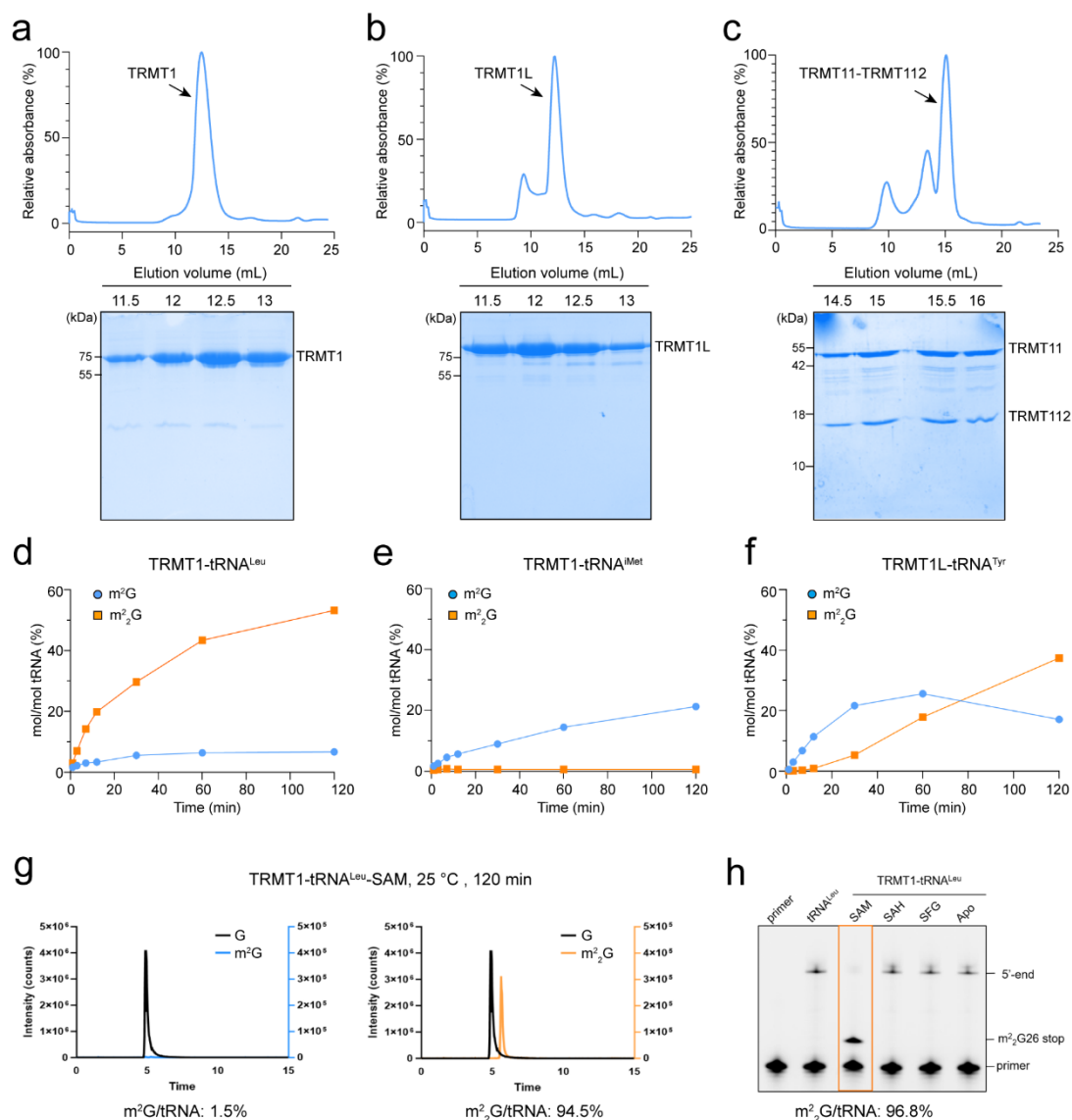
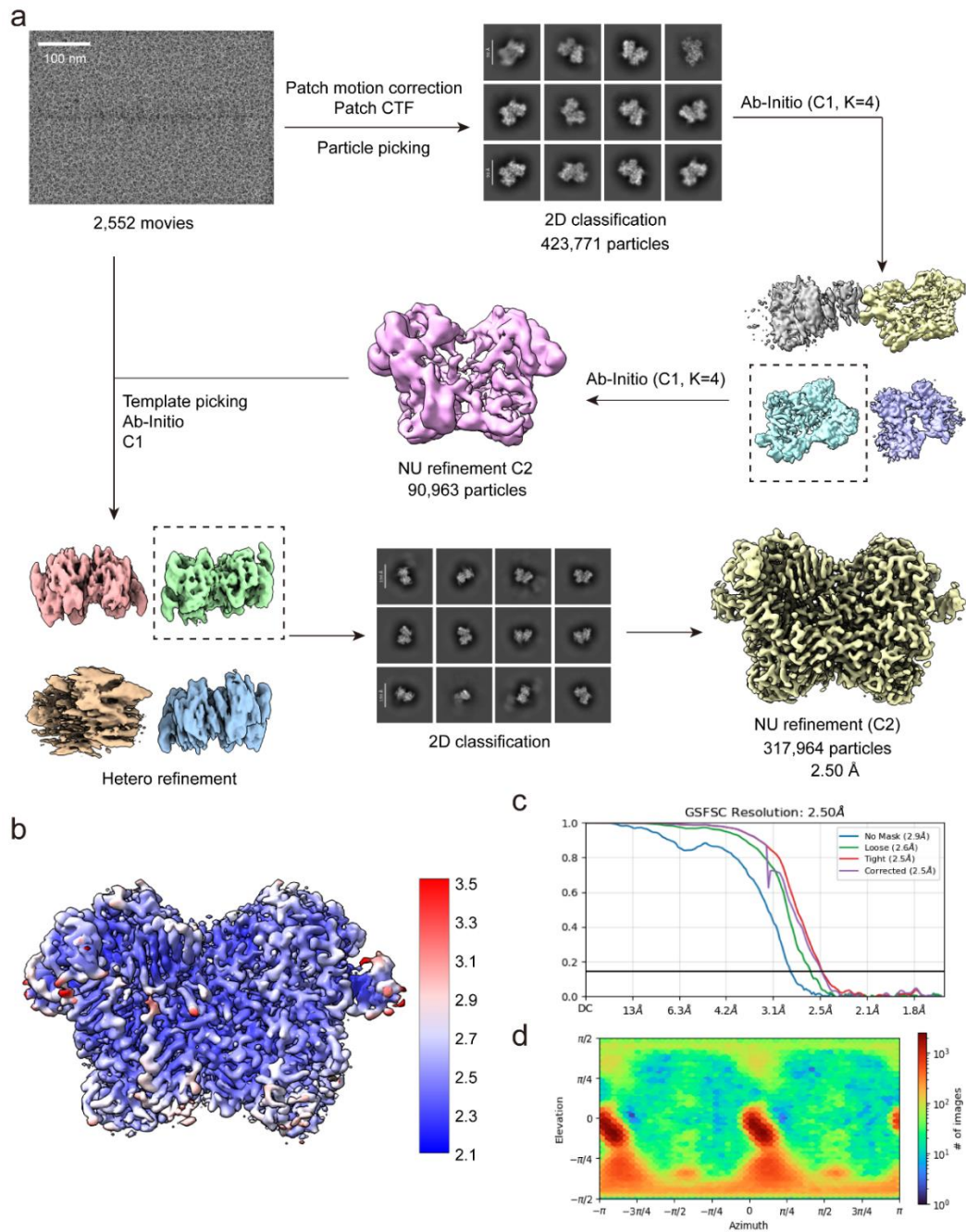


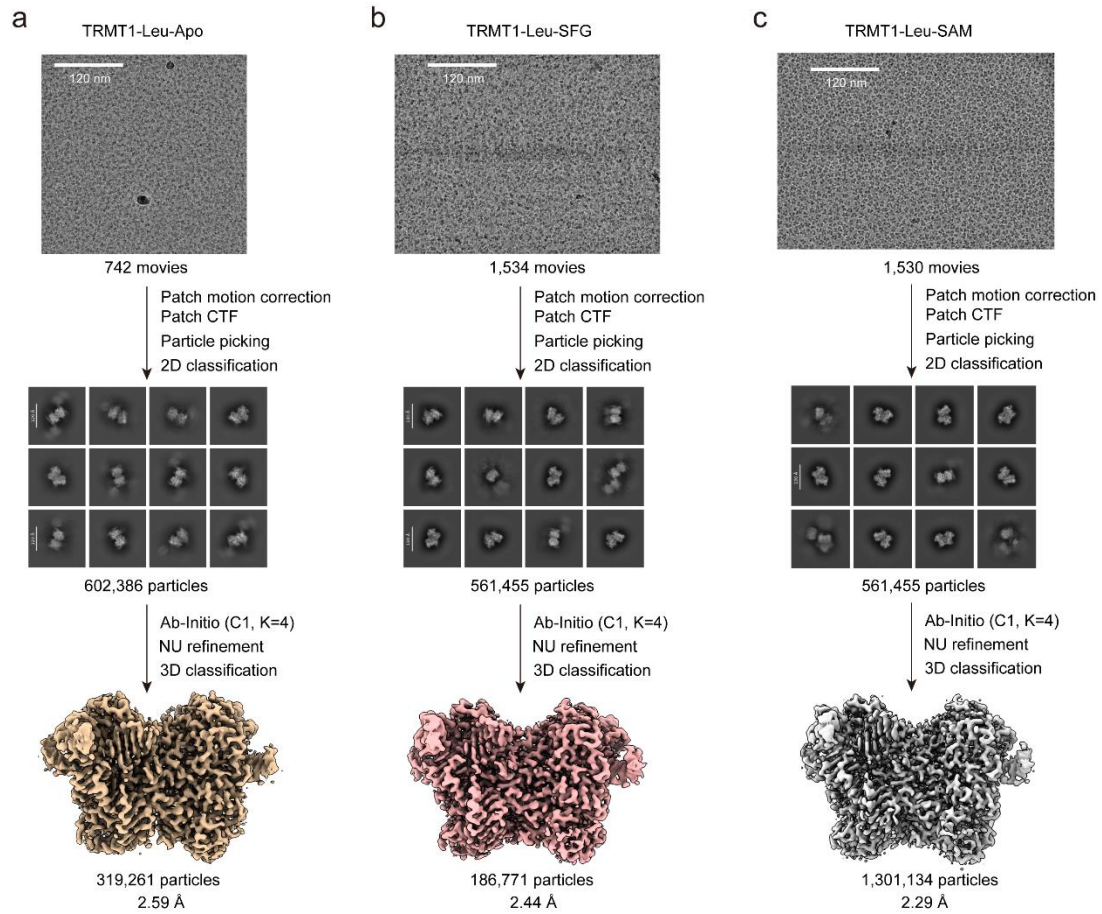
Supplementary Information



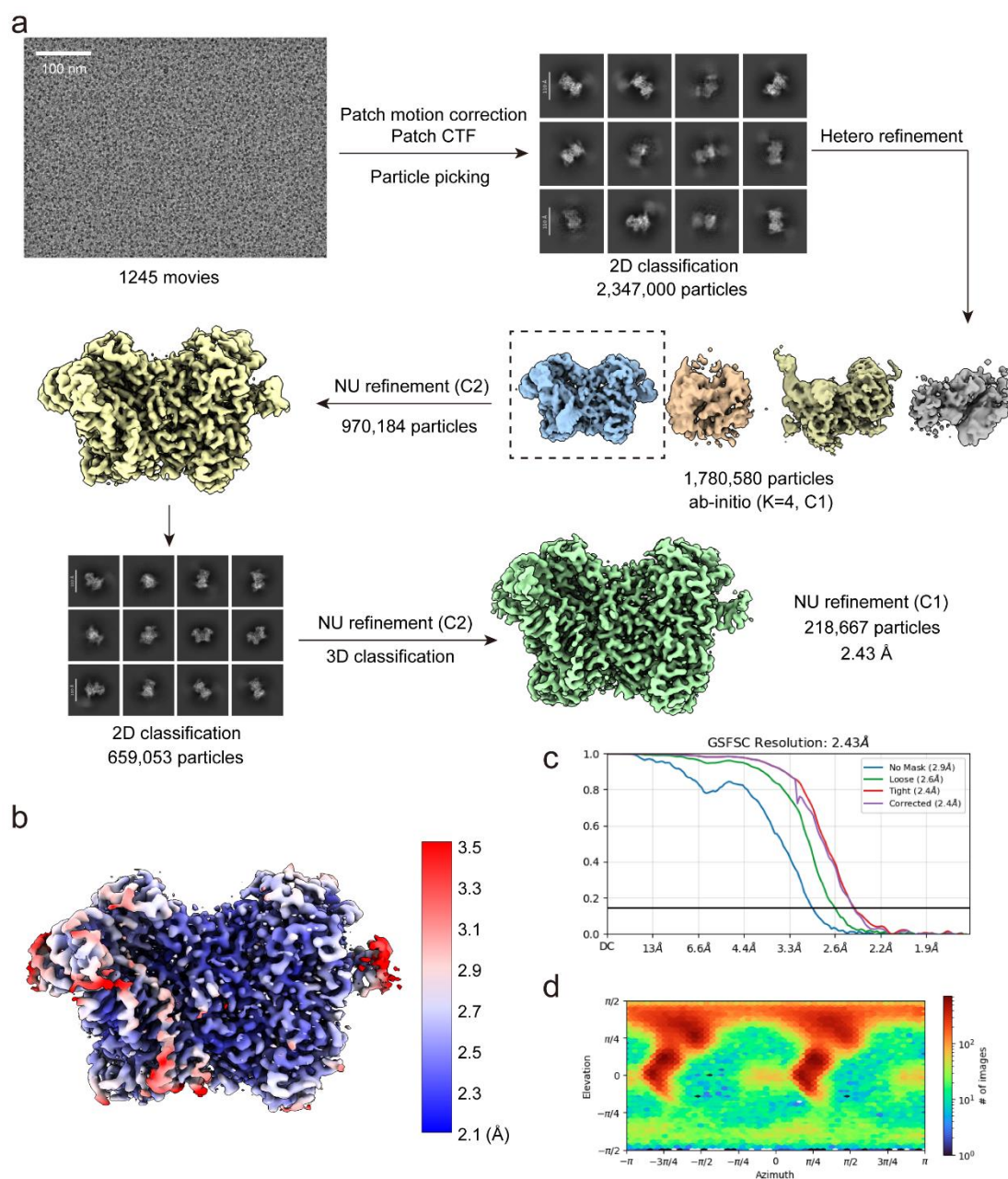
Supplementary Fig. 1 | Biochemical characterization of human tRNA N2-guanosine methyltransferases. **a-c**, Size-exclusion chromatography profiles and Coomassie-stained SDS-PAGE of purified TRMT1 (**a**), TRMT1L (**b**), and TRMT11-TRMT112 (**c**). **d-f**, Time-dependent formation of m^2G and m^2_2G for TRMT1-tRNA^{Leu} (**d**), TRMT1-tRNA^{iMet} (**e**), and TRMT1L-tRNA^{Tyr} (**f**), quantified by LC-MS/MS. **g**, Representative LC-MS/MS chromatograms after incubation of TRMT1 with tRNA^{Leu} and SAM at 25 °C for 120 min. Modification levels are indicated below. **h**, Primer-extension analysis of G26 modification in tRNA^{Leu}. Reactions were performed in the presence of SAM, SAH, SFG or no added cofactor. The m^2_2G26 -dependent termination product and the full-length extension product are indicated.



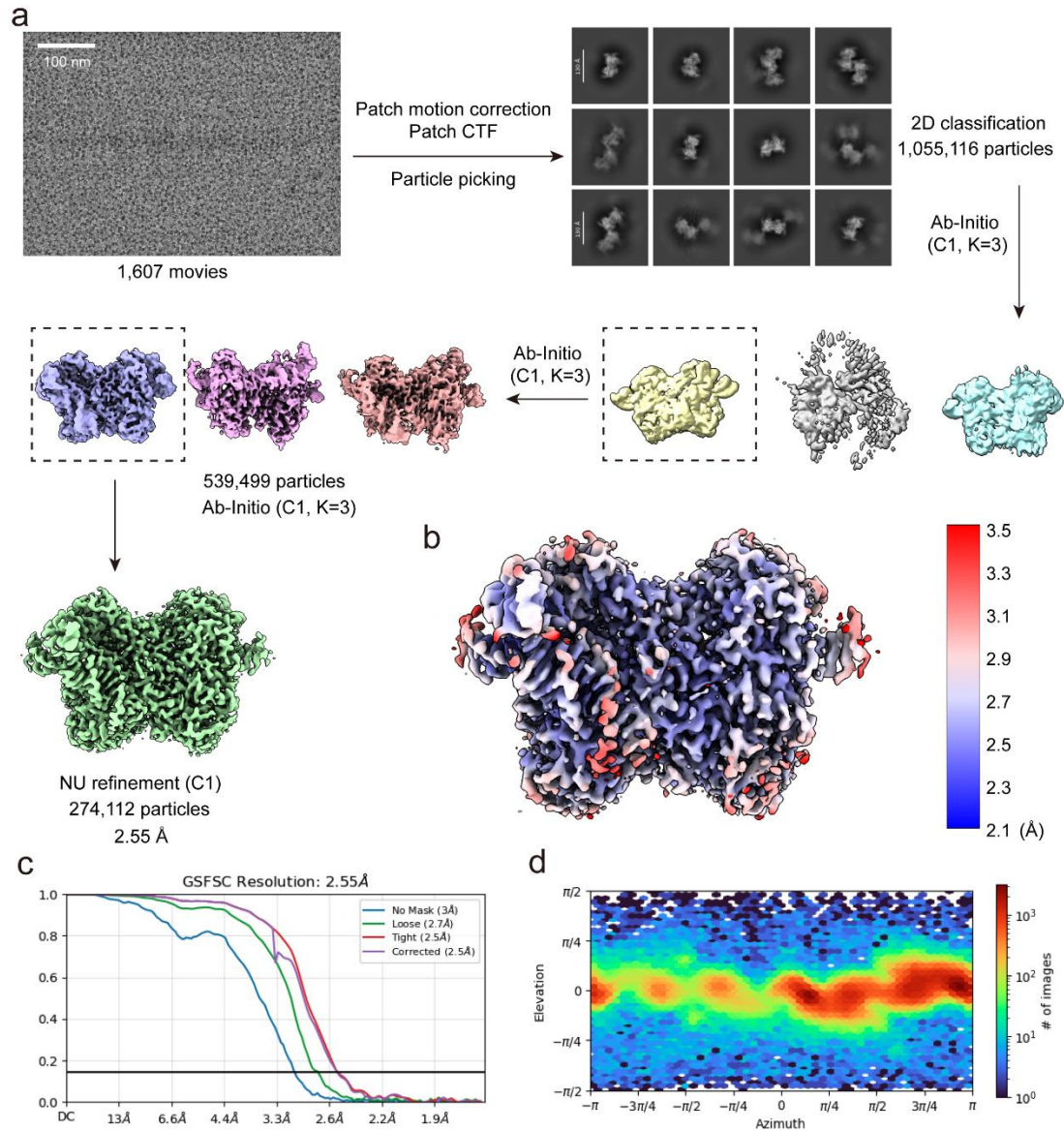
Supplementary Fig. 2 | Cryo-EM data processing and validation of the TRMT1-tRNA^{Leu}-SAH complex. **a**, Representative micrograph, 2D class averages, reconstruction workflow, and final refined map at 2.50 Å. Particle numbers at the indicated processing steps are shown. **b**, Final cryo-EM map colored according to local resolution. **c**, Gold-standard Fourier shell correlation (FSC) curves. The overall resolution was determined using the FSC = 0.143 criterion. **d**, Angular distribution of particles used for the final reconstruction.



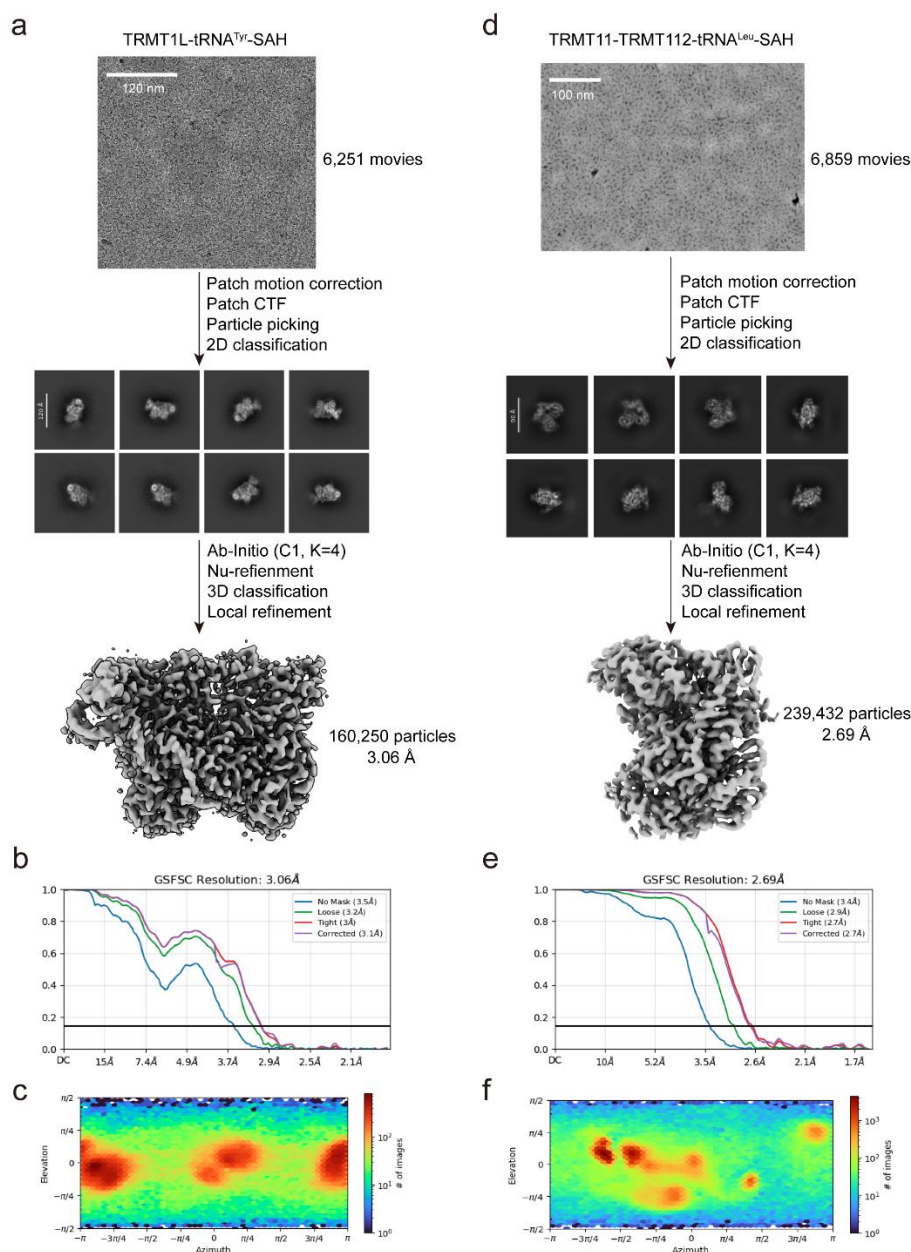
Supplementary Fig. 3 | Cryo-EM analysis of TRMT1-tRNA^{Leu} complexes in apo, SFG-bound, and SAM-reacted states. Representative micrographs, 2D class averages, cryo-EM data processing workflows, and final refined maps are shown for the apo (**a**), SFG-bound (**b**), and SAM-reacted (**c**) complexes. The final overall resolutions are 2.59 Å, 2.44 Å, and 2.29 Å, respectively. Particle numbers are indicated in the workflows.



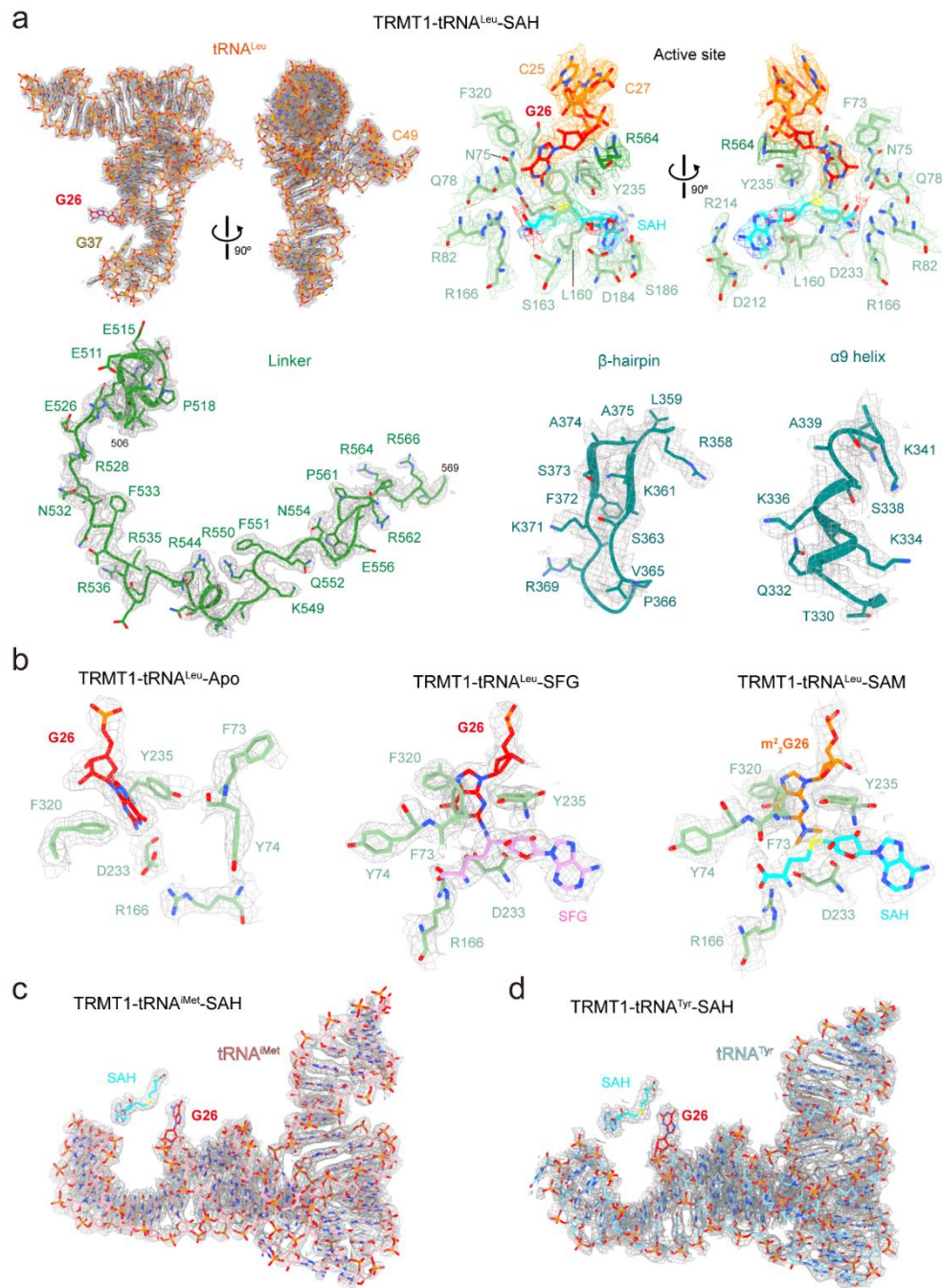
Supplementary Fig. 4 | Cryo-EM data processing and validation of the TRMT1-tRNA^{iMet}-SAH complex. **a**, Representative micrograph, 2D class averages, cryo-EM data-processing workflow, and final refined map at an overall resolution of 2.43 Å. **b**, Final cryo-EM map colored according to local resolution. **c**, Gold-standard FSC curves. The overall resolution was determined using the FSC = 0.143 criterion. **d**, Angular distribution of particles used for the final reconstruction.



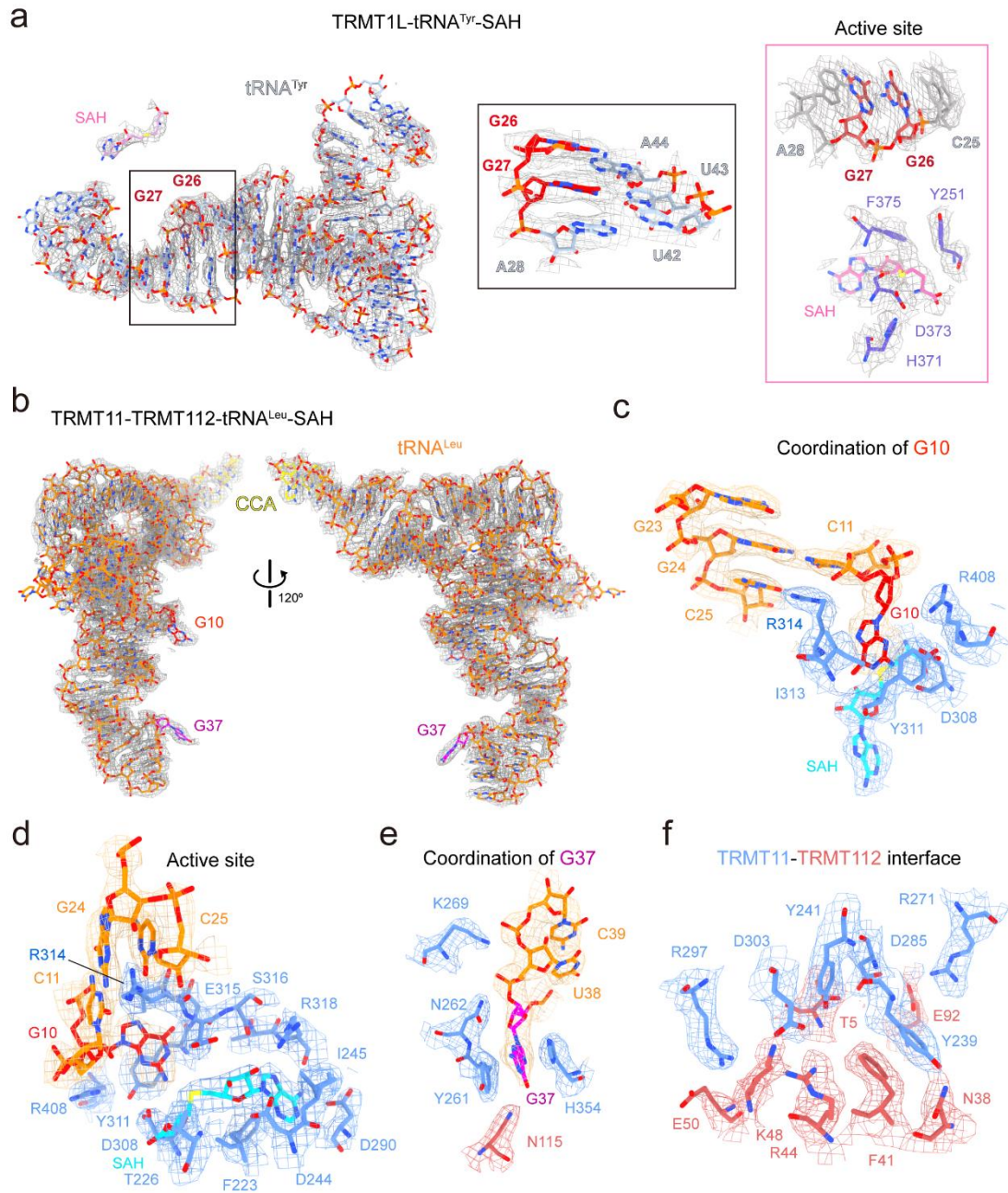
Supplementary Fig. 5 | Cryo-EM data processing and validation of the TRMT1-tRNA^{Tyr}-SAH complex. **a, Representative micrograph, 2D class averages, cryo-EM data-processing workflow and final refined map at an overall resolution of 2.55 Å. **b**, Final cryo-EM map colored according to local resolution. **c**, Gold-standard Fourier shell correlation (FSC) curve. The overall resolution was determined using the FSC = 0.143 criterion. **d**, Angular distribution of particles contributing to the final reconstruction.**



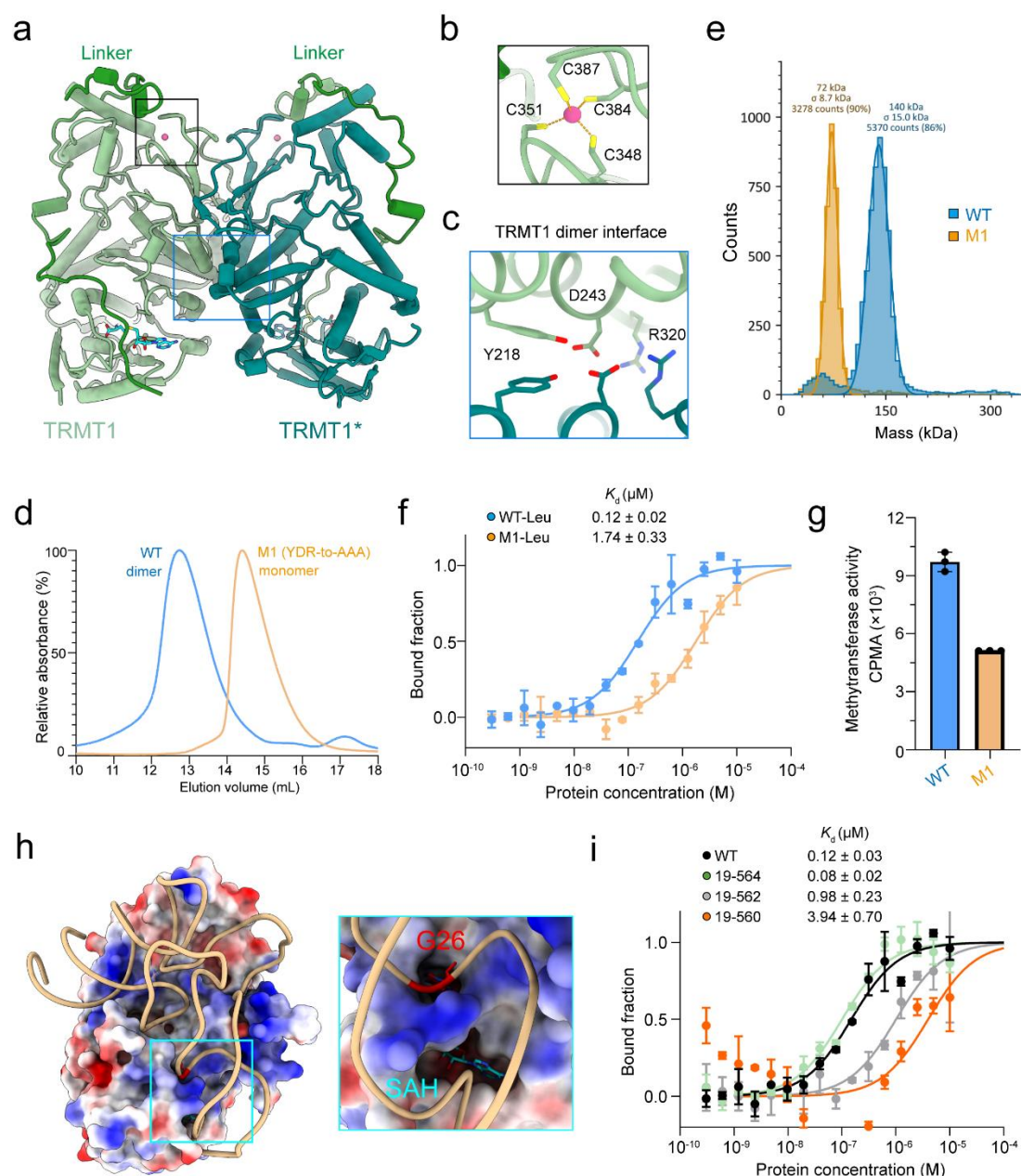
Supplementary Fig. 6 | Cryo-EM analysis of the TRMT1L-tRNA^{Tyr}-SAH and TRMT11-TRMT112-tRNA^{Leu}-SAH complexes. a,d, Representative micrograph, 2D class averages, cryo-EM data-processing workflow and final refined maps of the TRMT1L-tRNA^{Tyr}-SAH complex at an overall resolution of 3.06 Å (**a**) and the TRMT11-TRMT112-tRNA^{Leu}-SAH complex at an overall resolution of 2.69 Å (**d**). **b,e**, FSC curves for TRMT1L-tRNA^{Tyr}-SAH (**b**) and TRMT11-TRMT112-tRNA^{Leu}-SAH (**e**) complexes. **c,f**, Angular distribution of particles contributing to the final reconstructions of the TRMT1L-tRNA^{Tyr}-SAH complex (**c**) and TRMT11-TRMT112-tRNA^{Leu}-SAH complexes (**f**).



Supplementary Fig. 7 | Cryo-EM map quality of TRMT1-tRNA complexes. a, Cryo-EM density and model fit for the TRMT1-tRNA^{Leu}-SAH complex, with enlarged views of the active site, C-terminal linker, β -hairpin, and α 9 helix. **b,** Cryo-EM density around G26 in the apo, SFG-bound, and SAM-reacted complexes. G26, m²G26, SFG, and SAH are indicated. **c,** Cryo-EM density and model fit for the TRMT1-tRNA^{iMet}-SAH complex. **d,** Density and model fit for the TRMT1-tRNA^{Tyr}-SAH complex.



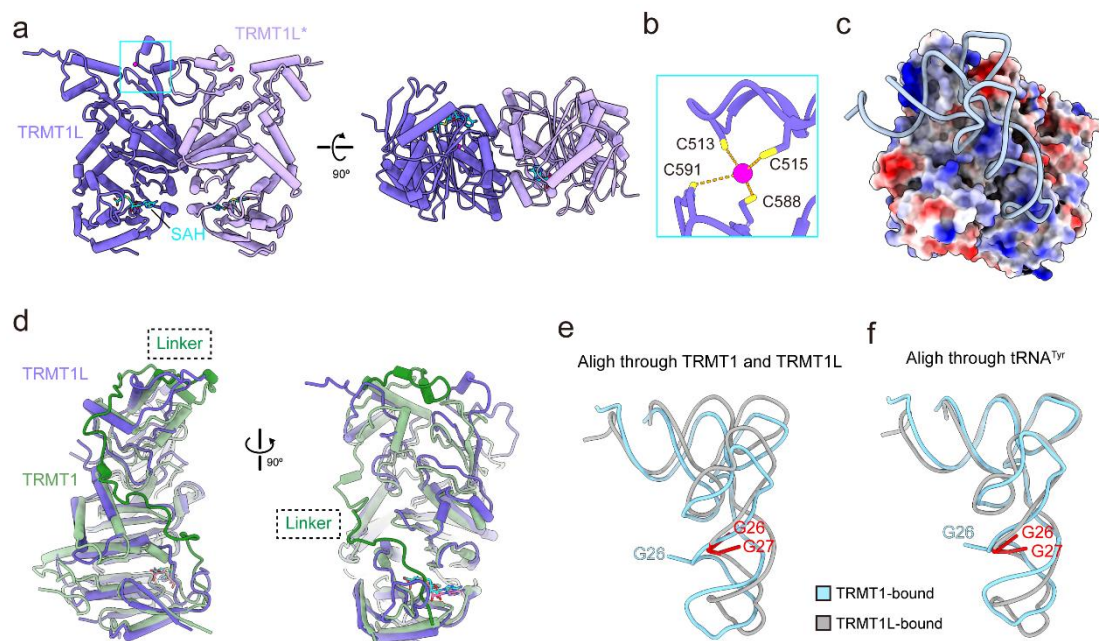
Supplementary Fig. 8 | Cryo-EM map quality of the TRMT1L-tRNA^{Tyr} and TRMT11-TRMT112-tRNA^{Leu} complexes. **a**, Cryo-EM density and model fit for the TRMT1L-tRNA^{Tyr}-SAH complex, with enlarged views of the G26–G27–A28 segment and the homologous catalytic pocket. **b**, Cryo-EM density and model fit for the TRMT11-TRMT112-tRNA^{Leu}-SAH complex in two views. **c**, Cryo-EM density around flipped G10. **d**, Cryo-EM density around the TRMT11 active site. **e**, Cryo-EM density and contacts around G37. **f**, Cryo-EM density at the TRMT11-TRMT112 interface. Residues and nucleotides are labeled in the panels.



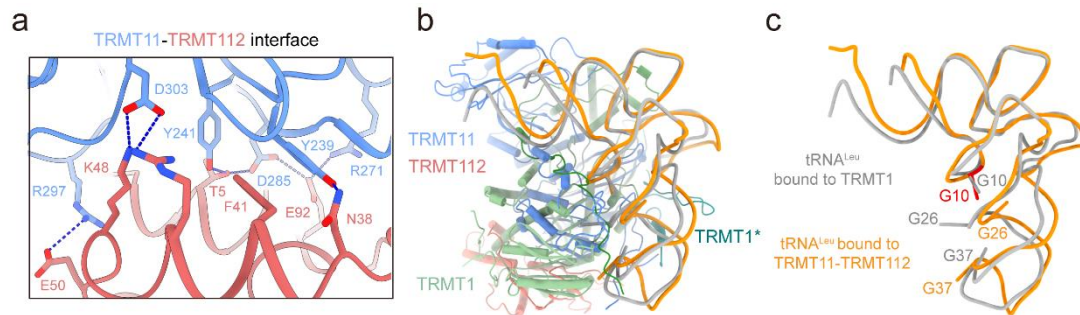
Supplementary Fig. 9 | TRMT1 dimerization and the C-terminal linker

contribute to tRNA recognition and catalysis. **a**, Overall structure of the TRMT1 homodimer with the C-terminal linkers highlighted. **b**, Close-up view of the zinc-coordination site formed by Cys348, Cys351, Cys384, and Cys387. **c**, Close-up view of the dimer interface centered on Tyr218, Asp243, and Arg320. **d**, SEC profiles of wild-type (WT) TRMT1 and the M1 mutant (Y218A/D243A/R320A). **e**, Mass-photometry distributions of WT TRMT1 and the M1 mutant. **f**, MST measurements of WT TRMT1 and M1 binding to tRNA^{Leu}. **g**, Methyltransferase activities of WT TRMT1 and M1. **h**, Electrostatic surface representation of the TRMT1-tRNA^{Leu}

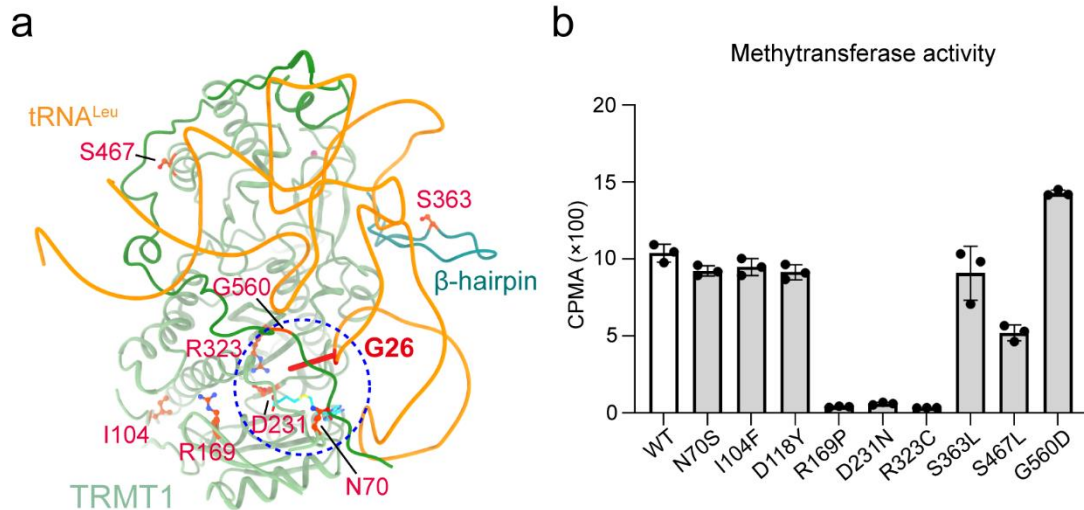
complex. The inset shows G26 and SAH accommodated in two adjacent pockets at the catalytic center. **i**, MST measurements of tRNA^{Leu} binding by WT TRMT1 and the indicated C-terminal truncations. Apparent K_d values are shown in the panels. The N-terminal mitochondrial targeting sequence (residues 1-19) was omitted from the recombinant proteins used in this study.



Supplementary Fig. 10 | Structural comparison of tRNA^{Tyr} recognition by TRMT1 and TRMT1L. **a**, Overall structure of the TRMT1L homodimer. **b**, Close-up view of the zinc-coordination site in TRMT1L. The zinc ion is coordinated by Cys513, Cys515, Cys588 and Cys591. **c**, Electrostatic surface representation of TRMT1L bound to tRNA^{Tyr}, showing the extended RNA-binding surface. **d**, Structural superposition of TRMT1 and TRMT1L. The methyltransferase domains are highly similar, whereas substantial structural differences are observed in the upper RNA-binding regions. The TRMT1-specific C-terminal linker is highlighted. **e**, Comparison of tRNA^{Tyr} binding orientations after superposition of TRMT1 and TRMT1L. tRNA^{Tyr} bound to TRMT1 and TRMT1L is shown in cyan and gray, respectively. Despite the conserved catalytic cores, the two tRNAs adopt markedly different orientations relative to the enzymes. The G26-G27 regions are indicated. Neither G26 nor G27 is flipped into the catalytic pocket in the structures shown. **f**, Superposition of the two bound tRNA^{Tyr} molecules after alignment on the tRNA itself. The overall tRNA conformations are highly similar, indicating that the different binding orientations observed in **(e)** primarily arise from distinct modes of enzyme-tRNA engagement rather than major conformational differences within the tRNA.



Supplementary Fig. 11 | The TRMT11-TRMT112 interface and distinct tRNA recognition by TRMT11-TRMT112 and TRMT1. **a**, Close-up view of the TRMT11-TRMT112 interface. Selected residues involved in the interaction are shown as sticks, and hydrogen-bonding or electrostatic interactions are indicated by dashed lines. **b**, Structural comparison of the TRMT11-TRMT112-tRNA^{Leu} and TRMT1-tRNA^{Leu} complexes. tRNA^{Leu} bound to TRMT11-TRMT112 is shown in orange, whereas tRNA^{Leu} bound to TRMT1 is shown in gray. The two tRNAs adopt markedly different binding orientations relative to their respective methyltransferase assemblies. **c**, Superposition of the two bound tRNA^{Leu} molecules shown in **(b)** after alignment on the tRNA itself. The positions of G10, G26 and G37 are indicated. The local differences around the tRNA core illustrate how the two enzyme systems present distinct regions of the same tRNA scaffold to their respective catalytic centers.



Supplementary Fig. 12 | Structural mapping and functional analysis of disease-associated TRMT1 variants. **a**, Mapping of disease-associated residues onto the TRMT1-tRNA^{Leu} structure. G26 and the β -hairpin are indicated. Gly560, Arg323, Asp231 and Asn70 are located near the active site, as indicated by blue dashed lines. **b**, Methyltransferase activities of WT TRMT1 and the indicated disease-associated variants, including N70S, I104F, D118Y, R169P, D231N, R323C, S363L, S467L, and G560D. Individual measurements are shown as dots.

Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics for TRMT1-tRNA^{Leu} complexes.

	TRMT1-Leu- APO (EMD-82508) (PDB 44CS)	TRMT1-Leu- SAH (EMD-69029) (PDB 23JQ)	TRMT1-Leu- SAM (EMD-69030) (PDB 23JR)	TRMT1-Leu- SFG (EMD-69032) (PDB 23JT)
Data processing				
Magnification	105,000×	105,000×	105,000×	105,000×
Voltage (kV)	300	300	300	300
Electron exposure (e-/Å ²)	50	50	50	50
Defocus range (μm)	1.2~1.8	1.2~1.8	1.2~1.8	1.2~1.8
Pixel size (Å)	0.93	0.82	0.82	0.82
Symmetry imposed	C2	C2	C2	C2
Initial particle images (no.)	1,151,511	3,897,680	3,674,184	3,352,153
Final particle images (no.)	319,261	317,964	1,301,134	186,771
Map resolution (Å)	2.59	2.50	2.29	2.44
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.4-2.8	2.2-2.7	2.1-2.7	2.2-2.6
Refinement				
Model resolution (Å)	2.7	2.6	2.4	2.5
FSC threshold	0.5	0.5	0.5	0.5
Map sharpening <i>B</i> factor (Å ²)	-68.1	-57.9	-62.5	-49.0
Model composition				
Non-hydrogen atoms	11116	11168	11172	11170
Protein residues	970	972	972	970
Ligands	2	4	4	4
<i>B</i> factors (Å²)				
Protein	37.46	27.14	35.53	37.99
Ligand	51.07	49.69	50.87	38.64
R.m.s. deviations				
Bond lengths (Å)	0.005	0.005	0.004	0.006
Bond angles (°)	0.625	0.561	0.852	0.889
Validation				
MolProbity score	1.77	1.30	1.56	1.17
Clashscore	5.78	4.15	6.29	3.66
Poor rotamers (%)	2.26	0.00	0.00	0.00
Ramachandran plot				
Favored (%)	96.88	97.51	96.67	97.92
Allowed (%)	3.12	2.49	3.33	2.08
Disallowed (%)	0.00	0.00	0.00	0.00

Supplementary Table 2. Cryo-EM statistics for TRMT1-tRNA^{Tyr}, TRMT1-tRNA^{iMet}, TRMT1L-tRNA^{Tyr} and TRMT11-TRMT112-tRNA^{Leu} complexes.

	TRMT1-Tyr- SAH (EMD-69026) (PDB 23JN)	TRMT1-iMet- SAH (EMD-69027) (PDB 23JO)	TRMT1L-Tyr- SAH (EMD-80920) (PDB 26VW)	TRMT11-112- Leu-SAH (EMD-69025) (PDB 23JM)
Data processing				
Magnification	105,000×	105,000×	105,000×	105,000×
Voltage (kV)	300	300	300	300
Electron exposure (e-/Å ²)	50	50	50	50
Defocus range (μm)	1.2~1.8	1.2~1.8	1.2~1.8	1.2~1.8
Pixel size (Å)	0.82	0.82	0.92	0.82
Symmetry imposed	C1	C1	C1	C2
Initial particle images (no.)	2,744,315	2,932,999	5,830,756	9,926,311
Final particle images (no.)	274,112	218,667	160,250	239,432
Map resolution (Å)	2.55	2.43	3.06	2.69
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.3-3.0	2.2-2.7	2.7-10.8	2.4-3.4
Refinement				
Model resolution (Å)	2.8	2.6	3.3	2.9
FSC threshold	0.5	0.5	0.5	0.5
Sharpening <i>B</i> factor (Å ²)	-80.3	-51.0	-80.9	-97.8
Model composition				
Non-hydrogen atoms	10701	10764	7834	6080
Protein residues	965	972	786	520
Ligands	4	4	4	1
<i>B</i> factors (Å²)				
Protein	29.88	46.51	48.08	49.84
Ligand	27.69	70.62	78.58	26.15
R.m.s. deviations				
Bond lengths (Å)	0.004	0.004	0.005	0.004
Bond angles (°)	0.875	0.518	0.694	0.863
Validation				
MolProbity score	1.52	1.34	2.12	1.49
Clashscore	4.91	3.27	10.84	4.99
Poor rotamers (%)	0.13	0.00	0.14	0.00
Ramachandran plot				
Favored (%)	96.13	96.57	89.38	96.50
Allowed (%)	3.87	3.43	10.62	3.50
Disallowed (%)	0.00	0.00	0.00	0.00