

Abstract

N²-methylguanosine (m²G) and N²,N²-dimethylguanosine (m²₂G) are conserved tRNA modifications installed at distinct positions. In human cells, TRMT11-TRMT112 modifies G10, TRMT1 modifies G26, and TRMT1L modifies G27. How these enzymes select different guanosines within the common L-shaped tRNA scaffold remains unclear. Here we report cryo-EM structures of human TRMT1, TRMT1L and TRMT11-TRMT112 bound to their tRNA substrates. TRMT1 forms a homodimer that creates composite RNA-binding surfaces, with a TRMT1-specific C-terminal Linker anchoring the G26-containing region. Disruption of dimerization or truncation of the Linker impairs tRNA binding and catalysis. Structures of TRMT1 in apo, sinefungin-bound and SAM-reacted states show that G26 flipping precedes cofactor binding, whereas cofactor engagement induces closure of the L1 loop and organizes the catalytic pocket. TRMT1L preserves a closely related catalytic geometry despite substitutions of several active-site residues, but engages tRNA^{Tyr} in a markedly different orientation. TRMT11-TRMT112 uses a distinct docking architecture in which the THUMP and methyltransferase domains of TRMT11 jointly position the tRNA. At the target site, Arg314 replaces the interaction of G10 with C25, while Tyr311 stabilizes the extruded G10 through aromatic stacking. Together, these structures reveal a general principle for site-selective N²-guanosine methylation: enzyme-specific global docking defines the target region, whereas local base remodeling and base extrusion enables catalytic selection.

Introduction

Post-transcriptional modifications expand the chemical and structural diversity of RNA¹⁻³. More than 170 modified ribonucleosides have been identified, and tRNAs are among the most heavily modified cellular RNAs⁴⁻⁹. A mature eukaryotic tRNA carries, on average, about 13 modified nucleotides. Modifications in the anticodon region tune decoding and translational fidelity, whereas modifications in the tRNA body stabilize the tertiary fold, promote maturation and aminoacylation, and protect tRNAs from aberrant cleavage or decay. Defects in these pathways are linked to neurological, developmental, metabolic and neoplastic disorders¹⁰⁻¹⁵.

The exocyclic N2 atom of guanosine can receive one or two methyl groups to form m²G or m²G. In human tRNAs, these modifications occur at several positions and are installed by distinct enzyme systems. THUMPD3-TRMT112 and TRMT11-TRMT112 generate m²G at G6/G7 and G10, respectively¹⁶⁻²⁰. TRMT1 and its paralog TRMT1L act near the junction of the D arm and anticodon stem. TRMT1 modifies G26 in many cytosolic and mitochondrial tRNAs²¹⁻²³, whereas TRMT1L has a narrower substrate range and catalyzes cytosolic tRNA^{Tyr} m²G27 formation²⁴⁻²⁶. G26 and G27 lie in the central tRNA core, where methylation can affect local base pairing and the geometry of the L-shaped fold²⁷⁻³⁰. Loss of TRMT1 perturbs protein synthesis and redox homeostasis and causes autosomal-recessive intellectual disability^{25,31,32}. Loss of TRMT1L reduces tRNA^{Tyr} abundance, impairs translation and sensitizes cells to oxidative stress²⁴⁻²⁶. Intriguingly, TRMT1 is also cleaved by the SARS-CoV-2 main protease (Mpro) at Gln530; the resulting N-terminal fragment has weak tRNA binding

and little methyltransferase activity³³⁻³⁵. These observations underscore the biological importance of N2-guanosine methylation while raising the question of how individual enzyme systems recognize their tRNA substrates.

Previous structures of archaeal and bacterial Trm1 proteins defined a conserved class-I methyltransferase fold and a putative guanine-binding pocket, but lacked substrate tRNA^{36,37}. Biochemical and structural studies of Trm11-Trm112 established an essential role for the partner protein and implicated the THUMP domain in RNA recognition³⁸⁻⁴⁰, yet the architecture of a TRMT11-TRMT112-tRNA complex has remained undefined. It has also been unclear how the closely related TRMT1 and TRMT1L proteins discriminate neighboring sites in the tRNA core.

Here we determine cryo-EM structures of three human tRNA N2-guanosine methyltransferase systems bound to their substrates. We first define how the TRMT1 homodimer recognizes an intact tRNA and how G26 is remodeled during catalysis. We then compare TRMT1 with TRMT1L and show that conserved catalytic cores are coupled to distinct RNA-binding architectures. Finally, we determine the human TRMT11-TRMT112-tRNA complex and define a different route for positioning G10. Together, the structures provide a framework for understanding how global tRNA recognition and local base remodeling cooperate to generate site selectivity.

Results

Human tRNA N2-guanosine methyltransferases use distinct architectures

TRMT1, TRMT1L and TRMT11-TRMT112 all modify the N2 atom of guanosine but target different positions in tRNAs (Fig. 1a). We purified the three enzyme systems to

homogeneity for biochemical characterization (Supplementary Fig. 1a-c). Both TRMT1 and TRMT11-TRMT112 displayed broad substrate specificity, modifying all four tRNAs tested, with the highest activity on tRNA^{Leu} (Fig. 1b). By contrast, TRMT1L showed detectable activity only toward tRNA^{Tyr}, revealing a much more restricted substrate preference²⁵. Product analysis revealed an additional layer of specificity. TRMT1 efficiently converted tRNA^{Leu} to m²G, whereas tRNA^{iMet} accumulated predominantly as the monomethylated m²G intermediate. TRMT1L-modified tRNA^{Tyr} showed transient accumulation of m²G followed by progressive formation of m²G (Supplementary Fig. 1d-f). Thus, the three enzyme systems differ in both substrate preference and the efficiency of the second methyl-transfer reaction.

We next reconstituted TRMT1-tRNA^{Leu}, TRMT1L-tRNA^{Tyr} and TRMT11-TRMT112-tRNA^{Leu} complexes in the presence of SAH and determined their structures by single-particle cryo-EM (Fig. 1c-e; Supplementary Figs. 2-8 and Supplementary Tables 1-2). TRMT1 forms a two-fold-related homodimer. Two tRNAs bind on opposite sides of the dimer, and each tRNA contacts both protomers (Fig. 1c). TRMT1L also assembles as a homodimer, with a single well-resolved tRNA^{Tyr} bound asymmetrically across the dimer (Fig. 1d). In contrast, TRMT11-TRMT112 forms a heterodimeric assembly in which the THUMP and methyltransferase domains of TRMT11, together with TRMT112, accommodate one tRNA (Fig. 1e). These structures immediately reveal three distinct solutions for docking the same L-shaped RNA scaffold.

TRMT1 dimerization creates a composite tRNA-binding surface

The TRMT1-tRNA^{Leu} structure shows that one tRNA spans both protomers of the homodimer (Fig. 2a,b). The catalytic protomer engages the G26-containing hinge and the proximal anticodon arm, whereas the partner protomer supports distal regions of the tRNA. This arrangement creates two equivalent composite RNA-binding surfaces within the dimer. The dimer interface is centered on Tyr218, Asp243 and Arg320 (Supplementary Fig. 9a-c). A triple mutant at these positions altered the size-exclusion profile, weakened tRNA^{Leu} binding and reduced methyltransferase activity (Supplementary Fig. 9d-g). Dimerization is therefore an integral part of substrate recognition.

TRMT1 engages the tRNA through several spatially separated surfaces, with the C-terminal linker (residues 506-569; hereafter referred to as the Linker) forming a prominent component of the recognition network (Fig. 2d-h; Supplementary Fig. 7a). At the acceptor arm, Arg544, Lys549 and Arg550 from the Linker contact the RNA. The D arm is recognized by Met262, Asn268, Arg285 and His288, together with Asn554 and Asn558 from the Linker. Around the G26-containing hinge, Linker residues Arg562, Arg564 and Arg566 contact the RNA, whereas Gln332 and Lys336 from the partner protomer engage the proximal anticodon region. Additional interactions involve the T arm and variable region. Most contacts are made with the phosphodiester backbone or ribose moieties rather than the bases, providing a structural basis for the ability of TRMT1 to accommodate tRNAs with diverse sequences.

Progressive truncation of the Linker caused a stepwise loss of tRNA binding and methyltransferase activity (Fig. 2i; Supplementary Fig. 9h,i). Constructs retaining residues 564-569 remained active, whereas truncation to residue 562 markedly impaired catalysis. Further shortening to residue 560, or to the SARS-CoV-2 Mpro cleavage product ending at Gln530, nearly abolished activity. These results identify the distal Linker as a critical RNA-engaging element for tRNA recognition. Loss of this segment therefore provides a direct structural explanation for TRMT1 inactivation following Mpro cleavage.

RNA truncation and deletion experiments provided complementary evidence for this recognition mode. Removal of major elements of the tRNA scaffold markedly impaired methylation, whereas several truncated substrates retained substantial activity (Fig. 2j). These results indicate that TRMT1 does not rely on a single short sequence for substrate recognition, but instead reads multiple structural features of the tRNA. Superposition with a reference tRNA further shows that the overall L-shaped fold is largely preserved, whereas the central region surrounding G26 undergoes local remodeling (Fig. 2c; Supplementary Fig. 7a). Thus, TRMT1 combines distributed recognition of the mature tRNA scaffold with local remodeling at the target site.

G26 flipping precedes cofactor-dependent active-site closure

In the TRMT1-tRNA^{Leu} complex, G26 is fully extruded from the tRNA core and inserted into a recessed pocket of the methyltransferase domain (Fig. 3a;

Supplementary Fig. 7a). The guanine base is surrounded by Phe73, Asn75, Gln78, Tyr235 and Phe320, whereas Asp233, Arg82 and Arg166 lie close to the substrate and cofactor. SAH occupies an adjacent pocket formed by the conserved methyltransferase fold. Together, the two pockets place the N2 atom of G26 close to the methyl-donor position of SAM (Supplementary Fig. 9h).

Structure-guided mutagenesis confirms the functional importance of this active-site network (Fig. 3b). Alanine substitutions of Phe73, Asn75/Gln78, Asp233, Arg82, Arg166, Asp184 or Asp231 markedly impaired methyltransferase activity. Mutation of Tyr235 had a more modest effect. By contrast, F320A increased activity, suggesting that Phe320 may constrain substrate positioning, access to the catalytic pocket or product release. Mutations at more peripheral positions produced intermediate effects. These results indicate that precise positioning of the target base is tightly coupled to cofactor recognition.

To follow local changes during catalysis, we determined TRMT1-tRNA^{Leu} structures without added cofactor (apo), with sinefungin (SFG), and after incubation with SAM (Fig. 3c-e; Supplementary Fig. 7b). G26 is already flipped into the active site in the apo complex, indicating that base extrusion does not require cofactor binding. In the SFG-bound state, the Phe73-containing L1 loop³⁷ moves toward G26 and closes over the catalytic pocket (Fig. 3e). After incubation with SAM, cryo-EM density at the target nucleotide is consistent with m²G26 formation, indicating a post-catalytic state with SAH occupying the cofactor pocket. Primer-extension analysis independently confirmed efficient m²G26 formation in the SAM-treated sample

(Supplementary Fig. 1h). The overall TRMT1-tRNA architecture remains largely unchanged among these states, whereas the major conformational rearrangement is confined to the active-site region.

These structures suggest an ordered sequence of events. TRMT1 first engages the tRNA and flips G26 into the catalytic pocket. Cofactor binding then induces L1 loop closure and organizes the active site for methyl transfer (Fig. 3e,f). Asp233 and Arg166 form the catalytic center and are positioned to facilitate proton transfer and methyl transfer, although the precise proton-transfer mechanism remains to be established. Notably, the same pocket accommodates both unmodified G26 and the dimethylated product. Whether the two methyl-transfer reactions occur processively remains unclear.

Conserved catalytic geometry and distinct tRNA docking by TRMT1 and TRMT1L

TRMT1 acts on several tRNA species. Structures of TRMT1 bound to tRNA^{Leu}, tRNA^{Tyr} and tRNA^{iMet} show nearly identical protein conformations and a common overall docking mode (Fig. 4a; Supplementary Fig. 7c,d). The central tRNA cores align well, with local differences near the G37 region and the variable loop (Fig. 4b). EMSA measurements yielded similar apparent affinities for the three substrates, with K_d values of about 0.18, 0.19 and 0.22 μ M, respectively. TRMT1L bound tRNA^{Tyr} with a comparable apparent K_d of about 0.15 μ M (Fig. 4c). Thus, stable association

with the tRNA scaffold is not sufficient to explain the large differences in catalytic output.

This conclusion is supported by the activity and product analyses. TRMT1 shows substantially different activities on tRNA^{Leu}, tRNA^{Tyr}, tRNA^{iMet} and mitochondrial tRNA^{Ala} despite similar binding to the structurally characterized substrates (Fig. 1b). LC-MS/MS further revealed efficient m²G formation on tRNA^{Leu} but predominantly m²G formation on tRNA^{iMet} (Supplementary Fig. 1d,e). Productive catalysis therefore depends on steps beyond initial binding, including local remodeling of the target nucleotide and proper organization of the catalytic pocket for the second methyl-transfer reaction

Comparison of the SAH-bound TRMT1-tRNA^{Tyr} and TRMT1L-tRNA^{Tyr} complexes reveals both conservation and divergence between the two paralogs (Fig. 4d-f; Supplementary Fig. 10). Their methyltransferase domains superimpose closely, and the overall geometry of the catalytic pocket is highly conserved. Notably, however, several residues surrounding the target-base pocket are replaced by chemically related residues in TRMT1L. Phe73, Tyr235 and Arg166 in TRMT1 correspond to Tyr251, Phe375 and His371 in TRMT1L, respectively, whereas the catalytic Asp233 is conserved as Asp373 (Fig. 4f). Thus, conservation of the active site is achieved at the level of overall geometry and chemical environment rather than strict residue identity. These substitutions may fine-tune the catalytic properties of the two paralogs without changing the overall architecture of the active site.

In contrast to the conserved catalytic core, the surrounding RNA-binding architecture differs substantially. The upper RNA-binding regions are more divergent, and the C-terminal Linker is unique to TRMT1 (Fig. 4f). When the proteins are aligned, the two bound tRNA^{Tyr} molecules adopt markedly different orientations (Fig. 4d). When the tRNAs themselves are aligned, however, their overall folds remain closely similar (Supplementary Fig. 10d-f). The difference therefore arises primarily from enzyme-directed docking rather than from large conformational changes within the tRNA. Strikingly, G26 is flipped into the catalytic pocket in the TRMT1-tRNA^{Tyr} complex, whereas both G26 and G27 remains outside the corresponding pocket in the TRMT1L-tRNA^{Tyr} complex (Fig. 4f). Thus, despite their closely related catalytic cores, the two paralogs differ in their ability to remodel the target region into a catalytically competent configuration. This difference may contribute to their distinct positional and substrate specificities.

TRMT11-TRMT112 uses a distinct tRNA-docking strategy to select G10

The TRMT11-TRMT112-tRNA^{Leu} structure reveals a third mode of tRNA recognition (Fig. 5a). TRMT11 contains an N-terminal THUMP domain and a C-terminal methyltransferase domain, whereas TRMT112 associates with the methyltransferase domain (Supplementary Figs. 11a). The tRNA spans both domains of TRMT11. Its 3'-CCA end is anchored by the THUMP domain, while the G10-containing core contacts the methyltransferase domain and is positioned above the catalytic center.

The THUMP domain forms a hydrophobic pocket that accommodates the 3'-CCA tail (Fig. 5b). Additional interactions from TRMT11 stabilize the acceptor arm and the region around G37 (Fig. 5c,d). Notably, G37 is sandwiched between Tyr247 and His340 of TRMT11 and is further stabilized by a hydrogen bond with Asn115 of TRMT112. This recognition mode provides a structural explanation for previous biochemical observations^{38,41}. Together, these distributed contacts, extending from the 3'-CCA terminus through the tRNA core to the G37 region, act as a molecular ruler that fixes the register of the bound tRNA and precisely positions G10 over the catalytic center. Structural comparison with TRMT1 shows that the two enzyme systems engage the same L-shaped tRNA scaffold through markedly different docking modes (Supplementary Fig. 11b,c).

At the catalytic center, G10 is completely extruded from the tRNA core and inserted into a recessed pocket of the TRMT11 methyltransferase domain (Fig. 5e-g). This base-flipping event is accompanied by a striking rearrangement around C25. Arg314 occupies the space vacated by G10 and forms two hydrogen bonds with C25, effectively substituting for the G10-C25 interaction and stabilizing the locally opened tRNA conformation. The extruded G10 is, in turn, stabilized within the catalytic pocket through π - π stacking with Tyr311 (Fig. 5e,5f). Asp308 and Arg408 are positioned nearby and contribute to the catalytic environment surrounding the target base. SAH occupies an adjacent cofactor-binding pocket, placing the N2 atom of G10 toward the methyl-donor position of SAM. These structural features suggest that Arg314 and Tyr311 act cooperatively to promote and stabilize G10 extrusion: Arg314

anchors the vacated C25, whereas Tyr311 captures the flipped guanine in the catalytic pocket.

Mutational analysis supports contributions from each recognition layer (Fig. 5h). Changes in the CCA-binding surface, distal RNA-contacting regions and the catalytic pocket reduced methyltransferase activity to different extents. Removal of TRMT112 caused a strong loss of activity. TRMT112 therefore contributes to more than complex stability; it helps build the substrate-recognition platform.

Despite its distinct global architecture, TRMT11-TRMT112 converges on the same local solution seen in TRMT1: extraction of one guanosine from the tRNA core and delivery of its N2 atom to a SAM-dependent catalytic pocket.

Discussion

Our structures provide a comparative view of three human tRNA N2-guanosine methyltransferase systems that act on the same overall RNA scaffold and catalyze closely related chemistry, yet modify distinct guanosines (Fig. 1). Despite their different architectures, a common principle emerges. Enzyme-specific interactions first dock the L-shaped tRNA and position a defined region near the catalytic center. Local remodeling then exposes the target guanosine and places its N2 atom in the methyl-transfer pocket. Site selectivity is therefore achieved through two coupled steps: global tRNA recognition and local nucleotide selection.

TRMT1 illustrates how distributed recognition can support broad substrate usage. The homodimer creates composite RNA-binding surfaces, and the TRMT1-

specific C-terminal Linker makes extensive contacts with the central tRNA region surrounding G26 (Fig. 2). Disruption of dimerization or progressive truncation of the Linker weakens tRNA binding and catalysis, indicating that substrate recognition depends on multiple spatially separated interactions rather than a short sequence motif (Fig. 2; Supplementary Fig. 9). This architecture also explains the loss of TRMT1 activity following SARS-CoV-2 Mpro cleavage. Cleavage at Gln530-Ala531 removes a substantial portion of the RNA-engaging Linker, and a cleavage-mimicking construct is nearly inactive.

The TRMT1 catalytic states further separate substrate docking from catalytic activation. G26 is already extruded in the apo complex, indicating that base flipping precedes cofactor binding. Cofactor engagement instead induces closure of the Phe73-containing L1 loop and organizes the catalytic pocket (Fig. 3). The SAM-reacted structure captures m²G26 and SAH without major rearrangement of the overall enzyme-tRNA assembly. Asp233 and Arg166 form key components of the catalytic center, although the detailed proton-transfer pathway remains to be defined. Notably, TRMT1 binds several tRNAs with similar affinities despite substantial differences in activity and product distribution. Catalytic specificity therefore arises largely after initial binding, through local remodeling and productive organization of the target nucleotide.

Comparison with TRMT1L shows how positional specificity can evolve while preserving the catalytic framework. The methyltransferase domains of TRMT1 and TRMT1L superimpose closely, and their active sites retain similar overall

geometry despite substitutions of several residues surrounding the target-base pocket. Phe73, Tyr235 and Arg166 in TRMT1 correspond to Tyr251, Phe375 and His371 in TRMT1L, respectively, suggesting conservation of the local chemical environment rather than strict residue identity (Fig. 4). In contrast, their RNA-binding architectures differ substantially. The TRMT1-specific Linker is absent from TRMT1L, and the two enzymes dock tRNA^{Tyr} in markedly different orientations. Consistent with this difference, G26 is flipped into the TRMT1 catalytic pocket, whereas both G26 and G27 remain outside the TRMT1L pocket. Thus, divergence in RNA docking is coupled to a distinct ability to remodel the target nucleotide into a catalytically competent state, providing a plausible basis for the restricted substrate specificity of TRMT1L^{24,25}.

TRMT11-TRMT112 uses a distinct global docking strategy but converges on a related local solution. The THUMP and methyltransferase domains of TRMT11 jointly position the tRNA, presenting the G10-containing core to the catalytic center (Fig. 5). G10 is then extruded from its interaction with C25. Strikingly, Arg314 occupies the vacated position and forms two hydrogen bonds with C25, whereas the flipped G10 is stabilized by π - π stacking with Tyr311. This arrangement suggests a base-substitution mechanism in which Arg314 stabilizes the opened tRNA core while Tyr311 captures the displaced guanine in the catalytic pocket. Thus, TRMT1 and TRMT11 dock the tRNA in very different orientations but both ultimately achieve site selection by extracting a defined guanosine from the structured tRNA core.

Disease-associated TRMT1 variants further illustrate the functional importance of this hierarchical architecture (Supplementary Fig. 12). Mutations such as R169P, D231N and R323C strongly impair methyltransferase activity and map to regions involved in catalytic organization or tRNA recognition.

Together, our results support a hierarchical model for site-selective tRNA N2-guanosine methylation. Global recognition determines which region of the common L-shaped scaffold is presented to the catalytic domain, whereas local remodeling and base extrusion determine which guanosine becomes catalytically competent. This combination of divergent tRNA-docking architectures with locally conserved catalytic strategies provides a structural explanation for how related methyltransferases achieve precise modification at G10, G26 and G27.

Methods

Cloning

Human TRMT1 (UniProt O60318-1) was cloned into pET-28a with an N-terminal 6×His tag. The N-terminal mitochondrial targeting sequence was omitted from recombinant TRMT1 constructs used for biochemical and structural studies. Human TRMT1L (UniProt Q5JVF3-1) was cloned into pET-28a with an N-terminal 6×His-SUMO tag. TRMT11 and TRMT112 were fused to N-terminal 6×His and Flag tags, respectively, and cloned into the two expression cassettes of pET-Duet for co-expression. Point mutants and truncations were generated by site-directed mutagenesis and verified by DNA sequencing.

Protein expression and purification

Proteins were expressed in *E. coli* Rosetta (DE3) cells grown in LB medium. Cultures were induced at an OD₆₀₀ of approximately 0.8 with 0.25 mM IPTG and incubated at 16 °C for 16 h. Cells were harvested and resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1.5 mM MgCl₂, 25 mM imidazole and 0.5 mM PMSF. Cells were disrupted by sonication. Clarified lysates were applied to Ni-NTA resin, washed six times with lysis buffer and eluted with GF150 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.5 mM MgCl₂) supplemented with 300 mM imidazole. For TRMT1L, the 6×His-SUMO tag was removed with Ulp1 protease. Proteins were further purified by heparin ion-exchange chromatography and size-exclusion chromatography (SEC) on a Superdex 200 Increase column equilibrated in GF150 buffer. Peak fractions were concentrated, flash-frozen and stored at -80 °C.

Preparation of tRNAs

DNA templates encoding the indicated human tRNAs were generated by PCR. In vitro transcription was performed with recombinant T7 RNA polymerase. Transcripts were extracted with phenol-chloroform, precipitated with sodium acetate and ethanol, and purified by denaturing polyacrylamide gel electrophoresis. Purified tRNAs were recovered from the gel, refolded before use and stored under RNase-free conditions.

Methyltransferase assay

Methyltransferase activity was measured in 25 mM HEPES-KOH, pH 7.9, 150 mM NaCl, 1.5 mM MgCl₂ and 2 mM DTT. Unless otherwise indicated, reactions contained 100 nM enzyme, 2 μM tRNA substrate, 10 μM SAM and 200 nM [³H]-SAM. Samples were incubated at 37 °C for 15 min and heated at 85 °C for 5 min to stop the reaction. RNA was recovered by phenol-chloroform extraction and ethanol precipitation. Tritium incorporation was quantified with a TriCarb 2910TR scintillation counter (PerkinElmer).

Nucleoside Mass spectrometry analysis

Purified tRNA (200 ng) was digested overnight at 37 °C with Benzonase (Yeasen), phosphodiesterase I and bacterial alkaline phosphatase in 4 mM ammonium acetate. Nucleosides were separated on an Atlantis Hilic Silica column (3 μm, 2.1×150 mm) and analyzed on an AB Sciex QTRAP 6500+ triple-quadrupole mass spectrometer in positive-ion multiple-reaction-monitoring mode. The nucleosides were quantified using the nucleoside-to-base ion mass transitions of 284.1 to 152.1 (G), 298.1 to 166.1 (m²G) and 321.1 to 180.2 (m²₂G). Quantification was performed by comparison with the standard curve obtained from pure nucleoside standards running in the same batch.

Electrophoretic mobility shift assay

Recombinant protein was serially diluted twofold from 2 μM in GF150 buffer. Each 10-μL binding reaction contained 20 nM tRNA. Samples were incubated at room temperature for 15 min and resolved on 6% native polyacrylamide gels in 0.5× TBE at 120 V for 25 min. Gels were stained with SYBR Gold and imaged on a ChemiDoc MP system. Band intensities were quantified with ImageJ.

Microscale Thermophoresis (MST)

5'-FAM-labeled tRNA was used at 20 nM and incubated with 16 twofold serial

dilutions of protein starting at 10 μ M in GF150 buffer supplemented with 0.05% Tween-20. Samples were loaded into capillaries and measured on a Monolith instrument. Binding curves were analyzed with MO.Affinity Analysis. Apparent dissociation constants were obtained from three measurements.

Primer extension assay

Cryo-EM reaction samples were incubated at room temperature for 1 h, diluted 20-fold with nuclease-free water and heated at 85 °C for 3 min. A 5'-FAM-labeled DNA primer complementary to tRNA^{Leu} was annealed to the RNA. Reverse transcription was performed with M-MLV (H-) reverse transcriptase in the presence of 0.5 mM dNTPs and 2.5 μ M primer at 50 °C for 30 min. Reactions were terminated with urea-PAGE loading buffer, heated at 95 °C for 5 min and resolved on 15% denaturing polyacrylamide gels. Products were imaged on an ImageQuant 800 system and quantified with ImageJ.

Cryo-EM sample preparation and data collection

TRMT1 (10 μ M) was mixed with tRNA (15 μ M) in the presence of 20 μ M SAM, SFG or SAH as indicated and incubated at 25 °C for 120 min. TRMT1-tRNA^{Tyr}, TRMT1-tRNA^{iMet}, TRMT1L-tRNA^{Tyr} and TRMT11-TRMT112-tRNA^{Leu} complexes were prepared under analogous conditions. Samples were applied to glow-discharged amorphous NiTi foil grids (Au300-1.2/1.3, Nanodim Tech.), blotted for 3 s and plunge-frozen in liquid ethane with a Vitrobot Mark IV operated at 8 °C and 100% humidity. Data were collected on a 300-kV Titan Krios microscope using EPU. Movies were recorded on a Gatan K3 detector behind a GIF Quantum energy filter with a 20-eV slit. The nominal defocus range was -1.2 to -1.8 μ m.

Cryo-EM data processing and model building

Movies were processed in cryoSPARC v4.2⁴². Particles were selected by template-based or Topaz picking⁴³, extracted and subjected to iterative two-dimensional classification, ab initio reconstruction, heterogeneous refinement and non-uniform

refinement. Local classification and refinement were used where needed to improve tRNA and active-site density. Final resolutions were estimated with the gold-standard Fourier shell correlation criterion at FSC = 0.143, and local resolution was calculated in cryoSPARC. Atomic models were built in Coot⁴⁴, refined in PHENIX⁴⁵ and validated with MolProbity⁴⁶. Dataset-specific particle numbers, map resolutions and refinement statistics are provided in Supplementary Tables 1-2.

Statistical analysis and reproducibility

All quantitative biochemical assays were performed with independent measurements as indicated in the corresponding figure legends. Methyltransferase activities were determined from independently prepared reaction mixtures, and individual measurements are shown as points in the figures. Microscale thermophoresis measurements were performed in three independent experiments, and apparent dissociation constants (K_d) were derived from the corresponding binding curves. Statistical analyses were performed using GraphPad Prism 8.

Data availability: The atomic coordinates and corresponding cryo-EM maps have been deposited in the Protein Data Bank and Electron Microscopy Data Bank, respectively. The accession codes are 44CS and EMD-82508 for the TRMT1-tRNA^{Leu}-Apo complex, 23JQ and EMD-69029 for the TRMT1-tRNA^{Leu}-SAH complex, 23JR and EMD-69030 for the TRMT1-tRNA^{Leu}-SAM complex, 23JT and EMD-69032 for the TRMT1-tRNA^{Leu}-SFG complex, 23JN and EMD-69026 for the TRMT1-tRNA^{Tyr}-SAH complex, 23JO and EMD-69027 for the TRMT1-tRNA^{iMet}-SAH complex, 26VW and EMD-80920 for the TRMT1L-tRNA^{Tyr}-SAH complex, and 23JM and EMD-69025 for the TRMT11-TRMT112-tRNA^{Leu}-SAH complex. Source Data are provided with this paper.

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Author contributions

X.G., Y.L. and J.D. designed and performed the experiments. R.L. and L.L. performed LC-MS/MS analyses. X.G. prepared cryo-EM samples and collected the cryo-EM data. XiaofeiG. and X.Z. processed the cryo-EM data and built the atomic models. All authors contributed to data analysis and interpretation. X.Z. supervised the project and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Ethics declarations

This study did not involve human participants, human clinical samples or animal experiments, and therefore did not require ethical approval.

Figure legends

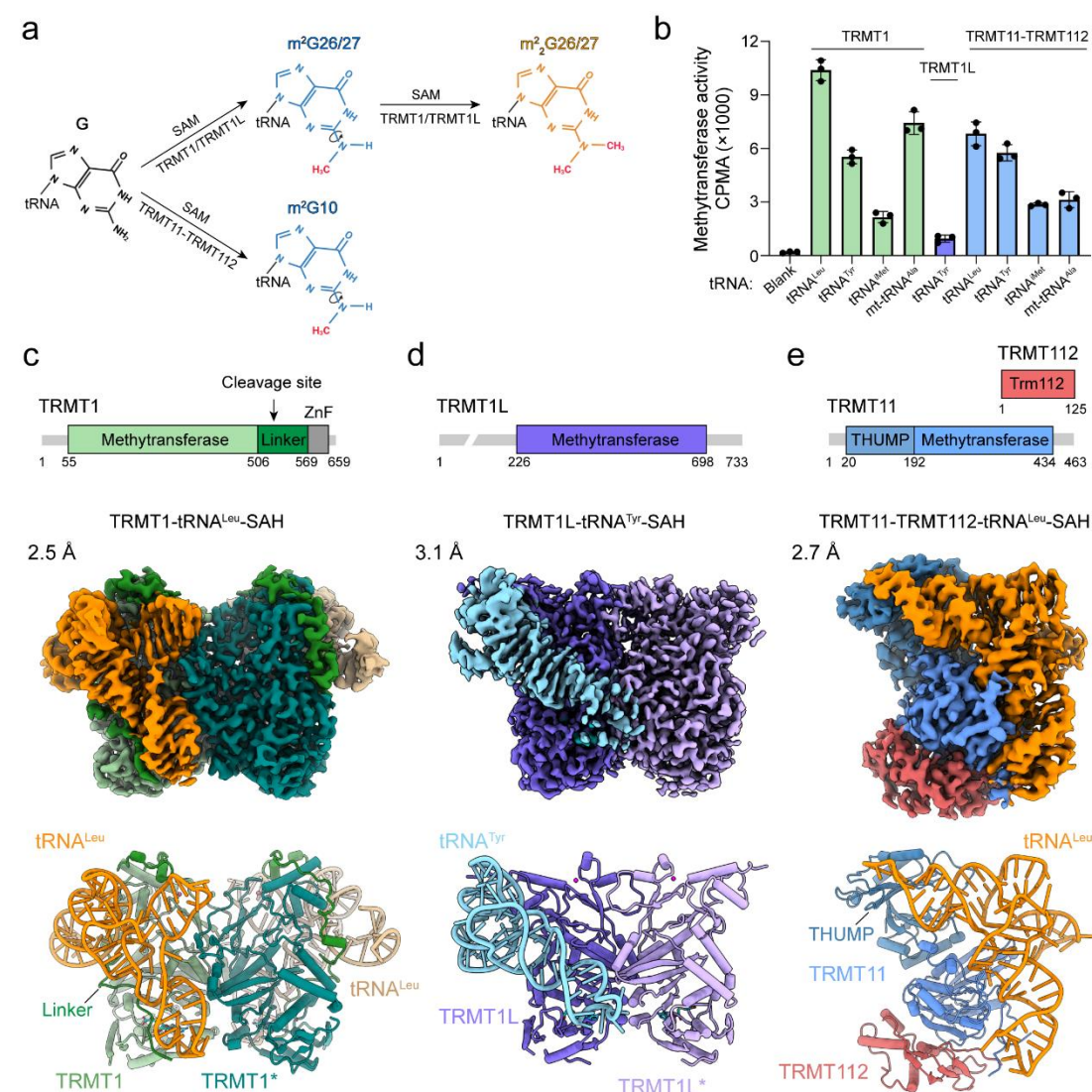
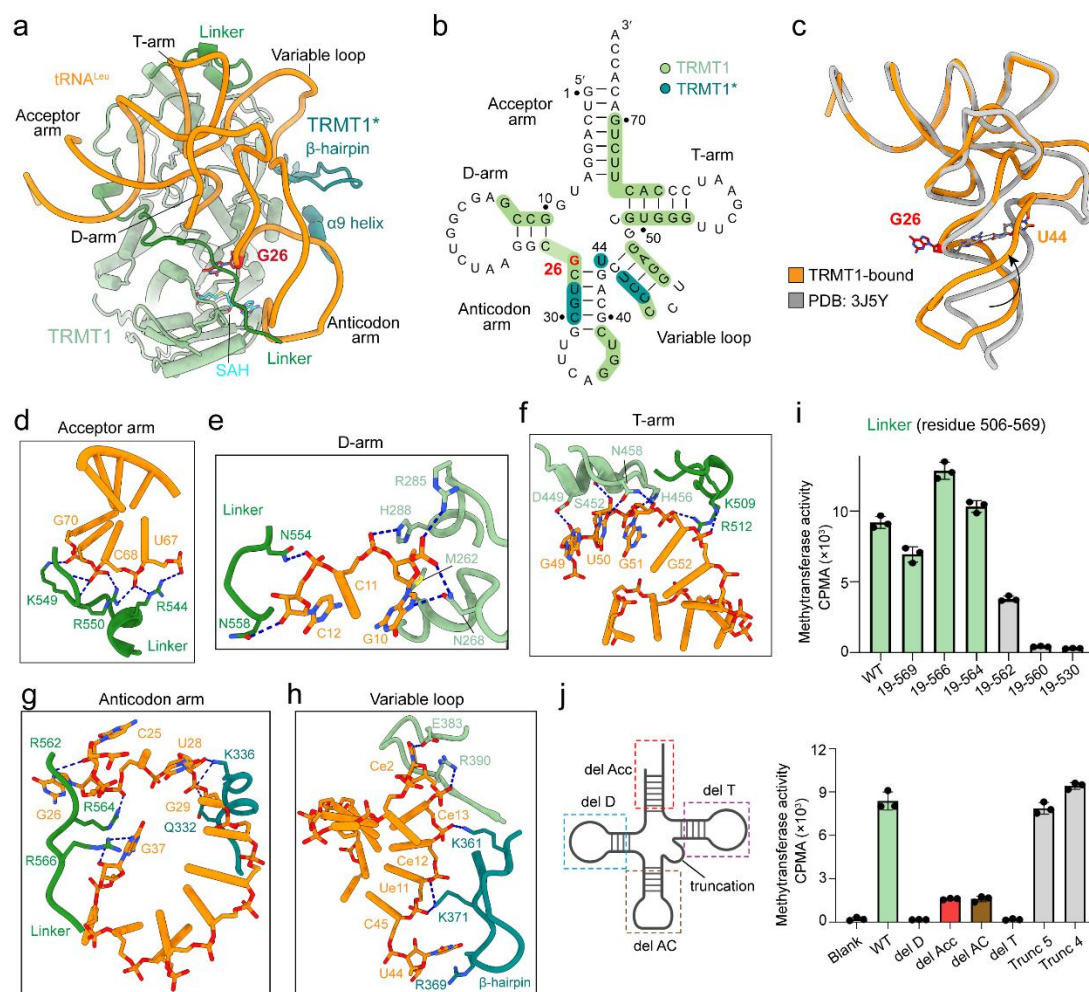


Figure 1 | Biochemical activities and overall structures of human tRNA N2-guanosine methyltransferases.

a, Reactions catalyzed by TRMT1/TRMT1L and TRMT11-TRMT112. TRMT1 and TRMT1L catalyze formation of m²G and m²G₂ at G26 and G27, respectively, whereas TRMT11-TRMT112 catalyze m²G formation at G10. SAM and SAH denote S-adenosyl-L-methionine and S-adenosyl-L-homocysteine. **b**, Methyltransferase activities of TRMT1, TRMT1L and TRMT11-TRMT112 toward the indicated tRNAs. Individual measurements are shown as dots. **c**, Domain organization of TRMT1 and

cryo-EM map and atomic model of the TRMT1-tRNA^{Leu}-SAH complex. The SARS-CoV-2 main protease (Mpro) cleavage site and the C-terminal zinc-finger are indicated. The two TRMT1 protomers are shown in green and teal, and the two tRNAs in orange and wheat. **d**, Domain organization of TRMT1L and cryo-EM map and atomic model of the TRMT1L-tRNA^{Tyr}-SAH complex. The two protomers are shown in purple shades and tRNA^{Tyr} in light blue. **e**, Domain organization of TRMT11 and TRMT112 and cryo-EM map and atomic model of the TRMT11-TRMT112-tRNA^{Leu}-SAH complex. The THUMP and methyltransferase domains of TRMT11 are shown in blue shades, TRMT112 in salmon, and tRNA^{Leu} in orange.

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617

618 **Figure 2 | A TRMT1 homodimer recognizes the L-shaped tRNA through**

619 **distributed contacts.**

620 **a**, Overall view of one composite tRNA-binding surface in the TRMT1-tRNA^{Leu}-SAH

621 complex. The catalytic protomer is shown in light green, the partner protomer in teal,

622 the C-terminal linker dark green, tRNA^{Leu} orange, G26 red and SAH cyan. **b**,

623 Cloverleaf representation of tRNA^{Leu} showing nucleotides contacted by the two

624 TRMT1 protomers. **c**, Superposition of TRMT1-bound tRNA^{Leu} with a reference

625 tRNA structure (gray; PDB 3J5Y⁴⁷), highlighting local remodeling around G26 and

626 U44. **d-h**, Close-up views of contacts with the acceptor arm (**d**), D arm (**e**), T arm (**f**),

627 anticodon arm and G26-containing region (**g**), and variable region (**h**). Dashed lines
628 indicate hydrogen-bonding or electrostatic contacts shown in the structural model. **i**,
629 Methyltransferase activities of wild-type TRMT1 and the indicated C-terminal
630 truncations. **j**, Schematic of the tRNA deletion and truncation constructs and their
631 methyltransferase activities. Individual measurements are shown as dots.

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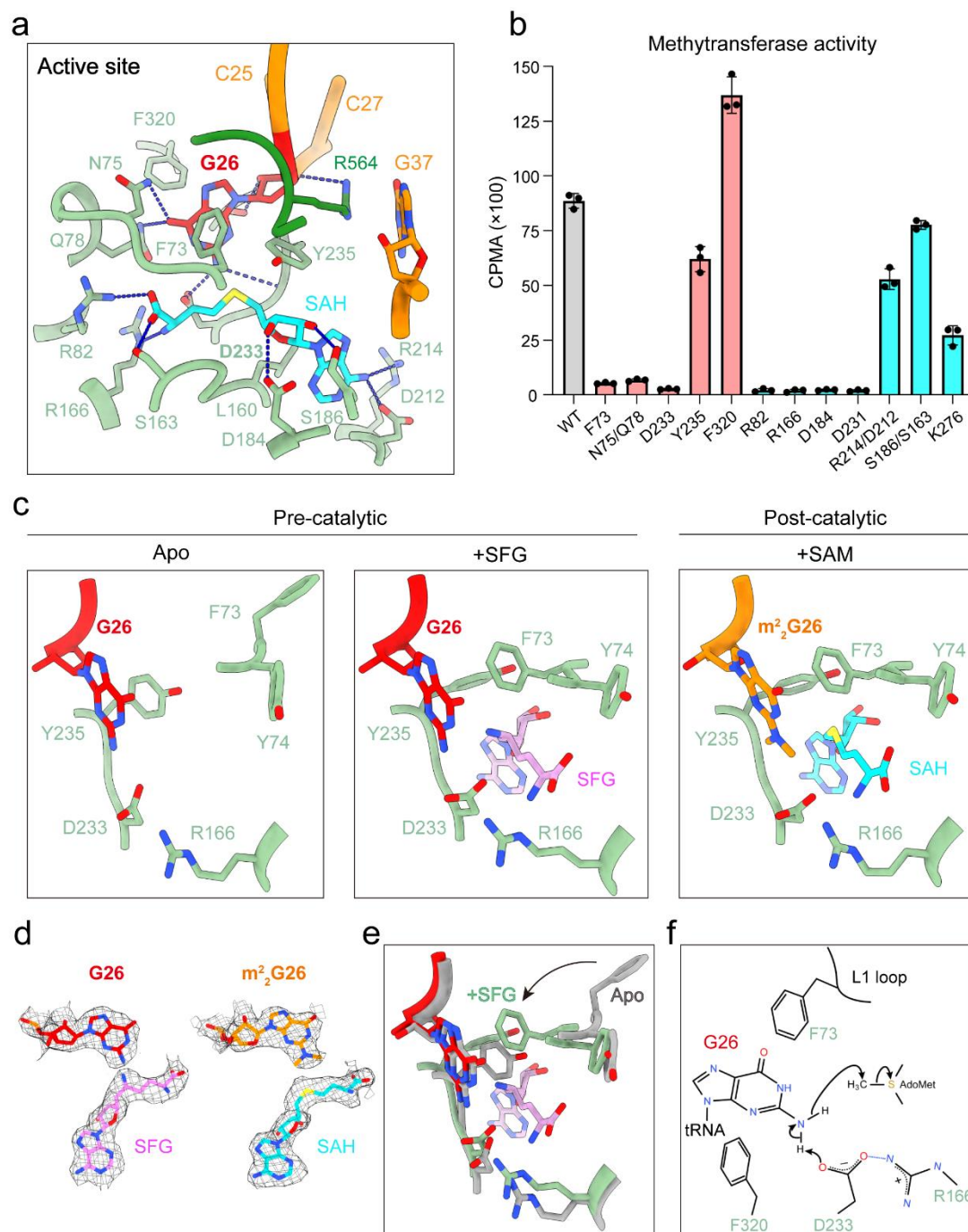


Figure 3 | Structural basis for G26 recognition and catalysis by TRMT1.

a, Close-up view of the TRMT1 catalytic center, showing the flipped G26 within the target-base pocket and SAH in the adjacent cofactor-binding pocket. G26 is shown in red, SAH in cyan and surrounding TRMT1 residues in green. Arg564 from the C-

terminal Linker and neighboring RNA nucleotides are also shown. **b**,
Methyltransferase activities of WT TRMT1 and the indicated active-site mutants.
Individual measurements are shown as dots. **c**, Comparison of the G26 pocket in the
apo, SFG-bound and SAM-reacted states. G26 is already flipped in the apo state,
whereas cofactor engagement induces closure of the Phe73-containing L1 loop. G26
is shown in red, m²G26 orange, SFG magenta and SAH cyan. **d**, Cryo-EM densities
for G26 and SFG in the pre-catalytic state and for m²G26 and SAH in the SAM-
reacted state. **e**, Superposition of the apo and SFG-bound active sites, highlighting
cofactor-dependent movement of the L1 loop toward the target base. **f**, Structure-
based model of G26 methylation by TRMT1. Asp233 and Arg166 constitute key
components of the catalytic center and are positioned to facilitate methyl transfer; the
precise proton-transfer pathway remains unresolved.

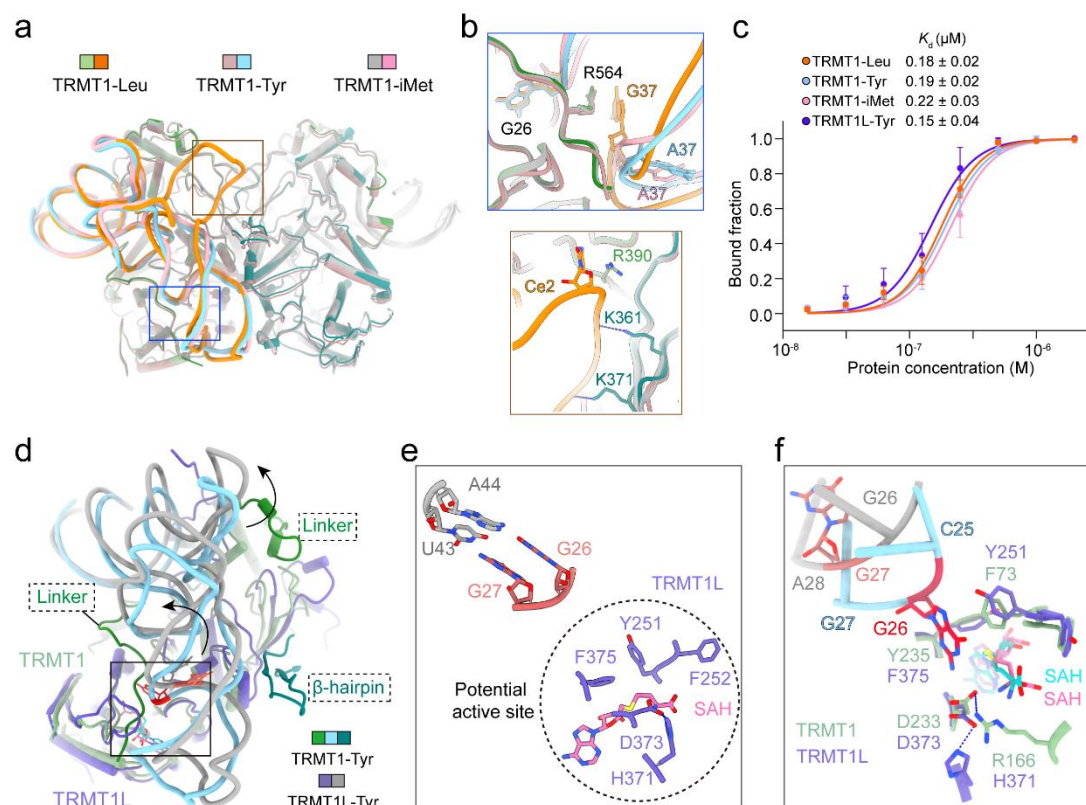


Figure 4 | Conserved catalytic geometry and distinct tRNA docking by TRMT1 and TRMT1L.

a, Superposition of TRMT1 complexes containing tRNA^{Leu}, tRNA^{Tyr} and tRNA^{iMet}, aligned on the TRMT1 dimer, showing a common overall docking mode. **b**, Close-up views of substrate-dependent differences near the G26-G37 region and the variable loop. **c**, EMSA measurements of TRMT1 binding to tRNA^{Leu}, tRNA^{Tyr} and tRNA^{iMet} and of TRMT1L binding to tRNA^{Tyr}. Apparent K_d values are indicated in the panel. **d**, Superposition of the TRMT1-tRNA^{Tyr} and TRMT1L-tRNA^{Tyr} complexes, highlighting differences in the upper RNA-binding regions and the TRMT1-specific C-terminal Linker. **e**, Close-up view of the G26-G27 region in the TRMT1L-tRNA^{Tyr} complex. The homologous catalytic pocket is indicated. Neither G26 nor G27 is inserted into the catalytic pocket, consistent with a substrate-engaged pre-catalytic state. **f**,

667 Superposition of the catalytic centers of TRMT1 and TRMT1L, showing conservation
668 of the overall active-site geometry despite substitutions of several pocket residues.
669 Phe73, Tyr235 and Arg166 in TRMT1 correspond to Tyr251, Phe375 and His371 in
670 TRMT1L, respectively. G26 is flipped into the catalytic pocket of TRMT1, whereas
671 G27 remains outside the corresponding pocket of TRMT1L.

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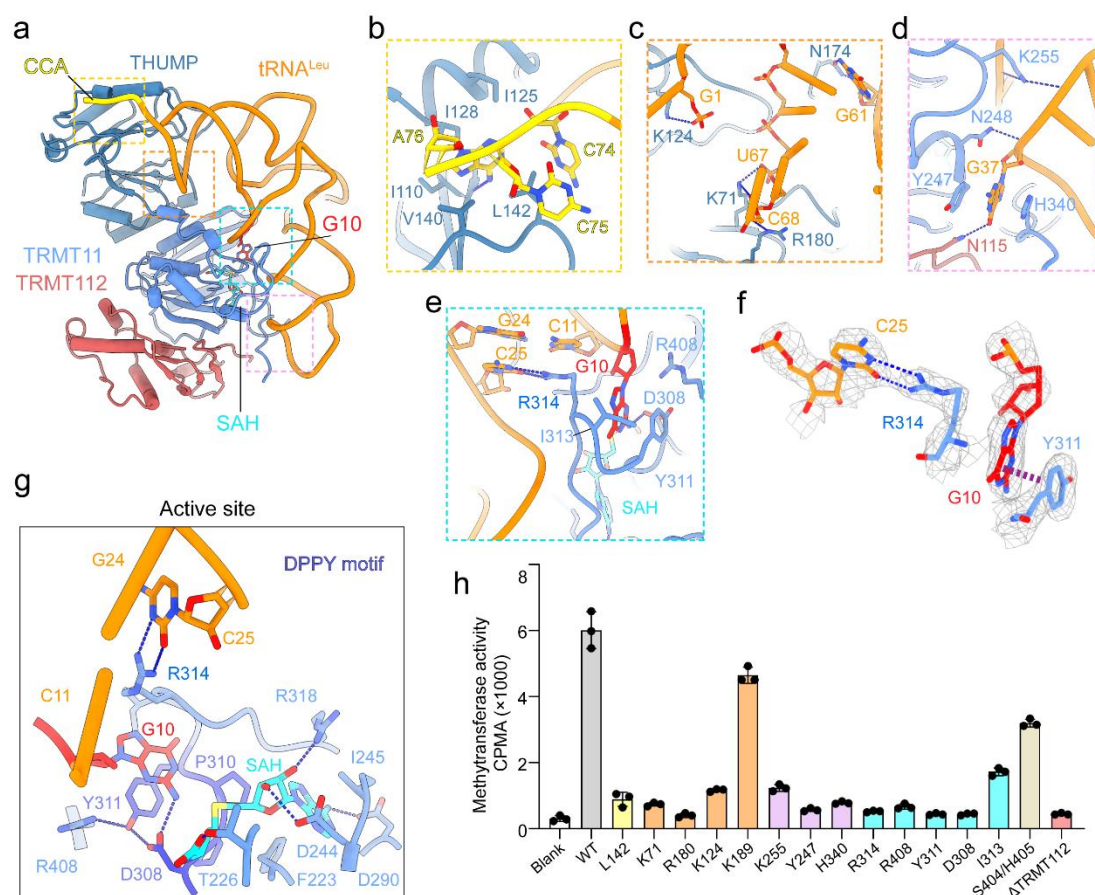


Figure 5 | TRMT11-TRMT112 uses a distinct recognition mode to position G10 for methylation.

a, Overall structure of the human TRMT11-TRMT112-tRNA^{Leu}-SAH complex. The TRMT11 THUMP domain is shown in dark blue, the methyltransferase domain in light blue, TRMT112 in salmon, tRNA^{Leu} in orange and SAH in cyan. The 3'-CCA terminus and G10 are indicated. **b**, Recognition of the 3'-CCA terminus by the TRMT11 THUMP domain. **c**, Contacts between TRMT11 and the acceptor-arm region. **d**, Recognition of the region around G37. G37 is sandwiched between Tyr247 and His340 of TRMT11 and further stabilized by a hydrogen bond with Asn115 of TRMT112. **e**, Local remodeling around G10. Arg314 occupies the position vacated by G10 and forms two hydrogen bonds with C25, effectively substituting for the G10-

686 C25 interaction, whereas the extruded G10 is stabilized by π - π stacking with Tyr311.

687 **f**, Cryo-EM density for the flipped G10 and surrounding active-site residues. **g**,

688 Detailed view of the catalytic center, showing the DPPY motif, G10, SAH and

689 residues surrounding the target base. **h**, Methyltransferase activities of wild-type

690 TRMT11-TRMT112, the indicated TRMT11 mutants and TRMT11 in the absence of

691 TRMT112. Individual measurements are shown as dots.

692