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**The structure of the human 80S ribosome at 1.9 Å resolution reveals
the molecular role of chemical modifications and ions in RNA**

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Abstract

The ribosomal RNA of the human protein synthesis machinery comprises numerous chemical modifications that are introduced during ribosome biogenesis. We present the 1.9 Å resolution cryo electron microscopy (cryo-EM) structure of the 80S human ribosome resolving numerous new rRNA modifications and functionally important ions such as Zn^{2+} , K^+ and Mg^{2+} including their associated individual water molecules. 2'-O-methylation, pseudo-uridine and base modifications were confirmed by mass spectrometry resulting in a complete investigation of the > 230 sites many of which could not be addressed previously. They choreograph key interactions within the RNA and at the interface with proteins, including at the ribosomal subunit interfaces of the fully assembled 80S ribosome. Uridine isomerisation turns out to be a key mechanism for U-A base pair stabilisation in RNA in general. The structural environment of chemical modifications & ions is primordial for the RNA architecture of the mature human ribosome, hence providing a structural framework to address their role in healthy context and in human diseases.

Introduction

Chemical modifications of ribosomal RNA are introduced during biogenesis of the ribosome and have an important role in translation regulation with medical relevance. For the human cytosolic ribosome, the vast majority of chemical modifications comprises 2'-O-methyl (2'-O-Me) alterations of the ribose moieties and pseudo-uridines (Ψ)^{1,2}. Such modifications have been characterized physicochemically or biochemically³⁻⁷, by RiboMethSeq^{8,9}, AlkAnilineSeq¹⁰, HydraPsiSeq¹¹, mass spectrometry¹² and listed in data bases such as Modomics¹³. In addition, other, yet less abundant modifications exist (~5%) such as m¹, m⁶ methylations etc. that occur on the nucleotide bases^{14,15}. There is growing evidence that such modifications can modulate ribosome function or cause antibiotic activity changes¹⁶⁻²⁷. The hypothesis is that specific changes on “specialized” ribosomes may occur in a particular cellular context²⁸⁻³¹. This has been observed recently for breast cancer in which certain 2'-O-Me sites are differently modified between tumors³², and similarly for pseudo-uridylation³³, hence suggesting an important role for modifications in cancer^{34,35} in relation with the fact that the human ribosome represents a promising anti-cancer target^{36,37}. Structural information in combination with biochemical characterisation of chemical modifications is essential to understand the underlying molecular mechanism and role in various rRNA nucleotide positions.

The human 80S ribosome comprises the 60S subunit, which encompasses three rRNAs (28S, 5.8S and 5S), and the 40S subunit that contains one single rRNA chain (18S). In an earlier study³⁸ we performed a first visualization and annotation of chemical modifications in the human ribosome structure determined by cryo electron microscopy (cryo-EM)³⁹ at an average resolution of ~3 Å. While numerous modifications could be

visualized, many more would be expected from data bases such as Modomics¹³ but could not be seen at the time, in particular 2'-O-Me and Ψ , the latter being limited in analysis by the possibility of resolving potentially associated water molecules; in addition, for some sites the chemical identity on nucleotide bases was unclear and remained to be addressed. Also, according to electrostatic potential (ESP) map calculations⁴⁰, some previously non-annotated sites on the 28S rRNA at the N7, O6 or O4 atoms of some guanines (G), adenines (A) or uridines (U), respectively, appeared more likely to instead represent Mg^{2+} ion positions but this remained to be confirmed experimentally; positive charges tend to generate larger densities in the cryo-EM map and may sometimes erroneously suggest the presence of a chemical bond. Better resolved maps would help overcoming such limitations. More generally, identifying the position of typically RNA-associated ions such as Mg^{2+} and K^+ and localizing water molecules in the structure would be important functionally. Using the latest-technology cryo electron microscope setups (see methods) combined with advanced image processing, structure sorting and extensive 2D & 3D focused classification & refinement procedures⁴¹⁻⁵¹ we now succeeded in breaking the 2 Å resolution barrier for the structural analysis of the 80S human ribosome, a non-symmetrical, rather flexible complex^{36,38,52,53} that compared to the bacterial ribosome comprises long disordered rRNA expansion segments and lacks strong stabilization when binding antibiotics/inhibitors. The structure provides unprecedented detailed insights into the overall RNA and protein structure of the human ribosome, the localisation of ions, ligands and water molecules and in particular chemical modifications of the rRNA in both 40S and 60S ribosomal subunits and their interfaces. In addition, to corroborate the identification of sites we used a mass spectrometry (MS) based method developed for the characterisation of RNA modifications⁵⁴, which was adapted and

extended to the analysis of rRNA modifications^{55,56}. Together, this allowed analysing the precise structural environment and function of chemical modification in the human 80S ribosome structure.

Results

High-resolution structure determination of the human ribosome

Human ribosomes were isolated from HeLa cells and flash-frozen for cryo-EM data collection (see methods & **Table 1**). Initial image processing including particle sorting and 3D classifications provided a first map at 2.3 Å resolution. A particular attention was then given towards refinement of parameters such as contrast transfer function (CTF) and higher-order aberrations using optic groups followed by particle polishing to account for minor imaging abnormalities and particle movements, reaching 2.0 Å resolution (**Extended Data Fig. 1**). Further focused and multi-body refinements (**Fig. 1a**) yielded 1.9 Å average resolution, with an average resolution of 1.9, 2.0 and 2.1 Å for the 60S ribosomal subunit and the body and head regions of the 40S ribosomal subunit, respectively. An additional final refinement on each body using partial signal subtraction further improved features in the cryo-EM map (**Extended Data Figs. 1 & 2**). Local resolution analysis shows that many regions extend to 1.7-1.8 Å resolution (**Fig. 1a**).

We then built an atomic model based on our previous lower resolution structure³⁸ (PDB ID 6QZP), but now refined in much more detail to account for precise side-chain conformations on ribosomal proteins and rRNA thanks to the high-resolution features of

the cryo-EM map (**Fig. 1b-d**; see methods). The map resolves numerous chemical modifications, ions and water molecules (annotated as described in the following), which were built and included in the atomic model and refined using the Phenix software⁵⁷ to obtain good geometric refinement parameters (**Table 1**).

Specific features and post-translational modifications

The structure provides unprecedented insights into many fine details. For instance (**Fig. 1c**), valine side-chain up and down conformations can be differentiated, side-chain conformations of isoleucine residues can be resolved because the orientation of methyl and ethyl moieties can be distinguished, and cis-prolines can be identified. High-resolution features can be observed on the ring structure of nucleotide bases and ribose moieties of the rRNA backbone (**Fig. 1d**). Chemical modifications can be found on numerous rRNA residues (as discussed later; **Supplementary Figs. 1 & 2**) but few are visible on the ribosomal proteins (this does not exclude the possibility that more exist) including methyl-arginine (ribosomal protein eS19), hydroxy-histidine (uL15, important for translational activity⁵⁸) and methyl-histidine (uL3) residues (**Extended Data Fig. 3**), consistent with post-translational modifications reported in the Uniprot database. A particularly interesting example is iso-aspartate found in the universally conserved ribosomal protein uS11 (**Fig. 1e**), a residue that contains an extra methylene moiety in the backbone and thereby modifies the local geometry; this modification has been observed in the *E. coli* ribosome⁵⁹ and in plants⁶⁰ (tomato; also visible in tobacco⁶¹ but not modelled), hence confirming the conservation of this rather peculiar post-translational

modification across species including human. The shorter methylcarboxylate side-chain of iso-Asp138 packs smoothly against the ribose moiety of G986 and forms a water-mediated hydrogen bond), which would not be possible with a standard aspartate residue (**Fig. 1e**). The functional meaning of this modification remains to be understood (for other proteins, it is linked to diseases^{62,63}), but structurally it stabilizes the interaction between the 135-140 extended loop region of uS11 and the single-stranded 986-989 region of the 18S rRNA on the 40S platform next to the tRNA exit (E) site and the 5' end of the mRNA channel.

Ions in the high-resolution structure of the human ribosome

Many additional small densities can be found in direct vicinity of nucleic acid bases, phosphate and ribose moieties and amino acids which potentially correspond to ions and water molecules (**Fig. 2**), and some polyamines (2 endogenous spermidine molecules as no polyamines were added in the buffer; **Extended Data Fig. 4**). Due to the complexity of the structure and the sheer amount of individually resolved densities we first placed ions and then searched for water molecules (see methods). To annotate ions in a reliable manner we considered both the strength of the map density and their characteristic coordination known from chemistry: tetrahedral for Zn^{2+} ions (typically located in a surrounding environment of Cys residues), octahedral for Mg^{2+} ions, and higher coordination (e.g. 8-12) for K^{+} ions (the latter show a stronger density than other ions; detailed below & see methods), hence considering distances and angular geometry for each ion type in its environment. Taken together, 9 Zn^{2+} , 294 octahedral Mg^{2+} and 107

K⁺ ions could be annotated (**Fig. 2a**), along with ~16000 water molecules (using SWIM⁶⁴ and Phenix^{57,65} software; see methods).

Coordinated Zn²⁺ ions can be seen in several ribosomal proteins (eL34, eL37, eL40, eL42, eL43, uS14, eS26, eS31). Several are very well resolved and show the characteristic tetrahedral configuration by 4 cysteine residues with a typical short distance of 2.3 Å between the cysteine sulphur atoms and the Zn²⁺ ion (**Fig. 2b**). Ribosomal protein eS27 exhibits a cysteine-preconfigured tetrahedral site (but no Zn²⁺ density is visible there, suggesting a weak binding site). With the exception of uS14, the Zn²⁺-binding sites are found primarily in eukaryote-specific ribosomal proteins. This suggests that the evolutionary addition of proteins in eukaryotes (as compared to bacterial ribosomes) required additional stabilization of the protein fold by a coordinating Zn²⁺ ion, possibly related with the fact that all the Zn-binding eukaryote-specific ribosomal proteins are surface-exposed (**Fig. 2b**) which may entail additional stabilization.

Mg²⁺ ions are readily identifiable thanks to their characteristic octahedral coordination with water molecules in 2.1 Å distance from the central ion (**Fig. 2c**). This is possible because the associated individual water molecules can often be resolved in the map or the octahedral hydration shell becomes visible at lower contour level when a given site is less stable (as illustrated by variable temperature factors). Hexahydrated Mg²⁺ ions were fitted as octahedrons into the cryo-EM map taking into account the possible substitution of one or more water molecules by a polar substituent (refinement of the octahedrons was restrained in Phenix, see methods). This leads to variable configurations within the octahedrons, either with 6 water molecules or variations with an increasing number of residues (e.g. 1, 2, 3 or 4) taking every time the place of one octahedron corner

and leaving respectively 5, 4, 3 or 2 water molecules (**Fig. 2c**). Substituents are found to be exclusively oxygen atoms, e.g. from negatively charged phosphate groups (which favours charge compensation), 2'-hydroxyl groups of ribose moieties and oxygen atoms of nucleotide bases and amino acids (**Fig. 2c**). The analysis confirms our previous ESP-annotated Mg^{2+} sites⁴⁰ that can now be resolved as such at N7, O6 and O4 positions of some G, A and U in the 28S rRNA (**Extended Data Fig. 5**) instead of an initially assumed chemical modification³⁸; these nucleotide base positions function as weak Lewis bases and therefore provide only partial charge compensation as compared to a phosphate group. Importantly, the possibility to resolve the associated water molecules throughout the structure reveals that in many cases the Mg^{2+} ion contacts to the neighbouring residues are water molecule mediated, *i.e.* even phosphate groups often interact with the Mg^{2+} ion in an indirect manner via a water molecule rather than directly as might be assumed classically (**Fig. 2c**). These Mg^{2+} ions generate interaction links between different structural elements (e.g. nucleotides G1880-A1669-U1319, A998-A1084-A1859, A1600-C2797 and U2852-A4496; 28S rRNA; **Fig. 2c**) that are relatively far away in sequence and in distance ($\sim 4\text{-}8$ Å, *i.e.* much beyond hydrogen bonding distance), hence stabilizing the rRNA fold.

The characteristic higher-order coordination of K^+ ions is visible as a recognizable feature (notably stronger density than a water molecule or other ions because of its larger atomic radius and positive charge, no close coordination by regular water molecules in contrast to octahedral Mg^{2+} , which at lower resolution - where water molecules cannot be seen may lead to falsely assigned K^+ ions) together with the more variable bonding distance and irregular geometry resembling a square anti-prismatic (**Fig. 2d**) as seen in potassium channels and in the *T. thermophilus* ribosome^{66,67}. K^+ ions are typically found

at interface areas between rRNA bases and amino acid side-chains (**Fig. 2d**), or involving 2'-OH ribose moieties (e.g. A363 in 28S rRNA, **Fig. 2d**), or stabilizing protein loops (Gly213 region of ribosomal protein uL2 which comprises 3 consecutive glycine residues; **Fig. 2d**). Their environment almost exclusively comprises oxygen atoms representing weaker Lewis bases (mostly amino acid backbone and nucleic acid keto groups and few hydroxyl groups, i.e. usually not phosphate groups). The role of K⁺ ions in stabilizing these structural elements - typically intrinsically less stable single-stranded rRNA or protein loop regions - implies also an important role in translation, which is consistent with the observation that ribosomes lacking K⁺ ions show reduced poly-Phe synthesis and increased thermal instability⁶⁸. In this context, there is a characteristic K⁺ binding site located at the PTC, which bridges G3907, G4393 and m⁵C4447 of 3 different 28S rRNA segments (**Fig. 2d**). The interaction with the phosphate moiety of m⁵C4447 creates a charge compensation, while coordinating with the 6-keto group of G4393 and the 6-keto and N7 positions of G3907. Interestingly, this K⁺ position is also observed in the bacterial ribosome structure⁶⁹, hence suggesting a conserved role across species. Furthermore, we could also identify K⁺ binding to the N7 position of G1484 showing some exchangeability between Mg²⁺ and K⁺ at this base moiety.

MS, rRNA sequencing & structural analysis of chemical modifications

RNA modifications are chemical changes that usually result in a mass shift, hence MS allowed to detect and determine the modifications placement on the RNA sequence. MS analysis of chemical modifications was done on the 18S, 28S, 5.8S and 5S rRNAs

using site-directed synthesized oligos combined with enzymatic cleavage with four different RNases (T₁, A, U₂ and MC1) and high-resolution LC-MS/MS analysis of RNA fragments allowed to determine the modified positions on the RNA sequence (see methods; **Extended Data Figs. 6 & 7; Supplementary Fig. 3**). 2'-O-Me sites were identified by MS and cross-validated with the cryo-EM structure, in particular for the sites not previously observed (e.g. Um428 from 18S or Um4498 from 28S rRNA). Ψ sites were identified by MS using specific acrylonitrile labelling to generate a corresponding mass shift; identified sites were confirmed in the structure based on the characteristic interaction of water molecules or rRNA phosphate groups of the rRNA backbone with the exposed N1 position of Ψ's. Sites not found by MS were annotated according to previous identifications^{9,11-13,31,70-73} and cross-checked in the structure.

To overcome confusing differences in rRNA sequence numbering as found in data banks we performed rRNA sequencing (based on reverse-transcription followed by PCR, see methods); this includes a sequence correction from G4910 to adenine in the 28S rRNA, which is visible also in the structure (**Extended Data Fig. 8**) hence providing a unique consistency validation between sequence, structure and MS analysis of the rRNA using the same sample. All chemical modifications were annotated on the rRNA sequences (**Supplementary Fig. 4**). This now allows an unambiguous annotation of rRNA residues both in the structure and with regards to chemical modifications found in the literature. This turns out to be greatly helpful to unify the entire annotation of modifications sites on the basis of a unique numbering (99 2'-O-Me sites, 109 Ψ, and 12 base modifications such as m¹acp³, m¹, m³ etc. for the structural data and respectively 6 and 9 seen only from the MS data; total of 235 sites including 105 2'-O-Me & 118 Ψ and 12 base modifications). The MS-identified 2'-O-Me sites and Ψ sites are overall

consistent with previous identifications^{9,11-13,31,70-73} (**Suppl. Data File 1**). New sites found by MS and also observed in the structure include for 18S rRNA Ψ63, Ψ100, Ψ300, Ψ1003, Ψ1360, Ψ1596. Those newly found by MS but not observable in the structure due to weakly resolved regions include for 18S rRNA Ψ366, Ψ514, Ψ556, Ψ667, Ψ769/770 (the only one on an rRNA expansion segment), Ψ804, Ψ1061, Ψ1186, and Gm1946 and Ψ5039 for 28S rRNA; Ψ897 listed in Modomics¹³ is not found by MS and is not visible in the structure; Ψ1136 found in Modomics is not found by MS but is visible in the structure. Together, the combined high-resolution structural analysis, sequencing and MS-based validation allows a full annotation of chemical modification sites.

The structure comprises in the 28S, 18S and 5.8S rRNA's, respectively, 62, 37 and 2 2'-O-Me and 58, 49 and 2 Ψ modifications, most of which are confirmed by our MS analysis and otherwise identified by previous studies^{9,11-13,31,70-73}. 5.8S rRNA carries 4 modifications 3 of which were found by MS, while none were found in the 5S rRNA (nor predicted nor visible in the structure). Compared to the set of modifications found by MS and/or previous studies, 12 2'-O-Me and 13 Ψ modifications are not visible in the structure (e.g. when less resolved in the periphery of the complex; 10 sites were found neither in the structure nor by MS, possibly owing to differences between cell lines). Interestingly, the MS data of the modification m¹acp³ψ1248 (18S rRNA) reveal an extra mass corresponding to a cyanoethylated acp3 adduct (see methods).

Ribose 2'-O-methyl modifications in the human ribosome

2'-O-methylations are found fairly widespread throughout the structure, with rather many in the 40S compared to the 60S ribosomal subunit, including clusters at functional centres such as the peptidyl transferase centre (PTC) and the decoding centre (DC), but only few on the solvent side of the 60S ribosomal subunit (**Fig. 3**). The 5.8S rRNA has two 2'-O-methylation sites as confirmed by MS (**Fig. 3b & c**), both of which do not show particular contacts to neighbouring residues unlike some sites in 18S and 28S rRNA; this may imply that other cellular components interact there, in particular when the sites are solvent-accessible as is the case for the 5.8S rRNA sites. However, many others do show contacts with neighbouring residues, which translate into van der Waals contacts due to the hydrophobic nature of a methyl group. Hydrophobic contacts typically involve hydrophobic side-chains of amino acids (**Fig. 3g, h, i, l**) or the aromatic face of nucleotide bases (**Fig. 3e, k**) or between several 2'-O-Me sites (**Fig. 3f, k**). However, van der Waals contacts may not necessarily represent hydrophobic contacts because they also exist with polar residues (**Fig. 3d, j**); also, an O-Me moiety would not be sufficiently polarized to form a hydrogen bond through the methyl group. However, closer inspection shows that without the methylation there would be a gap between polar residues (involving a free 2'-OH group), which would be too far ($>4 \text{ \AA}$) or inappropriate for a hydrogen bond donor/acceptor system. Hence, creating a van der Waals contact ($\sim 3.5\text{-}3.8 \text{ \AA}$ distance) with a methyl group appears to generate a better stabilization than leaving a gap with no hydrogen bond (e.g. **Fig. 3d, e, h, j, m**).

Interestingly, hydrogen bonds can in fact form with the oxygen atom of the 2'-O-Me group, for example with a free ribose 2'-OH group (**Fig. 3e, m**), a polar residue (**Fig.**

3h) or a water molecule (**Fig. 3i**). This is because a methoxy moiety keeps its hydrogen bond acceptor capability due to the 2 free electron pairs. Hence, a methylation removes the hydrogen bond donor activity of a 2'-hydroxy group but still maintains hydrogen bond acceptor activity. Combination of these capabilities thanks to the peculiar chemical properties of a methoxy group can create rather complex contact and interaction patterns, as exemplified by Gm644 and Cm1391 (18S rRNA, **Fig. 3e & h**) or Gm4228 (28S rRNA, **Fig. 3j**). Such series of contacts and interactions involving 2-O-Me moieties contribute to stabilize the conformation of numerous residues, both through van der Waals contacts and - unexpectedly - also hydrogen bonds through the oxygen atom of the methoxy moiety.

Pseudo-uridine modifications in the human ribosome

As opposed to 2'-O-Me sites which can be readily recognized in a structure, Ψ 's cannot be structurally distinguished from a U due to their isomeric nature in which the C5 and N1 atoms are swapped (**Fig. 4a**). Yet, Ψ sites identified by MS could be confirmed thanks to their specific property of having an exposed hydrophilic N1 position, which translates into the presence of a water molecule (that can be resolved in the high-resolution cryo-EM map), a phosphate group or other polar residue in hydrogen-bonding distance; by comparison, a non-modified U cannot form such interactions in this position. Thanks to this annotation, each site and its structural environment can now be analysed (**Fig. 4**), revealing a characteristic interaction pattern. Ψ 's typically interact via a water molecule with the phosphate group of the same residue (**Fig. 4d, f, h, l**), or with the

phosphate group of the n-1 residue (**Fig. 4e, j**) or with both phosphate groups (e.g. 5.8S rRNA, **Fig. 4b, c**), or only with the phosphate group of the n-1 residue (**Fig. 4e, j**). Interactions with the n-2 phosphate (**Fig. 4i**) or with other moieties such as ribose (**Fig. 4g**) or nucleotide bases (**Fig. 4k**, mediated by a water molecule) also exist but are rarer.

The majority of Ψ s (92 of the 109 visible in the structure) have a water molecule bound in the N1 position of the base (**Fig. 4 & Supplementary Figs. 1 & 2**). These water molecules stabilize the Ψ base conformation because of their interactions with neighbouring polar residues. Many Ψ s are in a single-stranded region of the rRNA and/or exhibit hydrogen bonds with a neighbouring nucleotide base. In this structural context, the water molecule-mediated stabilization of the base conformation helps to pre-configure its orientation in a favourable manner for interaction with a neighbouring base, in particular for A-U base pairs (**Fig. 4b, d-f, h, j, k, l**) and non-canonical base pairs such as U-U (**Fig. 4c, f**). The specific stabilization of A-U base pairing through a U- Ψ conversion is particularly frequent and occurs mostly in rRNA helical segments (**Fig. 4**). A-U pairs may benefit from additional hydrogen bonding opportunities because they involve only 2 hydrogen bonds as compared to G-C pairs that have 3. In conclusion, the introduction of a Ψ chemical modification indirectly stabilizes nucleotide base pairing. Consistently, the vast majority of Ψ N1 interactions occurs with rRNA nucleotides but not with amino acids. Overall, Ψ modifications cluster in the 40S ribosomal subunit and the 60S region proximal to the 40S, including at PTC & DC functional centres (**Fig. 4**), for example, the tip of H69 (28S rRNA) carries two universally conserved pseudo-uridines, m³ Ψ 3762 and Ψ 3764, located in the tRNA peptidyl site. In conclusion, the presence of the numerous Ψ 's in the structure creates many options for additional hydrogen bonding at the N1 of the modified base, which would otherwise not be available due to the hydrophobic

character of the C5 position in a U. As visible from the human rRNA structure, the Ψ can have a local stabilization effect on base pairs under variable configurations (Watson-Crick and Hoogsteen⁷³; **Fig. 4b-f,h,j-l**) including base triplets (**Fig. 4k**) and rRNA loops (**Fig. 4g,i**). The creation of additional hydrogen bonds collectively tends to stabilize the macromolecular assembly, consistent with the observation that Ψ -free ribosomes seem to be more flexible structurally⁷⁵.

Nucleotide base modifications and interfaces

The human rRNA comprises 12 nucleotide base modifications all of which were detected by MS (**Suppl. Data File 1**) and are visible in the structure (**Fig. 5**). The 28S and 18S rRNA's have 5 and 7 modifications respectively, but none are present in the 5 and 5.8S rRNA's. The cryo-EM map resolves individual methyl groups on the bases (**Fig. 5b-e, h, k**) or the conformation of the substituent, e.g. the methyl moiety at the N6 position of m⁶A4220 or m⁶A1832 (**Fig. 5b, k**) and the acetyl groups of cytosines C1842 and C1337 (**Fig. 5g, i**). The methyl groups often create stabilizing van der Waals contacts with neighbouring nucleotides (no amino acids involved; **Fig. 5b, c, e, h**) or increase base stacking as the methyl group is co-planar with the base (m⁵C3782, **Fig. 5d, k**). The hypermodified residue m¹acp³ Ψ 1248 (ref. 76) comprises base methylation and an aminocarboxypropyl (acp) moiety that is more flexible (**Fig. 5f**). Interestingly, its position appears to bridge the 40S head and body regions. Both m¹acp³ Ψ 1248 and m⁵C3782 are located in the tRNA peptidyl site and are likely to be in contact with the tRNA (note added in proof: as seen in a recent study⁷⁷). In addition, the 1248 modification appears

involved in cancer⁷⁸ and its absence affects 18S rRNA maturation⁷⁹, a mechanism that could make sure that only a properly modified 18S rRNA assembles into an initiation-competent 40S ribosomal subunit. The di-methylated m⁶₂A1850 and m⁶₂A1851 (18S rRNA, **Fig. 5j**) are essential for ribosome assembly⁸⁰. They are located on the 40S ribosomal subunit close to the rotation pivot point next to helix H69 (28S rRNA) corresponding to conformational changes and subunit ratcheting reminiscent of pre-/post-translocation states. m³U4530 is located at the PTC next to the entry of the peptide exit channel. Among the two acetylated residues (needed for 40S biogenesis⁸¹), ac⁴C1842 forms two hydrogen bonds with Arg2 (ribosomal protein eL41), one through its phosphate group and one through the acetyl moiety (**Fig. 5g**), thereby promoting inter-subunit interactions. The other acetylated residue, ac⁴C1337, is located at the entry site of the mRNA channel and might interact with the incoming mRNA (**Fig. 5i**).

Discussion

The present high-resolution structure of the human ribosome is a major advance in its observation at the molecular level providing unprecedented insights into its RNA architecture and function. Using latest-generation cryo electron microscope setups and advanced image processing including corrections of higher-order aberrations and focused refinement of separate structural segments has now allowed crossing the 2 Å resolution barrier on the full 80S human ribosome structure. With 1.9, 2.0 and 2.1 Å for the 60S ribosomal subunit and the body and head regions of the 40S ribosomal subunit, respectively, this represents a resolution improvement of 1 Å compared to our previous

study³⁸ and a subsequent study⁸² [note added in proof: and also slightly better resolved than the isolated 40S subunit (with no full ribosome) in a publication that appeared while the present work was under revision⁸³, which is overall consistent with the present findings]. This type of resolution range is a game changer at the level of observable details (see comparisons in **Extended Data Fig. 2**). This allows resolving unprecedented details on rRNA and ribosomal proteins, chemical modification of the rRNA, water molecules and various ions, but also several post-translational modifications on the ribosomal proteins. It provides the currently best resolved structure of the fully assembled human 80S ribosome including a detailed atomic model of the overall ribosomal RNA and proteins refined to good geometry (even cis-prolines could be built, which is only reasonably possible at such high resolution; **Table 1**). Thanks to the possibility to visualize water molecules it became possible to annotate pseudo-uridines (Ψ) in the structure; indeed, these uridine isomers cannot be identified in terms of their structure but instead can be indirectly recognized thanks to their resolved N1-associated water molecule.

2'-O-Me, ψ sites and nucleotide base modifications were analysed by advanced mass spectrometry of RNase-induced rRNA fragments. This allowed to confirm the chemical identity of the modification sites and served for cross-validation of the observations made in the structure; for the rare sites located in structurally ill-defined regions at the periphery, the MS data conveniently complete the picture and provide consistency across different biochemical methods used in the past^{9,11-13,31,70-73} (**Suppl. Data File 1**; slight differences could be due to the usage of different cell lines or different chemical identification methods). Together, this combined cryo-EM and MS analysis using the same sample for both methods provides the most complete annotation of

chemical modifications in human rRNA. Following our first visualization of chemical modifications in the human ribosome³⁸, the present study now resolves the chemical identity of those sites that remained to be characterized and it strongly extends the number of identified chemical modifications in the 3D structure (an additional 91 Ψ 's and 45 2'-O-methylations, confirmed by MS). With the exception of a few sites at the periphery of the structure, all 2'-O-Me, Ψ and base modifications can be seen. In total, 235 modifications were annotated: 220 rRNA modification sites were identified on the entire structure, including 99 2'-O methylations, 109 Ψ and 12 base modifications (the two Ψ m's and $m^1\text{acp}^3\Psi$ are counted twice due to their hyper-modifications) and an additional 15 sites were seen by MS but not observable in the structure being located at the periphery.

Chemical modifications found by MS and their corresponding sites in the structure are displayed together in **Fig. 6** (see also **Supplementary Figs. 1 & 2** for the detailed cryo-EM map and **Supplementary Fig. 3** for the list of modified fragments detected by MS). Their distribution in the structure is consistent with our observation that an evolutionary extended shell of rRNA modifications exists in eukaryotes³⁸. By contrast, the *E. coli* ribosome has much fewer sites (35), of which only 6 are conserved and 3 are chemically different (**Fig. 7**; post-translational modification on the ribosomal protein are also not well conserved, **Fig. 7b**); in *E. coli*, the rRNA modifications are predominantly base modifications (22; **Fig. 7a & b**), contrary to eukaryotes (**Supplementary Figs. 1 & 2** and **Suppl. Data File 1** for the list of modifications in ribosomes from different species). Similar considerations apply also to other bacterial species (e.g. *T. thermophilus*⁸⁴). Even though most rRNA modifications are not conserved *per se*, comparison with the *E. coli* ribosome structure⁵⁹ (**Fig. 7c**) shows that while the residue corresponding to $m^6\text{A1832}$

(A1500 in *E. coli*) is not methylated its neighboring nucleotide (m⁴Cm1402, corresponding to Cm1703 in human) is methylated instead (**Fig. 7c**). The fact that the methyl moiety is oriented in the same direction in space (possibly for an interacting factor) suggests the existence of a methyl-compensation effect to create a locally hydrophobic environment. The observed base methylation swap between 2 neighbouring residues across human and bacteria (**Fig. 7c**) suggests that the concept of co-evolution of key residues also exists for chemical modifications. Among eukaryotes, rabbit and plant ribosomes^{60,85} comprise chemical modifications similar to human but with some differences (summarized in **Suppl. Data File 1**). The overall distribution of modifications in the human ribosome covers functional centres such as PTC and DC but spreads out further around the tRNA binding sites (**Fig. 6**). Compared to its size (~1900 nucleotides), the 40S ribosomal subunit comprises a ~twice higher average occurrence of modification sites (103) than the 60S subunit (132) that has > 5000 nucleotides as compared to the 40S subunit (1870). The strong modification density in the 40S ribosomal subunit appears consistent with a tighter regulation mechanism, in particular during translation initiation and biogenesis. Interestingly, those in the 60S subunit are mainly located in the half oriented toward the 40S ribosomal subunit (**Fig. 6**), hinting at a 40S-associated allosteric regulatory role in the 60S subunit. This appears connected with the fact that tRNA binding and translocation and associated conformational changes occur precisely in these core regions of the ribosome, consistent with the observation that modifications may play a regulatory role at molecular level in the translation process^{16–27,86}.

Due to their opposite hydrophilic versus hydrophobic character, 2'-O-Me and Ψ modifications change the possible hydrogen bonding possibilities and van der Waals contacts. The N1-associated water molecule is positioned within the plane of the

nucleotide base and enables new hydrogen bond interactions with neighbouring residues. In particular, one can observe a recurrent stabilization of A-U(Ψ) base pairs through the stabilization of the Ψ base conformation through hydrogen bonding to its own phosphate group and/or to the neighbouring n-1 phosphate group. These specific interaction patterns would not be possible without the introduction of a Ψ modification in these sites. Similarly, the 2'-O-methylation converts a hydrophilic moiety into a partially hydrophobic group, which favours hydrophobic contacts to neighbouring hydrophobic groups, but yet keeping the hydrogen bond acceptor activity on the 2'-O position. The H-bonding peculiarities of 2'-O-Me and Ψ modifications therefore create a versatility as compared to non-modified nucleotides, which allows extending the repertoire of interactions directly relevant for molecular recognition mechanisms in the ribosome and in RNA in general, but also beyond in other RNA-nucleoprotein complexes. Finally, the frequent occurrence of Ψ 's allowed discovering that the stabilisation of uridine base conformations through isomerisation and H-bonding helps alleviating the intrinsically less stable A-U base pairing, thus compensating for the reduced amount of H-bonds in an A-U pair (2 H-bonds) as compared to the more stable G-C pair (3 H-bonds). This A- Ψ base pair stabilisation principle sets an interesting concept applicable to RNA in general.

The structure comprises numerous annotated ions and water molecules in their native environment, coordinated by amino acids and nucleotides, hence also clarifying fundamental aspects of chemistry underlying ribosome function. Ions such as Zn^{2+} , K^+ and Mg^{2+} and even their associated water molecules can be localized, revealing the important role that water molecules and ions play at the level of molecular recognition and structural stabilization of the RNA architecture and its interactions with ribosomal proteins and substrates. The characteristic octahedral hydration shell of Mg^{2+} ion

comprising 6 water molecules is often seen, but depending on local interactions one or several water molecules can be replaced by coordinating phosphate or ribose moieties of the rRNA or by coordinating amino acids, *i.e.* polar residues are seen to replace precise individual water molecules in Mg^{2+} octahedrons. However, it also turns out that many interactions are mediated by water molecules; for example, phosphate groups do not necessarily interact directly with Mg^{2+} ions in contrast to what could be commonly thought. The Mg^{2+} ion can adopt various configurations with varying numbers of associated water molecules. Mg^{2+} ions and their associated water molecules contribute to bridging structural elements within RNA and at protein-RNA interfaces and thereby enable ion- and water molecule mediated interactions of structural elements that would otherwise be too far from each other to interact. Zn^{2+} ions which are found mostly in the eukaryote-specific ribosomal proteins provide extra stabilization as these are surface-exposed proteins on the ribosome; this striking feature hints at an evolutionary predilection of recruiting Zn^{2+} -binding proteins to the surface of the translation machinery.

Finally, hydrogen bonds within Mg^{2+} octahedrons or K^+ ions often extend to nearby water molecules to form a network. This hydration shell is not only visible at the surface of the ribosome (as classically found for many protein structures in the PDB) but also in many cavities inside. Locally and collectively, the numerous ion and water molecule interactions with rRNA and ribosomal proteins contribute to stabilize the 3D structure by holding structural elements of the ribosome together, yet providing a sort of flexible, dynamic network that can fluctuate and adapt along with conformational changes of the ribosome during the translation process. Interestingly, some Mg^{2+} octahedrons and K^+ ions are found in the same position in human, yeast, plant and *E. coli* ribosomes

suggesting a conserved role, for example in the PTC (**Fig. 7d**). The existence of conserved sites for Mg^{2+} and K^+ ions throughout the ribosome indicates a certain requirement of rRNA for ion binding in specific sites to fold into its active state. In a general way, the manner by which ions such as hexa-hydrated Mg^{2+} clusters and K^+ interact within protein-RNA assemblies is of broader interest for the analysis of macromolecular complexes, but also from the chemistry point of view regarding coordination chemistry and catalysis.

Taken together, the integrated structural analysis cross-validated with extensive MS-analysis has fostered a unique synergy between structural and biochemical analysis of rRNA chemical modifications allowing a comprehensive analysis of their three-dimensional environment at the molecular level. This study represents currently the largest characterisation of a single set of RNA modifications in one single assembly (these structure & MS cross-validated data have now been integrated into the latest version of the Modomics database⁸⁷). Thanks to our rRNA sequencing we could now identify which rRNA variant is present in HeLa cells, which is important considering that HeLa cells are widely used and that differential variants exist in cancer cells. Being on the human system the findings are relevant for human health, but also for understanding translation mechanism in eukaryotes in general. As a mature 80S ribosome assembly the structure gives unique insights into each ribosomal subunit and their joint interface at which numerous ions and chemical modifications are located. This will help guiding future functional analysis of modification enzymes and help understanding human ribosome function in the healthy context and diseases because modifications (which are introduced during biogenesis) are involved in translation errors, accuracy loss and antibiotic activity changes. The present work thus paves the way towards functional assays regarding specific modification sites of interest to address the molecular mechanism underlying

dysregulation and human diseases considering the growing implications of the human ribosome as a cancer target^{32,33,36,37}. The structure will serve as a reference for the structure-function analysis of various functional complexes with translation factors, mRNA and tRNA and as a high-resolution template for molecular dynamics simulation calculations, modelling and drug design.

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Author Contributions

I.H., L.F. & S.H. performed sample preparation, L.F., S.H. & O.vL. image processing, A.L. & P. W. MS analysis and annotations, S.N.T.D. & O.vL. rRNA sequencing, C.B., L.F., S.H., O.vL. & B.P.K. structure refinement & model building, structural analysis and annotations. All authors analysed the data. B.P.K supervised the project and wrote the manuscript with input from all authors.

Competing Interest Statement The authors declare no competing financial interests.

Figure legends

Figure 1 High-resolution features, ions and water molecules in the human 80S ribosome

a Local resolution estimated with the Relion software before (top) and after (bottom) multi-body refinement (before final partial signal subtraction, see methods). The different masks used for the multi-body refinement are shown with dotted-lines.

b Global views of the 40S and the 60S regions illustrating the quality of the map (sigma level indicated in top right corners).

c Examples of several amino acids from ribosomal proteins uL2, eL32 and uL4. The resolution gives insight on the exact positioning of the di-methyl moiety of a valine side-chain (shown before and after flip and fitting), allows to see the zig-zag of an Arg side chain, allows to see the hole in the aromatic side chain of a Phe, gives the possibility to fit the ethyl and methyl moieties of an Ile and to distinguish a cis-Pro from a trans-Pro.

d Examples of nucleotides in the 28S and the 18S mRNA. The high-resolution features resolve the ring structure of purines and pyrimidines side-chains and in riboses.

e Example of a modified iso-Asp138 in ribosomal protein uS11 in the 40S region; wat, water molecule. An additional methylene moiety is visible in the backbone and the side-chain is shorter. The iso-Asp side chain interacts with the G986 ribose through an H-bond to stabilize the local region. In the bottom left corner, the Asp before modification is shown, making it impossible to fit in the cryo-EM map.

Figure 2 Assignment of ion coordinations in the human 80S ribosome

a Overall structure of the human ribosome with highlighted positions of Mg^{2+} , K^+ and Zn^{2+} ions.

b (*Left*) Overall positioning of the nine Zn^{2+} binding ribosomal proteins and (*right*) four examples of the Zn^{2+} binding sites in the 60S large ribosomal subunit and the 40S small ribosomal subunit. Respective σ -levels used to prepare figures and σ -levels at which the ions are still detectable are indicated.

c Selection of sites with octahedral water-coordinated Mg^{2+} ions. The number of coordinated water molecules can range from six to two depending on the number of direct interactions of the Mg^{2+} ions with oxygen atoms of ribosomal RNA or proteins. Mg^{2+} can coordinate ribosomal components directly or via a water molecule. Respective σ -levels used to prepare figures are indicated.

d Selection of sites that show coordinated K^+ ions. K^+ was found to have up to eight coordination partners, which include ribosomal proteins, water molecules or rRNA. The conserved K^+ ion in the PTC identified previously⁶⁹ was also detected in the present structure. Respective σ -levels used to prepare figures and σ -levels at which the ions are still detectable are indicated.

Figure 3 2'-O-methylation sites in the human 80S ribosome

a Annotation of 2'-O-Me modified nucleotides on the human 28S, 18S rRNA and 5.8S rRNA of the 40S and 60S ribosomal subunits, respectively. Positions of the 2'-O-methylated nucleotides inside the 80S ribosome are shown as green spheres; 18S rRNA is shown in yellow, 28S rRNA is shown in blue, ribosomal proteins are shown in transparent for clarity. Global front view of the 80S ribosome (left), side view of the

40S subunit (middle) and side view of the 60S subunit (right). Chemically modified nucleotides of the 5.8S rRNA are shown in green. For detailed maps of each site see **Supplementary Figs. 1 & 2**.

b-m Examples of 2'-O-Me sites (detailed hereafter). Hydrogen bonds (dotted lines) and van der Waals contacts (black arcs) are indicated.

b-c 2'-O-methylated nucleotides on 5.8S rRNA (2'-O-methylation are pointed by blue arrows).

d-h 2'-O-methylated nucleotides and their local environment on the 18S rRNA.

i-m 2'-O-methylated nucleotides and their local environment on the 28S rRNA. Water molecules are shown as red spheres.

k 28S rRNA site with 3 successive 2'-O-methylated nucleotides. These modifications are confirmed by mass spectrometry (spectrum on the right), which shows a deconvoluted MS/MS spectrum of $^{2363}[\text{Am}][\text{Gm}][\text{Cm}]\text{AAA}^{2369}\text{Up}$ with fragmentation assignments.

Figure 4 Pseudo-uridines in the human 80S ribosome

a Annotation of Ψ -modified nucleotides on the human 28S, 18S rRNA and 5.8S rRNA of the 40S and 60S ribosomal subunits, respectively. Positions of the pseudo-uridine nucleotides inside the 80S ribosome are indicated as red spheres. Chemically modified nucleotides of the 5.8S rRNA are shown in green. For detailed maps and hydrogen bonding distances of each site see **Supplementary Figs. 1 & 2**.

b-l Examples of a Ψ sites with the characteristic hydrogen-bonding in the N1 position. Water molecules interacting with the N1 atom of pseudo-uridines are shown with red spheres. Hydrogen bonds with water molecules are shown with black dotted lines and hydrogen bonds between bases are shown with blue dotted lines; van der Waals contacts are indicated as black arcs; full visualization of the water molecules at a lower map contour level is indicated in red in panels **c**, **f**, **l**.

b-c Pseudo-uridines on 5.8S rRNA.

d-g Pseudo-uridines and their local environment on the 18S rRNA.

f Pseudo-uridines 1003 and 1004 of the 18S rRNA. These were confirmed by mass spectrometry (spectrum on the right), as shown in the deconvoluted MS/MS spectrum of $^{1000}\text{CAU}[\Psi][\Psi]^{1005}\text{Gp}$ with fragmentation assignments; the $\text{ce}\Psi$ refers to the cyanoethylated adduct.

h-l Pseudo-uridines and their local environment on the 28S rRNA. Mg^{2+} ions are shown as green spheres and the K^+ ion interacting with the 2'-O-methylated pseudo-uridine 3818 ($\Psi\text{m}3818$; panel **i**) is shown as a purple sphere.

Figure 5

Nucleotide base modifications in the human 80S ribosome

a Annotation of nucleotide base modifications (purple spheres) on the human 28S, 18S rRNA and 5.8S rRNA of the 40S and 60S ribosomal subunits, respectively. For detailed maps of each site see **Supplementary Figs. 1 & 2**.

b-k Various nucleotide base modifications in 18S and 28S rRNA of the human ribosome. Hydrogen bonds with residues are shown with black dotted lines and hydrogen bonds between bases are shown with blue dotted lines; van der Waals contacts are shown as black arcs.

b Methylated adenosine 4220 (m^6A4220) of the 28S rRNA forms numerous van der Waals contacts.

c Methylated guanosine 4220 (m^7G1639) of the 18S rRNA forms van der Waals contacts with its environment.

d Methylated adenosine 4220 (m^5C3782) of the 28S rRNA forms an increased stacking interaction with its 2 neighboring nucleotide bases (left and right, rotated views).

e 2'-O-methylated guanosine 4392 (Gm4392) and methylated cytosine C4447 (m^5C4447) show base stacking that is extended to G4393.

f Chemically modified nucleotide m1acp3pseudo-uridine and its environment in the P-site. Density of the modification is shown in red at lower contour level to visualize the acp moiety. *Right*, deconvoluted MS/MS spectrum of $^{1246}AC[m^1acp^3\Psi]CAACAC^{1255}Gp$ from HeLa 18S rRNA with fragmentation assignments. The cem1acp3 Ψ refers to the cyanoethylated adduct.

g Acetylated cytosine 1842 (ac^4C1842) and its environment.

h 2'-O-methylated adenosine 1326 (Am1326) and methylated adenosine A1322 (m^1A1322) with their environment.

i Acetylated cytosine 1337 (ac^4C1337) positioned in the 40S ribosomal subunit in proximity of the mRNA channel entry.

j Pair of dimethylated adenines where stacking is increased and a van der Waals contacts are created with an rRNA loop (left and right, rotated views).

k m⁶A1832 increases stacking with its neighbor A1831 in the 18S rRNA.

Figure 6

Complete annotation of chemical modification in the human 80S ribosome through MS and structural analysis

Annotation of 2'-O methylations, Ψ and base modifications by MS (left and right tables for 18S and 28S/5.8S rRNA's, respectively, and on the 80S human ribosome structure (middle panels). RNAses that were used to identify the respective modifications are indicated. Colours codes for annotations are indicated. A complete list of sites analysed by MS and cryo-EM and the corresponding annotations, including a comparison with previously reported rRNA modifications from other species can be found in **Suppl. Data File 1**.

Figure 7

Comparison of chemical modifications and conserved ion sites in the human 80S and *E. coli* 70S ribosome

a Annotation of the 235 and 35 2'-O methylations, Ψ and base modifications in human 80S and *E. coli* 70S ribosomes, respectively. Colours codes for annotations are indicated.

b rRNA chemical modifications in *E. coli* compared to the human ribosome. Only few are conserved (green) and some are chemically different (blue). Post-translational modifications of ribosomal proteins are indicated in orange.

c Methylation swap between human and *E. coli* ribosomes; while in humans m⁶A1832 is modified, in *E. coli* the neighboring residue is methylated instead, suggesting a co-evolution effect of chemical modifications in this rRNA region.

d Examples of conserved ion positions at functional sites human versus *E. coli* ribosome. Left, the -1/-2 mRNA region (modelled) on the 40S subunit; right, two hexa-hydrated Mg²⁺ ions (green) and a K⁺ ion (next to G3907, as in **Fig. 2**) at the PTC and entry to the peptide exit channel.

Methods

Complex formation and cryo-EM data processing

Human 80S ribosomes were prepared from HeLa cells (certified as free of Mycoplasma using a polymerase chain reaction test) as described earlier⁵². Cryo-EM grids (carbon-coated Quantifoil R 2/2 copper grids, 300 mesh) were plunge-frozen using a Vitrobot IV (Thermo Fisher Scientific) operating at 95% humidity and 10°C.

Three data sets on the human 80S ribosome were used: data set 1 was collected on a Titan Krios G4 (equipped with a Selectris X energy filter and Falcon 4 camera; TFS factory in Eindhoven) with a pixel size of 0.72 Å as EER movie (total dose 40 electron / Å²) and 2 & 3 were collected on a CRYO ARM 300 (equipped with an Omega energy filter, Jeol, and a K3 camera, Gatan; RIKEN Osaka cryo-EM facility) at a pixel size of 0.82 Å as compressed .tif movies, respectively, both microscopes operating at 300 kV (ref. 88). The three data sets were first processed separately (see also **Table 1 & Extended Data Fig. 2**), including movie alignment using Relion's motion correction algorithm⁸⁹ based on MotionCor2 (ref. 90), CTF estimation using GCTF software⁹¹ implemented in Relion, particle picking using crYOLO⁹². All further steps were done in Relion⁸⁹. From the initially obtained particles sets (634 158 particles were obtained for data set 1, 484 393 for data set 2 and 149 674 particles for data set 3) 199 371, 229 233 and 105 596 particles were kept after 2D & 3D classifications. The full-sized particles were refined in 3D followed by CTF correcting for anisotropic magnification, beam tilt, trefoil, 4th order aberrations, defocus and astigmatism followed by Bayesian polishing. Particles from the three data sets were joined as distinct optics groups (merged data set) and refined together in 3D followed by a 3D global classification to 5 classes without alignment (**Extended**

Data Fig. 1). The largest class was kept (382 016 particles) and refined to 2.0 Å resolution. From this map three masks containing 60S large ribosomal subunit, 40S small ribosomal subunit body 40S small ribosomal subunit head and E-site tRNA were generated and extended by 12 pixels with a soft edge of 7 pixels with the *relion_mask_create* tool. These masks were used for focused refinement and the multi body refinement⁴¹⁻⁵¹. A final resolution of 1.9 Å was obtained for the 60S large ribosomal subunit, 2.0 Å for the 40S small ribosomal subunit body and 2.1 Å for the 40S small ribosomal subunit head as estimated from Fourier shell correlation calculations⁹³⁻⁹⁶ with the 0.143 cut-off criterion. Using partial signal subtraction on each body further improved the map (**Extended Data Fig. 2**), resolving for example a whole set of additional hexahydrated Mg²⁺ ions (particularly in the 40S ribosomal subunit). The post-processing procedure implemented in Relion⁸⁹ was applied to the final maps for masking, B-factor sharpening (-36.46, -37.99, -49.39 Å² for the 60S, 40S body and 40S head regions, respectively) using masks prepared from 15 Å lowpass filtered maps, that were shrunk by 3 pixels using a soft edge of 9 pixels. Local resolution estimation was done using the local resolution routine of Relion.

Atomic model building & refinement and localisation of rRNA modifications

The maps of the three sub-regions were combined into a single 80S map and an atomic model of the human ribosome (PDB: 6qzp³⁸) was placed as a rigid body into the map using Chimera⁹⁷. All positions for chemical modifications of the rRNA detected by the present MS analysis as well as previously reported rRNA modifications^{38,98,99} were manually cross-checked for presence in the structure, as summarized in **Suppl. Data File 1**. Chemical modifications were identified based on the presence of significant additional

densities in the 2'-OH position (indicated by cyan arrows in the figures) and nucleotide bases. The map contour level was adjusted individually as indicated by the local root mean square deviation from the mean value (similar to sigma levels) in each figure corner. Mg^{2+} , K^+ and Zn^{2+} ions were annotated in the structure based on their characteristic coordination patterns and coordination distances¹⁰⁰. Typical distances found for water molecules and ions are 2.3 Å for Zn^{2+} , 2.1 Å within the octahedron for Mg^{2+} and 2.6-3.2 Å range for K^+ , respectively, consistent with values known from chemistry and in proteins⁶⁴. Water-coordinated Mg^{2+} and modified amino acids and nucleotide templates were drawn using JLigand¹⁰¹ and their respective restraints for the atomic model fitting and refinements were generated. The model was subsequently refined in Phenix¹⁰² using restrained parameter refinement protocols (real space refinement, positional refinement, grouped B-factor refinement and simulated annealing as described^{36,53}). Finally, water molecules were picked using an automated procedure that combines density search and coordination with hydrophilic residues considering potential hydrogen bonds using SWIM⁶⁴ and Phenix^{57,65} software; we used the peak search implemented in Chimera using the segmentation-guided water and ion modelling (SWIM) through the Segger software⁶⁴ plugin to the UCSF ChimeraX¹⁰³ software. Water molecule annotation was done after having annotated (i) Zn^{2+} ions in their tetrahedral environment coordinated by cysteine residues, (ii) Mg^{2+} ions with their associated water molecules in an octahedral coordination, (iii) K^+ ions and (iv) water molecules at the N1 position of Ψ 's (if present, sometimes visible at lower contour level of the cryo-EM map). For regions with weaker cryo-EM density it cannot be excluded that some of the annotated water molecules are ions, but we refrained from annotating these else than as water molecules. The overall structure comprises 5783 nucleotides and 11440 amino acids from the 4 rRNAs and 80 ribosomal proteins, respectively (see statistics in **Table 1**). Images were prepared with ChimeraX 1.0 (ref. 103) and Pymol¹⁰⁴.

Mass spectrometry analysis

Chemicals and Reagents

Chemicals used were of analytical grade or high purity unless indicated otherwise. Water used to prepare sample solutions or buffers was obtained from Merck Millipore. Ammonium acetate (NH₄OAc), triethylamine (TEA), ZipTip®C18, Tri-Reagent® and acrylonitrile were purchased from Sigma-Aldrich. 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) was purchased from Honeywell. RNase T₁, RNase A and RNase H were purchased from ThermoFisher Scientific. RNase U₂ as well as RNase MC1 were homemade in 100 mM ammonium acetate (pH is not adjusted). Oligonucleotides used for RNA-DNA hybridization were synthesized by Eurofins Genomics.

Culture of HeLa cells and rRNA isolation

HeLa cells were obtained from a 10 L culture as described previously^{36,38,52,53}. Total RNA was extracted from the cells using Tri-Reagent following the manufacturer procedure. Next, the 28S and the 18S rRNA were isolated using size exclusion chromatography (Superose 6 Increase 10/300 GL column) by isocratic elution with 300 mM NH₄OAc at 0.5 mL/min on an ÄKTA purifier system (General Electric Healthcare). The 5.8S and 5S were isolated from the total RNA extract by 10% polyacrylamide gel. Gels were stained by ethidium bromide and the bands containing the rRNAs were visualized and excised under UV light. Gels bands were dried under vacuum.

Cyanoethylation of pseudo-uridines

Purified 28S and 18S rRNA samples were subjected to cyanoethylation following the procedure as described with few differences^{56,105}. The reaction was carried out with 10 μL of purified rRNA sample, plus 150 μL of 41% EtOH / 1.1 M TEAA (Triethylammonium acetate) (pH 8.5) and 10 μL of acrylonitrile. Specific labeling of pseudo-uridines was performed for 2 h at 70°C. Cyanoethylated rRNA samples were cooled down and precipitated by adding 40 μL 3M NH_4OAc , 40 μL H_2O and 600 μL EtOH 100% and stored at -20°C for at least 2h. Samples were centrifugated at 12 000 g for 10 min at 4°C. The supernatant was removed and the samples were washed with 75% EtOH. Pellets were dissolved in 300 mM NH_4OAc .

For the 5S and 5.8S rRNAs, the cyanoethylations were achieved in gel. In a tube containing the dried gel, 30 μL of 41% EtOH / 1.1 M TEAA (pH 8.5) plus 4 μL of acrylonitrile were added. Labelling was carried out at 70°C for 2 h. After derivatization, supernatant was removed and the gel were washed 3 times with 200 mM NH_4OAc . Pieces of gel were then dried under vacuum.

The modification $\text{m}^1\text{acp}^3\psi 1248$ (18S rRNA) reveals an extra mass corresponding to the addition of a cyanoethyl group. Considering that the only reactive nitrogen atom available is located on the modification, this indicates the formation of a cyanoethylated acp3 adduct due to the chemical treatment, which to our knowledge has not been observed before.

RNase H cleavage and gel electrophoresis

The specific cleavage of rRNA was done as described^{55,56}. Prior RNase H cleavage, rRNA ($\approx 10 \mu\text{g}$) and oligos ($\approx 10 \mu\text{g}$) were mixed and heated at 80°C for 2 min followed

by a cooling down to 50°C for DNA-RNA hybridization (see list of oligos in **Extended Data Fig. 7**). To obtain a specific cleavage, a chimeric oligo was also used (see spectrum in **Extended Data Fig. 6**). DNA-RNA duplexes were digested at 50 °C for 30 min by adding 1 µL of 10X RNase H buffer then 2.5 µL of 0.5 U/µL RNase H. After RNase H cleavage, the fragments of interest were isolated by 10% denaturing polyacrylamide gel. Gels were run at 16W for about 4 h. After migration, gels were stained by ethidium bromide and the bands containing the fragments of interest were visualized and excised under UV light. Bands were dried under vacuum. RNA & DNA oligos used for mass spectrometry analysis are listed in **Extended Data Fig. 7**. 18S, 28S, 5.8S & 5S rRNA fragments with 2'-O-Me's, ψ 's & nucleotide base modifications that were analyzed by mass spectrometry analysis are summarized in **Supplementary Fig. 3**.

RNA digestion and NanoLC-MS/MS of RNA oligonucleotides

Dried pieces of gel containing RNA were digested by adding 20 µL of RNase T₁ (1/1000) or 20 µL of RNase A (1/1000). Samples were then incubated at 55°C for 2h. In-gel digestions by RNase U₂ were carried out following previously described procedures^{106,107}. Briefly, 20 µL of 0.1 µg/µL U of RNase U₂ in 200 mM NH₄OAc (pH 5.3) samples were incubated at 55°C for 30 min. The MC1 digestions were carried out in solution for the 28S and 18S rRNAs because gel digestion did not work. Briefly, 20 µL of 0.1 µg/µL U of RNase MC1 in 100 mM NH₄OAc was added to 10 µg of rRNA. MC1 digestions were achieved in 37°C for 1 h. After digestion, the samples were desalted with ZipTip C18 using 200 mM NH₄OAc. Eluates were dried under vacuum and pellets containing RNase digestion products were resuspended in 3 µL of milliQ water prior injection. Separation of products were achieved on an Acquity peptide BEH C18 column

(130 Å, 1.7 µm, 75 µm × 200 mm) using a nanoAcquity system (Waters). The analysis was performed with an injection volume of 3 µL. To avoid RNase contamination, the chromatographic system was thoroughly washed when analyzing samples digested with another RNase. The column was equilibrated with buffer A containing 7.5 mM TEAA, 7.0 mM TEA and 200 mM HFIP at a flow rate of 300 nL/min. Oligonucleotides were separated using a gradient from 15% to 35% of buffer B (100% methanol) for 2 min followed by an increase of buffer B to 50% in 20 min. Column oven temperature was set at 65°C. Detection by MS and MS/MS were achieved using SYNAPT G2-S from Waters Corporation. The source setting used were: polarity mode: Negative ion; Capillary voltage: 2.6 kV; Sample cone voltage: 30 V; Source temperature: 130°C. The samples were analyzed over an m/z range from 550 to 1600 for the full scan, followed by fast data direct acquisition scan (Fast DDA). Collision-induced dissociation (CID) experiments were achieved using Argon gaz. Data were analyzed using the MassLynx software¹⁰⁸. Oligonucleotides sequences were confirmed by MS/MS deconvoluted spectra by following c or/and y ion series.

Sequencing of rRNA

To clarify sequence inconsistencies in data banks we performed sequencing of the 28S, 18S, 5.8S and 5S of the rRNAs to unify the annotation of nucleotides and chemical modifications. 5S, 5.8S, 18S and 28S rRNA samples purified from HeLa cell extract were reverse transcribed using transcriptase reverse transcriptase (Roche) and amplified by polymerase chain reaction (PCR) using Pfusion PCR polymerase (NEB) with specific primers or random hexamer primers. Here, total RNA samples were used for 5S and 5.8S rRNA. For the 18S and 28S rRNA the RNA was purified as described in the MS section.

The rRNA sequence was annotated with rRNA modifications found in the structure and detected by MS (**Supplementary Fig. 4**). Primers used for PCR are listed in **Extended Data Fig. 9**. The PCR products were sent for sequencing (Eurofins Genomics). Structure, MS and rRNA sequencing allowed a mutual validation and some sequence corrections as compared to the genomic sequence found in data banks (e.g. position 4910 in the 28S rRNA was found to be adenine instead of guanine, visible also in the structure; **Extended Data Fig. 8**).

Data availability

Atomic coordinates and cryo-EM maps have been deposited in the Protein Data Bank and EMDB under accession codes 8QOI and EMD-18539 (full 80S ribosome), EMD-18812, EMD-18813 and EMD-18814 for the focused refinements of the 60S ribosomal subunit, the 40S ribosomal subunit body and head regions, respectively, and EMD-18815 for the last global refinement of the 80S ribosome before the focused refinements. Mass spectrometry data have been deposited in the ProteomeXchange Consortium via the PRIDE data base <http://www.ebi.ac.uk/pride> (ref. 109) with the dataset identifiers PXD046739, PXD046743, PXD046744, PXD046747 for the 28S, 18S, 5.8S and 5S rRNA's, respectively. All other materials such as cells are available upon reasonable request.

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Human 80S
(EMDB-18539)
(PDB 8QOI)

Data collection and processing

Magnification	165 000 (Dataset 1) or 60 000 (Datasets 2-3)
Acceleration voltage (kV)	300
Electron exposure (e-/Å ²)	30 (Dataset 1) or 40 (Datasets 2-3)
Defocus range (µm)	-0.5 to -2
Pixel size (Å)	0.82
Symmetry imposed	C1
Initial particle images (no.)	534 200
Final particle images (no.)	382 016
Map resolution (Å)	1.9
FSC threshold	0.143
Map resolution range (Å)	512.0 - 1.7

Refinement

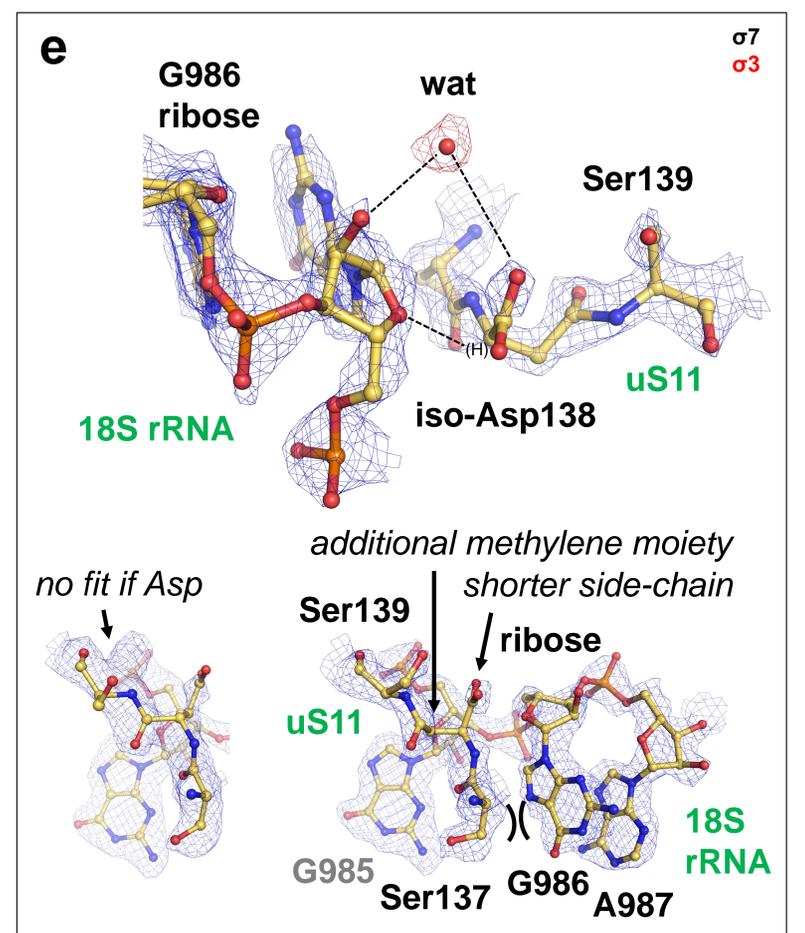
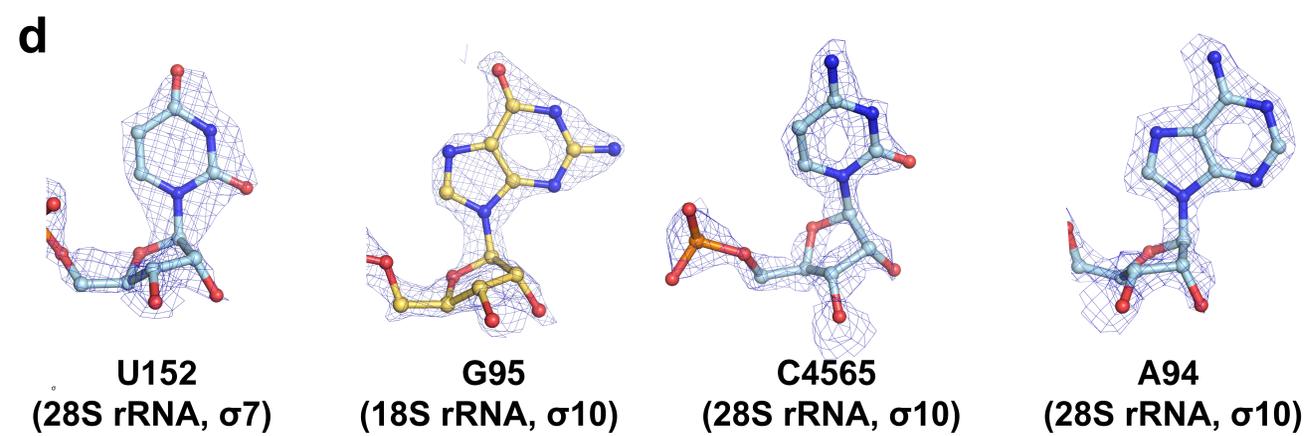
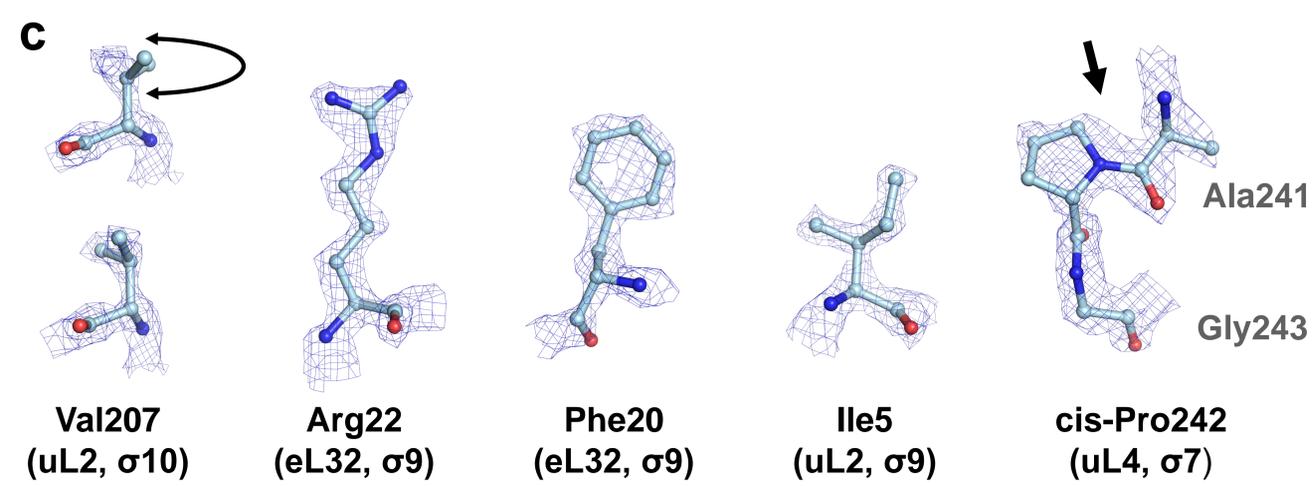
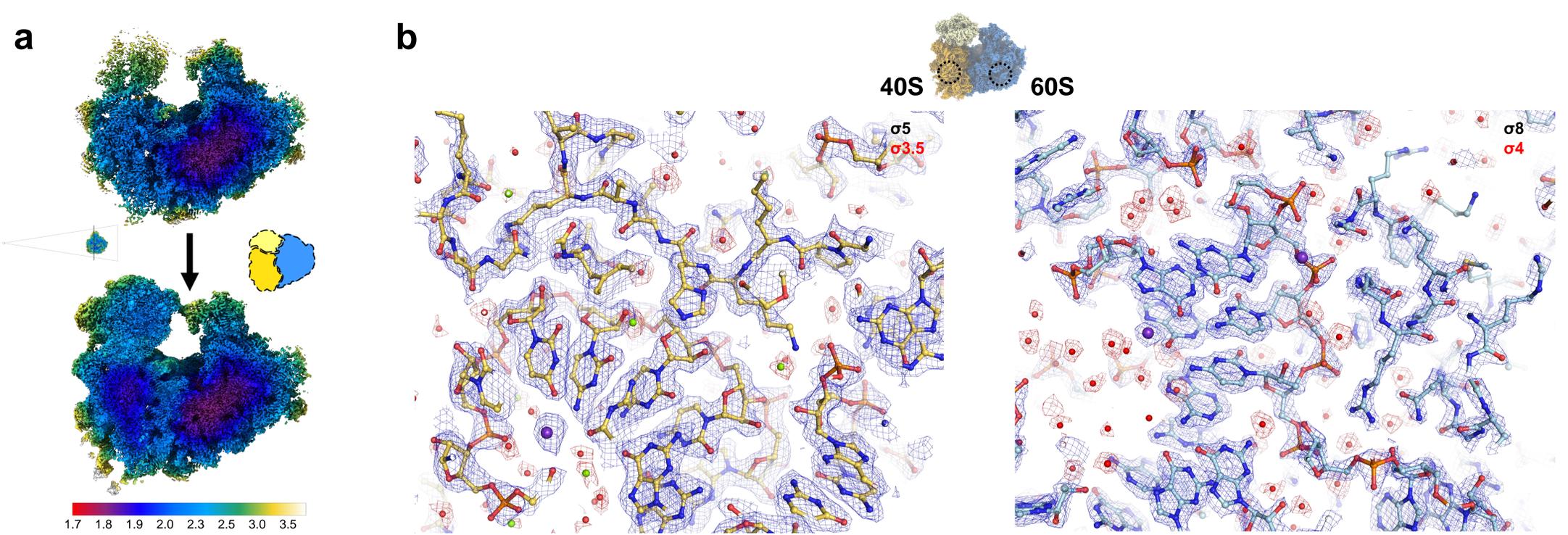
Initial model used (PDB code)	6QZP
Model resolution (Å)	1.9
FSC threshold	0.5
Model resolution range (Å)	7 - 1.7
Map sharpening <i>B</i> factor (Å ²)	-36.5 (60S); -38.0 (40S body); -40.4 (40S head)

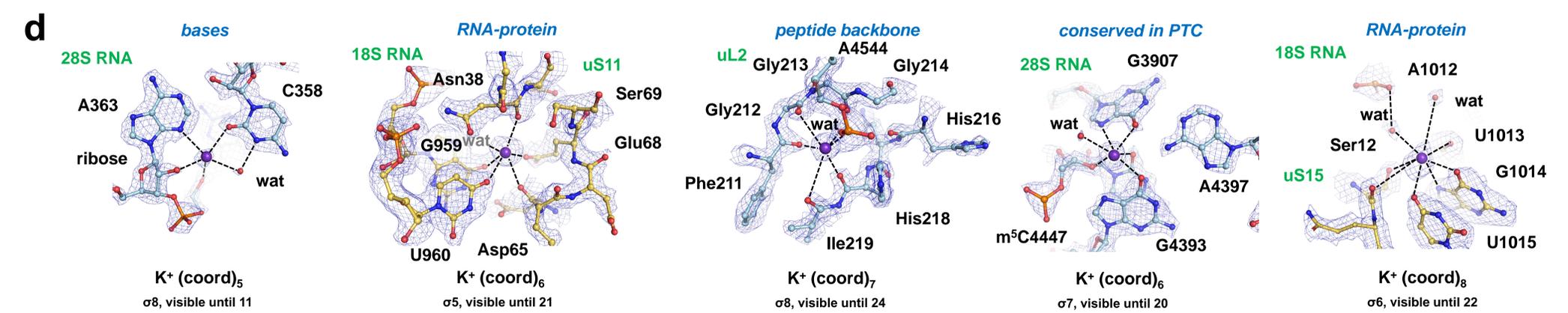
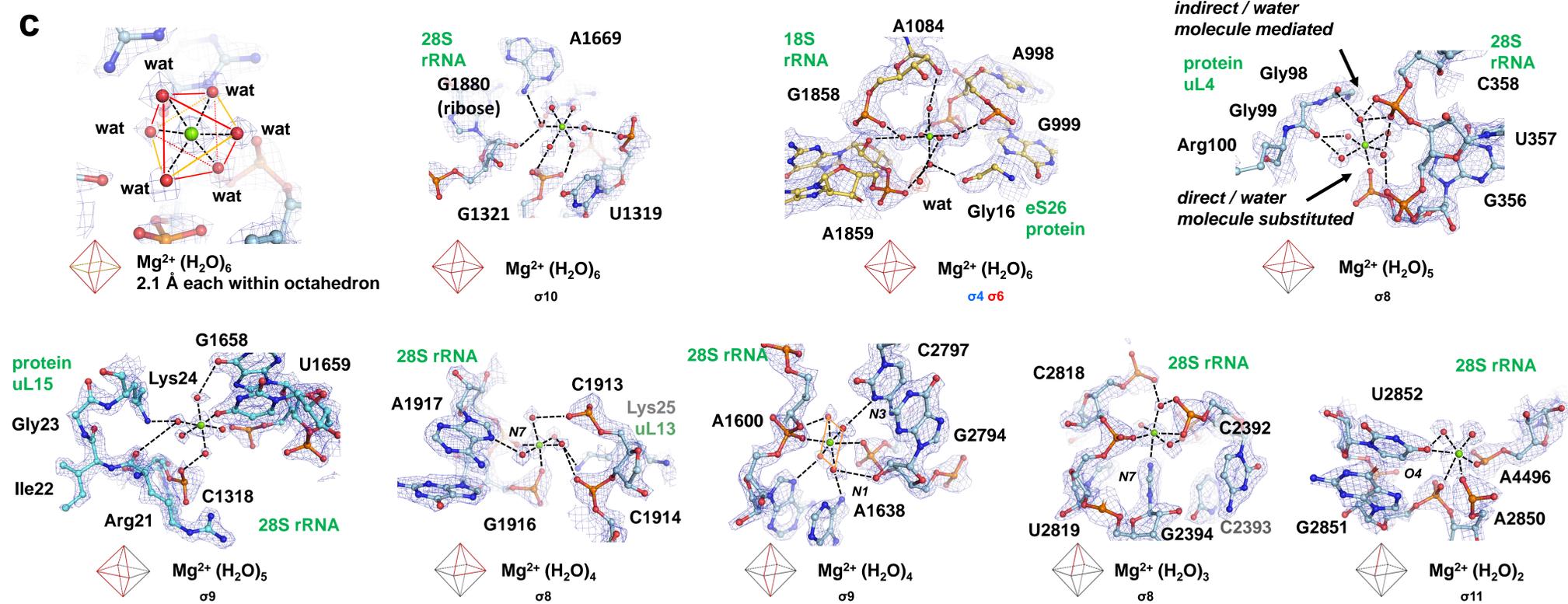
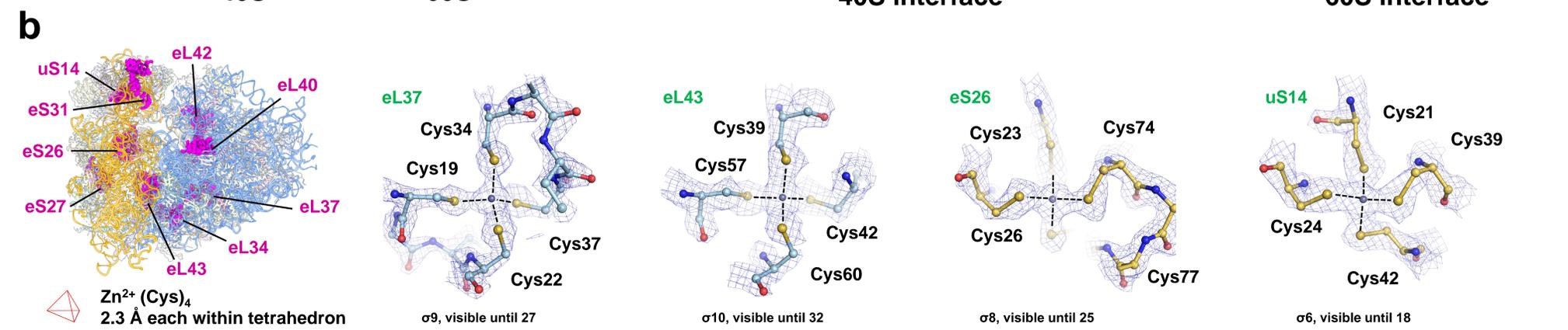
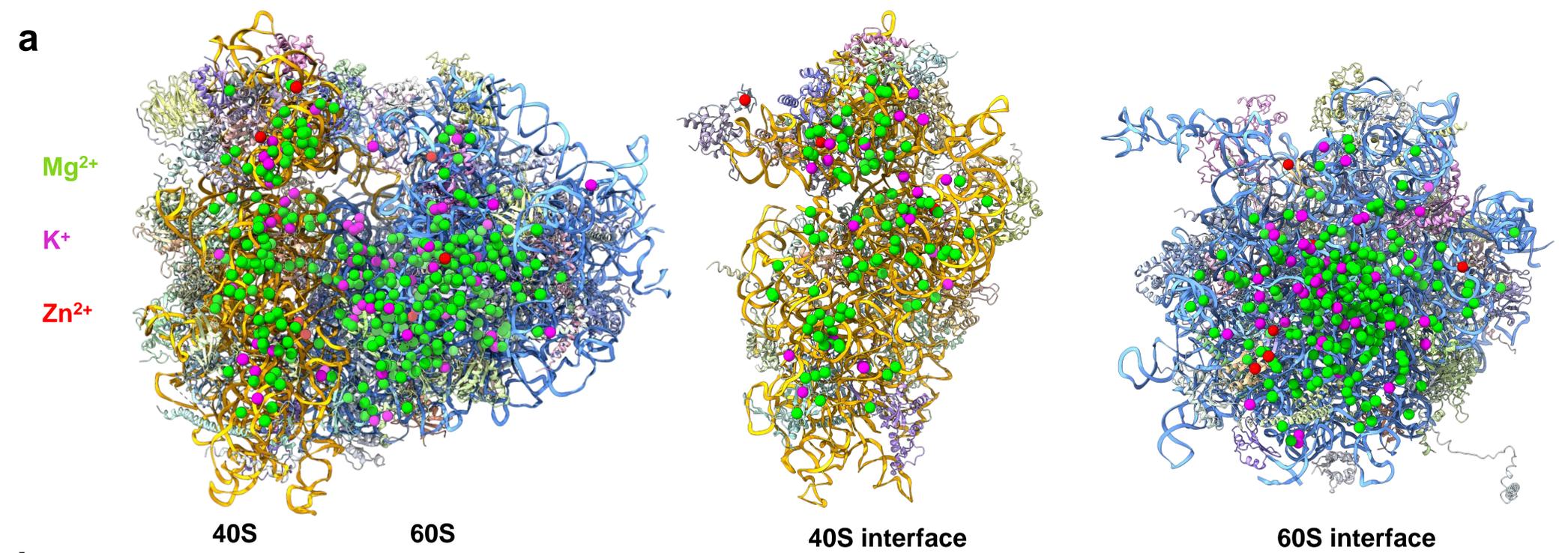
Model composition

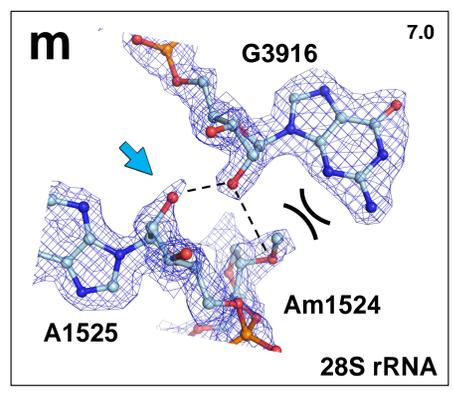
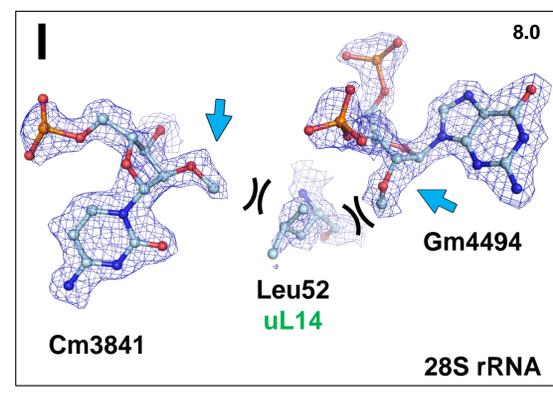
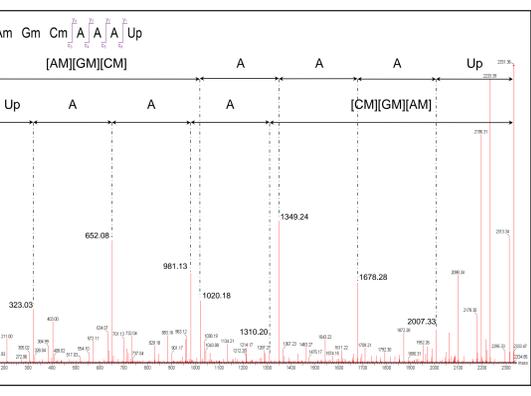
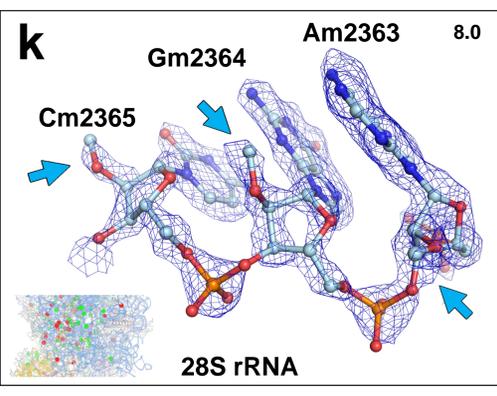
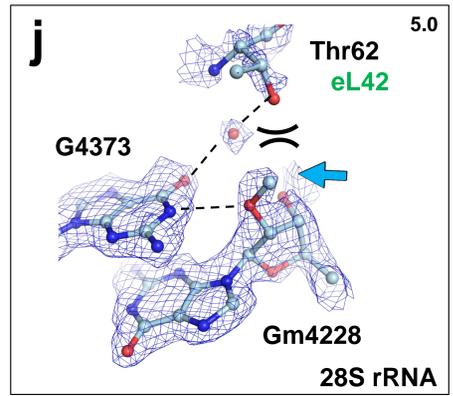
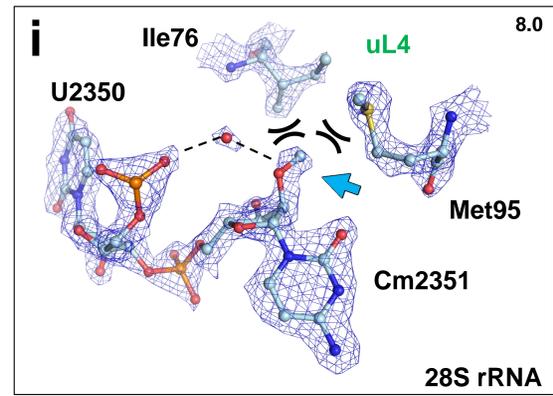
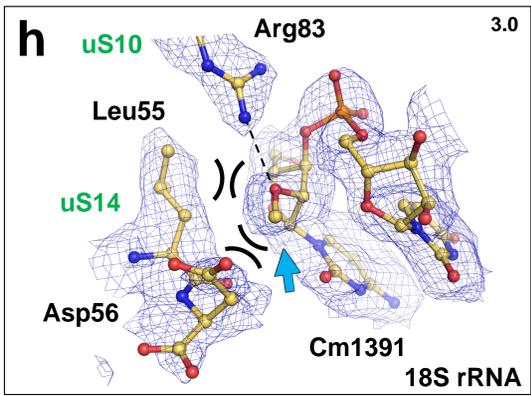
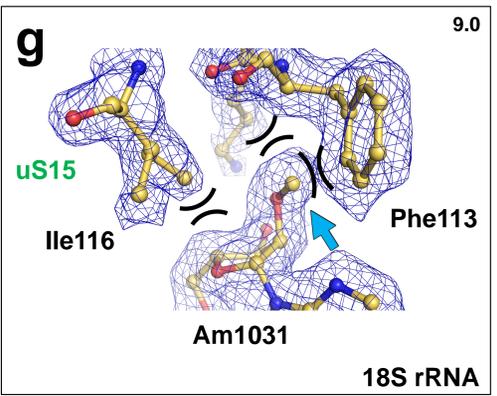
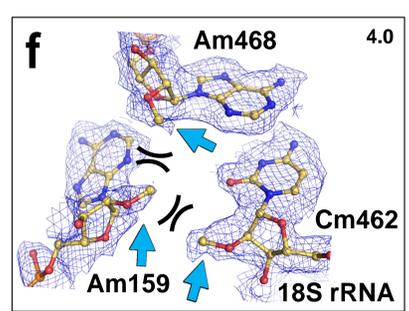
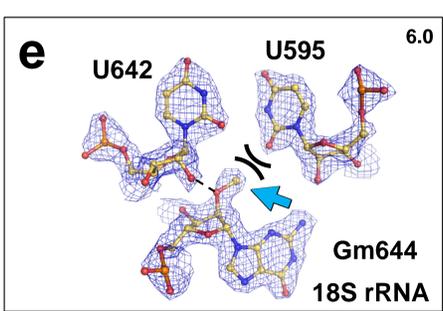
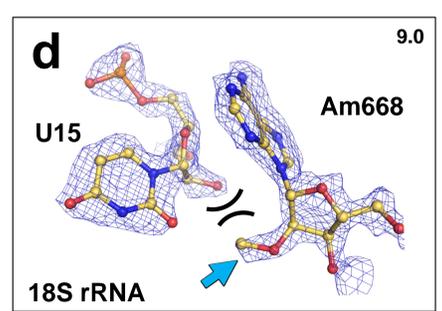
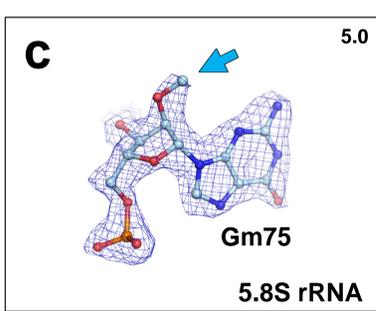
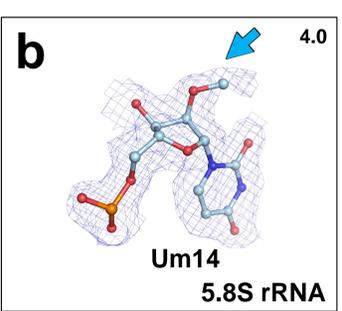
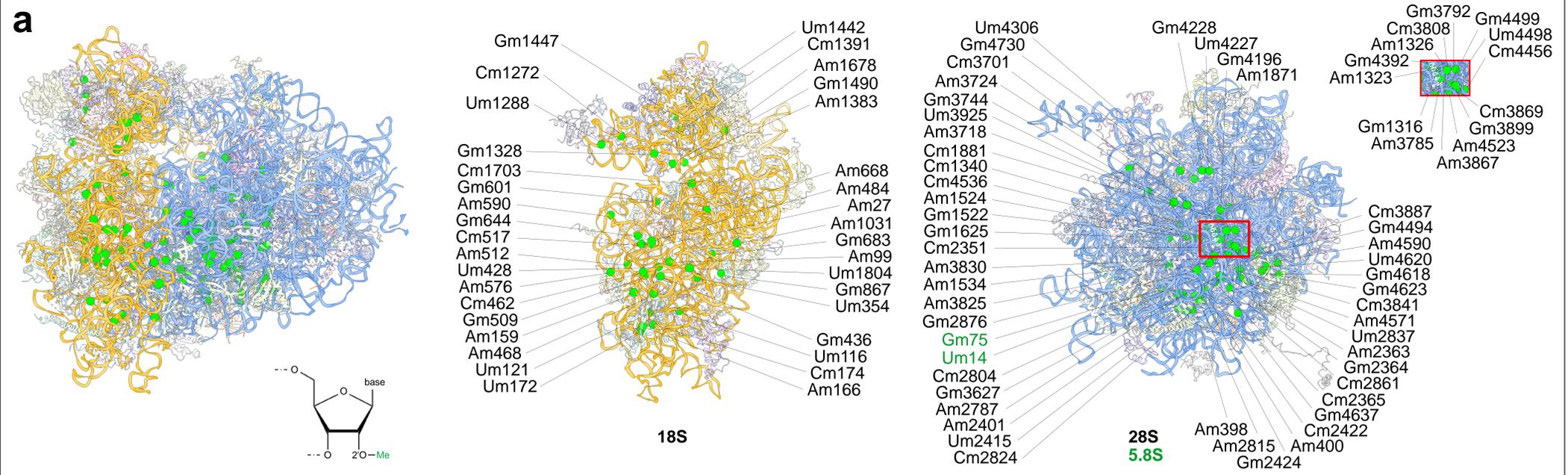
Non-hydrogen atoms	233 497
Protein residues	5 783

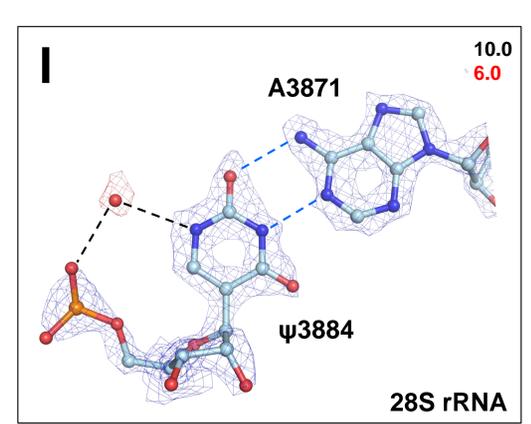
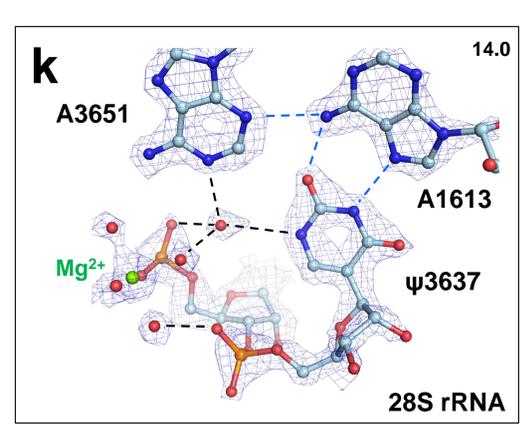
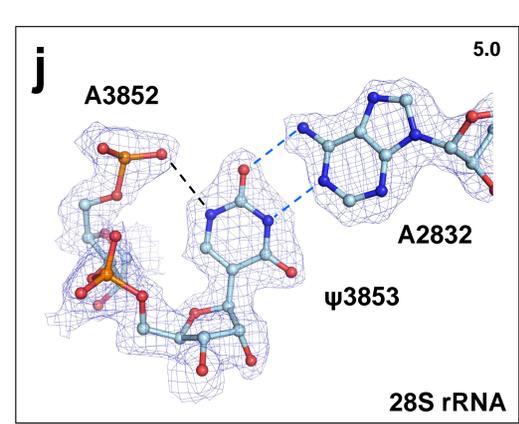
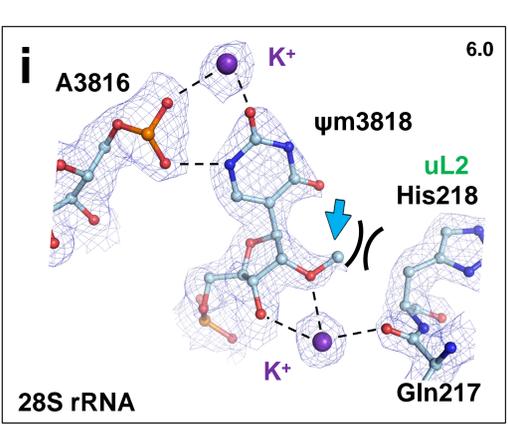
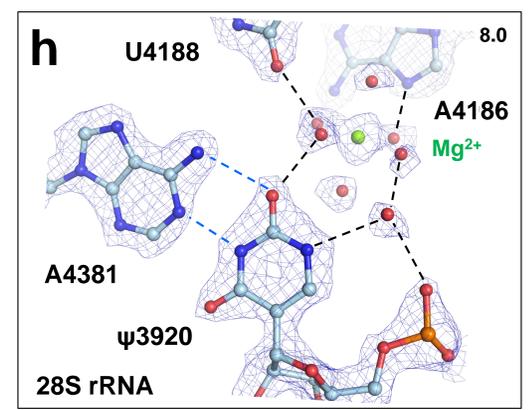
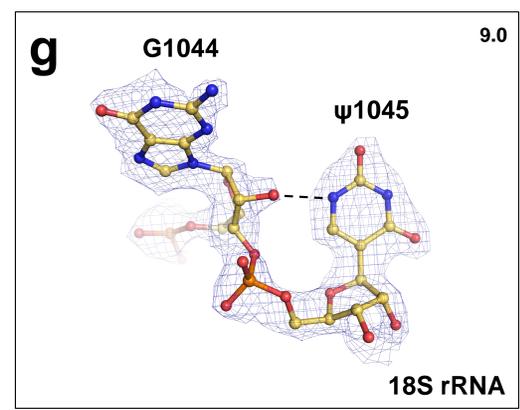
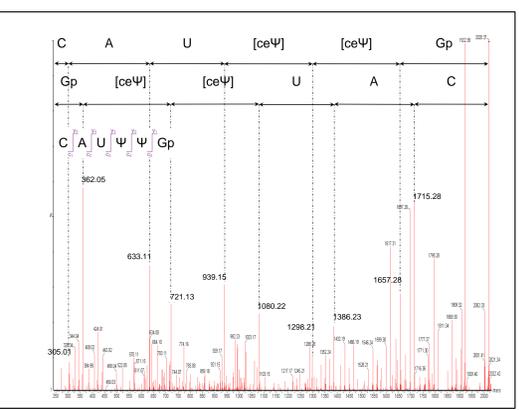
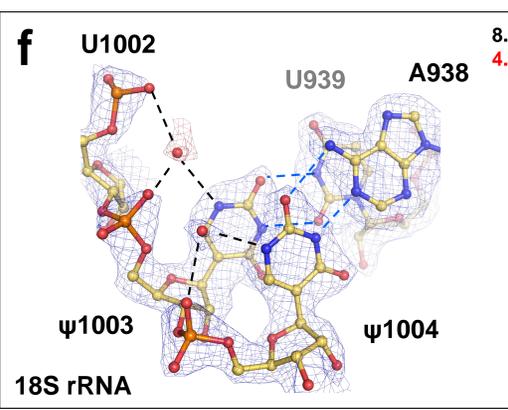
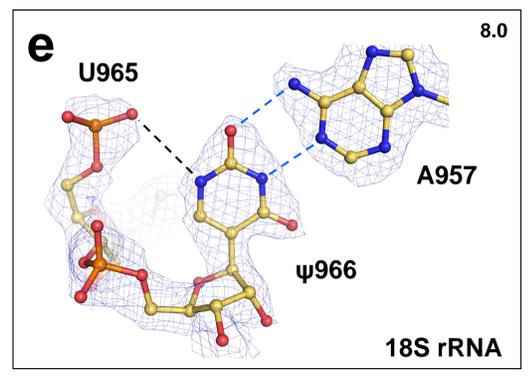
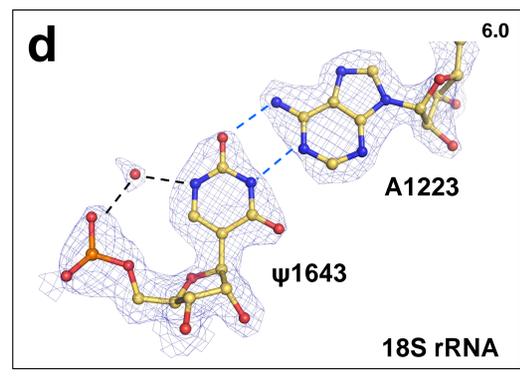
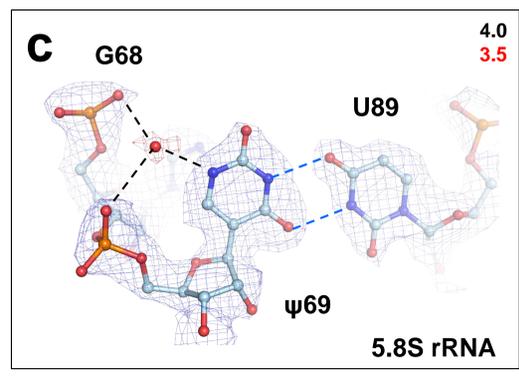
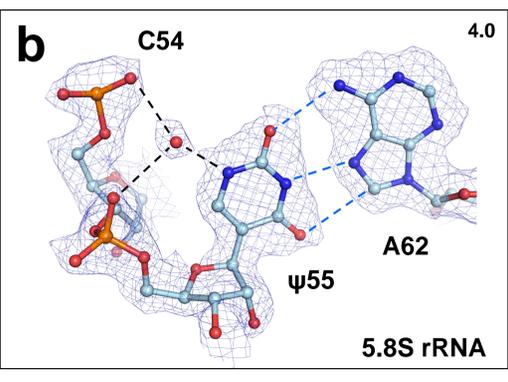
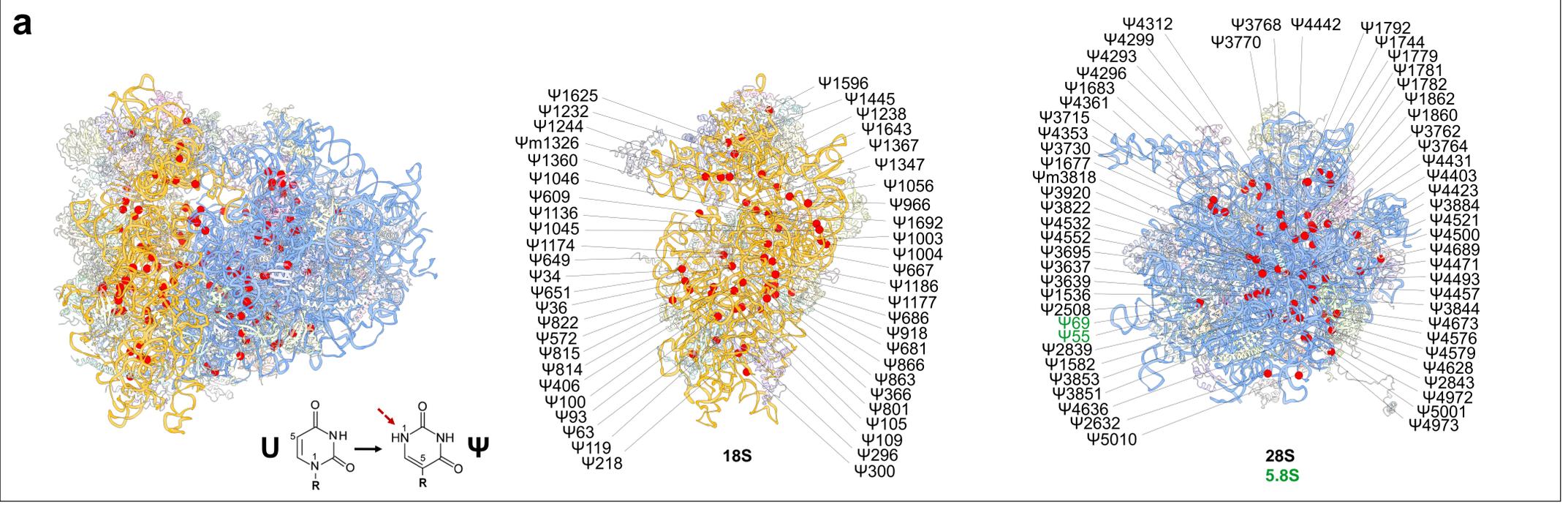
Nucleotides	11 440
Water molecules	16 411
Ions	410
Zn ²⁺	9
Mg ²⁺ (octahedral)	294
K ⁺	107
<i>B</i> factors (Å ²)	
Protein	23.8
RNA	32.1
Ligand	15.9
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.801
Validation	
<i>MolProbity</i> score	1.87
Clashscore	8.27
Poor rotamers (%)	1.74
Ramachandran plot	
Favored (%)	96.55
Allowed (%)	3.45
Disallowed (%)	0.11

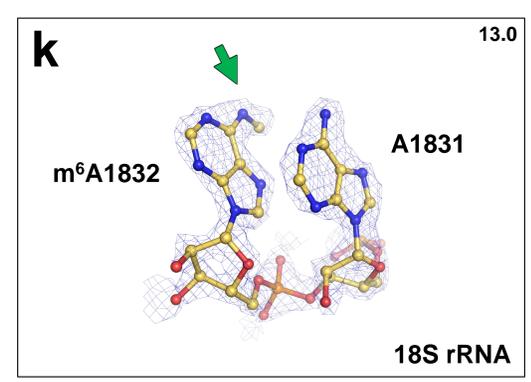
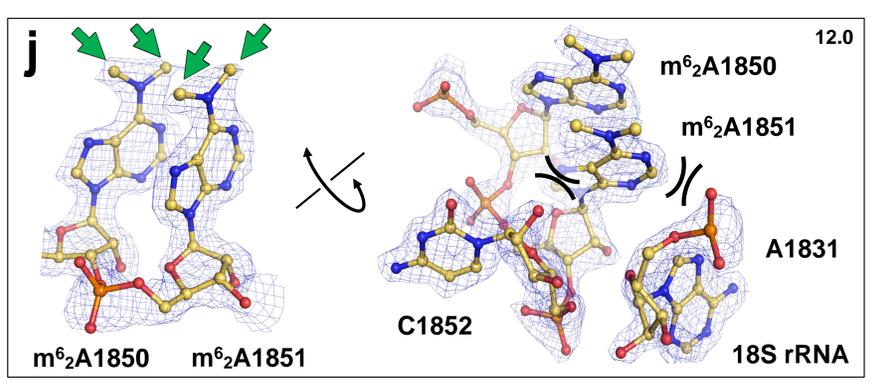
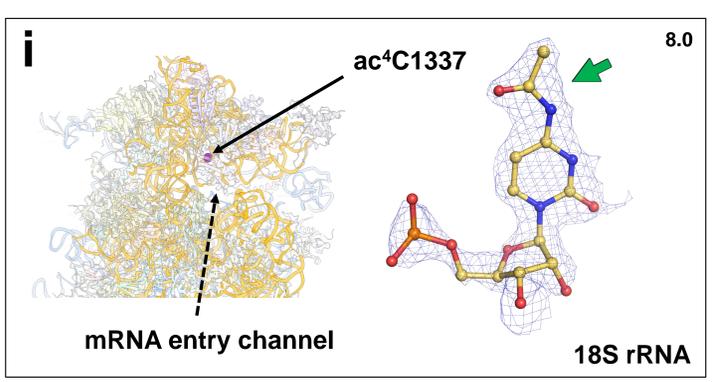
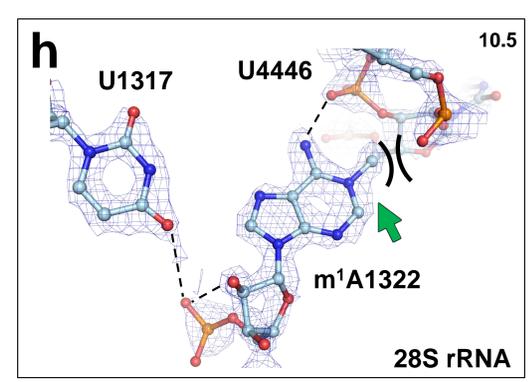
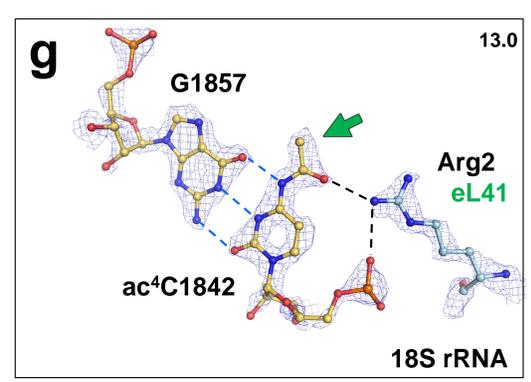
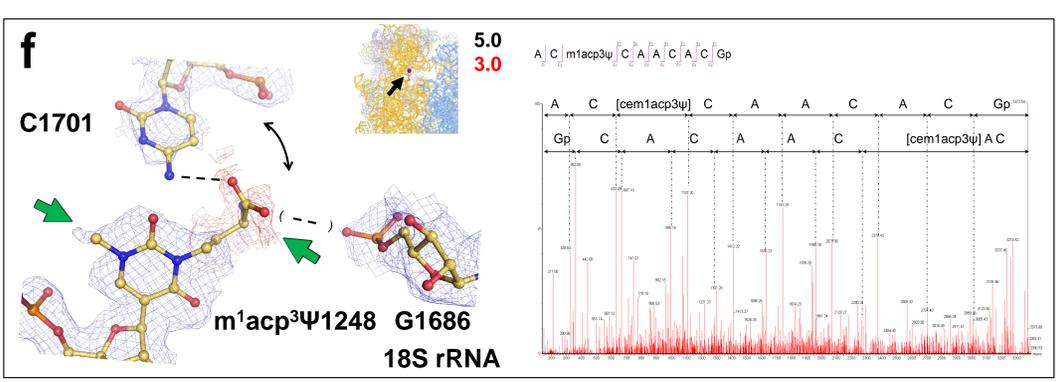
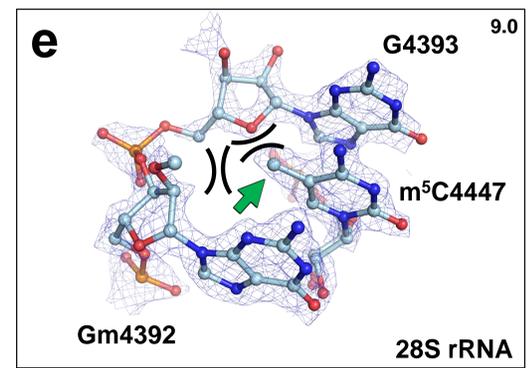
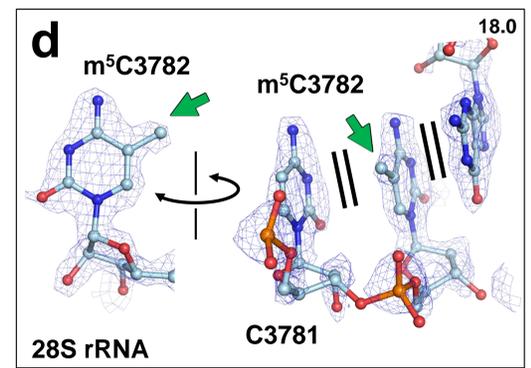
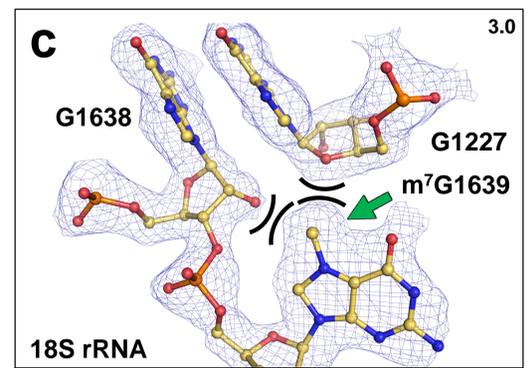
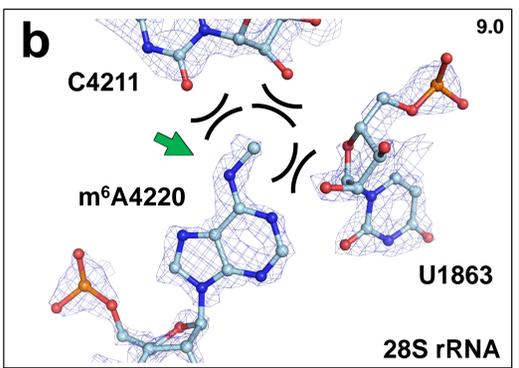
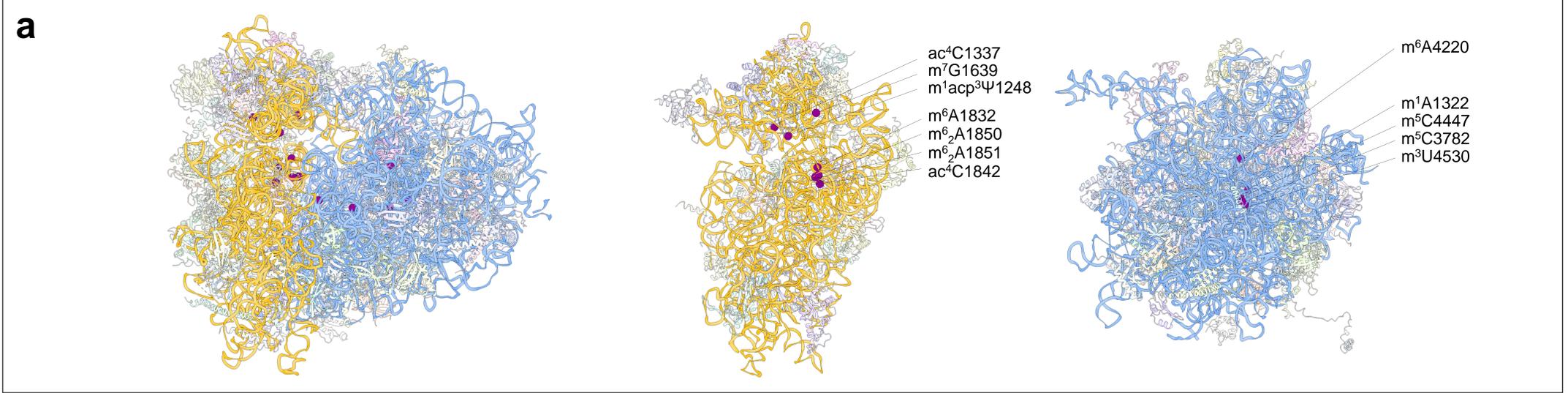
Table 1**Cryo-EM data collection, refinement and validation statistics**







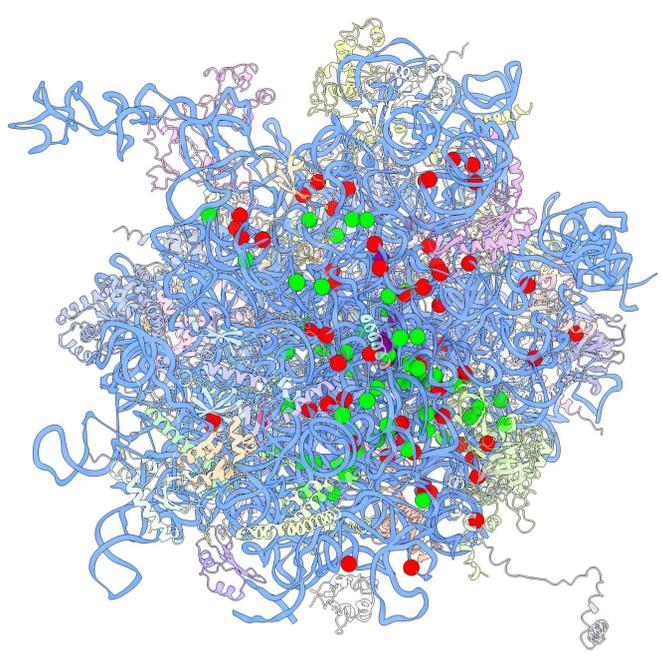
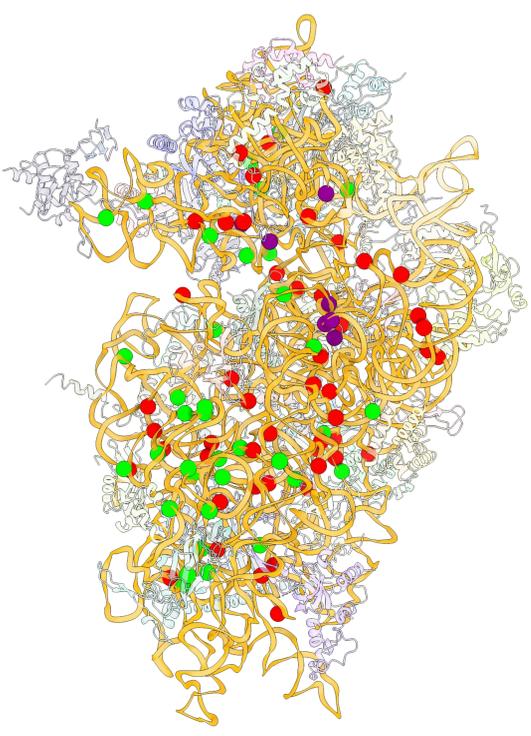
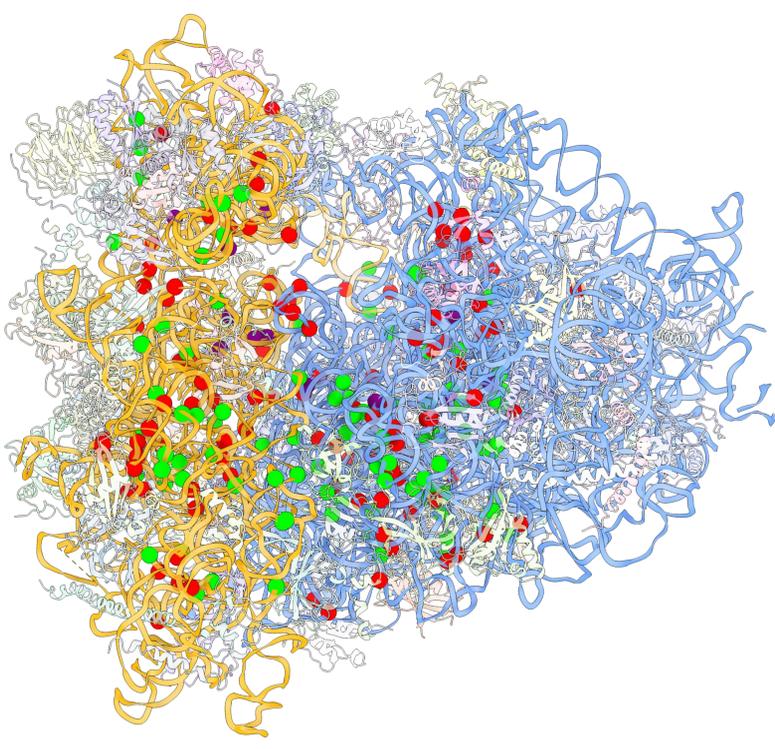




Position	3D structure	MS modif	RNase T1	RNase A	RNase U2	RNase MC1
Am27	Strong	[Am]27	T1			
ψ34	Strong, H ₂ O	[ψ]34	T1			
ψ36	Strong, H ₂ O	[ψ]36	T1			
ψ63	Strong, H ₂ O	[ψ]63		RA		
ψ93	Strong, H ₂ O	ND	T1			
Am99	Strong	[Am]99	T1		U2	
ψ100	Strong, H ₂ O	[ψ]100	T1		U2	
ψ105	Strong, H ₂ O	[ψ]105	T1			
ψ109	Strong, H ₂ O	[ψ]109	T1			
Um116	Strong	[Um]116	T1			
ψ119	Strong, P N-1	[ψ]119	T1			
Um121	Strong	[Um]121	T1			
Am159	Strong	[Am]159	T1	RA	U2	
Am166	Strong	[Am]166		RA		
Um172	Strong	[Um]172	T1	RA		
Cm174	Strong	[Cm]174	T1	RA		
ψ210	Not well resolved	[ψ]210		RA		
ψ218	Strong, H ₂ O	[ψ]218	T1			
ψ296	Strong, H ₂ O	[ψ]296	T1			
ψ300	Strong, H ₂ O	[ψ]300	T1			
Um354	Strong					
ψ366	Strong, H ₂ O	[ψ]366	T1			
ψ406	Strong, H ₂ O					
Um428	Strong	[Um]428	T1			
Gm436	Strong	[Gm]436		RA		
Cm462	Strong	[Cm]462	T1			
Am468	Strong	[Am]468		RA		
Am484	Strong	[Am]484	T1			
Gm509	Strong	[Gm]509		RA		
Am512	Strong	[Am]512		RA		
ψ514	No water molecule visible			RA		
Cm517	Strong	[Cm]517		RA		
ψ556	Not well resolved	[ψ]556	T1			
ψ572	Strong, H ₂ O	[ψ]572				
Am576	Strong					
Am590	Strong	[Am]590	T1	RA	U2	
Gm601	Strong	[Gm]601		RA		
ψ609	Strong, H ₂ O					
Cm621	Not visible					
Um627	Not well resolved	[Um]627	T1	RA		
Gm644	Strong	[Gm]644	T1			
ψ649	Strong, H ₂ O	[ψ]649	T1			
ψ651	Strong, H ₂ O	[ψ]651	T1			
ψ667	Weak, H ₂ O	[ψ]667	T1	RA		
Am668	Strong	[Am]668	T1	RA		MC1
ψ681	Strong, H ₂ O					
Gm683	Strong					
ψ686	Strong, H ₂ O	[ψ]686	T1			
ψ769/770	Not resolved (E565 region)	[ψ]769/770	T1			
Cm797	Not well resolved	[Cm]797		RA		
Um799	Weak	[Um]799	T1	RA		
ψ801	Strong, H ₂ O	[ψ]801	T1			
ψ804	No water molecule visible	[ψ]804	T1			
ψ814	Strong, H ₂ O	[ψ]814	T1	RA		
ψ815	Strong, H ₂ O	[ψ]815	T1			
ψ822	Strong, P 825	[ψ]822	T1			
ψ863	Strong, H ₂ O	[ψ]863	T1	RA		
ψ866	Strong, H ₂ O	[ψ]866	T1			
Gm867	Strong	[Gm]867	T1			
ψ897	At periphery	ND				
ψ918	Strong, H ₂ O	[ψ]918	T1			
ψ966	Strong, P N-1	[ψ]966	T1			
ψ1003	Strong, H ₂ O	[ψ]1003	T1			
ψ1004	Strong, H ₂ O	[ψ]1004	T1			
Am1031	Strong	[Am]1031		RA		
ψ1045	Strong, ribose 1044	[ψ]1045		RA		
ψ1046	Strong, H ₂ O					
ψ1056	Strong, H ₂ O					
ψ1061	Not well resolved	[ψ]1061	T1			
ψ1081	No interaction visible	[ψ]1081	T1			
ψ1136	Strong, H ₂ O	ND				
ψ1174	Strong, H ₂ O					
ψ1177	Strong, H ₂ O					
ψ1186	Strong, H ₂ O	[ψ]1186		RA		
ψ1232	Strong, H ₂ O					
ψ1238	Strong, H ₂ O					
ψ1244	Strong, H ₂ O					
Cm1272	Strong					
Um1288	Strong	[Um]1288		RA		
ψm1326	Strong, H ₂ O	[ψ]1326		RA		
Gm1328	Strong	[Gm]1328		RA		
ψ1347	Strong, H ₂ O					
ψ1360	Strong, H ₂ O	[ψ]1360	T1			
ψ1367	Strong, H ₂ O					
Am1383	Weak	[Am]1383				MC1
Cm1391	Strong	[Cm]1391	T1		U2	
Cm1440	Not visible					
Um1442	Strong	[Um]1442			U2	
ψ1445	Strong, H ₂ O	[ψ]1445			U2	
Gm1447	Strong	[Gm]1447		RA		
Gm1490	Strong	[Gm]1490	T1			
ψ1596	Strong, H ₂ O	[ψ]1596	T1			
ψ1625	Strong, H ₂ O	[ψ]1625	T1			
ψ1643	Strong, H ₂ O	[ψ]1643	T1			
Um1668	Not visible					
Am1678	Strong	[Am]1678	T1			MC1
ψ1692	Strong, H ₂ O	ND				
Cm1703	Strong	[Cm]1703			U2	
Um1804	Strong	[Um]1804	T1			
ψ1839	No water molecule visible	[ψ]1839			U2	

Position	3D structure	MS modif	RNase T1	RNase A	RNase U2	RNase MC1
m1acp3ψ1248	Weak	[m1acp3ψ]	T1			
ac4C1337	Strong	[ac4C]			U2	
m7G1639	Strong	[m7G]	T1	RA		
m6A1832	Strong	[m6A]	T1			
ac4C1842	Strong	[ac4C]	T1		U2	
m6(2)A1850	Strong	[m6,2A]	T1	RA	U2	
m6(2)A1851	Strong	[m6,2A]	T1	RA	U2	

18S rRNA



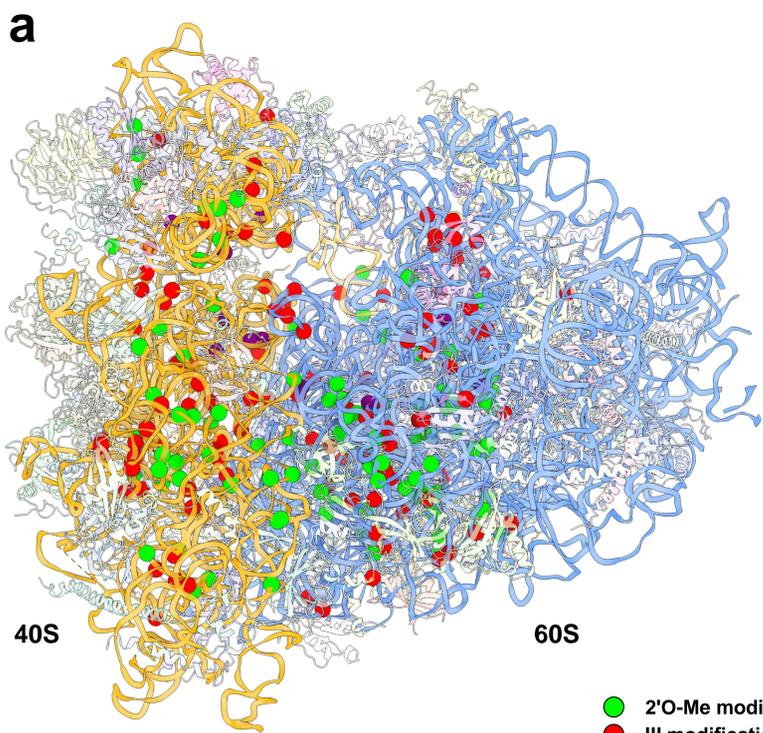
Visible in the structure
Not visible in the structure

ND, oligonucleotide found but modification not detected
Oligonucleotide carrying the modification not detectable by MS
New modified site not described in literature site identified by MS

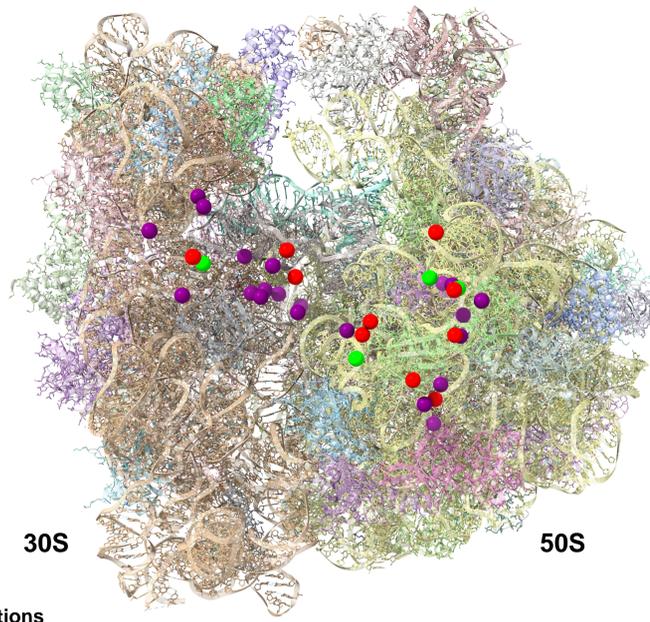
Not visible in MS nor structure, but found in other studies
Present in MS not visible in structure

- 2'O-Me modifications
- Ψ modifications
- nucleotide base modifications

Position	3D structure	MS modif	RNase T1	RNase A	RNase U2	RNase MC1
Am398	Strong	[Am]398		RA		
Am400	Strong	[Am]400		RA		
Gm1316	Strong	[Gm]1316	T1		U2	
Am1323	Weak	[Am]1323	T1			
Am1326	Strong	[Am]1326	T1			
Cm1340	Strong	[Cm]1340	T1			
Gm1522	Strong	[Gm]1522	T1	RA	U2	
Am1524	Strong	[Am]1524	T1	RA		
Am1534	Strong	[Am]1534	T1		U2	
ψ1536	Strong, H ₂ O	[ψ]1536	T1		U2	
ψ1582	Not visible					
Gm1625	Strong					
ψ1677	Weak, H ₂ O	[ψ]1677	T1			
ψ1683	Strong, H ₂ O	[ψ]1683		RA		
ψ1744	Strong, H ₂ O					
Gm1760	At periphery					
Um1773	At periphery	ND				
ψ1779	Weak, H ₂ O	[ψ]1779	T1			
ψ1781	Strong, H ₂ O	[ψ]1781	T1			
ψ1782	Strong, H ₂ O	[ψ]1782	T1			
ψ1792	Strong, H ₂ O					
ψ1860	Strong, H ₂ O	[ψ]1860	T1			
ψ1862	Strong, H ₂ O	[ψ]1862	T1			
Am1871	Strong	[Am]1871		RA		
Cm1881	Strong					
Cm2351	Strong	[Cm]2351	T1			
Am2363	Strong	[Am]2363	T1	RA	U2	MC1
Gm2364	Strong	[Gm]2364	T1	RA	U2	MC1
Cm2365	Strong	[Cm]2365	T1	RA	U2	MC1
Am2401	Strong	[Am]2401		RA		
Um2415	Weak	[Um]2415		RA		MC1
Cm2422	Strong	[Cm]2422	T1	RA		MC1
Gm2424	Strong	[Gm]2424	T1	RA		MC1
ψ2508	Strong, P N-1					
ψ2632	Strong, H ₂ O	[ψ]2632	T1			
Am2787	Strong	[Am]2787	T1			
Cm2804	Strong	[Cm]2804	T1			
Am2815	Strong	[Am]2815	T1			
Cm2824	Strong	[Cm]2824		RA		
Um2837	Strong	[Um]2837	T1			
ψ2839	Strong, H ₂ O					
ψ2843	Strong, H ₂ O					
Cm2861	Strong					
Gm2876	Strong					
Gm3627	Strong	[Gm]3627		RA		
ψ3637	Strong, H ₂ O					
ψ3639	Strong, H ₂ O					
ψ3695	Strong, H ₂ O	[ψ]3695	T1			
Cm3701	Strong	[Cm]3701	T1			
ψ3715	Strong, H ₂ O	[ψ]3715	T1			
Am3718	Strong	[Am]3718	T1	RA		
Am3724	Strong	[Am]3724		RA		
ψ3730	Strong, H ₂ O					
ψ3734	Not well resolved					
Gm3744	Weak	[Gm]3744	T1			
ψ3758	Not well resolved	[ψ]3758	T1	RA		
Am3760	Not well resolved	[Am]3760	T1		U2	
ψ3762	Strong, H ₂ O	[ψ]3762	T1		U2	
ψ3764	Weak, H ₂ O	[ψ]3764	T1		U2	
ψ3768	Weak, H ₂ O	[ψ]3768	T1		U2	
ψ3770	Strong, H ₂ O	[ψ]3770	T1		U2	
Am3785	Strong	[Am]3785	T1			
Gm3792	Strong	[Gm]3792	T1		U2	
Cm3808	Strong	[Cm]3808		RA		
ψm3818	Strong	[ψ]3818	T1	RA		
ψ3822	Strong, H ₂ O	[ψ]3822		RA		
Am3825	Strong					
Am3830	Strong	[Am]3830	T1	RA	U2	
Cm3841	Strong	[Cm]3841			U2	
ψ3844	Strong, H ₂ O	[ψ]3844			U2	
ψ3851	Strong, H ₂ O					
ψ3853	Strong, P N-1					
Am3867	Strong	[Am]3867	T1			
Cm3869	Strong	[Cm]3869	T1			
ψ3884	Strong, H ₂ O					
Cm3887	Strong	[Cm]3887		RA		
Gm3899	Strong	[Gm]3899		RA		
ψ3920	Strong, H ₂ O					
Um3925	Strong					
Gm3944	Not well resolved	[Gm]3944	T1	RA		
ψ3959	At periphery					
Gm4042	At periphery					
Cm4054	At periphery	[Cm]4054	T1			MC1
Gm4196	Strong	[Gm]4196		RA		
Um4227	Weak	[Um]4227	T1	RA		
Gm4228	Strong/weak	[Gm]4228	T1	RA		
ψ4293	Strong, ribose 4329					
ψ4296	Strong, H ₂ O					
ψ4299	Strong, H ₂ O					
Um4306	Strong	[Um]4306		RA		
ψ4312	Strong, H ₂ O	[ψ]4312	T1			
ψ4353	Strong, H ₂ O					
ψ4361	Strong, H ₂ O	[ψ]4361	T1			
Gm4370	Strong	[Gm]4370		RA		
Gm4392	Strong	[Gm]4392		RA		
ψ4403	Strong, H ₂ O					
ψ4420	Not well resolved	[ψ]4420	T1			
ψ4423	Weak, H ₂ O	[ψ]4423	T1			
ψ4431	Strong, H ₂ O					
ψ4442	Strong, H ₂ O	[ψ]4442	T1			
Cm4456	Strong	[Cm]4456	T1			U2
ψ4457	Strong, H ₂ O	[ψ]4457	T1			U2
ψ4471	Strong, H ₂ O					
ψ4493	Strong, H ₂ O					
Gm4494	Strong					
Um4498	Strong	[Um]4498	T1			U2
Gm4499	Strong	[Gm]4499	T1			U2
ψ4500	Strong, U3803	[ψ]4500	T1			U2
ψ4521	Strong, H ₂ O					
Am4523	Strong					
ψ4532	Strong, H ₂ O	[ψ]4532	T1			
Cm4536	Strong	[Cm]4536		RA		
ψ4552	Strong, H ₂ O					

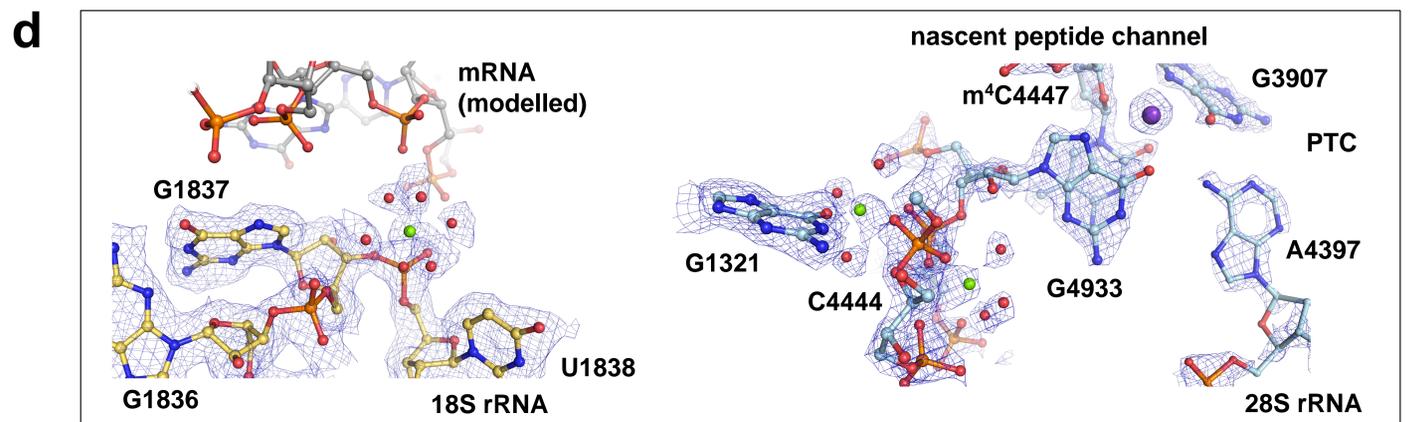
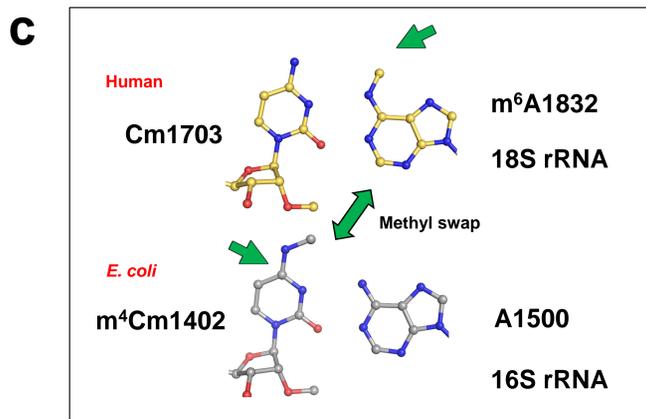


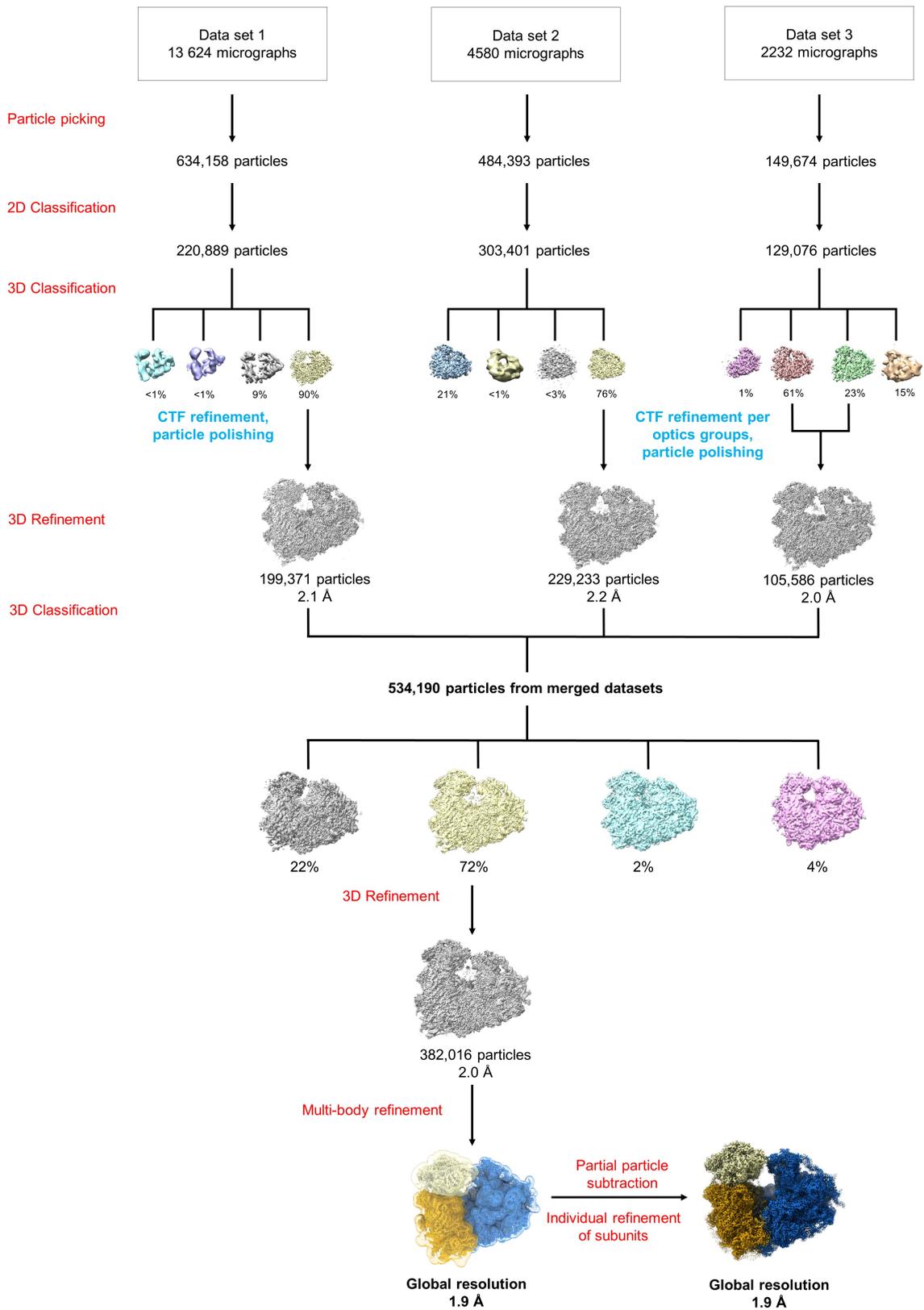
● 2'O-Me modifications
● Ψ modifications
● nucleotide base modifications



b

<i>E. coli</i>	Human	<i>E. coli</i>	Human
30S subunit	40S subunit	50S subunit	60S subunit
ψ516	U612, non-modified	m ⁷ G745	C1594, non-modified
m ⁷ G527	G623, non-modified	ψ746	G1595, non-modified
m ² G966	m ¹ acp ³ ψ1248, PTC, confirmed by MS	m ⁵ U747	U1596, non-modified
m ⁵ C967	C1249, non-modified	ψ955	G1864, non-modified
m ² G1207	C1501, non-modified	m ⁶ A1618	A2798, non-modified
m ⁴ Cm1402	Cm1703, swapped with m ⁶ A1832, absence of m ⁴ , confirmed by MS	m ⁷ G1835	G3698, non-modified
m ⁵ C1407	C1708, non-modified	ψ1911	U3758, non-modified
m ³ U1498	U1830, non-modified	m ³ ψ1915	ψ3762 (H69), absence of m ³ , confirmed by MS
m ² G1516	U1848, non-modified	ψ1917	ψ3764 (H69)
m ⁶ ,A1518	m ⁶ ,A1850, DC	m ⁵ U1939	U3786, non-modified
m ⁶ ,A1519	m ⁶ ,A1851, DC	m ⁵ C1962	G3809, non-modified
		m ⁶ A2030	A3876, non-modified
		m ⁷ G2069	U3915, non-modified
		Gm2251	Gm4196
		m ⁷ G2445	G4391, non-modified
Post-translational modifications		D2449	U4395, non-modified
iso-Asp119	iso-Asp138 (uS11)	ψ2457	ψ4403
mSAsp89 (D2T)	Asp114 (uS12), non-modified	Cm2498	C4444, non-modified
		m ⁷ A2503	A4449, non-modified
		ψ2504	U4450, non-modified
		Um2552	Um4498
		ψ2580	U4526, non-modified
		ψ2604	G4550, non-modified
		ψ2605	U4551, non-modified
		Post-translational modifications	
		mGln150 (uL3)	No match, different sequence
		HOArg81 (uL16)	No match, different sequence
		ThiopeptideM82	Met115 (uL16), non-modified





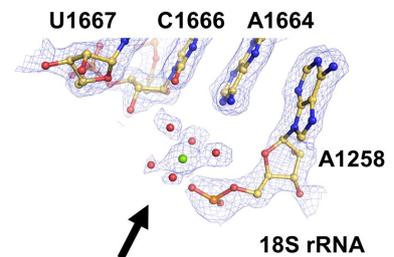
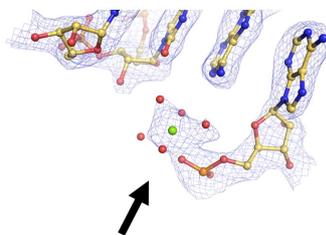
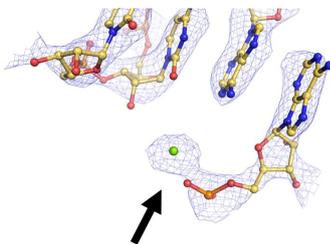
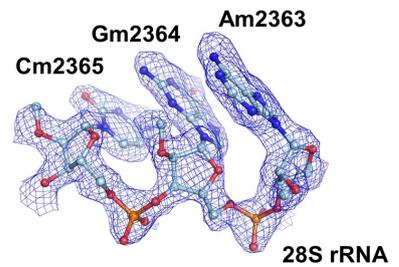
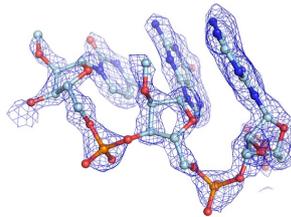
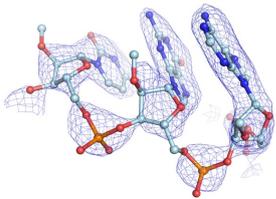
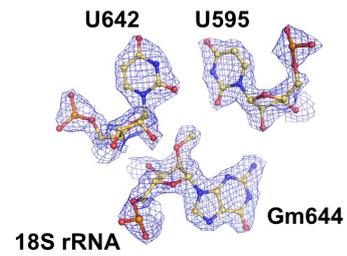
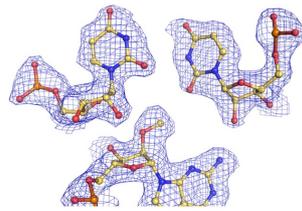
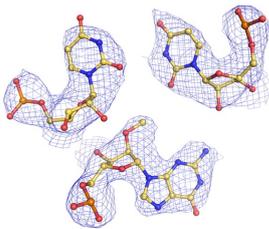
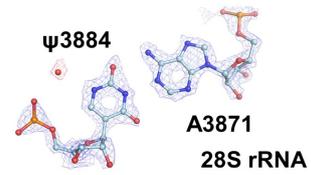
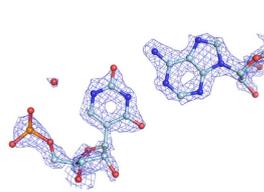
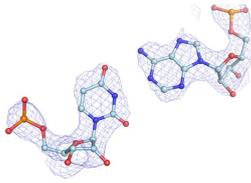
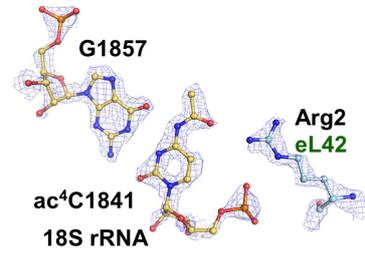
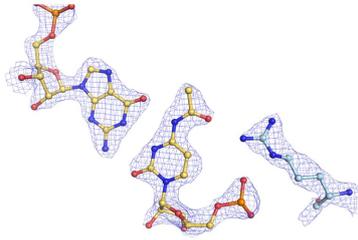
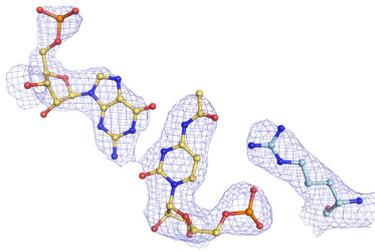
Particle sorting scheme

Extended Data Fig. 1

Previous study

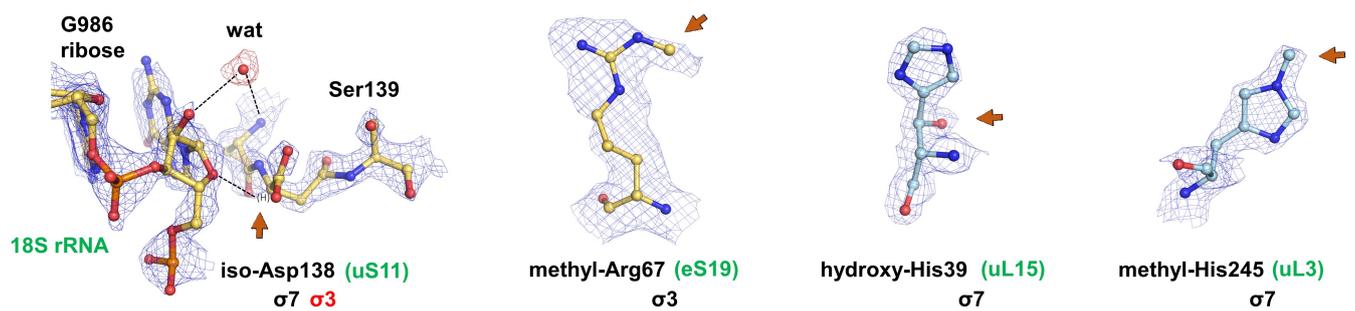
Current work
*After multibody
refinement*

Current work
*Final map after
partial signal
subtraction*



Resolution improvement of features in the cryo-EM map (present work compared to our previous study³⁸)

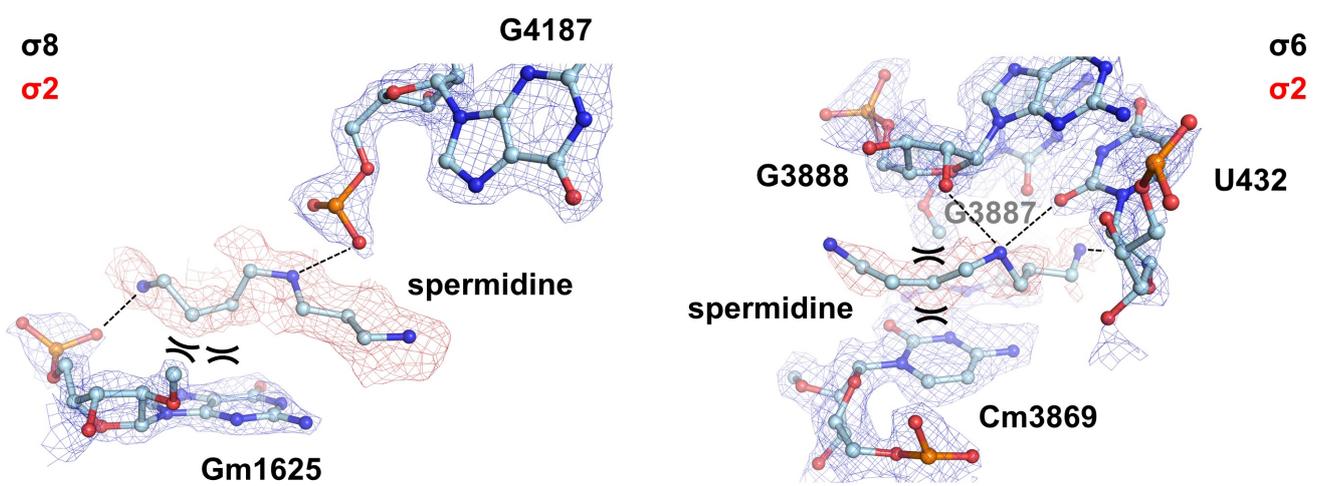
Extended Data Fig. 2



Post-translational modifications of ribosomal proteins in the human ribosome

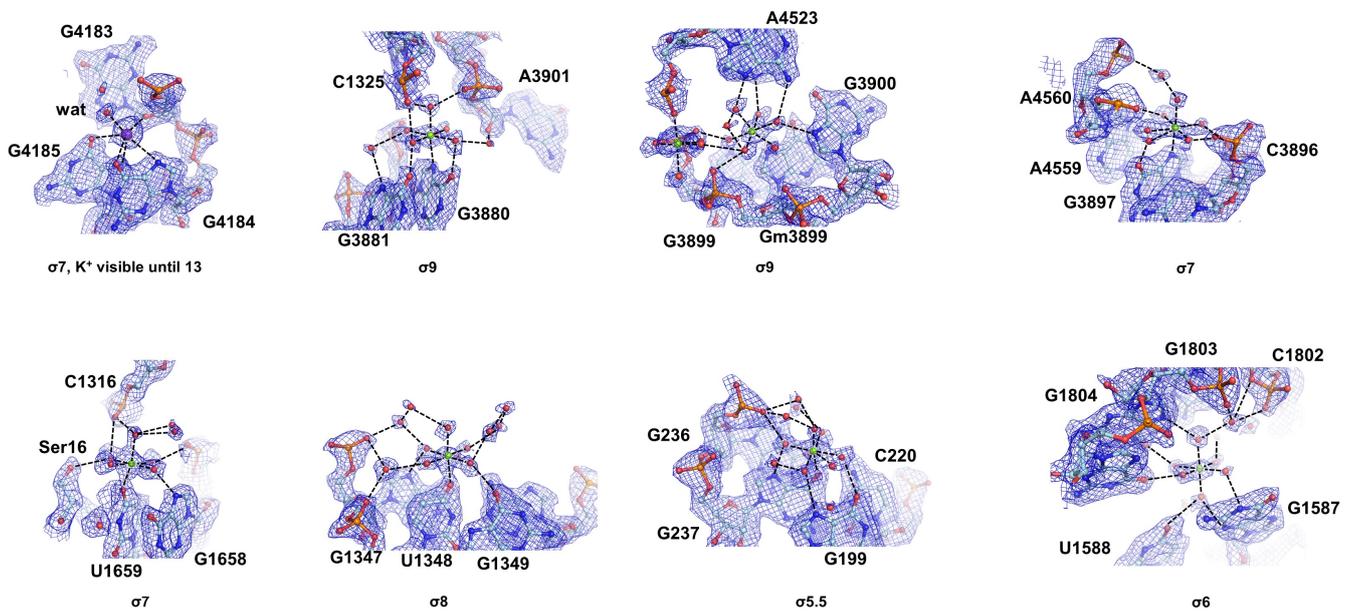
Iso-Asp138 (uS11), methyl-arginine (ribosomal protein eS19), hydroxy-histidine (uL15) and methyl-histidine (uL3).

Extended Data Fig. 3



Visualization of endogenous polyamines (2 spermidines) in the human ribosome

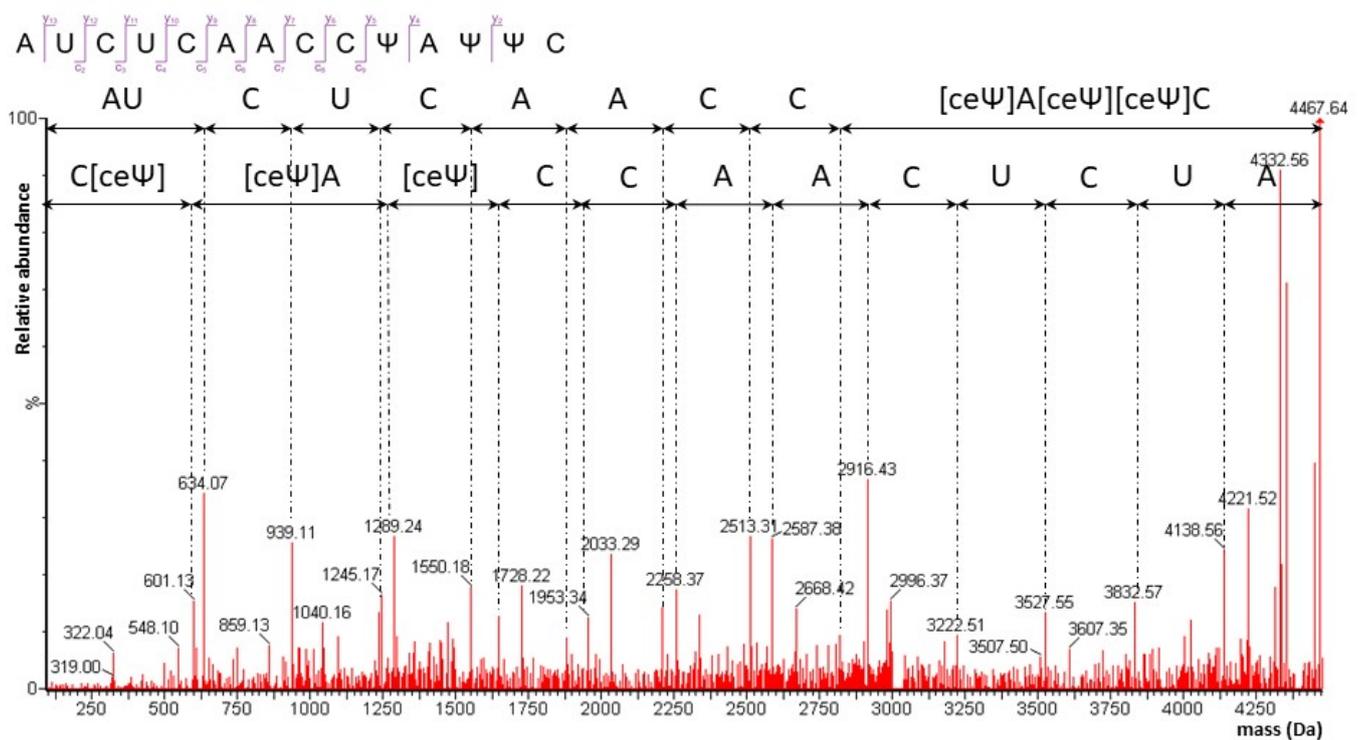
Extended Data Fig. 4



Assignment of octahedral Mg²⁺ coordinations with water molecules at N7, O6 and O4 positions of nucleotide bases

Confirmation of Mg²⁺-coordinated rRNA bases that were identified previously by electrostatic potential map calculations⁴⁰; respective σ -levels are indicated.

Extended Data Fig. 5



MS analysis using chimeric oligo nucleotides

Deconvoluted MS/MS spectrum of $^{1770}AUCUCAACC[\Psi]A[\Psi][\Psi]^{1783}C$ from HeLa 28S rRNA with fragmentation assignments.

Extended Data Fig. 6

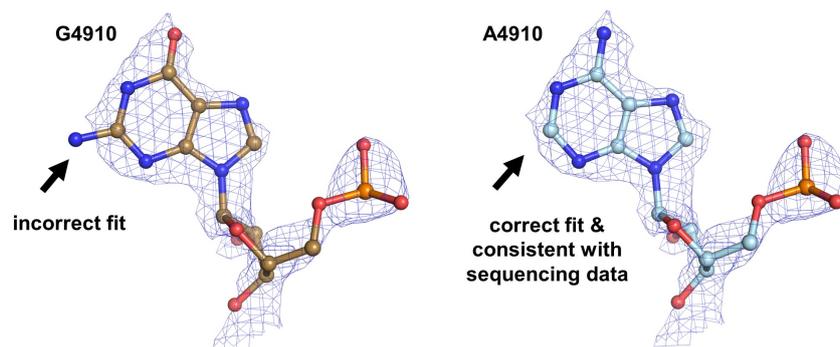
rRNA	Oligonucleotide name	Sequence (5'-->3')	GC-content
18S	18S 253	CCGCCCCCCCGGCCGGGGCCG	100%
18S	18S 253-528	GCCTCGAAAGAGTCCTGTAT	50%
18S	18S 528-727	AGGCAAGGGGCGGGGACGGG	80%
18S	18S 727-922	CGAATGCCCCCGGCCGTCCC	80%
18S	18S 922-1187	CCGTCAATTCCTTTAAGTTT	35%
18S	18S 1187-1661	GTTACCCGCGCCTGCCGGCG	80%

28S	28S 249	CCCGGGCCCCGGCGCGCCGGG	100%
28S	28S 249-505	CGGGGGGCGGGAAAGATCCG	75%
28S	28S 505-792	CCGGGGGGCGGAGACGGGGG	90%
28S	28S 792-1028	CGCGCCCCCGGGGGAGAC	90%
28S	28S 1028-1279	CGACGTGCGCCGCGACCCCG	85%
28S	28S 1279-1538	TGGCTTCGCCCTGCCAGGC	75%
28S	28S hyb 1538-1772	[Am][Am][Am][Gm][Um][Um][Um][Gm] [Am](GAAT)[Am][Gm][Gm][Um][Um][Gm][Am]	30%
28S	28S 1538-1815	CACGCCCGGCTCCACGCCAG	80%
28S	28S 1815-2061	GACGGCCGGGTATGGGCCCG	80%
28S	28S 2061-2329	TCTGCACCTGCGGCGGCTCC	75%
28S	28S 2329-2554	CTGGACGCCTCGCGGCGCCC	85%
28S	28S 2554-2837	TGCCGACTTCCCTTACCTAC	55%
28S	28S 2554-2881	CAGCCCTTAGAGCCAATCCT	55%
28S	28S 2837-3107	CCCCCGCCGCCGCCCCAC	95%
28S	28S 3107-3402	CCCCGCCCGCCGCCCGCCG	100%
28S	28S 3402-3663	GTCAACACCCGCCGCGGGCC	80%
28S	28S 3663-3915	GCCAGACTAGAGTCAAGCTC	55%
28S	28S 3663-3959	GGGGGCCCTCCCACTTATTCT	60%
28S	28S 3915-4157	GGCACTGTCCCCGGAGCGGG	80%
28S	28S 4157-4416	AAGCGACGTCGCTATGAACG	55%
28S	28S 4157-4500	CGTTCCCTATTAGTGGGTGA	50%
28S	28S 4391-4643	CCGCCACAAGCCAGTTATCC	60%
28S	28S 4416-4643	CGCCCCATTGGCTCCTCAGC	70%
28S	28S 4643-4876	CGTTTCCCAGGACGAAGGGC	65%

RNA & DNA oligos used for mass spectrometry analysis

Deoxyribonucleotides are in parenthesis; Nm corresponds to 2'-O-methylated nucleotides.

Extended Data Fig. 7



Correction of the rRNA reference sequence as found by sequencing and confirmed from the structure and MS analysis

A4910 is labeled in red in the 28S rRNA sequence (see **Supplementary Fig. 4**).

Extended Data Fig. 8

18S_1F	CCTGGTTGATCCTGCCAGTAGC	18S_160R	CCACAGTTATCCAAGTAGG
18S_544_F	TAACAATACAGGACTCTTTCG	18S-972_R	TCCAAGAATTCACCTCTAGC
18S_1600_F	TAACCCGTTGAACCCCA TTCGTGAT	18S-1581_R	GTTACCCGCGCCTGCCGGCG
		18S_1760_R	CCGCCCCCGGCGGGGCCG
		18S_1860_R	TAATGATCCTCCGCAGGTTCAAC

28S_1_F	CGCGACCTCAGATCAGACGTGG	28S-450-R	ACTGCGCGGACCCACCCGTTT
28S_510_F	ATCTTCCCGCCCCCGTTCCT	28S_1300_R	TTTCAAGACGGGTCGGGTGGGTAGCCGACGT
28S_1500_F	GAACATGCCTGGGCAGGGC	28S-2260-R	TCCTACTCGTCGCGGGTAGCGTCCGCGGGGCT
28S_2030_F	AATCAACTAGCCCTGAAAATGGATG	28S_2750_R	CGGCGCGAGATTTACACCCTCTC
28S-2900-F	ATAAGGATTGGCTCTAAGGGCTGGGTCTG GT	28S-3270-R	ATCCACGGGAAGGGCCCGGCTCGCGTCCAGA
28S-3620_F	GACCAGGGGAATCCGACTGTTTAATT	28S_3600_R	CACCAGTTCTAAGTCGGCTGCTAG
28S-4440_F	AAGCGTTCATAGCGACGTCGCTTTTTGAT	28S_5060_R	GACAAACCCTTGTGTCGAGGGC
28S_2750_F	GAGAGGGTGTAATCTCGCGCCG	28S_5000_R	GCTACGTACGAAACCCCGACC
		28S_4690_R	ATAATCCACAGATGGTAGCTTCGCCCCATT

5.8S_1_F	CGACTCTTAGCGGTGGATCACTC	5.8S_157_R	AAGCGACGCTCAGACAGGC
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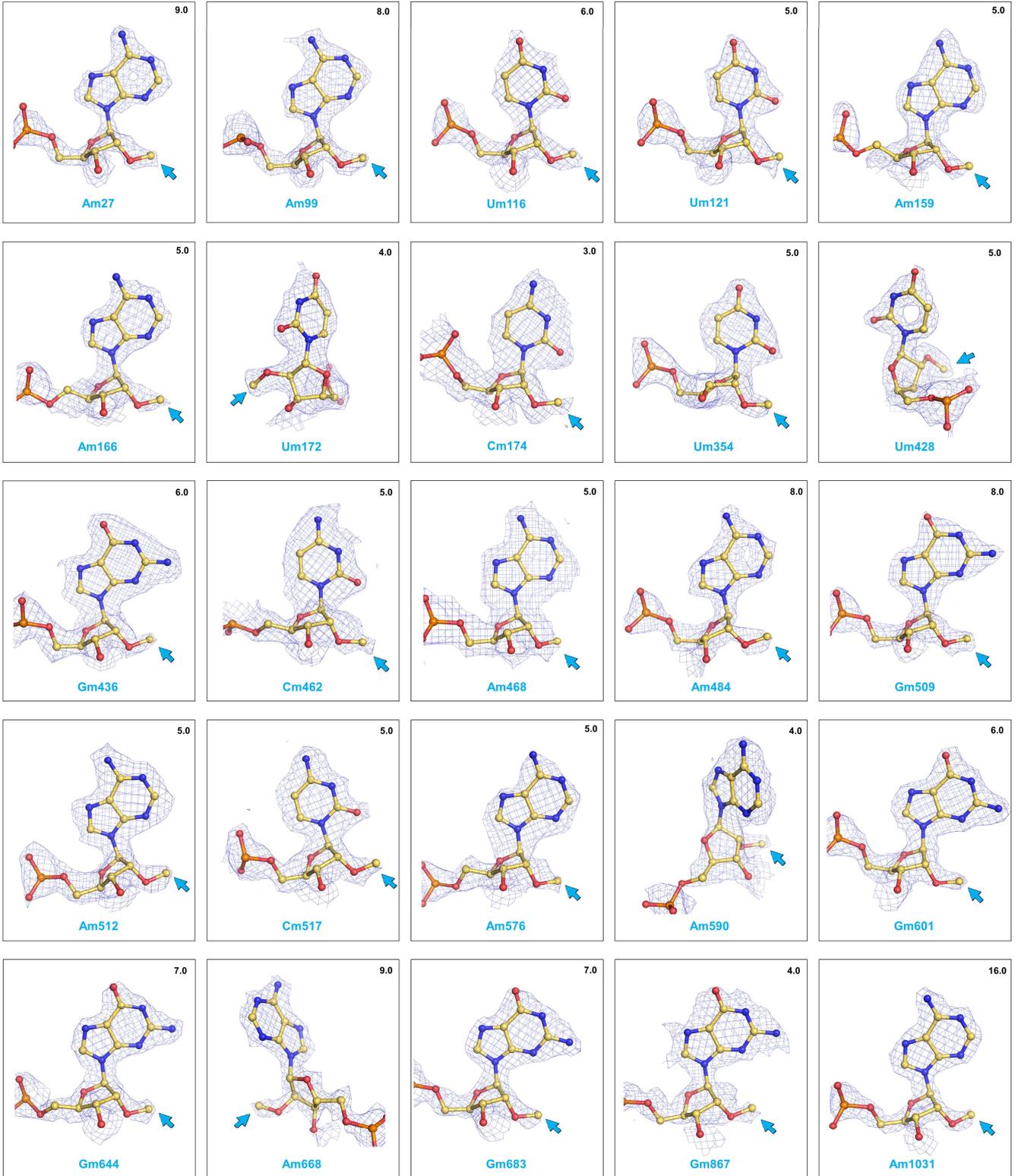
5S_1_F	GTCTACGGCCATACCACCTG	5S_120_R	AAGCCTACAGCACCCGGTATTC
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Primer sequences used for human 18S, 28S, 5.8S and 5S rRNA sequencing

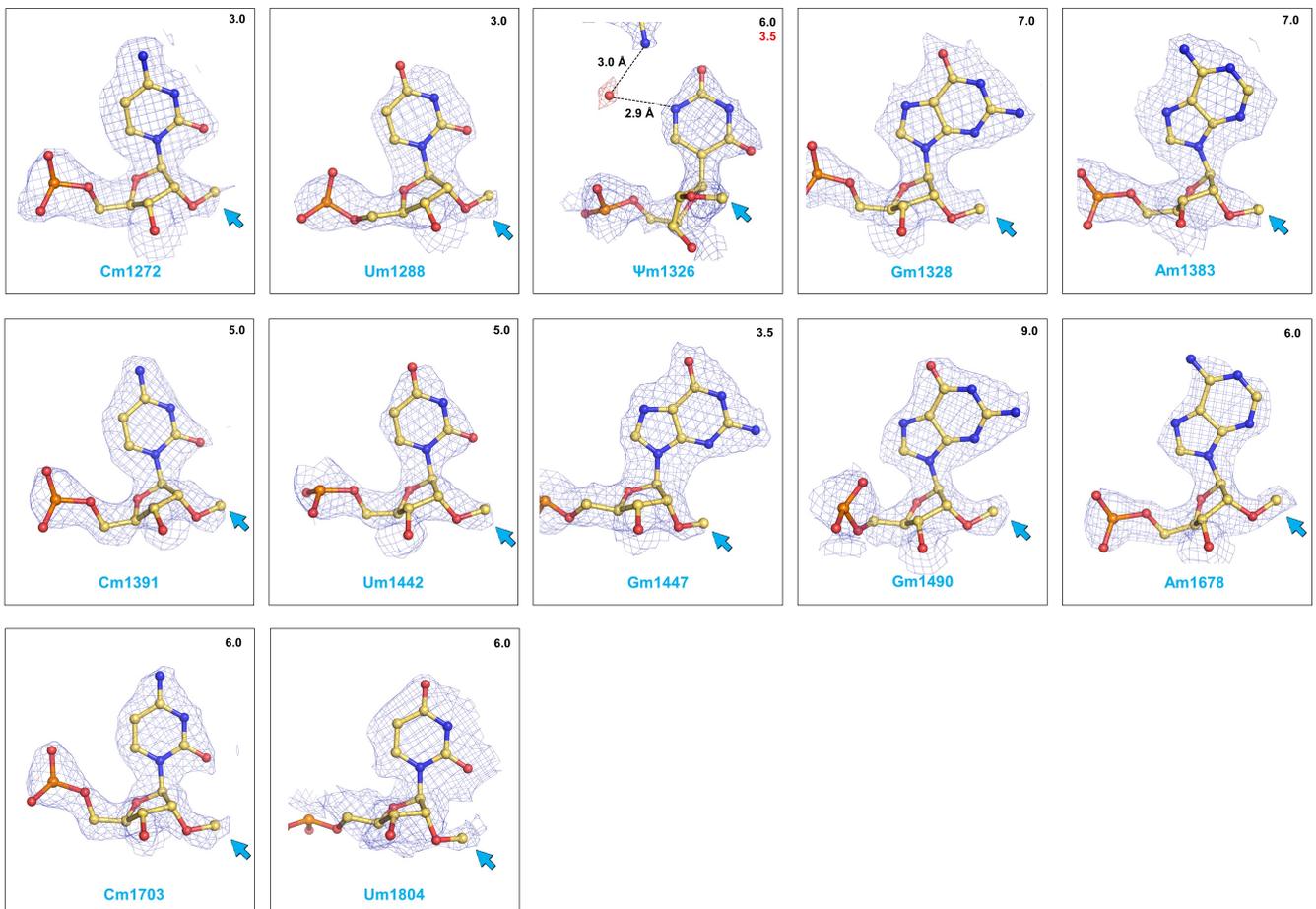
Oligonucleotide primers used for PCR.

Extended Data Fig. 9

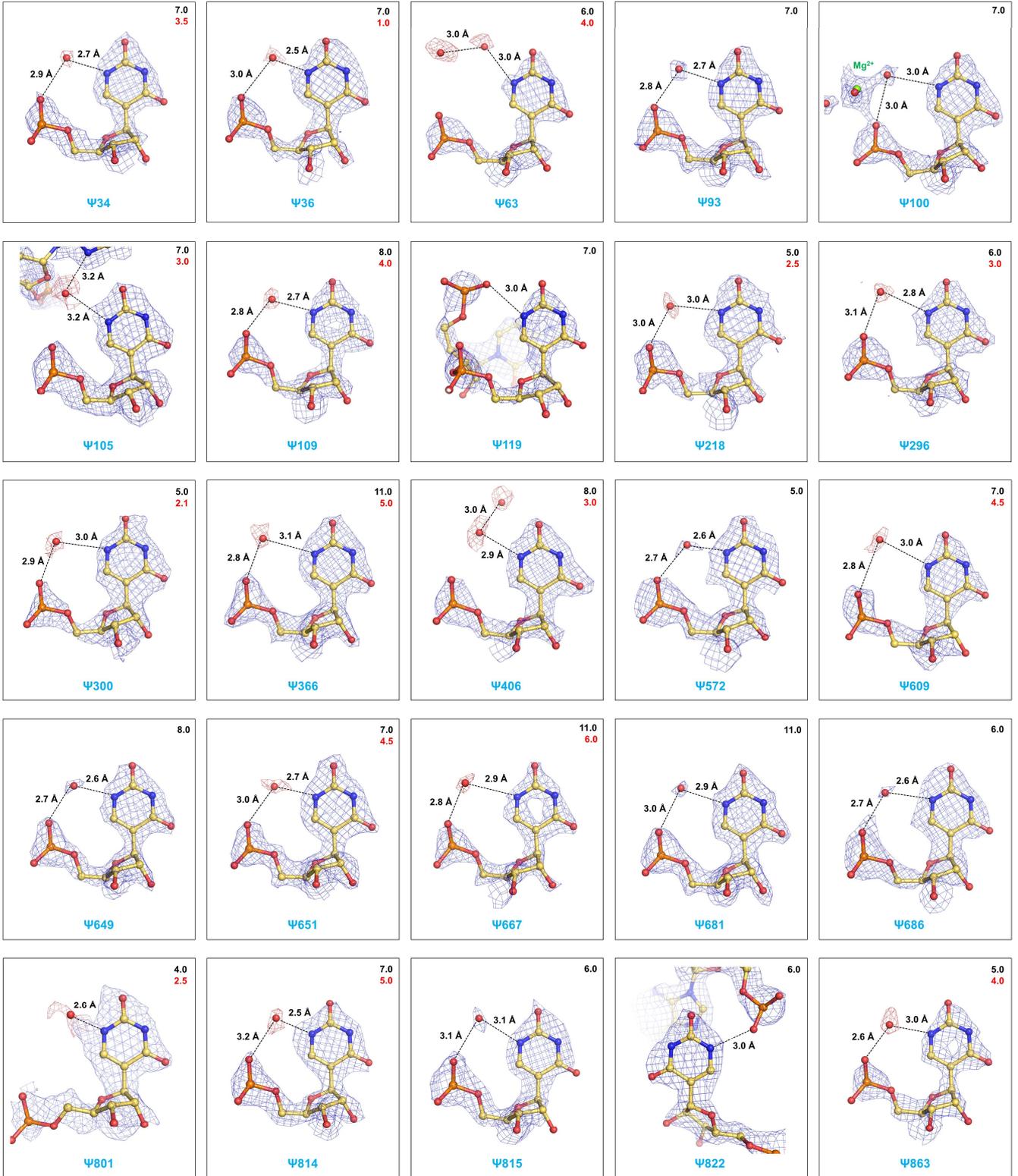
18S rRNA – 2'-O-methylations



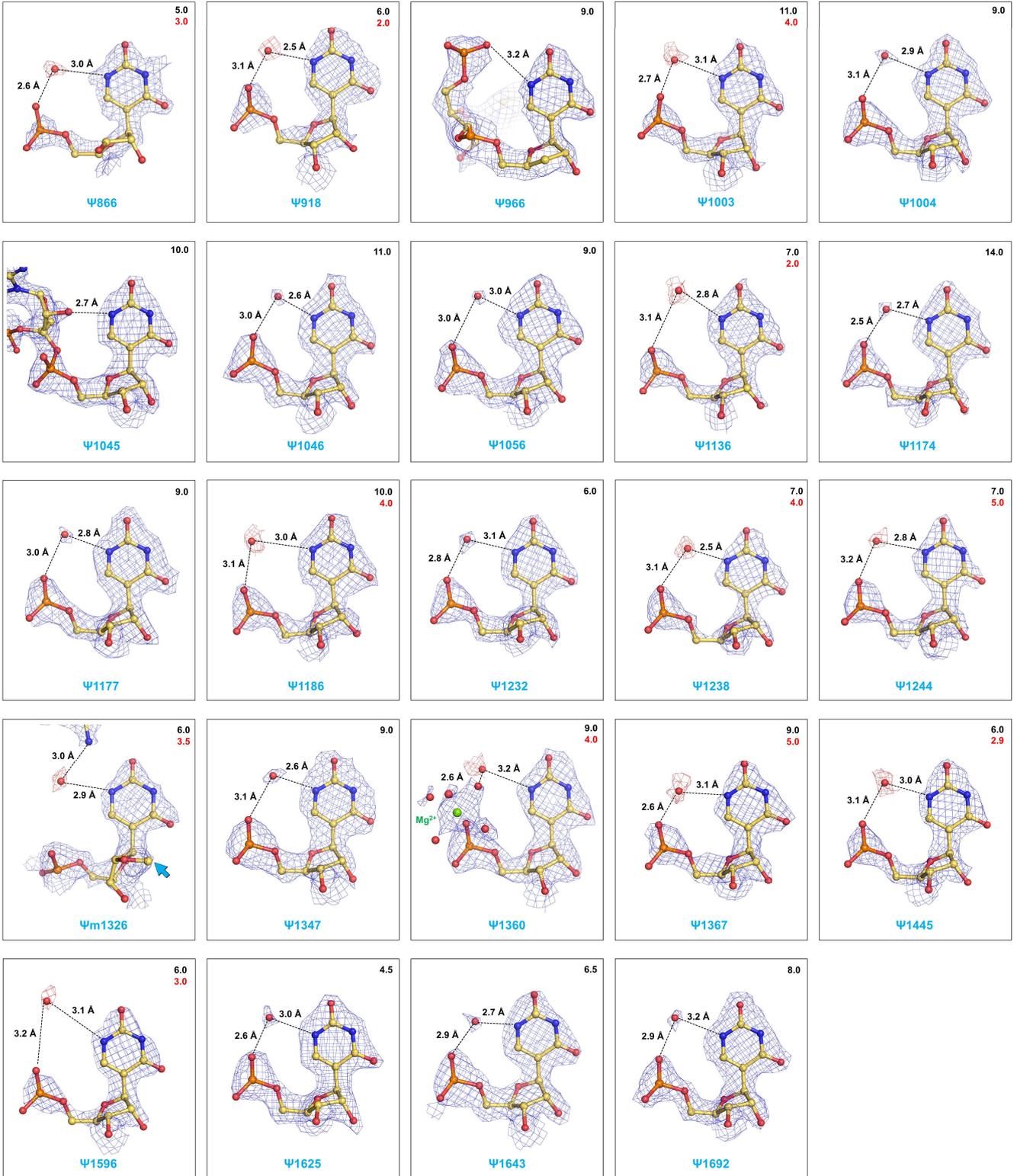
18S rRNA – 2'-O-methylations (cont.)



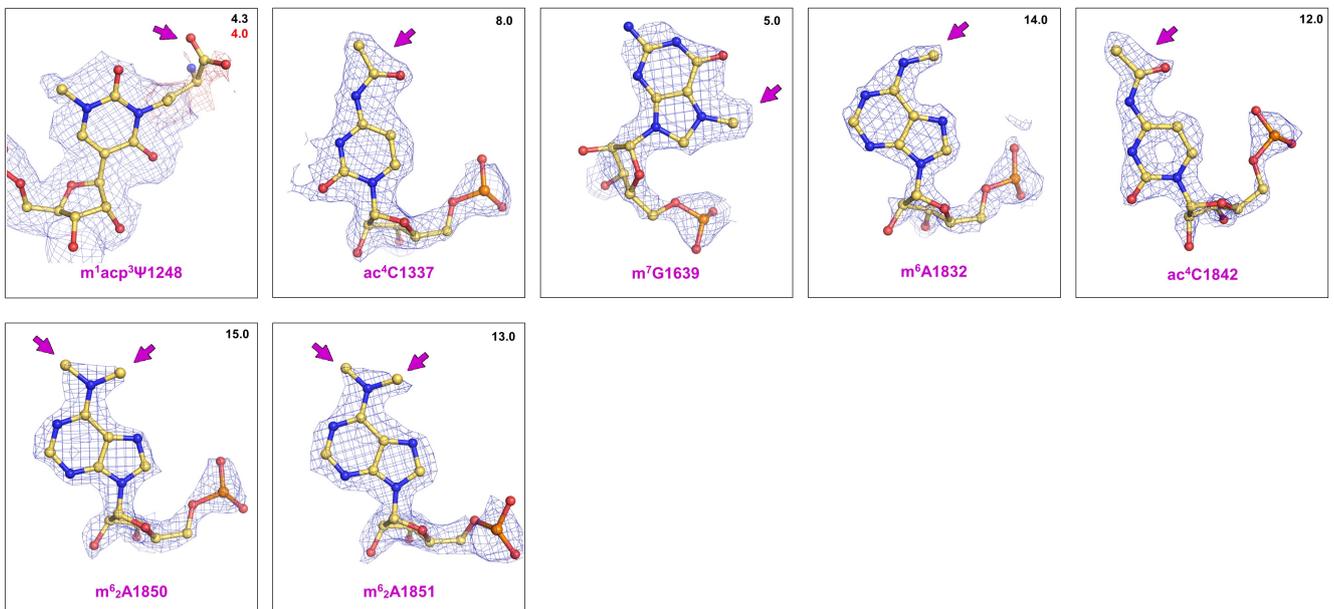
18S rRNA – pseudo-uridines



18S rRNA – pseudo-uridines (cont.)



18S rRNA – base modifications

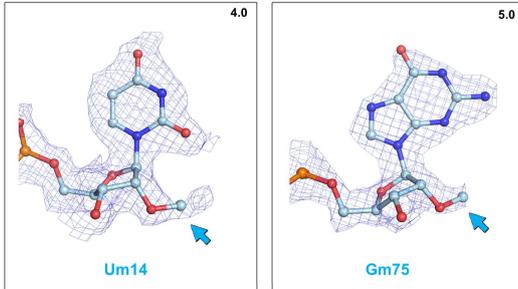


Chemical modifications seen in the cryo-EM map (40S ribosomal subunit)

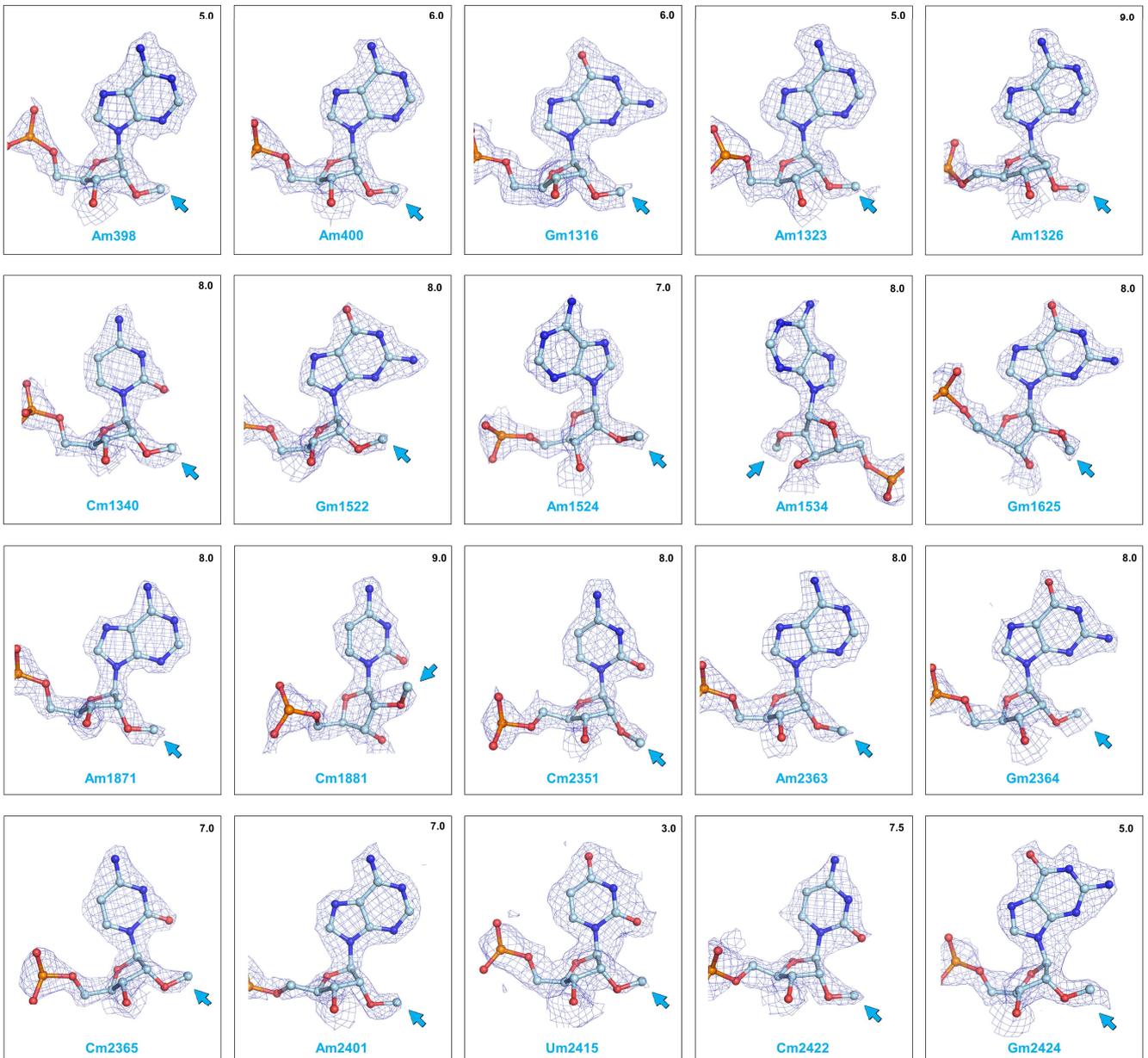
2'-O-Me's, ψ 's & nucleotide base modifications in the 40S ribosomal subunit (human 18S rRNA).

Supplementary Fig. 1

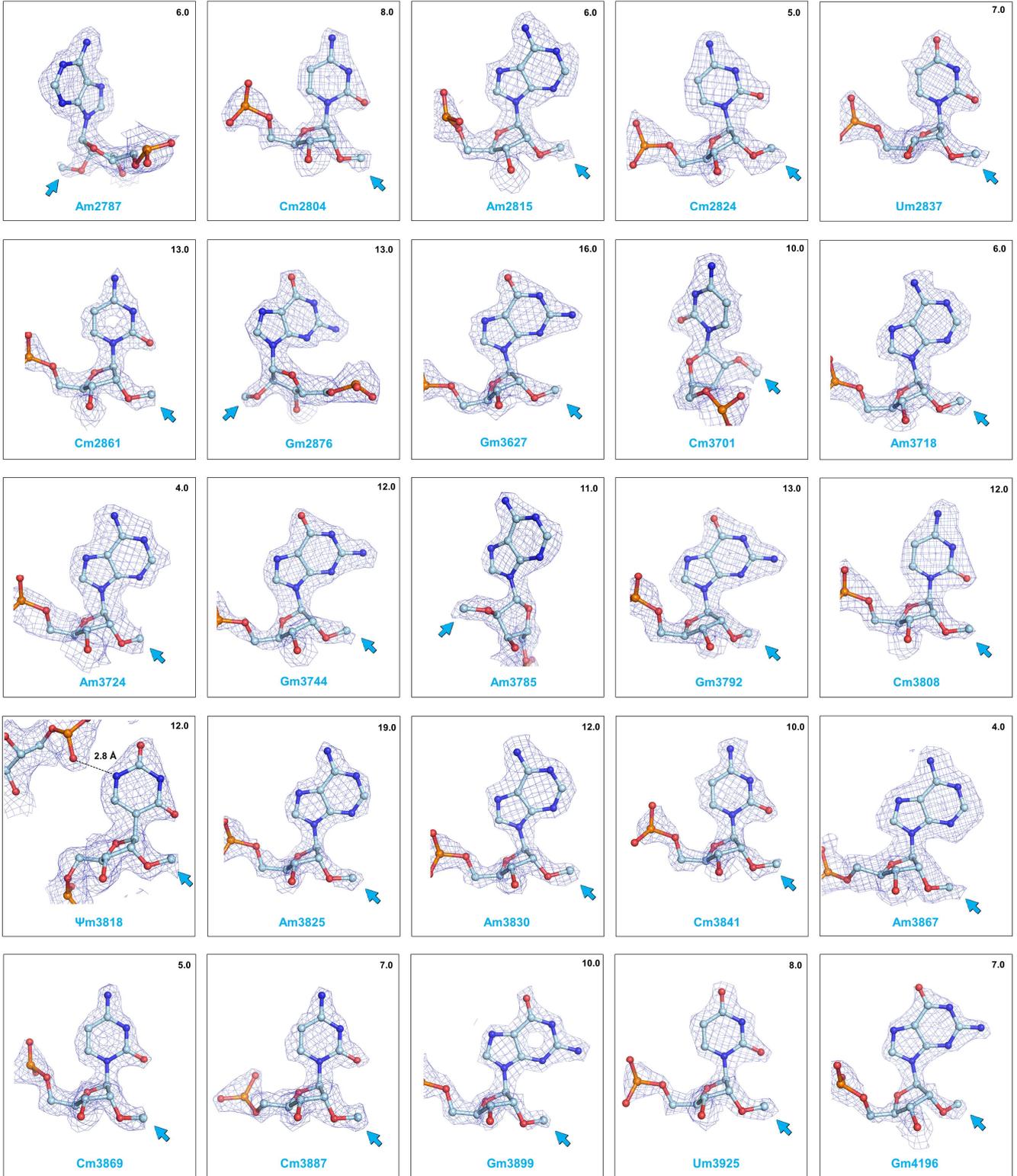
5.8S rRNA – 2'-O-methylations



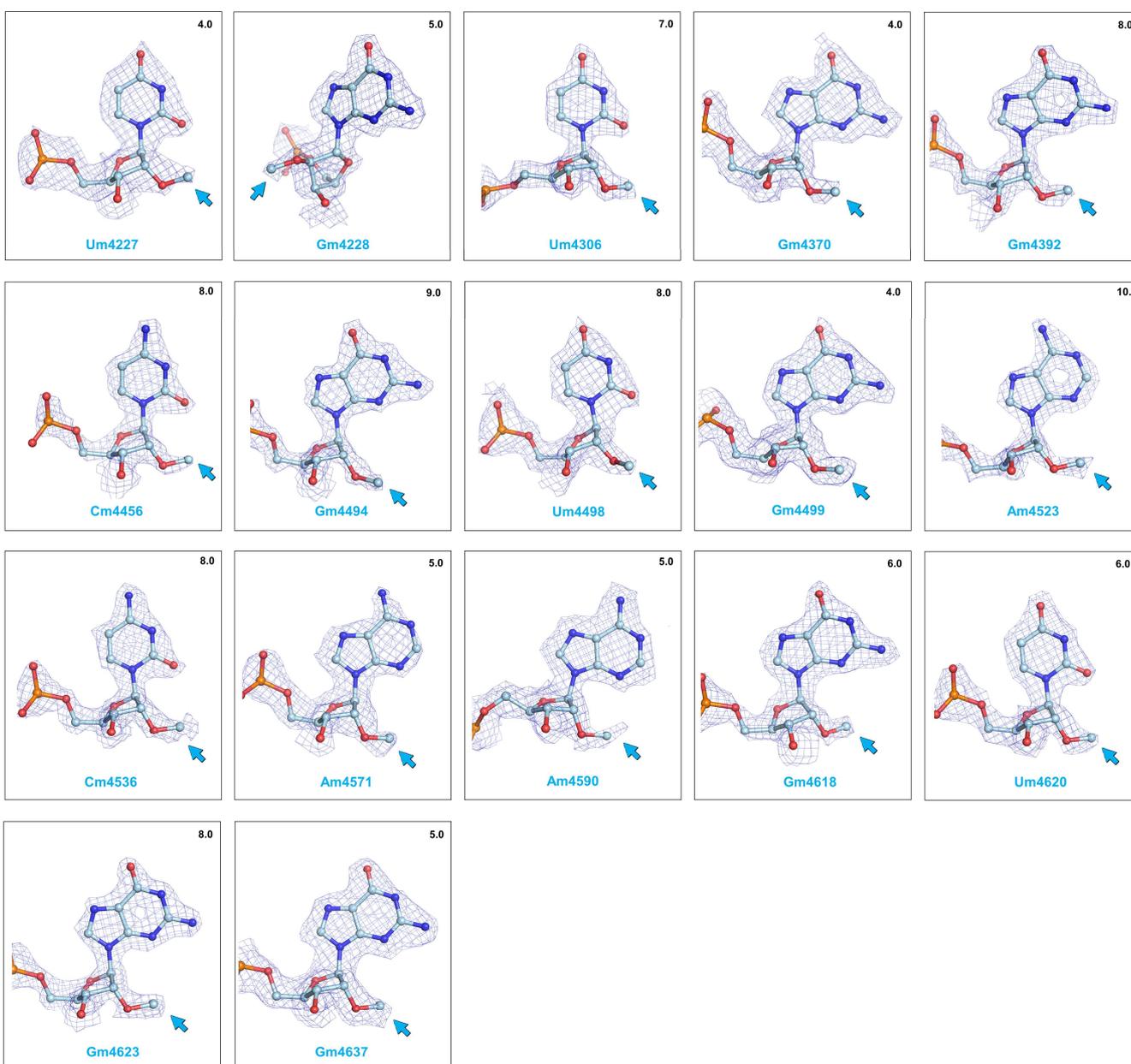
28S rRNA – 2'-O-methylations



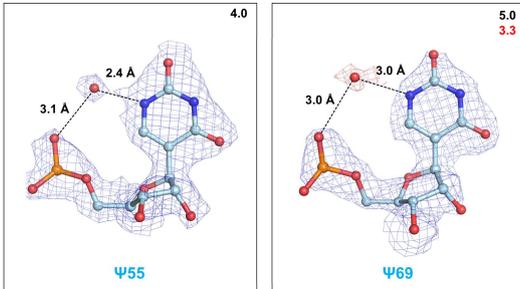
28S rRNA – 2'-O-methylations (cont.)



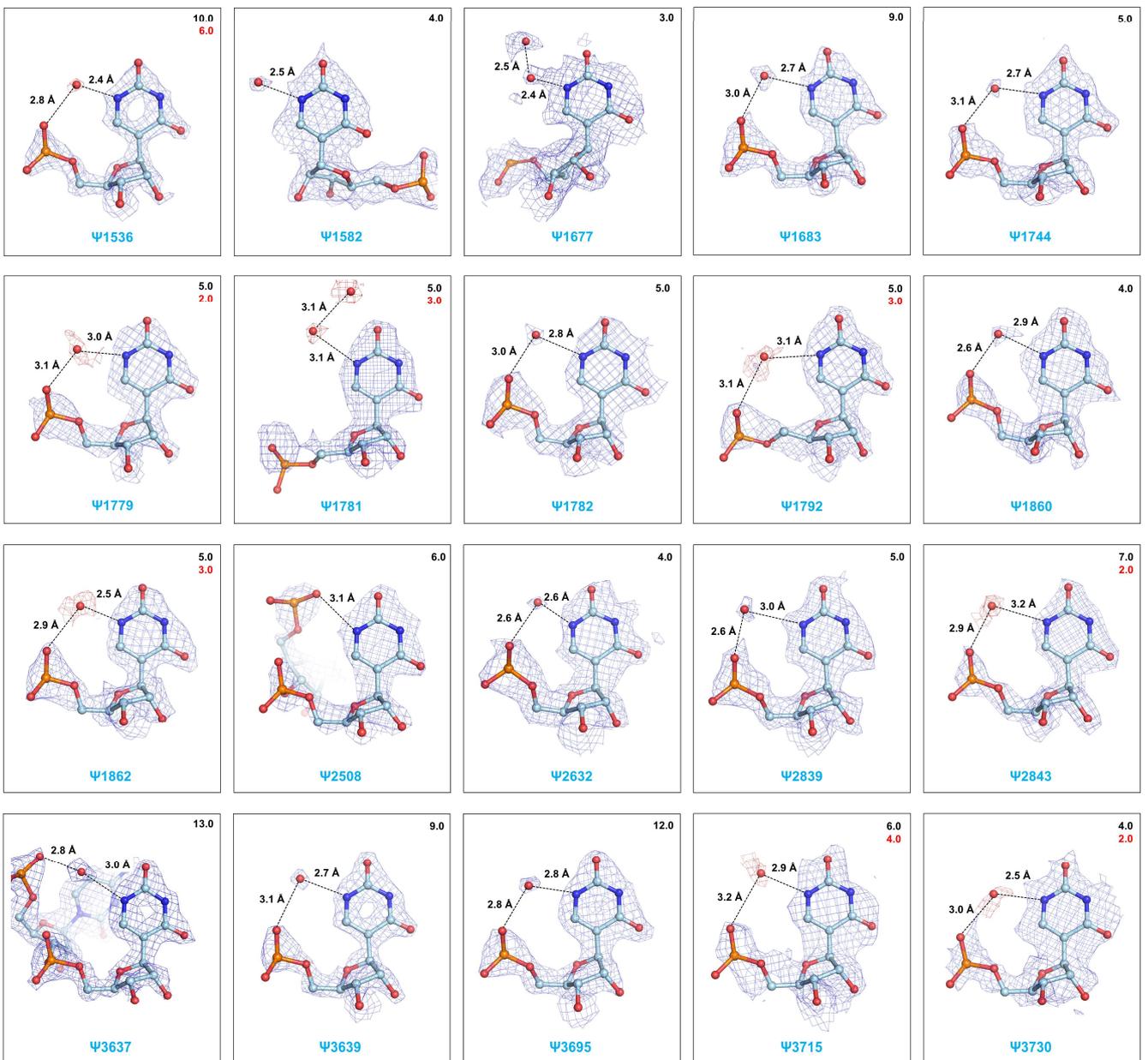
28S rRNA – 2'-O-methylations (cont.)



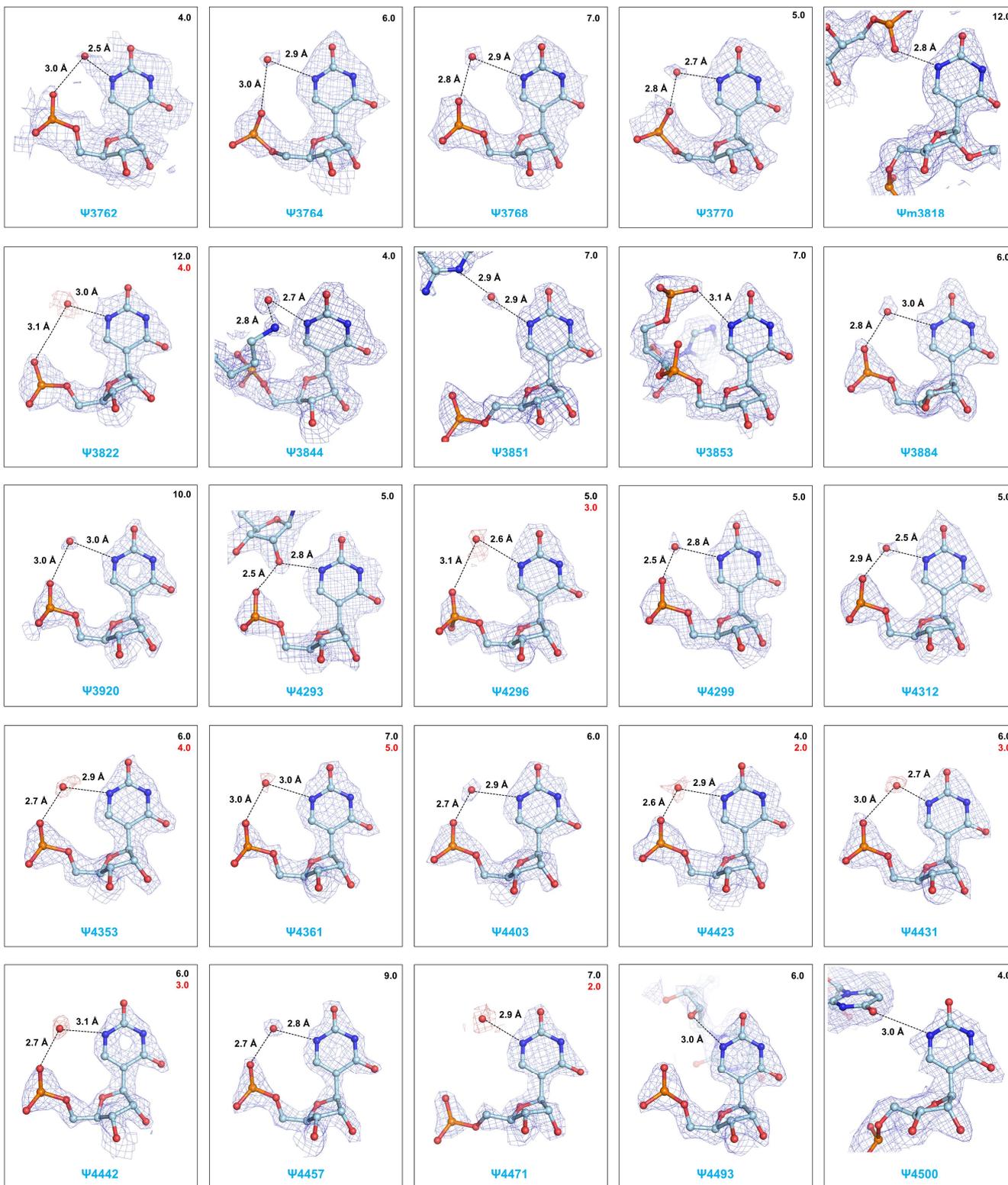
5.8S rRNA – pseudo-uridines



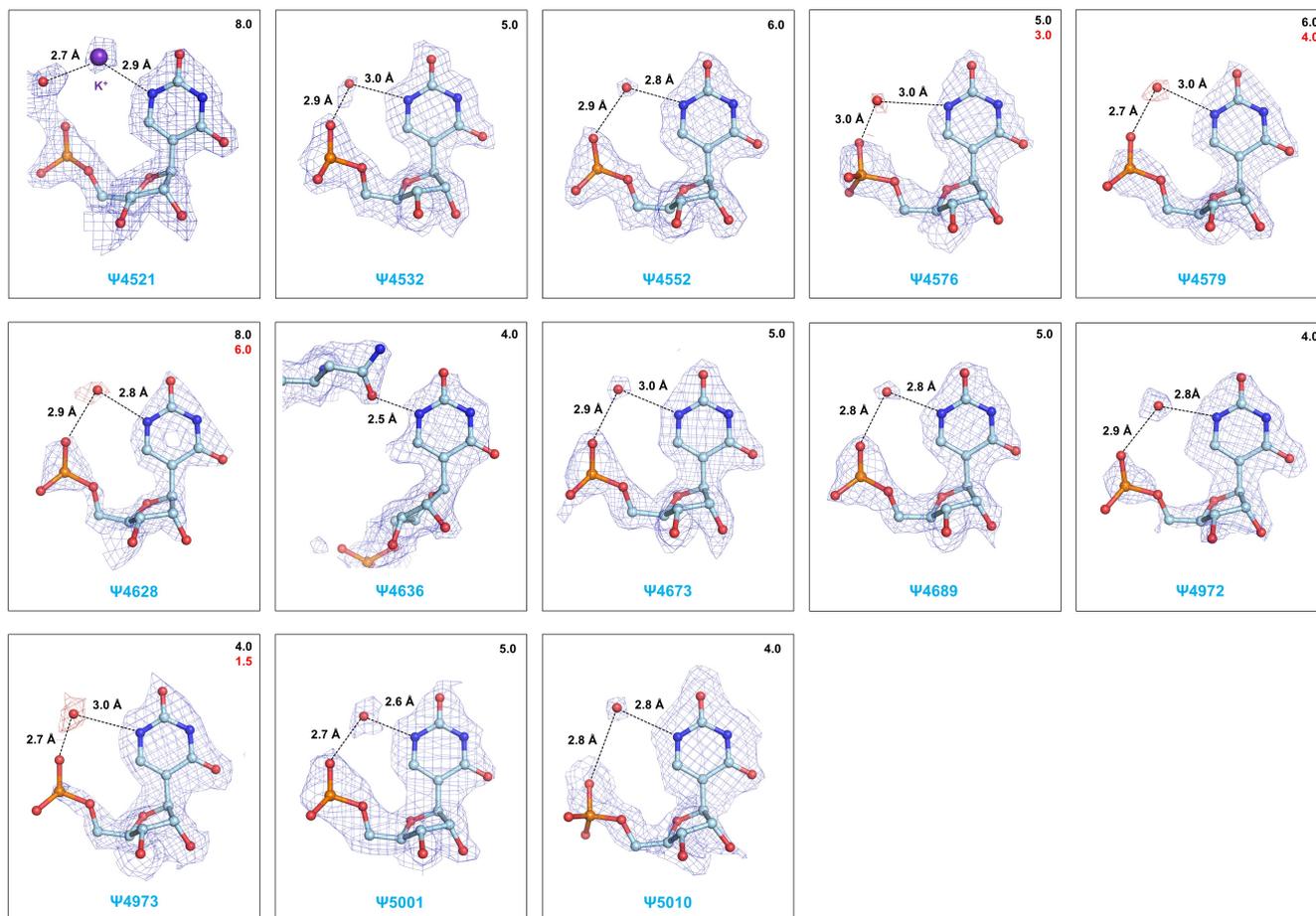
28S rRNA – pseudo-uridines



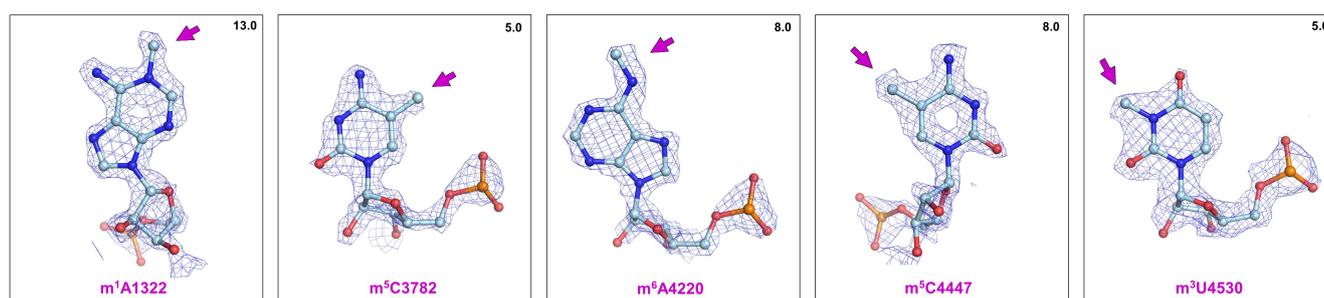
28S rRNA – pseudo-uridines (cont.)



28S rRNA – pseudo-uridines (cont.)



28S rRNA – base modifications



Chemical modifications seen in the cryo-EM map (60S ribosomal subunit)

2'-O-Me's, ψ 's & nucleotide base modifications in the 60S ribosomal subunit (human 5.8S & 28S rRNA).

Supplementary Fig. 2

MS analysis of 2'-O-Me and ψ modifications in the 18S rRNA

18S	RNase T ₁		RNase A		RNase U ₂		RNase MC1	
modification	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)
Am27	CAU[Am]UGp	975.13 (-2)						
ψ 34	[ceY]C[ceY]CAAAGp	1338.19 (-2)						
ψ 36	[ceY]C[ceY]CAAAGp	1338.19 (-2)						
ψ 63			AAG[ceY]ACp	1006.14 (-2)				
Am99	CUC[Am][ceY]UAAUACAGp	1403.87 (-3)			CUC[Am][ceY]UA>p	1125.64 (-2)		
ψ 100	CUC[Am][ceY]UAAUACAGp	1403.87 (-3)			CUC[Am][ceY]UA>p	1125.64 (-2)		
ψ 105	CUC[Am]UUAUA[ceY]CAGp	1403.85 (-3)						
ψ 109	[ceY]UAUGp	830.59 (-2)						
Um116	U[Um]CC[ceY]U[Um]Gp	1291.15 (-2)						
ψ 119	U[Um]CC[ceY]U[Um]Gp	1291.15 (-2)						
Um121	U[Um]CC[ceY]U[Um]Gp	1291.15 (-2)						
Am159	UA[Am]UUCUAGp	1445.67 (-2)			[Am]UUCUA>p	946.6 (-2)		
Am166	AG[Am]GCp	841.62 (-2)						
Um172	CUAA[Um]A[Um]AUGp	1077.47 (-3)	AA[Um]A[Um]AUp	1138.7 (-2)				
Cm174	CUAA[Um]A[Um]AUGp	1077.47 (-3)	AA[Um]A[Um]AUp	1138.7 (-2)				
ψ 210			GGGGGGGA[ceY]p	1039.51 (-3)				
ψ 218	CA[ceY]UUAUACAGp	1078.16 (-3)						
ψ 296	ACUC[ceY]AGp	1147.22 (-2)						
ψ 300	A[ceY]AACCUCGp	1464.29 (-2)						
ψ 366	CCCUAUCAAC[ceY]UUCGp	1586.96 (-3)						
Um428	AU[Um]CCGp	963.12 (-2)						
Gm436	GGAGA[Gm]GGAGCp	1245.55 (-3)						
Cm462	CUACCACAU[Um]CAAGp	1487.21 (-3)						
Am468			AAGG[Am]AGGCp	1515.79 (-2)				
Am484	C[Am]AAUUAACCACUCCCG>p	1339.68 (-4)						
Gm509			GGGGA[Gm]GUp	1367.75 (-2)				
Am512	[Am]G[ceY]GA[Um]GAAAAUp	1450.54 (-3)						
ψ 514	[Am]G[ceY]GA[Um]GAAAAUp	1450.54 (-3)						
Cm517	[Am]G[ceY]GA[Um]GAAAAUp	1450.54 (-3)						
ψ 556	UAA[ceY]UGp	995.12 (-2)						
Am590	[Am]UCCAUUGp	1280.66 (-2)	GAGG[Am]Up	1014.68 (-2)	[Am]UCCA>p	793.09 (-2)		
Gm601			GGAG[Gm]GCp	1194.73 (-2)				
Um627	[Um]AAUUCAGp	1445.19 (-2)	GG[Um]AAUp	995.17 (-2)				
Gm644	CUCCAAU[Um]Gp	1179.49 (-3)						
ψ 649	UA[ceY]A[ceY]UAAAGp	1111.81 (-3)						
ψ 651	UA[ceY]A[ceY]UAAAGp	1111.81 (-3)						
ψ 667	U[ceY][Am]AAAAGp	1342.7 (-2)	[ceY][Am]AAAAGCp	1342.25 (-2)				
Am668	U[ceY][Am]AAAAGp	1342.7 (-2)	[ceY][Am]AAAAGCp	1342.25 (-2)			U[Am]AAAAGC>p	1306.68 (-2)
ψ 686	A[ceY]CUUGp	983.11 (-2)						
ψ 769/770 ^a	CUC[ceY]UAGp or CUCU[ceY]AGp	1135.7 (-2)						
Cm797			GAAG[Um]G[Um]Up	1327.23 (-2)				
Um799	[Um]U[ceY]AC[ceY]UUGp	1475.76 (-2)	GAAG[Um]G[Um]Up	1327.23 (-2)				
ψ 801	[Um]U[ceY]AC[ceY]UUGp	1475.76 (-2)						
ψ 804	[Um]U[ceY]AC[ceY]UUGp	1475.76 (-2)						
ψ 814	AAAAAA[ceY][ceY]AGp	1127.22 (-3)	GA[Um]AAAA[ceY]p	1347.26 (-2)				
ψ 815	AAAAAA[ceY][ceY]AGp	1127.22 (-3)						
ψ 822	UG[ceY]UCAAGp	1484.78 (-2)						
ψ 863	AA[ceY]AA[ceY][Gm]Gp	1377.29 (-2)	AGGAA[ceY]p	1026.19 (-2)				
ψ 866	AA[ceY]AA[ceY][Gm]Gp	1377.29 (-2)						
Gm867	AA[ceY]AA[ceY][Gm]Gp	1377.29 (-2)						
ψ 918	AU[ceY]AAGp	1006.68 (-2)						
ψ 966	AAAU[ceY]CUUGp	1465.28 (-2)						
ψ 1002/1003 ^b	CAU[ceY][ceY]Gp	1009.68 (-2)						
ψ 1004	CAU[ceY][ceY]Gp	1009.68 (-2)						
Am1031			AAGA[Am]Cp	998.21 (-2)				
ψ 1045			GGAGG[ceY]p	1042.18 (-2)				
ψ 1061	A[ceY]ACCGp	994.18 (-2)						
ψ 1081	ACCA[ceY]AAACGp	1093.21 (-3)						
ψ 1186			[ceY]GAAACp	1006.2 (-2)				
Um1288			AGGA[Um]Up	995.16 (-2)				
ψ m132e ^b			GG[Um]G[Um]Up	1018.17 (-2)				
Gm1328			GG[Um]G[Um]Up	1018.17 (-2)				
ψ 1358/1359/1360 ^a	AUU[ceY]Gp	830.63 (-2)						
Am1383							UAACGA[Am]CGAGAC>p	1413.83 (-3)
Cm1391	ACU[Um]UGp	963.17 (-2)			CU[Um]UGG>p	962.11 (-2)		
Um1442					CU[Um]CU[ceY]A>p	1114.13 (-2)		
ψ 1445					CU[Um]CU[ceY]A>p	1114.13 (-2)		
Gm1447			A[Gm]AGGGACp	1351.27 (-2)				
Gm1490	CAAUAA[Um]Gp	1093.48 (-3)						
ψ 1596	AACCCAU[ceY]CGp	1179.22 (-3)						
ψ 1625	CAAUUAU[ceY]CCCCAUGp	1594.87 (-3)			U[ceY]CCCCA>p	1106.14 (-2)		
ψ 1643	[m ^b G]AAU[ceY]CCCAG>p	1089.47 (-3)						
Am1678	AUU[Am]AGp	987.14 (-2)					U[Am]AGUCCC>p	1271.16 (-2)
Cm1703					CCG[Um]CCGUCGUA>p	1374.17 (-3)		
Um1804	AACU[Um]Gp	975.12 (-2)						
ψ 1839					U[Y]UC[ac4C]G>p	983.1 (-3)		

^aThe position of the cyanoethylation could not be determined by fragmentation spectra, but the residues are **resolved** in the structure: ψ 1358/1359/1360; ψ 1002/1003.

^bThe cyanoethylated precursor ion was absent from the MS analysis suggesting a Um instead of ψ m.

MS analysis of 2'-O-Me and ψ modifications in the 28S rRNA

28S	RNase T ₁		RNase A		RNase U ₂		RNase M1	
modification	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)
Am398			GAAG[Am]G[Am]GAGUp	1239.84 (-3)				
Am400			GAAG[Am]G[Am]GAGUp	1239.84 (-3)				
Gm1316	ACCC[Gm]UCUUGp	1062.13 (-3)			CCC[Gm]UCUUG>p	1420.15 (-2)		
Am1323	[m ¹ A][Am]AC[Am]CGp	1164.68 (-2)						
Am1326	[m ¹ A][Am]AC[Am]CGp	1164.68 (-2)						
Cm1340	U[Cm]UAACACGp	1444.69 (-2)						
Gm1522	ACCC[Gm]A[Am]AGp	1482.72 (-2)	[Gm]A[Am]AGUp	1178.17 (-2)	CCC[Gm]Ap	809.61 (-2)	UAGGACCC[Gm]A[Am]AGA>p	1533.56 (-3)
Am1524	ACCC[Gm]A[Am]AGp	1482.72 (-2)	[Gm]A[Am]AGUp	1178.17 (-2)			UAGGACCC[Gm]A[Am]AGA>p	1533.56 (-3)
Am1534	A[Am]C[ceY]AUUp	1166.17 (-2)			[Am]C[ceY]Ap	676.10 (-2)		
ψ1536	A[Am]C[ceY]AUUp	1166.17 (-2)			[Am]C[ceY]Ap	676.10 (-2)		
ψ1677	UUUCCC[ceY]CAGp	1062.11 (-3)						
ψ1683			AGGA[ceY]p	861.61 (-2)				
ψ1779	AUCUCAACC[ceY]A[ceY][ceY]C	1488.51 (-3)						
ψ1781	AUCUCAACC[ceY]A[ceY][ceY]C	1488.51 (-3)						
ψ1782	AUCUCAACC[ceY]A[ceY][ceY]C	1488.51 (-3)						
ψ1860	CCAC[ceY]U[ceY]UGp	978.11 (-3)						
ψ1862	CCAC[ceY]U[ceY]UGp	978.11 (-3)						
Am1871			[Am]GAACp	833.63 (-2)				
Gm1946			AGAAAAG[Gm]Up	1508.23 (-2)				
Cm2351	AU[Cm]UUGp	963.6 (-2)						
Am2363	U[Am][Gm][Cm]AAAUUCAAACG>p	1374.93 (-4)	[Am][Gm][Cm]AAUUp	1165.18 (-2)	U[Am][Gm][Cm]Ap	836.13 (-2)	U[Am][Gm][Cm]AAA>p	1156.17 (-2)
Gm2364	U[Am][Gm][Cm]AAAUUCAAACG>p	1374.93 (-4)	[Am][Gm][Cm]AAUUp	1165.18 (-2)	U[Am][Gm][Cm]Ap	836.13 (-2)	U[Am][Gm][Cm]AAA>p	1156.17 (-2)
Cm2365	U[Am][Gm][Cm]AAAUUCAAACG>p	1374.93 (-4)	[Am][Gm][Cm]AAUUp	1165.18 (-2)	U[Am][Gm][Cm]Ap	836.13 (-2)	U[Am][Gm][Cm]AAA>p	1156.17 (-2)
Am2401			GG[Am]GAAGGUp	1130.82 (-3)				
Um2415			G[Um]GAACp	994.64 (-2)			UG[Um]GAACAG[Cm]A[Gm]U>p	1421.2 (-3)
Cm2422	[Cm]A[Gm]UUGp	990.12 (-2)	AG[Cm]A[Gm]Up	1001.64 (-2)			UG[Um]GAACAG[Cm]A[Gm]U>p	1421.2 (-3)
Gm2424	[Cm]A[Gm]UUGp	990.12 (-2)	AG[Cm]A[Gm]Up	1001.64 (-2)			UG[Um]GAACAG[Cm]A[Gm]U>p	1421.2 (-3)
ψ2632	UUUCUUU[ceY]UCUUUUp	1462.81 (-3)						
Am2787	UACCC[Am]UAUCCGp	1268.15 (-3)						
Cm2804	UCU[Cm]CAAGp	1280.16 (-2)						
Am2815	AAC[Am]Gp	833.63 (-2)						
Cm2824			GG[Cm]AUUp	830.12 (-2)				
Um2837	AACAA[Um]Gp	1151.16 (-2)						
Gm2876	UAACUUC[Gm]Gp	1453.17 (-2)						
Gm3627			AGG[Gm]GAAUp	1351.69 (-2)				
ψ3695	AUU[ceY]CUUp	1136.13 (-2)						
Cm3701	CC[Cm]AGp	809.61 (-2)						
ψ3715	[ceY]CA[Am]AGp	1013.15 (-2)						
Am3718	[ceY]CA[Am]AGp	1013.15 (-2)	A[Am]AGUp	834.11 (-2)				
Am3724			GA[Am]GAAUUp	1335.68 (-2)				
Gm3744	[Gm]UAAACGp	1159.15 (-2)						
ψ3758	[ceY]A[Am]C[ceY]AUUp	1345.68 (-2)	GGGAG[ceY]p	1042.15 (-2)				
Am3760	[ceY]A[Am]C[ceY]AUUp	1345.68 (-2)			[Am]C[ceY]A[ceY]GA>p	1183.66 (-2)		
ψ3762	[ceY]A[Am]C[ceY]AUUp	1345.68 (-2)			[Am]C[ceY]A[ceY]GA>p	1183.66 (-2)		
ψ3764	UA[Am]C[ceY]A[ceY]Gp	1345.68 (-2)			[Am]C[ceY]A[ceY]GA>p	1183.66 (-2)		
ψ3768	AC[ceY]C[ceY]CUUAAAGp	1197.49 (-3)			C[ceY]C[ceY]CUUA>p	1286.15 (-2)		
ψ3770	AC[ceY]C[ceY]CUUAAAGp	1197.49 (-3)			C[ceY]C[ceY]CUUA>p	1286.15 (-2)		
Am3785	C[m ¹ C]AA[Am]UUp	1146.16 (-2)						
Gm3792	CCUC[Gm]UCAUCUAAUAGp	1348.92 (-4)			CUC[Gm]UCA>p	1106.65 (-2)		
Cm3808			GA[Cm]GCP	829.61 (-2)			UAGUGA[Cm]GCGCA>p	1301.83 (-3)
ψ3822			GAA[ceYm]GGAUp / GAA[Um]GGA[ceY]p	1358.68 (-2)				
Am3830	[Am]UUUCCACUUp	1056.8 (-3)	GAG[Am]Up	842.11 (-2)	[Am]UUUCCA>p	1098.63 (-2)		
Cm3841	U[Cm]CC[ceY]ACCUACUA[ceY]CCAG>p	1436.92 (-4)						
ψ3844	U[Cm]CC[ceY]ACCUACUA[ceY]CCAG>p	1436.92 (-4)						
ψ3851	U[Cm]CCUACCUAC[ceY]AUCCAG>p	1423.69 (-4)						
ψ3853	U[Cm]CC[ceY]ACCUACUA[ceY]CCAG>p	1436.92 (-4)						
Am3867	AAACCAC[Am]Gp	1467.72 (-2)						
Cm3869	[Cm]CAAGp	821.62 (-2)						
Cm3887	GG[Cm]GGAUUp	1339.68 (-2)						
Gm3899	GG[Gm]GAAAGAAGCp	1459.53 (-3)						
Um3925	AC[Um]CUAG>p	1118.63 (-2)						
Gm3944	AA[Gm]AGp	853.63 (-2)	GAA[Gm]AGACp	1343.21 (-2)				
Cm4054	AAAUACCA[Cm]UACUCUG>p	1269.66 (-4)					UACCA[Cm]UAC>p	1415.66 (-2)
Gm4196			G[Gm]GGCp	857.63 (-2)				
Um4227	[Um][Gm]UCCUAAAGp	973.12 (-3)	AGG[Um][Gm]Up	1010.13 (-2)				
Gm4228	[Um][Gm]UCCUAAAGp	973.12 (-3)	AGG[Um][Gm]Up	1010.13 (-2)				
ψ4299	A[ceY]UUUCAGp	1300.64 (-2)						
Um4306			AG[Um]ACp	822.11 (-2)				
ψ4312	AA[ceY]ACAGp	1170.67 (-2)						
ψ4361	UUU[ceY]AAGp	1148.13 (-2)						
Gm4370			AGGA[Gm]GUp	1187.16 (-2)				
Gm4392			AG[Gm]GAUUp	1014.64 (-2)			UACCACAG[Gm]GA>p	1194.49 (-3)
ψ4420	U[ceY]CAUUG	1147.64 (-2)						
ψ4423	UUCA[ceY]UAG	1147.64 (-2)						
ψ4442	A[ceY]CCUU[m ¹ C]Gp	1295.15 (-2)						
Cm4456	[Cm][ceY]CUUCCUUAUUAUUG>p	1578.19 (-3)			[Cm][ceY]CUUCCUA>p	1419.16 (-2)		
ψ4457	[Cm][ceY]CUUCCUUAUUAUUG>p	1578.19 (-3)			[Cm][ceY]CUUCCUA>p	1419.16 (-2)		
Um4498	AU[Um][Gm][ceY]UACCCACUAAUAG>p	1525.68 (-4)			U[Um][Gm][ceY]UCAp	1150.15 (-2)		
Gm4499	AU[Um][Gm][ceY]UACCCACUAAUAG>p	1525.68 (-4)			U[Um][Gm][ceY]UCAp	1150.15 (-2)		
ψ4500	AU[Um][Gm][ceY]UACCCACUAAUAG>p	1525.68 (-4)			U[Um][Gm][ceY]UCAp	1150.15 (-2)		
ψ4532	[m ¹ U]U[ceY]AGp	837.61 (-2)						
Cm4536			AGA[Cm]Cp	821.62 (-2)				
Am4590	UA[Am]UCCUG>p	1271.66 (-2)						
Gm4618	[Gm]U[Um]CA[Gm]ACAU[ceY]UG>p	1418.19 (-3)			[Gm]U[Um]CAp	817.61 (-2)		
Um4620	[Gm]U[Um]CA[Gm]ACAU[ceY]UG>p	1418.19 (-3)			[Gm]U[Um]CAp	817.61 (-2)		
Gm4623	[Gm]U[Um]CA[Gm]ACAU[ceY]UG>p	1418.19 (-3)						
ψ4628	[Gm]U[Um]CA[Gm]ACAU[ceY]UG>p	1418.19 (-3)						
ψ4636	UA[ceY][Gm]UUG>p	1001.12 (-2)						
Gm4637	UA[ceY][Gm]UUG>p	1001.12 (-2)						
ψ4673	CUACCA[ceY]CUUp	1069.8 (-3)						
ψ4972	CUAAACCA[ceY][ceY]CGp	1306.84 (-3)						
ψ4973	CUAAACCA[ceY][ceY]CGp	1306.84 (-3)						
ψ5039	AUCUA[ceY]UGp	1300.64 (-2)						

MS analysis of 2'-O-Me and ψ modifications in the 5.8S rRNA

5.8S	RNase T ₁		RNase A		RNase U ₂		RNase MC1	
modification	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)
Um14			GG[Um]GGAUp	1175.69 (-2)				
ψ 55	C[ceY]Gp	1026.14 (-1)						
ψ 69								
Gm75	AAUU[Gm]CAGp	1312.17 (-2)			UU[Gm]CA>p	801.59 (-2)		

MS analysis of nucleotide base modifications in the 18S rRNA

18S	RNase T ₁		RNase A		RNase U ₂		RNase MC1	
modification	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)
m ² acp ³ ψ 1248	AC[ce ^m acp ³ Y]CAACACGp	1123.5 (-3)						
ac ⁴ C1337					C[ac ⁴ C]GUUCUUA>p	1426.69 (-2)		
m ⁷ G1639	[m ⁷ G]AAU[ceY]CCCAG>p	1089.47 (-3)	GAG[m ⁷ G]AAUp	1179.22 (-2)				
m ⁵ A1832	UA[m ⁵ A]CAAGp	1151.18 (-2)						
ac ⁴ C1842	UUUC[ac ⁴ C]Gp	965.6 (-2)			UUUC[ac ⁴ C]G>p	956.59 (-2)		
m ⁵ ₂ A1850	[m ⁵ ₂ A][m ⁵ ₂ A]CCUGp	995.67 (-2)	G[m ⁵ ₂ A][m ⁵ ₂ A]Cp	690.14 (-2)	UG[m ⁵ ₂ A][m ⁵ ₂ A]CCUGCGGAA>p	1425.55 (-3)		
m ⁵ ₂ A1851	[m ⁵ ₂ A][m ⁵ ₂ A]CCUGp	995.67 (-2)	G[m ⁵ ₂ A][m ⁵ ₂ A]Cp	690.14 (-2)	UG[m ⁵ ₂ A][m ⁵ ₂ A]CCUGCGGAA>p	1425.55 (-3)		

MS analysis of nucleotide base modifications in the 28S rRNA

28S	RNase T ₁		RNase A		RNase U ₂		RNase MC1	
modification	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)	Sequenced fragment	measured m/z (z)
m ¹ A1322	[m ¹ A][Am]AC[Am]CGp	1164.68 (-2)						
m ³ C3782	C[m ³ C]AA[Am]UGp	1146.16 (-2)						
ψ m3818	AA[ceYm]Gp	696.1 (-2)	GAA[ceYm]GGAUp / GAA[Um]GGA[ceY]p	1358.68 (-2)				
m ⁴ A4220	UA[m ⁴ A]CGp	822.11 (-2)						
m ³ C4447	A[ceY]CCUU[m ³ C]Gp	1295.15 (-2)						
m ³ U4530	[m ³ U]U[ceY]AGp	837.61 (-2)						

Mass spectrometry analysis of 18S, 28S, 5.8S & 5S rRNA fragments with 2'-O-Me's, ψ 's & nucleotide base modifications

Supplementary Fig. 3

2001 GAGUGUGUAA CAACUCACCU GCCGAUCA CUAGCCUGA AAAUGGAUG CGCUGGAGCG UCGGGCCAU ACCCGCCGU CGCCGGCAGU CGAGAGUGGA

2101 CGGGAGCGGC GCGCGCGCG CGCGUGUGGU GUGCGUCGGA GGGCGCGGC GCGCGCGCG GCGGGGUGU GGGGUCCUUC CCCC GCCC

2201 CCCCCACGC CUCCUCCCU CCUCCGCC ACGCCCGCU CCCC GCCC GGAGCCCGC GGACGCUACG CCGCAGCAG UAGGAGGCG GCUCGGUGA

2301 GCCUUGAAGC CUAGGGCGCG GCGCGGGUG GAGCCCGCG AGGUGCAGAU CmUUGGUGUA GUAmGmCmAAUA UUCAACGAG AACUUUGAAG GCCAAGUGG

2401 AmGAAGGGUUC CAUGUmGAACA GmAGmUUGAAC AUGGGUCAGU CGGUCCUGAG AGAUGGGCGA GCGCCGUUC GAAGGACCG GCGAUGGCC CCGUUGCCU

2501 CGGCCGAWCG AAAGGGAGUC GGUUCAGAU CCCC GAUCC GAGUGGCG AGAUGGGCG CCGAGGCGU CCAGUGCGGU AACGCGACG AUCCCGGAGA

2601 AGCCCGCGG AGCCCCGGG AGAGUUCUCU UUCUUCUGU AAGGGCAGG CCCCUGGAA UGGGUUCGCC CCGAGAGAGG GCGCCGUGCC UUGGAAAGCG

2701 UCGCGGUUC GCGCGGUC GUGAGCUCU CGCUGGCCU UGAAAUCG GGGGAGAGG UGUAAAUC GCGCGGGCC GUACCCAmUAU CCGCAGCAGG

2801 UCUmCAAGGU GAACAmGCCUC UGGCmAUUGU GAACAAUmGWA GGWAAGGAA GUCGGCAAG CmGGAUCCGUA ACUUCGmGGAU AAGGAUUGG UCUAAGGGU

2901 GGGUCGGUC GCGUGGGCG CGAAGCGGG CUGGGCGCG GCCCGGGC GACGAGGCG CCGCCGCC CCCACGCC GGGCACCCC CUCGCGGCC

3001 UCCCCGCC CACCCCGC GCGCCGUC CUCUUC CCCCCGCC CUCUCUCU CUCUCUCC CGCUCCCGU CCUCCCCCU CCCC GGGG

3101 GCGCGGUG GGGCGCGG GGGGGGAGA AGGGUCGGG CGGAGGGG CCGCGCGG CCGCCCGG GCGCGCGG CGGGGACG GUCCCCCG

3201 AGGGGGCCC GGGCACCG GGGGCCGG GCGCGCGGA CUCUGGAGC GAGCCGGCC CUUCCGUGG AUCGCCAG CUGCGCGGG CGUCGCGCC

3301 GCCCCGGG AGCCCGCG GCGCCGCG GCCCCCCC CCACCCAG UCUCGUCG CCGCGUCG CUGGGGGCG GGAGCGGUC GGGCGCGG

3401 GUCGGCGGG GCGGGGGCG GCGGUUCGU CCCCCGCC UACCCCCG GCGCGUCG CCCCCGU CCCCCUC CUCGGCGCG GGGCGCGG

3501 GCGGAGGCG GCGGAGGCG CGCGGGCG UCCCCCGC GGGUCCGCC CCGGGGCC CGGUCCCG CCGCGCCU CCUCGCGGG CGCUAGCAG

3601 CCGACUAGA ACUGGUGCG ACCAGGmGAA UCCGACWGU UAAUAAAAC AAAGCAUCG GAAGGCCCG GCGGGGUGU GACGGAUGU GAUWUCG

3701 CmAGUUCUC AAUGWCAmAG UGAAmGAAUW CAAWGAAGCG CGGmUAAAC GCGGGWAm CWAWGAWCW CUUAAGGUAG CCAAmUGCCU CmUCAUCUA

3801 UUAGUGAmC GCAUGAAWmG AWGAAmCGAm UUCCACUG CmCWACCUAC WAWCCAGCA AACCAmGmC AAGGAACCG GCUWGGmGA AUCAGGGmG

3901 AAAGAAGAC CUGUUGAGCW UGACUmCUAGU CUGGCACGG GAAmAGACAU GAGAGGWA GAUAAGUG GAGGCCCG GCGCCCCC GGUGUCCCG

4001 CGAGGGCCC GGGCGGGU CCGCCGCC UCGGGCGC CmGUGAAUA CCAmUACUC GAUCGUUUU UCACUGACC GGUAGGCG GGGGCGAGC

4101 CCCGAGGGC UCUCGUUC GCGCCAAG GCCCGCGC GCGCGCGG GCGCGACC GCUCCGGGA CAGUGCCAG UGGGAGUU GACUGmGGC

4201 GUACACCUU CAAACGUAA CGCAGUmGmUC CUAAGCGAG CUCAGGAGG ACAGAAACU CCCUGGAGC AGAAGGGCA AAGCUCU GAWCUWGAW

4301 UUCAGUmACGA AWACAGACG UGAAAGCG GCCUCAGAU CCUUCUGACC UUWUGGUUU WAAAGCAGm GUGUCAGAA AGUUAACA GmGAUAACU

4401 GCWUGGCG GCCAAGCW CAWAGCAGC WCGCUUUUG AWCCUUCGA UCGGmWCU CCUAUCAU WWAAGCAGAA UUCACCAAG GUWmGAUUmGmW

4501 UCACCCACUA AUAGGGAAC WAmGCUGGU UWAGAmCmGUC GUGAGACAG UWAGUUUAC CCUACUGAU AmUGUWUGWU GCCAUGGUAm UCCUGCUCAG

4601 UACGAGAGGA ACCGAGmUUm CAmACAUWUG GUGUAWmUG. UUGCGAGG AGCCAAUG GCGAAGCU CAWCUUGGG AUUAUGACW AACCCUCUA

4701 AGUCAGAUC CCGCCAGG GGAACGAUC GGCAGCGC GAGGCCUC GUUGGCCUG GAUAGCCGU CCCCCCUG UCCCCCGG CGGGCGCCC

4801 CCCCCUCA CCGCCCCG GCGCGGGGA GGGCGGUC CCGCCCGC GCGGGACC GGGUCCGUG CCGAGUGCC UUCGUCCUG GAAACGGGC

4901 GCGCGGGA AGCGCGCG CCCCUGCC GUCACGACC GCAGUUCGU GGGGAACUG GCGUAAAC AWWCUAGAG GACCUCUUC UGGGCGGG

5001 WUUCGUACW AGCAGAGCAG CUCCUCGCU GCGAUUAU UGAAAGUCAG CCCUCGAC AAGGUUUU C

5.8S rRNA sequencing with annotations of chemically modified nucleotides

1 CGACUCUAG CCGUmGGAUCA CUCGGUCGU GCGUGAUGA AGAACGCAG UAGCWGCGAG AAUUAUGW AAUUmCAGGA CACAUUGAUC AUCGACACU

101 CGAACGCACU UCGGGCCCCG GGUUCCUCC GGGGCUACG CUGUCUGAG GUCGCUU

5S rRNA sequencing (contains no chemical modifications)

1 GUCUACGGCC AUACCACCU GAACGCGCC GAUCUCGUC GAUCUCGAA GCUAAGCAG GUCGGGCCUG GUUAGUACU GGAUGGGAGA CCGCCUGGA

101 AUACGGGGUG CUGUAGGCUU

18S, 28S, 5.8S & 5S rRNA sequencing with annotations of chemically modified nucleotides

A4910 is indicated in red (see **Extended Data Fig. 8**); sequences in grey were not covered.

Supplementary Fig. 4