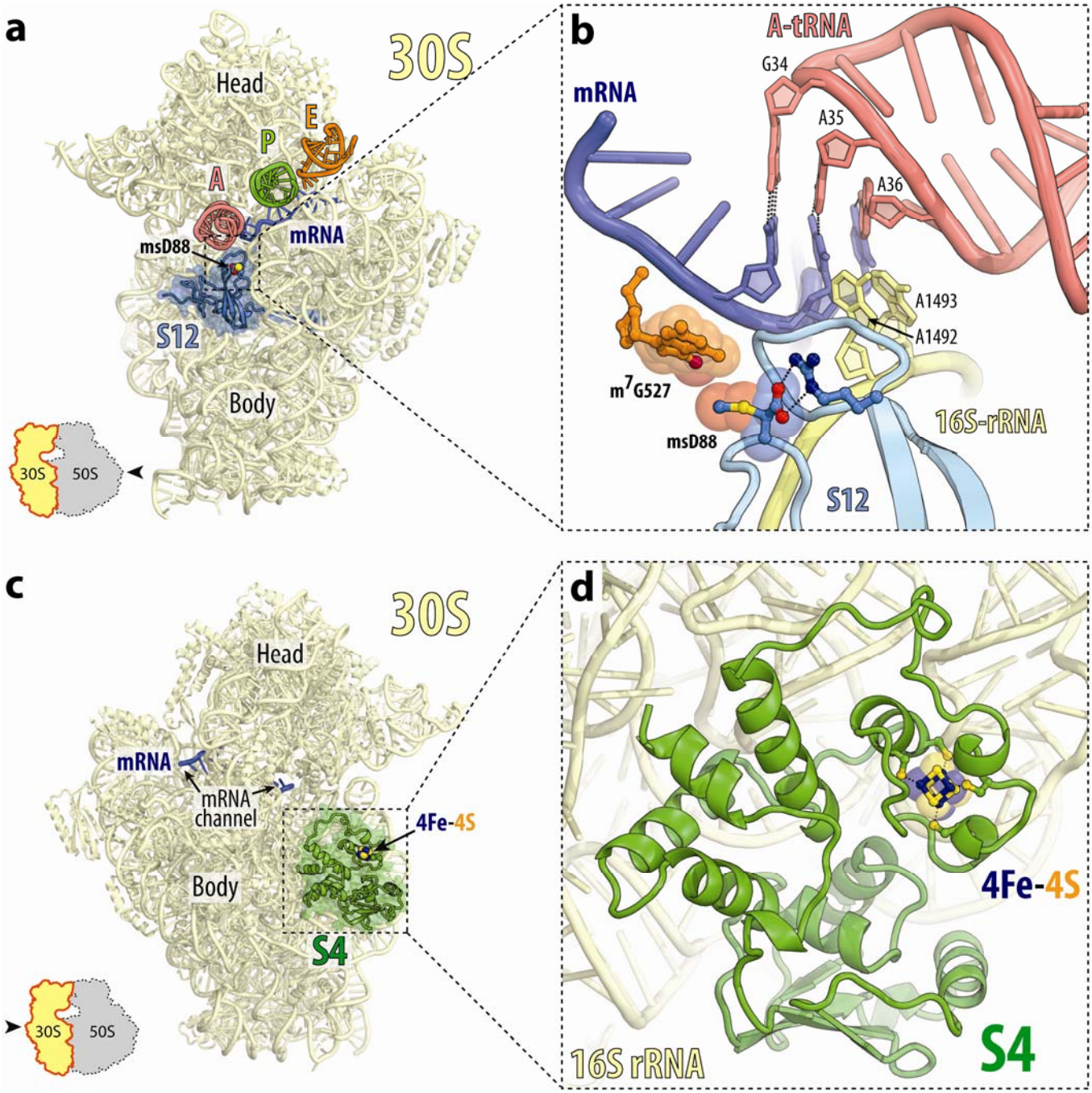
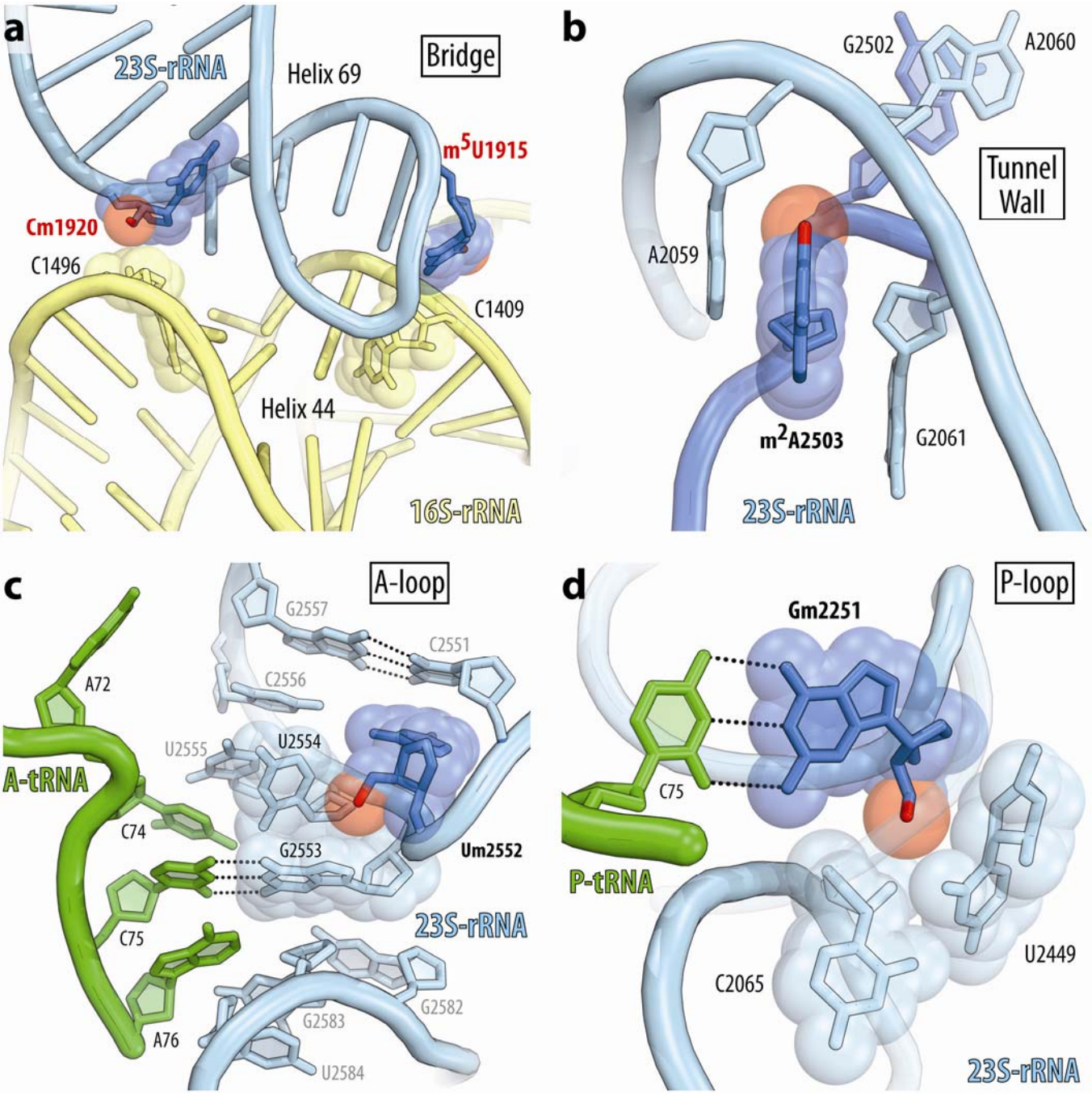


Supplementary Figure 1

Post-translational modification of ribosomal protein S12 and prosthetic group of protein S4.

(**a, b**) β-methyl-thiolation of Asp88 (ms-Asp88) in ribosomal protein S12 is a bacteria-specific and essential modification with unknown role in protein synthesis (Kowalak, J.A. & Walsh, K.A. Beta-methylthio-aspartic acid: identification of a novel posttranslational modification in ribosomal protein S12 from Escherichia coli. *Protein Sci*. 5, 1625-1632, 1996). We show that this post-translational modification establishes an additional RNA-protein contact, mediated by the N7-methyl group of the modified nucleotide m^7G527. This interaction, observed in a close proximity to the decoding center, suggests that the essential function of msD88 might be related to the

assembly of the decoding center and that the effect of msD88 on the ribosome structure may depends on methylation of m^7G527. (**a**) The 30S subunit is shown in light yellow with protein S12 highlighted in light blue. (**b**) Close-up view of panel (**a)**. (**c, d**) The iron-sulfur cluster coordinated by protein S4 has been recently identified in a crystallographic study of *T. thermophilus* ribosomes (for details see Online Methods section in (Polikanov, Y.S., Steitz, T.A. & Innis, C.A. A proton wire to couple aminoacyl-tRNA accommodation and peptide-bond formation on the ribosome. *Nat. Struct. Mol. Biol*. 21, 787-793, 2014)). It is located in the vicinity of the mRNA entrance tunnel on the cytoplasmic side of the small ribosomal subunit. The covalent association of the 4Fe-4S cluster with protein S4 is mediated by four cysteine residues (Cys9, Cys12, Cys26, and Cys31) in the N-terminal globular domain. Presence of the iron-sulfur cluster within *T. thermophilus* ribosomes is consistent with metabolism of these bacteria that require high concentration of iron for optimal growth. It also explains why samples of pure ribosome have a yellow/red color. Importantly, it has been reported previously that protein S4 can associate with RNA polymerase *in vivo* (Torres, M., Condon, C., Balada, J.M., Squires, C. & Squires, C.L. Ribosomal protein S4 is a transcription factor with properties remarkably similar to NusA, a protein involved in both non-ribosomal and ribosomal RNA antitermination. *Embo J*. 20, 3811-3820, 2001). Although the function of this prosthetic group has not been examined, it is appealing to propose that protein S4 can mediate the ribosome interaction with RNA-polymerase during the coupled transcription-translation, where iron-sulfur cluster stabilizes the complex at high temperatures. Location of protein S4 on the 30S subunit is viewed from the cytoplasm with the 50S subunit removed, as indicated by the inset. (**c**) The 30S subunit is shown in light yellow with protein S4 highlighted in green. The mRNA is shown in blue, and arrows are pointing to its entrance and exit channels. (**d**) Close-up view of panel (**c**).



Supplementary Figure 2

Modified nucleotides form molecular contacts between the ribosomal subunits and within the ribosome interior.

(**a**) 2'-O-methyl groups of m^5U1915 and Cm1920 of the 23S rRNA at the interface between the ribosomal subunits. Being specific to *T. thermophilus*, these modifications might adjust the strength of inter-subunit interactions at high temperatures. (**b**) m^2A2503 methylation extends the stacking surface between the nucleotides A2059 and A2503 that maintains the fold of the two single-stranded rRNA segments forming the wall of the peptide exit tunnel. (**c**) The 2'-O-methyl group of the Um2552 of the 23S rRNA introduces hydrophobic contacts in the A-loop with the key residue G2553 that is involved in accommodation of the aminoacyl-tRNA. (**d**) Similarly to the A-loop,

the 2’-O-methyl group of Gm2251 fills the cavity between the sugar of C2065 and the base of U2449 in the P-loop of the 23S rRNA.

I. SUPPLEMENTARY TABLES

Supplementary Table 1 | Summary of rRNA modifications within the *T. thermophilus* ribosome. *T. thermophilus* ribosomes comprise 23 nucleotide modifications, five of which are conserved across bacteria, archaea and eukaryotes and five are specific to *T. thermophilus* ribosomes (compared to *E. coli* ribosomes and highlighted below in red). Interactions formed by rRNA modifications are briefly summarized using *E. coli* numbering of rRNA nucleotides.

Modifications of 16S rRNA

Thermus thermophilus	*Escherichia coli*	Name	Conservation	Vicinity to the functional centers	Interactions
Ψ500	Ψ516	Pseudouridine	B	mRNA channel latch	-
m^7G511	m^7G527	N7-Methylguanosine	B	Decoding center	Interacts with the modified msAsp88 of ribosomal proteins S12
m^2_2G944	m^2_2G966	N2-Dimethylguanosine	B	P site	Directly contacts the P-site wobble base-pair (tRNA ribose) Interacts with Lys127 of protein S9
m^5C945	m^5C967	5-Methylcytidine	B	P site	Extends m^2_2G944-m^5C945 stacking (m^2_2G944 directly interacts with the mRNA/tRNA duplex or with the pY)
m^2G1189	m^2G1207	N2-Methylguanosine	B	mRNA channel latch	-
m^5C1383	C1400	5-Methylcytidine	B	P-site	Contacts the wobble base-pair of the P-site mRNA/tRNA duplex
m^4Cm1385	m^4Cm1402	N4,O2'-Dimethylcytidine	B/BAE	P site	Contacts mRNA or protein pY
m^5C1387	C1404	5-Methylcytidine	B	P site	Mediates C1386-C1387 stacking at the point of h44 deformation
m^5C1390	m^5C1407	5-Methylcytidine	B	P site	Extends U1389-C1390 stacking at the point of h44 deformation
m^3U1476	m^3U1498	N3-Methyluridine	B	P site	Oriented towards the C1386 WC-edge at the helix-loop junction
G1494	m^2G1516	N2-Methylguanosine	B	-	-
m^6_2A1496	m^6_2A1518	N6-Dimethyladenosine	BAE	A/P site	Extends stacking between m^6_2A1518 and m^6_2A1519 within h45 hairpin
m^6_2A1497	m^6_2A1519	N6-Dimethyladenosine	BAE	A/P site	Extends stacking between m^6_2A1518 and m^6_2A1519 within h45 hairpin

Modifications of 23S rRNA

Thermus thermophilus	*Escherichia coli*	Name	Conservation	Vicinity to the functional centers	Interactions
G791	m^1G745	N1-Methylguanosine	B	-	-
A792	Ψ746	Pseudouridine	B	-	-
U793	m^5U747	5-Methyluridine	B	-	-
C999	Ψ955	Pseudouridine	B	-	-
A1663	m^6A1618	N6-Methyladenosine	B	-	-
G1865	m^2G1835	N2-Methylguanosine	B	-	-
Ψ1932	Ψ1911	Pseudouridine	B	Helix 69	-
m^5U1936	m^3Ψ1915	5-Methyluridine	B	Helix 69	Extends U1915-A1916 stacking and faces h44 of the small subunit
Ψ1938	Ψ1917	Pseudouridine	B	Helix 69	-
Cm1941	C1920	2'-O-Methylcytidine	B	Helix 69	Directly contacts h44 of the small ribosomal subunit
m^5U1960	m^5U1939	5-Methyluridine	B	-	Extends A1937-U1939 stacking at the helix-bulge border
m^5C1963	C1942	5-Methylcytidine	B	A/P site	Extends C1941-C1942-U1943 stacking within the H70-H71 helical junction
m^5C1983	m^5C1962	5-Methylcytidine	B	A/P site	Extends G1935-C1962 stacking within the H70-H71 helical junction
A2051	m^6A2030	N6-Methyladenosine	B	-	-
G2090	m^7G2069	N7-Methylguanosine	B	-	-
Gm2262	Gm2251	2'-O-Methylguanosine	BAE	P site	Intercalates between the ribose of C2065 and the base of U2449 (G2251 is a key residue which base-pairs with the CCA-end of the P-site tRNA)
G2456	Gm2445	N2-Methylguanosine	B	-	-
U2460	D2449	5,6-Dihydrouridine	B	-	-
U2468	Ψ2457	Pseudouridine	B	-	-
C2509	Cm2498	2'-O-Methylcytidine	B	-	-
m^2A2514	m^2A2503	2-Methyladenosine	BAE	Peptide tunnel	Extends A2059-A2503 stacking between two segments of single-stranded rRNA
U2515	Ψ2504	Pseudouridine	B	-	-
Um2563	Um2552	2'-O-Methyluridine	BAE	A site	Intercalates between the bases of U2554 and G2553 (G2553 is a key residue which base-pairs with the CCA-end of the A-site tRNA)
U2591	Ψ2580	Pseudouridine	B	-	-
U2615	Ψ2604	Pseudouridine	B	-	-
Ψ2616	Ψ2605	Pseudouridine	B	-	Coordinates water molecule between the base and the phosphate using pseudouridine-specific H-bonds

Supplementary Table 2

Data collection and refinement statistics

	70S–pY[a]	70S–mRNA–tRNAs[a]
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions		
a, *b*, *c* (Å)	209.16, 448.37, 618.12	209.70, 450.05, 624.09
Resolution (Å)	309–2.30 (2.36–2.30)	312–2.50 (2.56–2.50)
R_{merge}	15.1 (186.6)	14.1 (111.6)
$I / \sigma I$	8.50 (0.85)[b]	8.27 (0.90)[c]
Completeness (%)	97.6 (81.4)	97.9 (88.5)
Redundancy	5.60 (4.70)	3.28 (2.52)
Refinement		
Resolution (Å)	173.22–2.32	122.01–2.50
No. reflections	2,467,089	1,962,003
R_{work} / R_{free}	20.69 / 24.67	23.07 / 28.07
No. atoms		
Protein	93.016	90.976
Ligand/ion	194.892	203.092
Water	9.516	5.058
B factors		
Protein	59.5	62.8
Ligand/ion	54.9	59.1
Water	43.8	46.3
r.m.s. deviations		
Bond lengths (Å)	0.006	0.006
Bond angles (°)	1.151	1.063

Values in parentheses are for highest-resolution shell.
[a]A single crystal was used to obtain the structure.
[b]$I/\sigma I$ = 2 at 2.5-Å resolution
[c]$I/\sigma I$ = 2 at 2.7-Å resolution