Supplementary Information

Atomic mutagenesis reveals A2660 of 23S ribosomal RNA as key to EF-G GTPase activation

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1) Supplementary Results:

Supplementary Figure 1: 23S rRNA constructs and uncoupled EF-G GTPase activities of wild type ribosomes. (a) Schematic representation of the secondary structure of *T. aquaticus* 23S rRNA (center) highlighting the positions of the natural 5’- and 3’- ends in helix 1 (H1). For the ΔGAC mutant (left), helices 42-44 were deleted (green). The additional nucleotides that were added during clone construction into 23S rRNA are in bold lower case letters. To construct the ΔSRL mutant (right), a circularly permuted 23S rRNA was generated by connecting the natural ends in H1 and introducing new endpoints at positions 2685 (5’) and 2639 (3’), respectively. The deleted 23S rRNA fragment, encompassing the SRL (H95), that was provided as synthetic RNA oligomer *in trans* during *in vitro* reconstitution, is shown in red. The EF-G footprinting sites in the GAC (1067, 1069) and the SRL (2655, 2660, 2661, 2662) are indicated. (b) A representative time course of a ribosome-dependent uncoupled EF-G GTPase reactions. The quantified amounts of EF-G-dependent hydrolyzed GTP (pmol GTP/pmol ribosome) obtained in the presence of native *T. aquaticus* ribosomes (closed diamonds) or ribosomes containing reconstituted 50S carrying the wild type cp-23S rRNA (cp2685-2684) transcripts (open diamonds) were plotted as a function of time.
Supplementary Figure 2: Activities of ribosomes carrying the ΔGAC deletion in 23S rRNA. (a) Time course of ribosome-triggered uncoupled EF-G GTPase in the presence of ribosomes containing reconstituted 50S subunits carrying the wild type 23S rRNA transcript (filled circles) or the ΔGAC deletion (open circles). The curves represent the mean and standard deviation of two independent time course experiments. (b) The same ribosomal particles as in (a) were also tested in the poly(U)-directed poly(Phe) in vitro translation assay. In ribosomes carrying the wild type 23S rRNA (filled circles), almost 50 pmol [³H]-L-phenylalanine (specific activity 640 cpm/pmol) were incorporated per pmol 70S particle (right y-axis). The amount of active ribosomes was estimated by the quantity of added native E. coli 30S subunits (see Methods) and therefore give the theoretical maximal amount of available 70S particles.
**Supplementary Figure 3:** Peptidyl transferase activity of 70S ribosomes containing chemically engineered 50S subunits. The same ribosomal particles that were used in the EF-G GTPase assays (see Fig. 3 of the main text) were tested in parallel in the single-turnover puromycin reaction (right part). The amount of reaction product catalyzed by ribosomes carrying the cp-23S rRNA wild type control cp2685-2684 (cp_wt) was taken as 1.0. This product yield was compared to ribosomes containing reconstituted 50S subunits with the ΔSRL deletion mutant or that carried the ΔSRL construct with the compensatory synthetic RNA fragment encoding the wild type sequence of the SRL (ΔSRL+wt) or harboring the purine modification at position A2660 (ΔSRL+Pu). The peptidyl transferase activity of the ΔGAC mutant 50S was compared to reconstituted ribosomes carrying the wild type 23S rRNA *in vitro* transcript (set to 1.0). The mean and standard deviation of at least two independent puromycin reactions are shown.

**Supplementary Figure 4:** GTPase activity of ribosome-bound LepA (EF4). Analogous to the multiple-turnover GTPase assay shown in Fig. 3 of the main text, chemically-engineered ribosomes carrying the wild type SRL sequence (black; wt) or purine (yellow; Pu), 6-methyl-purine (purple; m^6^Pu) or inosine (green; I) at position 2660 were incubated with *T. aquaticus* LepA and γ-[³²P]-GTP. The data points represent the mean and the standard deviations of three independent time course experiments, whereas the amount of hydrolyzed GTP after 30 minutes with ribosomes carrying the wt RNA oligonucleotide was taken as 1.0.
Supplementary Figure 5: Single-turnover EF-G GTPase assay in the presence of thiostrepton. Chemically engineered ribosomes containing 50S particles with the ΔSRL mutant 23S rRNA construct or carrying the compensating wild type (wt) synthetic SRL RNA-oligomer, or the SRL oligo with purine (Pu) at position 2660 were employed in an EF-G GTPase assay in the presence of 2 µM thiostrepton 1 for 3 minutes. The mean and standard deviation of three GTPase assays are shown.
Supplementary Figure 6: Structural probing of ribosomes containing reconstituted 50S subunits. Reverse transcriptase analysis of cp-23S rRNA extracted from wild type (cp_wt) or from ΔSRL 50S subunits probed with dimethyl sulfate (+ DMS). A, C denote dideoxy sequencing lanes. Bases labeled with asterisks are residues that have been shown in an E. coli ΔSRL 50S construct by Lancaster et al. (2008) to possess enhanced reactivity in the deletion mutant as compared to the wild type control. In the T. aquaticus ΔSRL 50S used in this study, we see an unchanged DMS probing pattern and thus no indication for severe 50S assembly defects in the ΔSRL 50S used.
Supplementary Figure 7: Proposed mechanism of activation of the EF-G GTPase involving A2660 of the SRL of 23S rRNA. Access (green arrow) of the supposedly catalytic EF-G residues His-84 (E.coli EF-Tu nomenclature is used in the figure. The corresponding residue in T. thermophilus EF-G is His-87) in the switch II region (orange) to the hydrolytic water (blue), that in turn attacks the γ-phosphate of GTP, is controlled by the ‘hydrophobic gate’ formed by amino acids from the switch I (Ile-60; magenta) and the P-loop (Val-20; brown) regions (Ile-63 and Ile-21 in T. thermophilus EF-G). Structural changes in the G-domain of ribosome-bound EF-G that open the ‘hydrophobic gate’ and re-position His-84 are triggered by the 23S rRNA nucleotide A2660 of the SRL. These structural rearrangements either involve a steric clash of the A2660 exocyclic N6 amino group or a specific π–stacking interaction of the nucleobase with critical residues in the G-domain (black arrows; question marks).
Supplementary Figure 8: Binding activity of EF-G to reconstituted ribosomes. These are the full, uncut gels used to create the images shown in Figure 4a of the main text. The silver stained EF-G and L2 bands were quantified and the amount of ribosome-bound EF-G was calculated relative to L2. The relative binding values of reconstituted ribosomes carrying wild type T7 transcripts (the cp-23S rRNA variant cp2685-2684 (cp_wt), or transcripts with genuine ends (wt)) were compared to ribosomes lacking the GTPase associated center (ΔGAC) or the sarcin-ricin loop (ΔSRL), as well as to ribosomes harboring the synthetic SRL-oligomer with the wild type sequence (ΔSRL+wt), a purine (ΔSRL+Pu) or the abasic site analog (ΔSRL+rab) at A2660. The reconstitution reaction in the absence of 23S rRNA (no 23S rRNA) served as negative control. The mean of three independent experiments are shown below the respective lanes. The relative EF-G binding efficiency in the presence of reconstituted ribosomes carrying wild type 23S rRNA transcripts was taken as 1.00. M, protein length marker; 70S, total ribosomal proteins of native ribosomes from *T. aquaticus*. 
2) Supplementary Methods:

i) EF-G dependent GTP hydrolysis assays:
Even though the employed ribosomes, ribosomal proteins (T. aquaticus) as well as EF-G (T. thermophilus) were from thermophilic bacteria, in all the in vitro systems used the best ratio between EF-G dependent GTP hydrolysis and un-catalyzed background GTP hydrolysis was obtained sharply at 37 °C.

ii) Structural probing:
100 pmol cp2685-2684 (cp_wt) or 100 pmol cp2685-2639 (ΔSRL) were in vitro reconstituted to 50S particles and purified via 10-40 % sucrose gradient centrifugation as described. 50S subunits were precipitated form the sucrose fractions by the incubation with 3 volumes EtOH for 60 min at – 80 °C and resuspended in reconstitution buffer. 50S were reassociated to 70S ribosomes by the incubation with an equal amount of native T. aquaticus 30S subunits for 10 min at 40 °C. 10 pmol reassociated 70S were subsequently programmed with 30 µg poly(U), combined with 10 pmol deacylated tRNA and incubated for 15 min at 37 °C in 26.7 µl reconstitution buffer. Structural probing was initiated by the addition of 1.1 µl dimethyl sulphate (DMS; diluted 1:8 in EtOH) for 30 minutes at 20 °C. The reaction was stopped by the addition of ½ volume stop mix (1 M Tris/Cl pH 7.5, 1 M β-mercaptoethanol, 0.1 M EDTA) and the rRNAs were subsequently purified and used as template for primer extension analysis as described. The following DNA primers were used:

\[5'-TGGTCTTCCGGTTGCTTAC-3'; \ 5'-CTTCTTCAGCCCCAGGA-3';\]
\[5'-TCCTGGCTGTCTTCGCGCGC-3'; \ 5'-AAGCCCCGATCATTTTC-3'\]

iii) Synthesis of 6-methylpurine nucleoside phosphoramidite 4

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**General.** $^1$H, $^{13}$C and $^{31}$P NMR spectra were recorded on a Bruker DRX 300 MHz instrument. The chemical shifts are reported relative to TMS and referenced to the residual proton signal of the deuterated solvent CDCl$_3$: 7.26 ppm for $^1$H NMR spectra, 77.0 ppm for $^{13}$C spectra. $^{31}$P-shifts are relative to external 85% phosphoric acid. $^1$H- and $^{13}$C-assignments were based on COSY and HSQC experiments. MS experiments were performed on a Finnigan LCQ Advantage Max ion trap instrumentation in the positive-ion mode. UV-spectra were recorded on a Varian Cary 100 spectrophotometer at room temperature. Reaction control was performed via analytical thin-layer chromatography (TLC, Marchery-Nagel) on silica plates with fluorescent indicator. Flash column chromatography was carried out on silica gel 60 (70-230 mesh). Chemical reagents and solvents were purchased from commercial suppliers (Sigma-Aldrich, Acros) and used without further purification.
Organic solvents for reactions were dried overnight over freshly activated molecular sieves (4Å). All reactions were carried out under argon atmosphere.

Compound 1 was synthesized from inosine in 4 steps according to the literature.

6-Methyl-9-[5-O-(4,4’-dimethoxytrityl)-β-D-ribofuranosyl]purine (2)

To a solution of compound 1 (556 mg; 2.09 mmol) in dry pyridine (8.0 mL), N,N-dimethylformamide dimethyl acetal (333 µL; 2.51 mmol) was added and the mixture was stirred for 2 h at room temperature. Then, the solvent was evaporated and the residue was coevaporated twice with pyridine to remove residual methanol. The crude intermediate was redissolved in dry pyridine (8.0 mL) and treated with 4,4’-dimethoxytrityl chloride (778 mg; 2.30 mmol) in three portions over a period of 1 h. The resulting orange solution was stirred for 1 h at room temperature and the reaction progress was monitored by TLC. After completed conversion, methanol was added (1 mL) and the mixture was evaporated and coevaporated with toluene and dichloromethane. The residue was dissolved in dichloromethane and washed with 5% citric acid, water and sat. sodium bicarbonate solution. The organic phase was dried over Na₂SO₄, filtrated and evaporated. The crude product was purified by column chromatography on SiO₂ (CH₂Cl₂/CH₃OH, 98/2 – 95/5 v/v).

Yield: 852 mg of 2 as white foam (72 %);

TLC (CH₂Cl₂/CH₃OH, 94/6): Rₜ = 0.45;

¹H-NMR (300 MHz, CDCl₃): δ 2.89 (s, 3H, C(6)-CH₃); 3.04 (s, 1H, HO-C(3’)); 3.27 (dd, J = 2.4, 10.5 Hz, 1H, H1-C(5’)); 3.45 (dd, J = 2.9, 10.5 Hz, 1H, H2-C(5’)); 3.76 (s, 6H, 2x OCH₃); 4.45 (m, 2H, H-C(4’) + H-C(3’)); 4.87 (m, 1H, H-C(2’)); 5.71 (s, br, 1H, HO-C(2’)); 6.01 (d, J = 5.7 Hz, 1H, H-C(1’)); 6.72 (d, J = 8.7 4H, H-C(ar)); 7.16 (m, 9H, H-C(ar)); 8.30 (s, 1H, H-C(8)); 8.81 (s, 1H, H-C(2)) ppm;

¹³C-NMR (75 MHz, CDCl₃): δ 19.32 (6-CH₃); 55.10 (2x OCH₃); 63.38 (C(5’)); 71.96 (C(3’)); 75.24 (C(2’)); 85.32 (C(4’)); 86.56 (C(ar)); 89.91 (C(1’)); 113.09, 126.84, 127.76, 127.93, 129.89 (C(ar)); 133.35; 135.40; 135.43; 142.51 (C(8)); 144.29; 149.69; 151.61 (C(2)); 158.47; 159.59 ppm;

ESI-MS (m/z): [M+H]⁺ calcd for C₃₂H₃₂N₄O₆, 569.63; found 569.16; [M+Na]⁺ calcd 591.61; found 591.20.
$^1$H NMR (300 MHz, CDCl$_3$):

$^{13}$C NMR (75 MHz, CDCl$_3$):
Compound 2 (189 mg; 0.332 mmol) was dissolved in a mixture of N-ethyldiisopropylamine (190 µL; 1.162 mmol) and dry dichloroethane (1.3 mL). Then, dibutyltin dichloride (112 mg; 0.365 mmol) was added and the mixture was stirred for 3 h at room temperature. After the mixture was heated to 80°C for 2 min, [(triisopropylsilyl)oxy]methyl chloride (82 mg; 0.365 mmol) was added dropwise and heating was continued for 20 min. The reaction solution was quenched by addition of methanol, diluted with dichloromethane and washed with ½ sat. sodium bicarbonate solution. The organic phase was dried over sodium sulfate, filtrated over celite and evaporated. The crude product was purified by column chromatography on SiO2 (hexane/EtOAc, 60/40 – 50/50 v/v).

Yield: 87 mg of 3 as white foam (35 %) and 53 mg of the 3'-isomer (21%);

TLC (hexane/EtOAc, 30/70): Rf = 0.52;

$^1$H-NMR (300 MHz, CDCl$_3$): δ 1.03 (m, 21H, 3x SiCH(CH$_3$)$_2$); 2.85 (s, 3H, C(6)-CH$_3$); 3.03 (s, br, 1H, HO-CH(3')); 3.34 (dd, J = 4.1, 10.3 Hz, 1H, H1-C(5')); 3.49 (dd, J = 3.2, 10.3 Hz, 1H, H2-C(5')); 3.78 (s, 6H, 2x OCH$_3$); 4.30 (m, 1H, H-C(4')); 4.56 (m, 1H, H-C(3')); 4.96-5.14 (m, 3H, H-C(2') + OCH$_2$O); 6.22 (d, J = 5.4 Hz, 1H, H-C(1')); 6.79 (d, J = 8.7 Hz, 4H, H-C(ar)); 7.33 (m, 7H, H-C(ar)); 7.42 (d, J = 6.9 Hz, 2H, H-C(ar)); 8.21 (s, 1H, H-C(8)); 8.75 (s, 1H, H-C(2)) ppm;

$^{13}$C-NMR (75 MHz, CDCl$_3$): δ 11.85 (Si(CH(CH$_3$)$_2$)$_3$); 17.77 (Si(CH(CH$_3$)$_2$)$_3$); 19.48 (6-CH$_3$); 55.16 (2x OCH$_3$); 63.41 (C(5')); 71.01 (C(3')); 81.82 (C(2')); 84.31 (C(4')); 86.59 (C(ar)$_3$); 87.02 (C(1')); 90.82 (OCH$_3$Si) 113.16, 126.88, 127.83, 128.15, 130.04 (C(ar)); 133.67; 135.67; 135.73; 142.70 (C(8)); 144.55; 150.31; 152.31 (C(2)); 158.54; 159.39 ppm;

ESI-MS (m/z): [M+H]$^+$ calcd for C$_{60}$H$_{45}$N$_4$O$_7$Si, 756.00; found 755.26; [M+Na]$^+$ calcd 777.98; found 777.27.
$^1$H NMR (300 MHz, CDCl$_3$):

$^{13}$C NMR (75 MHz, CDCl$_3$):
6-Methyl-9-{5-O-(4,4’-dimethoxytrityl)-2-O-[(triisopropylsilyl)oxy]methyl-β-D-ribofuransoyl 3-(2-cyanoethyl)-N,N-diisopropylphosphoramidite}purine (4)

Compound 3 (85 mg; 0.133 mmol) was dissolved in a mixture of N-ethyldimethylamine (128 µL; 1.13 mmol) in dry dichloromethane (3.4 mL). After 15 minutes at room temperature, (2-cyanoethyl)-N,N-diisopropylchlorophosphoramidite (42 mg; 0.170 mmol) was added slowly and the solution was stirred at room temperature for 2.5 hours. The reaction mixture was diluted with dichloromethane and washed with ½ sat. sodium bicarbonate solution. The organic phase was dried over sodium sulfate, filtered and evaporated. The crude product was purified by column chromatography on SiO₂ (hexane/ethylacetate, 8/2 – 7/3 v/v (+0.5% NEt₃)).

Yield: 63 mg of 4 as white foam (59 %);

TLC (hexane/EtOAc, 50/50): Rf = 0.47;

¹H-NMR (300 MHz, CDCl₃): δ 0.87 (m, 21H, 3x SiCH(CH₃)₂); 1.08-1.20 (m, 12H, 2x CH(CH₃)₂); 2.39 (t, J = 6.6 Hz, 1H, POCH); 2.65 (t, J = 6.3 Hz, 1H, POCH); 2.84 (s, 3H, C(6)-CH₃); 3.36 (m, 1H, H1-C(5′)); 3.54-3.65 (m, 4H, H₂-C(5′), 2x CH(CH₃)₂, CNCH); 3.77, 3.78 (2s, 6H, 2x OCH₃); 3.87 (m, 1H, CNCH); 4.39 (m, 1H, H-C(4′)); 4.70 (m, 1H, H-C(3′)); 4.93 (m, 2H, OCH₂O); 5.22 (m, 1H, H-C(2′)); 6.19 (m, 1H, H-C(1′)); 6.78 (m, 4H, H-C(ar)); 7.28 (m, 9H, H-C(ar)); 8.17, 8.19 (2s, 2H, H-C(8)); 8.69, 8.71 (2s, 2H, H-C(2)) ppm;

³¹P-NMR (121 MHz, CDCl₃): δ 150.8, 151.6 ppm;

ESI-MS (m/z): [M+H]⁺ calcd for C₅₃H₇₃N₆O₈PSi, 956.21; found 955.31; [M+Na]⁺ calcd 978.19; found 977.25.
$^1$H NMR (300 MHz, CDCl₃):

$^{31}$P NMR (121 MHz, CDCl₃):
Solid-phase synthesis, deprotection and purification of oligonucleotides

RNA 2'-O-TOM standard nucleoside phosphoramidite building blocks and the corresponding polystyrol-supports (80 μmol/g; PS200) were purchased from ChemGenes and GE Healthcare. The phosphoramidite of the ribose abasic site analog was obtained from Glen Research. The 6-methylpurine nucleoside phosphoramidite was synthesized as described above.

All oligonucleotides were synthesized on a Pharmacia Gene Assembler Special following standard synthesis protocols. Detritylation (2.0 min): dichloroacetic acid/1,2-dichloroethane (4/96); coupling (3.0 min): phosphoramidites/acetonitrile (0.1 M x 120 µl) were activated by benzylthiotetrazole/acetonitrile (0.3 M x 360 µl); capping (3 x 0.4 min): A: 4-(dimethylamino)pyridine/acetonitrile (0.5 M), B: Ac2O/sym-collidine/acetonitrile (2/3/5), A/B = 1/1; oxidation (1.0 min): I2 (10 mM) in acetonitrile/sym-collidine/H2O (10/1/5). Solutions of amidites, tetrazole solutions and acetonitrile were dried over activated molecular sieves (4Å) overnight. All sequences were synthesized trityl-off.

Oligoribonucleotides were deprotected with CH3NH2 in EtOH (8 M, 0.65 mL), CH3NH2 in H2O (40%, 0.65 mL) for 6-7 h at room temperature. After the solution was evaporated to dryness, the 2'-O-silyl ethers were removed by treatment with tetrabutylammonium fluoride trihydrate (TBAF.3H2O) in anhydrous THF (1 M, 0.95 mL) for at least 12 h at 37°C. The reaction was quenched by the addition of triethylamonium acetate buffer (1 M, pH 7.0, 0.95 mL). The volume of the solution was reduced to 1 ml and directly applied on a HiPrep™ 26/10 desalting column (GE Healthcare). The crude RNA was eluted with H2O and subsequently evaporated to dryness.

Analysis of crude oligonucleotides after deprotection was performed by anion-exchange chromatography on a Dionex DNA Pac®PA-100 column (4 x 250 mm) at 80°C. Flow rate: 1 mL min⁻¹; eluant A: 25 mM Tris-HCl (pH 8.0), 10 mM LiClO₄; eluant B: 25 mM Tris-HCl (pH 8.0), 500 mM LiClO₄; gradient: 0-60% B in A within 45 min; UV-detection at 265 nm. Crude RNA products were purified on a semipreparative Dionex DNA Pac®PA-100 column (9 x 250 mm). Flow rate: 2 mL min⁻¹; gradient: Δ5-10% B in A within 20 min. Fractions containing oligonucleotide were loaded on a C18 SepPak®Plus cartridge (Waters/Millipore), washed with 0.1–0.2 M (Et3NH)HCO3 and H2O, eluted with H2O/CH3CN and lyophilized to dryness.

The purified oligonucleotides were characterized by mass spectrometry on a Finnigan LCQ Advantage MAX ion trap instrument connected to an Amersham Ettan micro LC system (negative-ion mode with a potential of -4 kV applied to the spray needle). LC: Sample (250 pmol of oligonucleotide dissolved in 20 µL of 20 mM EDTA solution; average injection volume: 10–20 µL); column (Amersham µRPC C2/C18; 2.1 x 100 mm) at 21°C; flow rate: 100 µL min⁻¹; eluant A: 8.6 mM TEA, 100 mM 1,1,1,3,3,3-hexafluoro-2-propanol in H2O (pH 8.0); eluant B: methanol; gradient: 0-100% B in A within 30 min; UV detection at 254 nm.
Supplementary References:


