Supporting Information

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SI Text

Yeast Strains. The haploid strain LY512 (MAT **a** ade2 can1 his3 leu2 trp1 ura3 OST4::FLAG3-KANMX6 STT3::MBP-HISMX6) (1) was used for the purification of OT. To generate a Sbh1-FLAG construct, PCR was carried out by using the pFA6a-FLAG3-HIS3MX6 as a template, which was a kind gift from Rolf Sternglanz (Stony Brook University). The primers were 5′-TTTCTGTTGT-TGCATTACATGTTATTTCTAAAGTTGCCGGTAAGTTA-TTTCGGATCCCCGGGTTAATTAA-3′ and 5′-GTTTTGTC-AAATAGGGTGGATAAAAGCTGAATCATTACTGAAGA-AAATTCGAATTCGAGCTCGTTTAAAC-3′. The PCR product was used to transform W303–1a (MAT **a** ade2 can1 his3 leu2 trp1 ura3) by homologous recombination. The recombination was confirmed by PCR. The resulting transformant was designated as YH1 (MAT **a** ade2 can1 his3 leu2 trp1 ura3 SBH1::FLAG-HISMX6). The YH1 strain did not show any growth defects at 30 °C or 37 °C.

Preparation of the Ribosomes. All buffers contained 1 mM phenylmethylsulfonylfluoride. WT yeast strain W303–1a (MAT **a** ade2 can1 his3 leu2 trp1 ura3) were lysed in 10 mM Tris·HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA by beating in an equal volume of glass beads. The homogenate was clarified by centrifugation at 30,000 × *g* for 60 min. The supernatant was layered onto a 10% sucrose cushion in 10 mM Tris·HCl (pH 7.4), 500 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA and centrifuged at 100,000 × *g* for 3 h. The use of high salt (500 mM KCl) removes strongly associated proteins from ribosomes (2). The pellet was resuspended in 10 mM Tris·HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA frozen in liquid nitrogen and stored as crude ribosomes at −80 °C. Crude ribosomes (100 μL) were layered onto 10 mL of a 10–30% sucrose linear gradient in 10 mM Tris·HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA and spun at 100,000 × *g* for 3 h. The gradient was fractionated in 500-μL aliquots from top to bottom, and the fractions with maximal A_{260} were pooled as 80S ribosome. The 80S ribosome was diluted 2-fold with 10 mM Tris·HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA, sedimented at 200,000 × *g* for 2 h, and resuspended in the dilution buffer. The final suspension was frozen in liquid nitrogen and stored at −80°C. Purity of the ribosomes was examined by SDS/PAGE/silver staining and rRNA analysis.

OT Activity Assay. Fifty to 100 ng of purified OT was incubated in 100 μL of assay buffer [20 mM Tris·HCl (pH 7.4), 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 15 μM TAMRA-Arg-Asn-Ala-Thr-Ala-Arg-COOH, 0.25 mg/mL phosphatidylcholine, 0.1% Tx-100, 8.6 μM dolichylpyrophosphoryloligosaccharide (Dol-PP-OS), and 5% dimethyl sulfoxide]. The reaction was stopped by the rapid addition of 100 μL of ice-cold 5% Nonidet P-40 followed by addition of 1 mL of Con A buffer (20 mM Tris·HCl (pH 7.4), 500 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.5% Nonidet P-40] and placed on ice. Con A beads (100 μL of 50% slurry in Con A buffer) were added to the reaction and then the mixture was incubated for 30 min at 4 °C with gentle agitation. After washing the beads 3 times with 1 mL of cold Con A buffer, 50 μL of ConA buffer was added to the pellet, the suspension was transferred to a 96-well microplate (Costar 3601; Corning) and the fluorescent intensity (Ex: 544 nm and Em: 590 nm) of the N-glycosylated TAMRA-glycopeptide was measured with a Fluostar Optima fluorescent spectrometer (BMG Laboratories).

Con A Pull-Down Assay. The ribosomes (1.7 pmol) were incubated with either 7.2 pmol of OT and 36 pmol of the Sec61 complex or 36 pmol of the Sec61 complex on ice for 30 min, and then further incubated for 30 min at room temperature. The mixture was loaded onto a 10–40% linear sucrose gradient (3.5 mL) and spun at 200,000 × *g* at 4°C for 2 h. The fractions (350 μL/fraction) were manually taken from top to bottom of the gradient. The fraction was analyzed by A_{260} measurement. Fifty microliters of each fraction was stored for Western blot analysis. Fractions (7–9 tubes) that showed a peak of A_{260} were pooled, diluted 4-fold with 20 mM Hepes·NaOH (pH 7.8), 100 mM NaCl, 0.1% deoxy Big CHAP, 2 mM $MnCl_2$, and 2 mM $MgCl_2$, and then sedimented at 200,000 × *g* for 2 h. The pellet was resuspended in 100 μL of resuspension buffer [20 mM Hepes·NaOH (pH 7.8), 100 mM NaCl, 0.1% deoxy Big CHAP, 2 mM $MnCl_2$, 2 mM $MgCl_2$, and 2 mM $CaCl_2$], and the solution was incubated with 5 μL of Con A beads at 4 °C for 30 min. It should be noted that the Con A beads were pretreated with 0.5 mL of resuspension buffer containing 2 mg/mL lipid-free BSA (Sigma A0281) for 1 h at room temperature and washed 3 times with 50 μL of resuspension buffer. Con A beads incubated with the pellet suspension were washed twice with 50 μL of resuspension buffer. The beads were boiled in 200 μL of 1× SDS sample buffer for 3 min. Ten percent of the sample was analyzed by SDS/PAGE/Western blotting with anti-MBP, anti-Sec61, and anti-Rpl3 antibodies.

EM and Image Processing. The ribosome–OT complex that was prepared at a molar ratio of 1:3, respectively, was negatively stained for EM analysis. For negative staining, a drop of 4.5 μL of the reconstituted ribosome–OT complex sample was applied to a glow-discharged 300-mesh copper grid covered with a thin layer of carbon film. After incubating on carbon film for 1 min, excess solution was removed by blotting with a piece of filter paper. The grid was then stained sequentially by using 2 4.5-μL drops of 2% (wt/vol) uranyl acetate solution with 1-min staining each time. Excess stain was removed by blotting, and then the grids were quickly dried by argon flow. Images were recorded under low-dose conditions (10 e/Å^2) in a JEOL JEM 1200EX electron microscope operating at 120 kV at a magnification of ×40,000 and an underfocus of 1.2–1.5 μm. The images were recorded on Kodak SO-163 negative films, digitized with a Nikon Supercool scanner 8000ED, and processed by using EMAN (3). Only particles with the side views clearly showing binding of OT to ribosomes were picked for further analysis. Raw particles (785) were boxed out from 20 electron micrographs, low-pass-filtered at 15 Å, and classified. 2D class averages were visually compared with projections of the 3D cryoEM map of the yeast ribosome–translocon complex (EM data bank accession code EMD-1076).

1. Li H, Chavan M, Schindelin H, Lennarz WJ, Li H (2008) Structure of the oligosaccharyl transferase complex at 12-Å resolution. *Structure (London)* 16:432–440.
2. Inada T, et al. (2002) One-step affinity purification of the yeast ribosome and its associated proteins and mRNAs. *RNA* 8:948–958.
3. Ludtke SJ, Baldwin PR, Chiu W (1999) EMAN: Semiautomated software for high-resolution single-particle reconstructions. *J Struct Biol* 128:82–97.

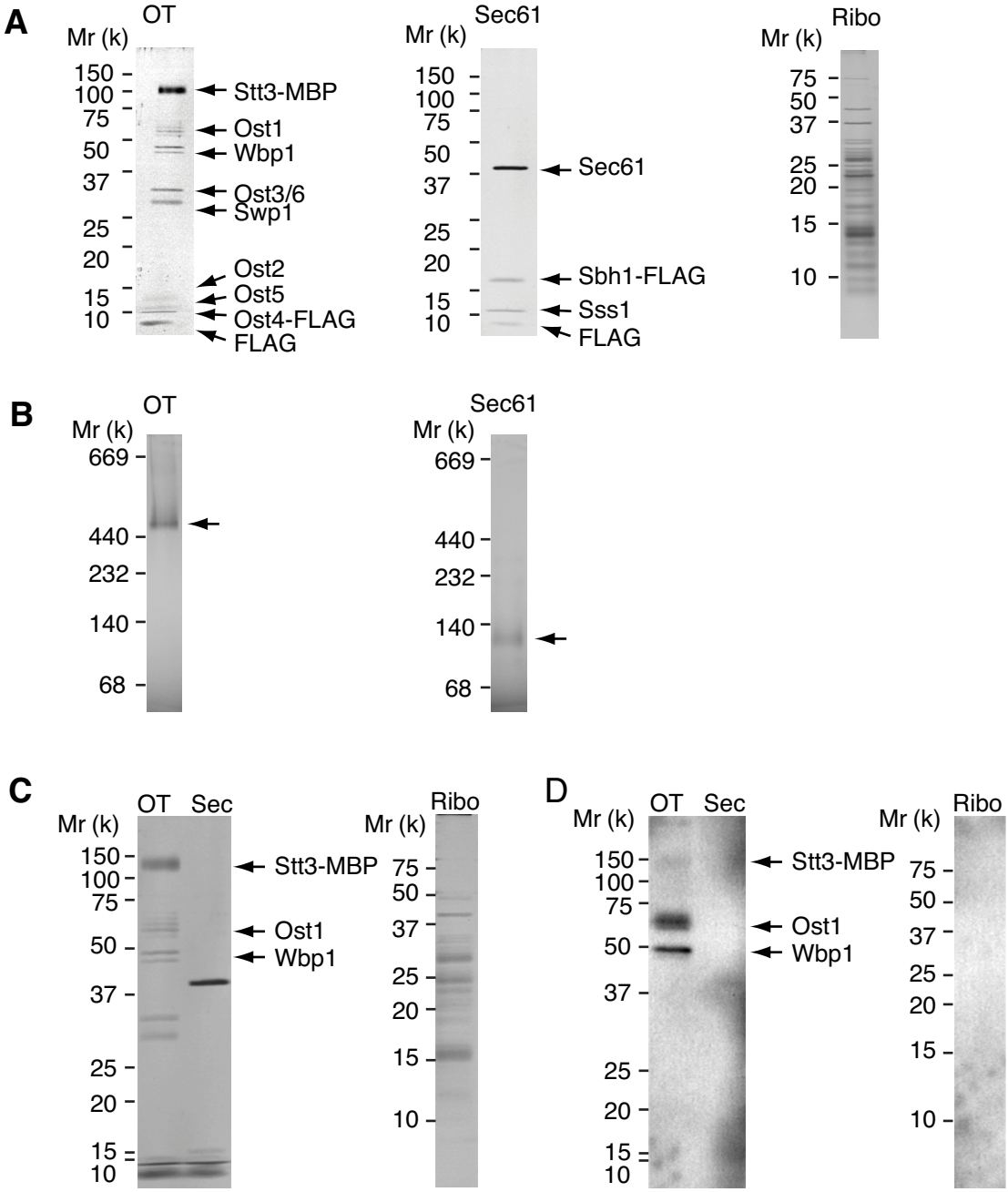

Fig. S1. Electrophoresis analyses of OT, the Sec61 complex, and ribosome. (*A*) SDS/PAGE of purified OT (80 ng/lane) (*Left*), the Sec61 complex (190 ng/lane) (*Center*), or ribosome (800 ng) (*Right*). (*B*) Blue native-PAGE of OT (*Left*) and the Sec61 complex (*Right*). All gels were stained with silver. (*C* and *D*) OT (100 ng, O), the Sec61 complex (100 ng, S), and ribosome (400 ng, R) were analyzed by SDS/PAGE/silver staining (*C*) and Con A blotting (*D*). Glycoproteins bound to Con A (Stt3-MBP, Ost1 and Wbp1) are indicated by arrows.

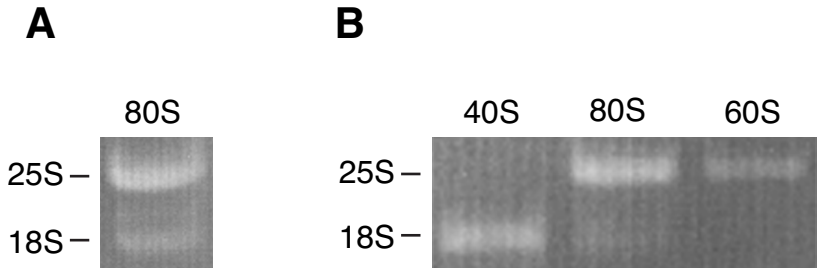

Fig. S2. Analysis of rRNA in 80S ribosome and the 40S and 60S ribosomal subunits. (*A*) The rRNA was extracted from the 80S ribosome (3 pmol) by the chloroform/phenol method, and then analyzed by 1% agarose gel electrophoresis. The rRNA was visualized by ethidium bromide. (*B*) The rRNA was extracted from the 60S (3 pmol) and the 40S (7 pmol) ribosomal subunits and analyzed as described in *A*. The rRNA extracted from the 80S ribosome (3 pmol) was loaded as a standard.

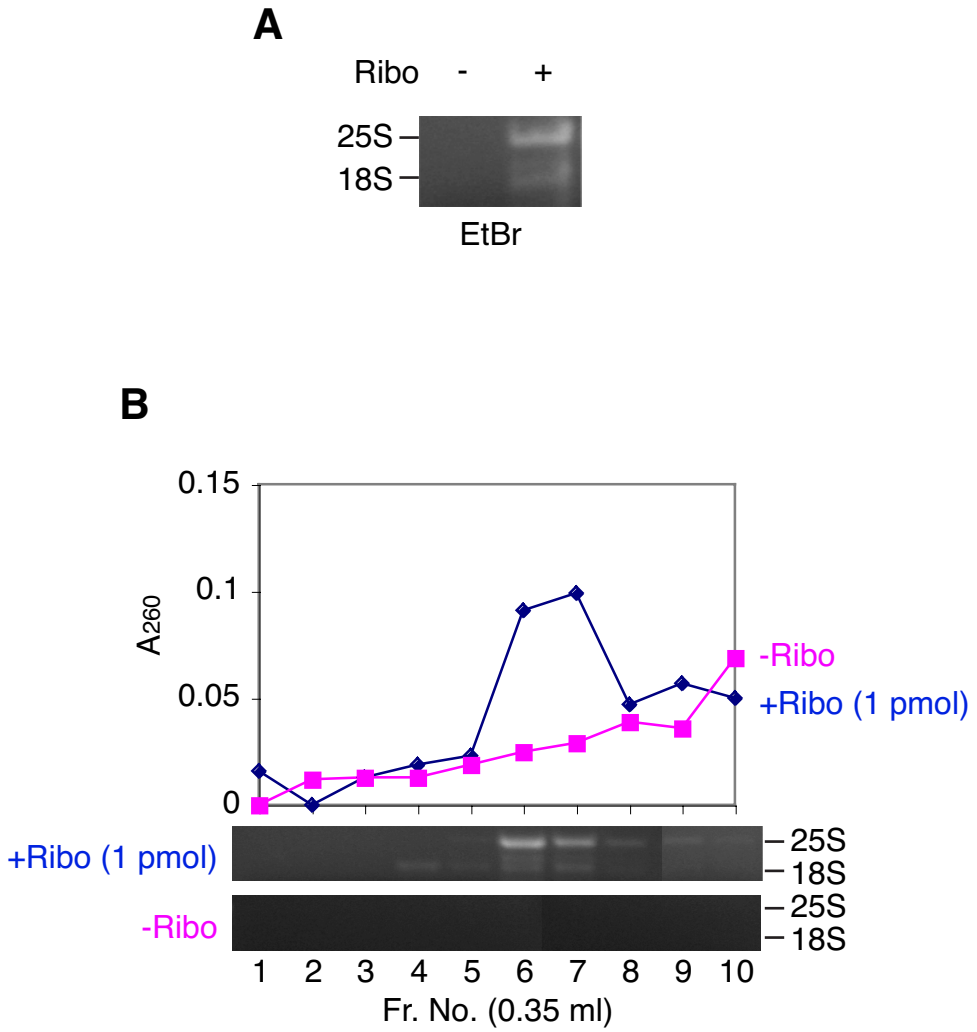

Fig. S3. Sedimentation analysis of the purified ribosome. One picomol of the purified ribosome or buffer alone [20 mM Hepes·NaOH (pH 7.8), 100 mM NaCl, 0.1% deoxy Big CHAP, 2 mM $MnCl_2$, and 2 mM $MgCl_2$] was incubated on ice for 30 min and then further incubated at room temperature for 30 min. The ribosomes were loaded onto 3.5 mL of either a 20% sucrose cushion (*A*) or onto a 10–40% linear sucrose density-gradient (*B*) in 20 mM Hepes·NaOH (pH 7.8), 100 mM NaCl, 0.1% deoxy Big CHAP, 2 mM $MnCl_2$, and 2 mM $MgCl_2$ followed by ultracentrifugation at 200,000 $\times$ *g* for 2 h. (*A*) After the centrifugation, rRNA were extracted from the pellet fraction by the chroloform/phenol method, electrophoresed in 1% agarose gel, and visualized by ethidium bromide (EtBr) staining. (*B*) The fraction (0.35 mL/fraction) was manually taken from top to bottom and the UV absorption at 260 nm was measured for each fraction. The rRNA were extracted from 270 μL of each fraction by the chroloform/phenol method and analyzed as described in *A*.

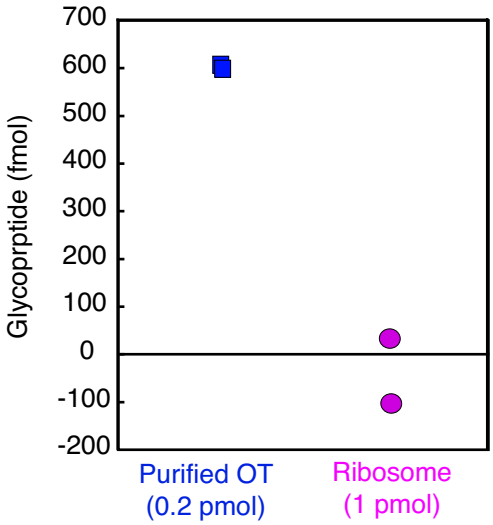

Fig. S4. Purified ribosome contains no detectable OT activity. OT activity was measured with 0.2 pmol of purified OT ($n = 2$) and 1 pmol of purified ribosome ($n = 2$). The reaction was stopped at 15 min. Individual data are represented.

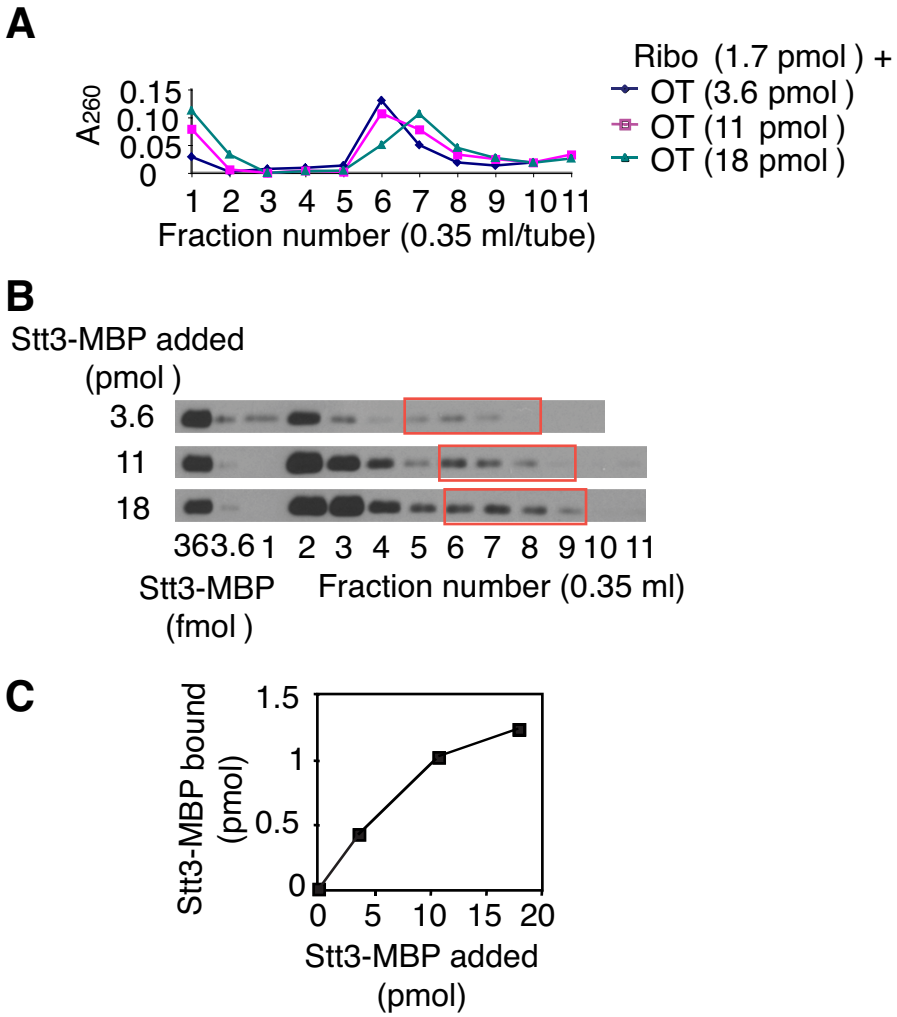

Fig. S5. Binding assay of various amounts of OT to a fixed amount of ribosome. (*A* and *B*) The binding of various amounts of OT (3.6–18 pmol) to a fixed amount of ribosomes (1.7 pmol) was analyzed by a 10–40% linear sucrose density-gradient followed by measuring the A_{260} (*A*) and by quantitative Western blot analysis using anti-MBP antibody (*B*). Stt3-MBP comigrating with ribosomal fractions was interpreted as the ribosome-bound Stt3-MBP (red box in *B*). (*C*) It was then further quantified.