The ribosome is a ribonucleoprotein machine responsible for protein synthesis. In all kingdoms of life it is composed of two subunits, each built on its own ribosomal RNA (rRNA) scaffold. The independent but coordinated functions of the subunits, including their ability to associate at initiation, rotate during elongation, and dissociate after protein release, are an established model of protein synthesis. Furthermore, the bipartite nature of the ribosome is presumed to be essential for biogenesis, since dedicated assembly factors keep immature ribosomal subunits apart and prevent them from translation initiation. Free exchange of the subunits limits the development of specialized orthogonal genetic systems that could be evolved for novel functions without interfering with native translation. Here we show that ribosomes with tethered and thus inseparable subunits (termed Ribo-T) are capable of successfully carrying out protein synthesis. By engineering a hybrid rRNA composed of both small and large subunit rRNA sequences, we produced a functional ribosome in which the subunits are covalently linked into a single entity by short RNA linkers. Notably, Ribo-T was not only functional but coordinated functions of the subunits, including their ability to associate at initiation, rotate during elongation, and dissociate after protein release, are an established model of protein synthesis. Furthermore, the bipartite nature of the ribosome is presumed to be essential for biogenesis, since dedicated assembly factors keep immature ribosomal subunits apart and prevent them from translation initiation. Free exchange of the subunits limits the development of specialized orthogonal genetic systems that could be evolved for novel functions without interfering with native translation. Here we show that ribosomes with tethered and thus inseparable subunits (termed Ribo-T) are capable of successfully carrying out protein synthesis. By engineering a hybrid rRNA composed of both small and large subunit rRNA sequences, we produced a functional ribosome in which the subunits are covalently linked into a single entity by short RNA linkers. Notably, Ribo-T was not only functional in vitro, but was also able to support the growth of Escherichia coli cells even in the absence of wild-type ribosomes. We used Ribo-T to create the first fully orthogonal ribosome–messenger RNA system, and demonstrate its evolvability by selecting otherwise dominantly lethal rRNA mutations in the peptidyl transferase centre that facilitate the translation of a problematic protein sequence. Ribo-T can be used for exploring poorly understood functions of the ribosome, enabling orthogonal genetic systems, and engineering ribosomes with new functions.

The random exchange of ribosomal subunits between recurrent acts of protein biosynthesis presents an obstacle for making fully orthogonal ribosomes, a task with important implications for fundamental science, bioengineering, and synthetic biology. Previously, it was possible to redirect a subpopulation of the small ribosomal subunits from translating indigenous mRNAs to instead translating a specific mRNA by placing an alternative Shine–Dalgarno sequence in a reporter mRNA and introducing the complementary changes in the anti-Shine–Dalgarno region in 16S rRNA. This enabled selection of mutant 30S subunits with new decoding properties. However, because large subunits freely exchange between native and orthogonal small subunits, creating a fully orthogonal ribosome has been impossible, thereby limiting the engineering of the 50S subunit, including the peptidyl transferase centre (PTC) and the nascent peptide exit tunnel, for specialized new properties.

The orthogonality of the full ribosome could be hypothetically achieved by linking the small and large subunit rRNA into a continuous molecule. A successful chimaeric 16S–23S construct must (1) properly interact with the ribosomal proteins and biogenesis factors for functional ribosome assembly; (2) avoid RNase degradation; and (3) have a linker(s) sufficiently short to ensure subunit cis-association, yet long enough for minimal interference with subunit movement required for translation initiation, elongation, and peptide release. In the native ribosome, the ends of 16S and 23S rRNA are too far apart (>170 Å) to be connected with a nuclease-resistant RNA linker. Therefore, we considered an alternative design in which the 23S rRNA would be ‘grafted’ into the 16S rRNA with the bridges connecting 16S and 23S rRNA sequences located across the rim of the subunits interface. To identify potential linking sites, we connected the native 23S rRNA ends that are proximal to each other, and generated new termini at different locations (Fig. 1a). This circular permutation approach has been successfully exploited in vitro previously, and a subsequent pilot study showed that three 23S rRNA circular permutation variants could assemble into a functional subunit in vivo. We prepared a comprehensive collection of 91 circularly permuted 23S (CP23S) rRNA mutants with new ends placed at nearly every hairpin (Fig. 1b). The CP23S sequences were introduced in place of the wild-type 23S rRNA gene of the pAM552 plasmid (Fig. 1a, Extended Data Figs 1a and 2), and the resulting constructs were transformed in the Escherichia coli SQ171 cells lacking chromosomal rRNA alleles. Twenty-two constructs were able to replace the resident plasmid pCSacB carrying the wild-type rRNA operon (Fig. 1b, Extended Data Fig. 2d, e and Extended Data Table 1). Most of the viable circularly permuted variants had new 23S rRNA ends at the subunit solvent side, including several locations close to the interface rim (Fig. 1c).

One of the viable mutants (CP2861, Fig. 1b) had 23S rRNA ends within the loop of helix 101 (H101), located in the ribosome near the apex loop of the 16S rRNA helix 44 (h44) (Figs 1c and 2c). Because the length of h44 varies among different species, and its terminal loop sequence can tolerate alterations, h44 was a promising site for grafting the CP2861 23S rRNA and generating a hybrid 16S–23S rRNA molecule (Fig. 2a–c). In the chimaeric rRNA, the processing sequences flanking the mature 16S rRNA would remain intact for proper maturation of the 16S rRNA termini, whereas endonuclease processing signals of 23S rRNA would be eliminated, thereby preventing its cleavage from the hybrid molecule.

The RNA linkers must span the 30–40 Å distance between h44 and H101 loops and allow for ~10 Å subunit ratcheting during protein synthesis (Fig. 2c and Extended Data Fig. 3). Being unable to estimate the optimal length of the linkers accurately, we prepared a library of constructs, pRibo-T, in which the length of two tethers—T1 connecting 16S rRNA G1453 with 23S rRNA C2858, and T2 linking 23S C2857 with 16S G1454—varied from 7 to 12 adenine residues (Supplementary Table 2). Notably, plasmid exchange in SQ171 cells yielded several slowly growing colonies, and the pattern of extracted RNA showed a single major rRNA species corresponding to the 16S–23S chimaera instead of the individual 16S and 23S bands (Fig. 2d). This result suggested that translation in these cells was carried out exclusively by Ribo-T, and revealed for the first time that the bipartite nature of the ribosome is dispensable for successful protein synthesis and cell viability.

---

1Center for Pharmaceutical Biotechnology – m/c 870, University of Illinois at Chicago, 900 South Ashland Avenue, Chicago, Illinois 60607, USA. 2Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208, USA. 3Chemistry of Life Processes Institute, Northwestern University, 2170 Campus Drive, Evanston, Illinois 60208, USA. *Present address: Institut de Biologie et Chimie des Protéines, UMR5086 CNRS-Université Lyon 1, 7 passage du Vercors, 69627 Lyon, France.

*These authors contributed equally to this work.
The linker combinations 8A/9A or 9A/8A (for T1/T2) were found in the six best-growing clones. The first combination showed slightly faster growth than the second. By passaging cells in liquid culture for approximately 100 generations, we isolated faster growing mutants. One such clone, SQ171fg/pRibo-T (for fast growing), exhibited better growth characteristics and shorter doubling time (70 ± 2 min) (Extended Data Fig. 4c). PCR and primer extension analysis showed the lack of wild-type DNA and rRNA, respectively, confirming that every ribosome in this strain was assembled with the tethered rRNA (Extended Data Fig. 4b, c). Because the pRibo-T plasmid from the SQ171fg clone was unaltered, we sequenced the entire genome and found a nonsense mutation in the ybeX gene encoding a putative Mg\(^{2+}/\text{Co}^{2+}\) transporter, and a missense mutation in the rpsA gene encoding ribosomal protein S1 (Extended Data Fig. 4d, e). Either one of these mutations or their combined effect must account for the faster growth of SQ171fg/pRibo-T cells (henceforth called Ribo-T cells).

To establish that protein synthesis in Ribo-T cells was carried out by ribosomes with tethered subunits, we carefully examined the integrity of Ribo-T RNA. Analysis of Ribo-T preparations in a denaturing gel showed only very faint 16S and 23S-like RNA bands (marked by asterisks in Extended Data Fig. 5a), possibly reflecting the linker cleavage either in the cell or during Ribo-T isolation. In most of the multiple Ribo-T preparations, these cleavage products accounted for less than 4% of the total Ribo-T RNA. In some of the preparations, these bands were completely absent (for example, lane Ribo-T(1) in Extended Data Fig. 5a), showing that more than 99% of Ribo-T remained intact. Consistently, primer extension across the T1 and T2 linkers did not show any major stops attesting to the general stability of the oligo(A) connectors (Extended Data Fig. 5d). Protein synthesis rate in Ribo-T cells reached 50.5 ± 3.5% of that in cells with wild-type ribosomes (Extended Data Fig. 6a) and thus cannot be accounted for by a small fraction of Ribo-T with cleaved tethers. Unequivocal proof of active Ribo-T translation in vivo came from analysis of polysomes prepared from Ribo-T cells, in which intact 16S–23S hybrid RNA (rather than the products of its cleavage) was associated with the heavy polysomal fractions (Fig. 2e). This result provided clear evidence that intact Ribo-T composed of covalently linked subunits is responsible for protein synthesis in the Ribo-T cells. 2D-gel analysis showed that most of the proteins present in SQ171 cells that express wild-type ribosomes are efficiently synthesized in the Ribo-T cells (Extended Data Fig. 6).

We isolated ribosomes with tethered subunits from Ribo-T cells and characterized their composition and properties. The tethered ribosome contains an apparently equimolar amount of SS rRNA and the full complement of ribosomal proteins in quantities closely matching the composition of wild-type ribosome (Extended Data Fig. 5 b, c). Chemical probing showed that the rRNA hairpins h44 and H101 remain largely unperturbed, while both linkers were highly accessible to chemical modification, indicating that they are solvent-exposed (Extended Data Fig. 7).

Sucrose gradient analysis of Ribo-T showed that at 15 mM Mg\(^{2+}\) most of the ribosomal material sedimented as a 70S peak with a minor faster-sedimenting peak, which may represent Ribo-T dimers owing to cross-ribosome subunit association at a high Mg\(^{2+}\) concentration (Fig. 3a). At lower Mg\(^{2+}\) concentration (1.5 mM), when the native ribosome completely dissociates into subunits, Ribo-T still sediments as a single peak with an apparent sedimentation velocity of 65S (Fig. 3a). The distinctive resistance of Ribo-T to subunit dissociation offers a venue for isolating Ribo-T if it is expressed in cells concomitantly with wild-type ribosomes.

We then tested the activity of Ribo-T in the PUREXpress in vitro translation system lacking native ribosomes\(^1\). Ribo-T efficiently synthesized the 18-kilodalton (kDa) dihydrofolate reductase or superfolder green fluorescence protein (sGFP)\(^1\) (Fig. 3b). The rate of Ribo-T-catalysed protein synthesis reaches approximately 45% of that of the wild-type ribosomes (Fig. 3b). To assess which translation step is the most problematic for Ribo-T, progression of Ribo-T through a short synthetic gene\(^1\) was analysed by toe-printing (Fig. 3c). A more pronounced band of the ribosomes at the open reading frame start codon indicated that Ribo-T is impaired in translation initiation at a step subsequent to the start codon recognition. Although the true nature of this effect will require further investigation, it is unlikely to reflect a lower affinity of Ribo-T for initiation factors because higher concentrations of IF1, IF2 and IF3 could not rescue the initiation defect (data not shown).
To enable a fully orthogonal ribosome–mRNA system, we next engineered a Ribo-T version (oRibo-T) committed to translation of a particular orthogonal cellular mRNA. The wild-type 16S anti-Shine–Dalgarno region was altered from ACCUCCUUA to AUUGUGGUA (ref. 3) producing a poRibo-T1 construct. When poRibo-T1 was introduced in *E. coli* carrying the *sf-gfp* gene with the Shine–Dalgarno sequence CACCAC cognate to oRibo-T (Extended Data Fig. 1c, pLpp5oGFP), notable sfGFP expression was observed (Extended Data Fig. 8a), demonstrating the activity of oRibo-T.

Ribosomes prepared from poRibo-T1-transformed cells (containing a mixture of wild-type ribosomes and oRibo-T) translated an orthogonal *sf-gfp* gene in a cell-free system (green dotted line in Extended Data Fig. 8b). However, because the orthogonal *sf-gfp* transcript is the only mRNA available during *in vitro* translation and no native mRNA engage wild-type 30S subunits, a fraction of orthogonal sfGFP biosynthesis is accounted for by wild-type ribosomes (pink dotted line in Extended Data Fig. 8b). Therefore, to isolate oRibo-T1 activity *in vitro*, we used the A2058G mutation in the 23S rRNA portion of oRibo-T, which rendered
Figure 3 | Functional characterization of Ribo-T. a, Sucrose gradient analysis of wild-type ribosomes (top) and Ribo-T (bottom) under 15 mM MgCl$_2$ (solid line) or 1.5 mM MgCl$_2$ subunit dissociating conditions (dotted line). The peak marked with grey arrow and ‘X’ may represent Ribo-T dimers. The result was qualitatively verified in an independent experiment performed at Mg$^{2+}$ concentrations 1.5 mM and 10 mM. b, In vitro translation of proteins by isolated Ribo-T. Top, SDS–PAGE analysis of the dihydrofolate reductase (DHFR) protein synthesized in the Aribosome PURExpress system supplemented with purified wild-type ribosomes or Ribo-T (T); wild-type ribosomes provided with the kit (WT*) were used as a control. The transcription–translation reaction was carried out in the presence of [35S]$\text{-}$methionine in the absence or presence of 50 mM erythromycin (ERY). The A2058G mutation in Ribo-T renders the Ribo-T–driven translation resistant to the antibiotic. The ‘no erythromycin’ samples are a representative result of two independent biological experiments. Bottom, time course of sfGFP protein expression in the Aribosome PURExpress system supplemented with purified wild-type (black) or Ribo-T (grey) ribosomes. The $k_{on}$ rates (385 ± 13 relative fluorescent units (RFU) min$^{-1}$ (mean ± s.d.) for wild-type, 177 ± 6 RFU min$^{-1}$ for RiboT) were determined from the initial slopes. The activity of both ribosomes was fully inhibited by 50 µg ml$^{-1}$ chloramphenicol (time points indicated by x). Each curve is an average of two independent biological replicates, with error bars indicating the s.d. c, Toeprinting analysis of translation of a 20-codon synthetic gene RST1 (ref. 15) by wild-type ribosomes or Ribo-T. The antibiotic thiostrepton (THS), present at 50 µM, arrests the initiating ribosome at the start codon ($\text{AUG}$) (black arrowhead). The threonyl-tRNA synthetase inhibitor borrelidin (BOR) arrests translation at the fourth codon of RST1 mRNA (grey arrowhead)$.^{19}$ The position of a toeprint band that would correspond to the ribosome that has reached the RST1 stop codon is shown by an open arrowhead. A more pronounced toeprint band at the start codon in the samples lacking thiostrepton indicates that Ribo-T departs from the initiation codon slower than wild-type ribosomes. A weaker borrelidin–specific band observed in the Ribo-T sample suggests that under our experimental conditions, fewer Ribo-T compared to wild-type ribosomes were able to reach the fifth codon, apparently owing to slower initiation.

During subsequent experiments, we fortuitously isolated a mutant version of the poRibo-T1 plasmid (poRibo-T2) that contained a single mutation in the P$_2$ promoter that improved its transformation properties and was used thereafter (Extended Data Fig. 9).

We next demonstrated the evolvability of oRibo-T by selecting the gain-of-function mutations in the PTC, which could facilitate translation of a problematic protein sequence by the ribosome. The SecM polypeptide presents a classic example of an amino acid sequence for which translation is problematic for the ribosome$.^{11}$ The expression of the essential SecA secretion ATPase is controlled by programmed ribosome stalling at the Pro166 codon of secM. Translation arrest ensues because specific interactions of the SecM nascent chain with the ribosomal exit tunnel impair the PTC function, preventing the transfer of the 165-amino-acid-long peptide to the incoming polytRNA (Pro-tRNA). Several mutations in the ribosomal exit tunnel (for example, A2058G) have been previously identified as relieving translation arrest possibly by disrupting the interactions between the nascent chain and ribosome, and rRNA residues in the PTC A-site have been proposed to have a key role in the mechanism of ribosome stalling$.^{18–20}$ However, exploring the role of the PTC in the mechanism of the translation arrest has been impossible so far because of the lethal nature of PTC mutations$.^{21,22}$

We therefore asked whether the PTC A-site mutations can relieve SecM–induced translation arrest. Our interest in testing the use of oRibo-T for manipulating the ribosomal A-site was additionally fuelled by future prospects of engineering ribosomes capable of programmed polymerization of unnatural amino acids and backbone–modified analogues. To search for SecM arrest bypass mutations, we removed the A2058G mutation from poRibo-T2 and prepared a library of plasmids with mutations at two 23S residues, A2451 and C2452. These residues form the amino acid binding pocket in the PTC A-site$.^{10,23}$ (Fig. 4b), and their mutations are dominantly lethal in E. coli$.^{22}$ We also engineered an orthogonal SecM–based reporter, poSMI (Fig. 4a and Extended Data Fig. 1d), encoding the SecM arrest sequence fused in frame with lacZa gene$.^{19}$ (Fig. 4a).

Notably, when the C41(DE3) cells capable of α-complementation were transformed first with the poSMI reporter and then with the poRibo-T2 (A2451N/C2452N) mutant library, some of the colonies gained blue colour on indicator plates (Fig. 4c), demonstrating read-through of the SecM arrest sequence in some of the mutants. Sequencing 15 blue colonies showed that they all carried a C2451–
cells although to a lesser extent. These results suggested that the SecM-arrest caused by the SecM sequence.

protein synthesis but also gained the ability to bypass translation arrest caused by the SecM sequence.

We verified in vitro the discovered role of A2451 in the mechanism of SecM translation arrest by testing the translation of the orthogonal secM-lacZa gene by isolated oRibo-T with and without the A2451C mutation. To assure oRibo-T activity only, the pactamycin-resistance mutation G693A (refs 16, 17) was introduced into the 16S segment of oRibo-T constructs, and cell-free translation in the PURExpress system was carried out in the presence of pactamycin. Only a small fraction of original oRibo-T was able to bypass the SecM arrest signal and synthesize the full-size hybrid protein (Fig. 4e, lane oRibo-T/A2451). By contrast, the A2451C mutant was able to bypass the SecM arrest site twice as efficiently as the unmodified oRibo-T (Fig. 4e, lane oRibo-T/C2451), confirming that the selected (and otherwise lethal) mutation in the PTC has improved the ability of oRibo-T
to polymerize a polypeptide sequence problematic for wild-type ribosomes. These results provide the first, to our knowledge, direct experimental evidence of a direct involvement of the PTC A-site in the mechanism of nascent peptide-dependent ribosome stalling, and suggest that interactions between the proline moiety of Pro-tRNA and the A-site rRNA residues are crucial for the SecM-induced translation arrest.

By engineering a ribosome with inseparable tethered subunits, and demonstrating its functionality in vivo and in vitro, we have revised one of the key concepts of molecular biology: that successful expression of the genome requires reversible association and dissociation of the ribosome into individual subunits. Although the ability of translation initiation by 70S ribosome at leaderless mRNAs or via scanning re-initiation has been previously demonstrated [2, 3], it was surprising that Ribo-T would be active enough to express the entire bacterial genome at a sufficient level for active cell growth and proliferation. This finding in turn made possible a fully orthogonal and dedicated gene expression system in the cell in which an entire specialized ribosome, not just the mRNA-interacting small subunit, is dedicated to the translation of a defined genetic template. As a proof of principle we showed that oRibo-T can be used for studying in cells mutations of functionally crucial rRNA residues that are dominantly lethal, a task that would be difficult or impossible to achieve in any other system. This shows that Ribo-T may find important implications in exploring poorly understood functions of the ribosome in protein synthesis. Furthermore, the opportunity provided by the oRibo-T system to modify the catalytic properties of the protein synthesis machine opens exciting prospects for engineered ribosomes with principally new properties.

Online Content
Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 28 January; accepted 26 June 2015.
Published online 29 July 2015.


Supplementary Information
is available in the online version of the paper.

Acknowledgements We thank I. Ntai for mass spectrometry analysis, K. N. Swonger, C. Burghard, E. M. Fukui, V. Raghavan and N. Aleksashin for help with some experiments, K. Ito for providing the sequence of the pNH122 secM-lacZa reporter, Y. Polikanov for help in preparing ribosome images, J. Lee for assistance in genome sequence analysis, S. Sothivelam and J. Marks for discussions and suggestions, and N. Vázquez-Laslop for advice on the project and critical reading of the manuscript. This work was supported by the Defense Advanced Research Projects Agency (N66001-12-C-2411), the National Science Foundation grants MCB-0943393 (to M.C.J.) and MCB-1244455 (to A.S.M.) and the David and Lucille Packard Foundation Fellowship (2011-37152) (to M.C.J.).

Author Contributions M.C.J. and A.S.M. designed the study, analysed results, and wrote the paper. C.O. and E.D.C. designed and performed experiments and analysed data. T.S. and T.F. performed experiments.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.

Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.S.M. (shura@uic.edu) or M.C.J. (mcjw@northwestern.edu).
Preparation of circularly permuted variants of the 23S rRNA. The A2058G mutation was introduced into the pAM552 plasmid (Extended Data Fig. 1a) by inverse PCR using primers 5'-GCGCTTGGGCGGGGTTAGTAC-3' and 5'-GGTGAC CCGGGGAAAGACCAAGGACCGGGTTAC-3' (the underlined sequence is complementary to the second primer and the mutation is shown by italicized bold character) followed by re-circularization by Gibson assembly reaction (all primers used in this study were synthesized by Integrated DNA Technology). A 23S-A2058G gene with native 5' and 3' ends linked by a GAGA tetra-loop was generated by inverse PCR using primers 5'-GGTGAGCTACCGGCGGTTAC-3' and 5'-GGTGACCGGGGTTAGTAC-3' (GGAGGAGGTTAAGGCTACCTTCTTTTGC-3' was added homology to the T7-Flag-4 vector underlined) and Gibson-assembled with the T7-Flag-4 vector amplified by the primers 5'-AAGATTCATCTCGAGAGTG-3' and 5'-GAAAAGCAGGTTGTGTCTC-3'. The cloned circularly permuted 23S rRNA gene in the resulting plasmid pCP23S-EagI containing a pBR322 origin of replication and KanR selective marker (Extended Data Fig. 2) was fully sequenced.

The pCP23S-EagI plasmid was then digested with EagI (New England Biolabs) for 1 h at 37°C, and the circularly permuted 23S RNA (CP23S) gene was isolated from a SYBR Safe-stained 0.7% agarose gel using a EZ.N.A. Gel Extraction kit (Omega). CP23S rRNA was circuclarized by T4 polynucleotide ligase (New England Biolabs) in a 50 μl reaction with 2.5 μg ml⁻¹ DNA for 14 h at 15°C, followed by heat inactivation at 75°C for 10 min. The reaction was diluted 1:100 for use as a template in the PCR reactions for generating the circular permuted (Extended Data Fig. 2).

Ninety-one CP23S mutants were designed by introducing new 23S rRNA 5' and 3' ends at most of the apex loops and some internal loops of rRNA helices to assure spatial proximity of the new rRNA termini in the fully assembled 50S ribosomal subunit. Each CP23S rRNA gene was PCR-cloned in a 40 μl reaction using Phusion High Fidelity DNA polymerase (New England Biolabs), with primer pairs shown in Supplementary Table 1, and 4 μl of the 1:100 diluted 23S circular ligation reaction as template. Each primer pair adds to the 5' and 3' ends of the amplified CP23S gene 20-base-pair (bp) of homology to the 23S rRNA processing stem and did not affect growth of SQ171 cells expressing the wild-type CP23S rRNA gene (see above and Extended Data Fig. 2a) by using the primers introducing poly-A linkers into the permuted 23S rRNA gene (Extended Data Fig. 2e).

To minimize PCR errors in generating the vector backbone, which carried 16S rRNA sequences, and prevent carry-through of the wild-type product was purified using E.Z.N.A. Cycle Pure kit (Omega) and the size of the 16S-23S rRNA gene 20-base-pair (bp) of homology to the 23S rRNA processing stem was verified by PCR using a mixture of three primers: primer 1 (5'-GCGGTACCTCGATGCTG-3') corresponding to the sequence of the 16S rRNA gene at 580 bp, primer 2 (5'-GCTGAGTTACCGGCTACTTTTTTG-3') containing the sequence of the 23S rRNA gene at 2863 bp, and primer 3 (5'-GGTTCAGTTTTGGATC-3') corresponding to the sequence of the 16S/23S intergenic spacer 139–116 bp upstream from the 23S rRNA gene in rrnB (Extended Data Fig. 2e). The combination of the 1 and 3 produces a 207-bp PCR product with the wild-type rrn operon is present; the combination of primers 1 and 2 produces a 112-bp PCR band on the template with circularly permuted 23S rRNA gene (Extended Data Fig. 2e). To reduce the number of false-negative CP23S rRNA variants, the experiment was repeated one more time using de novo Gibson assembly reactions with the CP23S rRNA constructs that failed to replace pCSacB in the first experiment. Two of the remaining CP23S functional constructs were sequenced to confirm their identity. Altogether, 22 CP23S rRNA variants were able to replace pCSacB in the SQ171 cells. CP23S identity was confirmed by plasmid sequencing. Growth rates were analysed on Biotek Synergy H1 plate readers in 96-well flat-bottom plates (Costar) in 100 μl LB with 50 μg ml⁻¹ carbenicillin. Doubling times and final A600 max after 18 h are shown in Extended Data Table 1.

Construction of prlBo-T. To avoid generation of mutations in the 23S rRNA gene during PCR amplification for Gibson assembly, the 23S rRNA gene variant circularly permuted at H101 (corresponding to CP2861 from Fig. 1) was first cloned into the pcUG18 vector. For that, the 23S rRNA gene circularly permuted at H101 was PCR-amplified from circularized 23S rRNA gene prepared in the circular permutation study (see above and Extended Data Fig. 2a) by using the high-fidelity AccuPrime Taq polymerase (Life Technologies) and primers containing BamHI restriction sites (shown in bold) 5'-TATTTTAGACCTGGGTATGC TTGCGATCTAAGGCCTTTATAC-3' and 5'-TTTTAGAGCTCCAGACCGAC-3' corresponding to the sequence of the 16S/23S intergenic spacer 139–116 bp upstream from the 23S rRNA gene in rrnB (pUC23S) was fully sequenced to verify the lack of mutations in the 23S rRNA gene.

For preparation of prlBo-T (Extended Data Fig. 1b), pAM552-A23S-AflII plasmid (see above) served as a recipient for the CP2861 23S rRNA gene. The plasmid backbone was prepared by PCR-amplifying the plasmid pAM552- A23S-AflII digested with AflII and cloned in dephosphorylated BamHI-cut pUC18 plasmid. A plasmid containing 2863 23S rRNA (pUC23S) was used for transformation into the E. coli POP2136 electrocompeent cells, plating on LB agar plates supplemented with 50 μg ml⁻¹ carbenicillin, and grown at 30°C. Plasmids from several colonies were isolated and fully sequenced. The resulting pAM552-A23S-AflII plasmid contains the 16S rRNA, 23S processing stems with an added AflII restriction site, SS rRNA, and β-lactamase resistance gene and ColE1 ori (Extended Data Fig. 2). Vector backbone was prepared by digesting pAM552-A23S-AflII with AflII restriction enzyme at 37°C for 2 h and purification using an E.Z.N.A. Cycle Pure kit.

All the CP23S plasmids were assembled in parallel by Gibson assembly reaction (Extended Data Fig. 2) in a 96-well PCR plate. For each CP23S target, 50 ng of AflII-digested purified backbone was added to threefold molar excess of the PCR-amplified and purified CP23S insert. Gibson assembly mix (15 μl) was added, the final volumes brought to 48 μl with nuclease-free water, and incubated at 50°C for 1 h in the PCR machine. No CP23S insert was added to the negative control reaction. To check the efficiency of DNA assembly, 2 μl of selected assembly reactions were transformed into electroporation competent POP2136 cells. After 1 h recovery at 37°C in SOC media, a quarter of each transformation was plated on LB-agar plates supplemented with 50 μg ml⁻¹ carbenicillin and grown for 20 h at 30°C. A typical CP23S assembly reaction generated 30–120 POP2136 colonies with the control reaction generating only few colonies.

Testing CP23S rRNA constructs. Transformation of SQ171/pSacB rubidium chloride-resistant cells was carried out in a 96-well plate. Two microplates of the Gibson Assembly reactions were added to 20 μl competent cells in the pre-chilled 96-well plate (45°C for 5-min incubation in a water bath, 45°C for 1 min on ice, 45°C for 10 min). The plate was incubated overnight at 37°C with shaking at 600 r.p.m. on a microplate shaker. Forty microplates of medium were then transferred from each well to the wells of another 96-well plate containing 120 μl SOC supplemented with 100 μg ml⁻¹ ampicillin and 0.25% sucrose. The plate was incubated overnight at 37°C with shaking at 600 r.p.m. A 96-pin replicator was used to spot aliquots of the cultures onto a rectangular LB agar plate containing 100 μg ml⁻¹ ampicillin, 5% sucrose and 1 mg ml⁻¹ erythromycin. The plate was incubated overnight at 37°C and the appearance of Amp'/Ery' transformants was recorded. The completeness of the replacement of the wild-type pCSacB plasmid with the plasmids carrying circularly permuted 23S rRNA gene was verified by PCR using a mixture of three primers: primer 1 (5'-GCGGTACCTGGGTATGC TTGCGATCTAAGGCCTTTATAC-3') complementary to the 23S rRNA segment 50–69; primer 2 (5'-TTTTAGAGCTCCAGACCGAC-3') containing the sequence of the 23S rRNA gene at 2863–2882, and primer 3 (5'-GAGAACCGACGTTGGATCATTGATTG-3') corresponding to the sequence of the 16S/23S intergenic spacer 139–116 bp upstream from the 23S rRNA gene in rrnB.
ATAGCCGGGTTTGAAGGCA(A):-5’GGAGGGGGCTTTCTCCTTTGT (forward primer with tether T2). The PCR reaction, which was catalysed by Phusion High Fidelity DNA polymerase, was carried out under the following conditions: 98°C for 2 min followed by 30 cycles of (98°C, 30 s; 62°C, 30 s; 72°C, 2 min) followed by 2 min for 5 min. The resulting 4.6-kilobase (kb) PCR fragment was treated with DpnI for 4 h at 37°C and purified using Wizard SV Gel and PCR Clean-Up Kit (Promega). The PCR-amplified plasmid backbone and the gel-purified CP2861 23S rRNA gene fragment were combined in a Gibson Assembly reaction. Five microlitres of the reaction mixture was transformed into 50 µl electrocompetent POP2136 E. coli cells. Cells were plated onto LB/agar plate supplemented with 100 µg ml⁻¹ ampicillin. After 24 h incubation at 30°C, the colonies appeared. Seventeen colonies were picked, grown in LB/ampicillin at 30°C, plasmids were isolated and linkers were sequenced using the primers 5’-GAACCTTACCTGGTCTTGACATC-3’ (corresponding to the 16S rDNA sequence 976-988) and 5’-ATATCCAGGGGCTTTGTTG-3’ (corresponding to the 23S rDNA sequence 2476–2495) to verify the complexity of the linker library (Supplementary Table 2). All the colonies were then washed off the plate and total plasmid was extracted and used to transform SQ171-competent cells.

Functional replacement of the wild-type ribosome by Ribo-T. SQ171 cells carrying the pScSb plasmid, which carries the wild-type rrnB operon, were transformed with the total pRibo-T preparation isolated from the POP2136 cells. In brief, 250 ng of plasmid preparation were added to 250 µl of ribudil-chloroform-ethanol precipitate and were incubated for 45 min on ice, 45 s at 37°C, and 2 min on ice followed by addition of 1 ml SOC medium and incubation at 37°C for 2 h with shaking. A 150-µl aliquot of the culture was transferred to 1.85 ml SOC supplemented with 100 µg ml⁻¹ ampicillin and 0.25% sucrose (final concentrations) and grown overnight at 37°C with shaking. Cells were spun down and plated on an LB agar plate containing 100 µg ml⁻¹ ampicillin, 5% sucrose and 1 mg ml⁻¹ erythromycin. Eighty of the colonies that appeared after 48-h incubation of the plate at 37°C were inoculated in 2 ml LB supplemented with 100 µg ml⁻¹ ampicillin and grown for 48 h. The growth rate of ∼30 clones that managed to grow during that period was then assessed in LB/ampicillin medium in the 96-well plate. Plasmids were isolated from six faster growing clones and linkers were sequenced. The linker T1 in five sequenced clones was composed of 9 adenines and linker T2 was composed of 8 adenines, while one clone had the reverse combination. Total RNA was extracted from these clones using RNeasy Mini Kit (Qiagen) and analysed by agarose electrophoresis. The successful replacement of the wild type pScSb plasmid with the pRibo-T plasmids carrying Ribo-T was verified by PCR using primers 5’-GACAGTCGGTCCCTATCTG-3’ (corresponding to the 23S rDNA sequence 2599–2618) and 5’-TTAAGGCTTACGGTCTTGTTG-3’ (corresponding to the 23S rDNA sequence 2878–2900) and additionally verified by primer extension on the total cellular rRNA as indicated in the Extended Data Fig. 4. The growth of the cells was monitored at 37°C in 150 µl of LB supplemented with 100 µg ml⁻¹ ampicillin in the wells of a 96-well plate in the TECAN microplate reader (15 min orbital shaking with a 3-mm amplitude) supplemented with 100 µg ml⁻¹ spectinomycin. The growth rate of SQ171/pAM522 cells showed that radioactivity curve plateaus after 120 s of incubation of cells with [35S]-methionine.

Exponential cultures (250 ml) of the SQ171g strain transformed with either pAM522 (wild-type) or pRibo-T8/9 as described26. RNA was phenol extracted, precipitated as previously described and resolved by electrophoresis in a denaturing 6% (acrylamide-bis-acrylamide ratio 1:19, w/w) polyacrylamide gel (for the 5S rRNA analysis) or 4% (acrylamide-bis-acrylamide ratio 1:29, w/w) polyacrylamide gel (for the analysis of large rRNAs). The associated ribosomal proteins were analysed by mass spectrometry at the Proteomes Center of Excellence, Northwestern University. Ribosomes were precipitated by incubation in 20% trichloroacetic acid at 4°C overnight and centrifugation at 14,000g for 10 min. Precipitated ribosomes were washed once with cold 10% trichloroacetic acid and twice with acetone. The pellet was air-dried for 20–10 min before resuspension in 20 µl 8 M urea. Proteins were reduced with 10 mM dithiothreitol and cysteine residues alkylated with 50 mM iodoacetamide in the final volume of 160 µl. Sequencing-grade trypsin (Promega) was added at a 1:50 enzyme-protein ratio, and after overnight digestion at room temperature, the reaction was stopped by addition of formic acid to 1%. After digestion, peptides were desalted using C18 Spin columns (Pierce, 98870) and lyophilized. Amino-reactive tandem mass tag (TMT) reagents (126/127, Thermo Scientific, 90065) were used for peptide labelling. The reagents were dissolved in 41 µl acetonitrile and added to the lyophilized peptides dissolved in 100 µl of 100 mM triethylmonium bicarbonate. After 1 h at room temperature, the reaction was quenched by adding 8 µl of 5% hydroxyamine. After labelling, the two samples under analysis were mixed in 1:1 ratio. Peptides were desalted using C18 ZipTip Pipette Tips (EMD Millipore) and resuspended in 30 µl of solvent A (95% water, 5% acetonitrile, 0.2% formic acid).

Peptides were analysed by nanoelectrospray ionization on an Orbitrap Elite mass spectrometer (Thermo Scientific). Proteome Discoverer (Thermo Scientific) and the Sequest algorithm were used for data analysis. Data were searched against a custom database containing UniProt entries using E. coli taxonomy, allowing three missed cleavages, 10 p.p.m. precursor tolerance, and carbamidomethylation of cysteine as a static modification. Variable modifications included oxidation of methionine, TMT of lysine and amino-terminal TMT. For quantification via the reporter ion the intensity of the signal closest to the theoretical m/z, within a ±10 p.p.m. window, was recorded. Reporter ion intensities were adjusted based on the overlap of isotopic envelopes of all reporter ions as recommended by the manufacturer. Only peptides with high confidence were used for quantification. Ratios of 126/127 were normalized based on median.

 Succrose gradient analysis of ribosomes and ribosomal subunits. Wild-type 70S ribosomes or Ribo-T isolated from SQ171g cells as described above were diluted approximately 70-fold in high Mg²⁺ buffer (20 mM Tris-HCl, pH 7.5, 150 mM MgCl₂) supplemented with 1 mM MgCl₂, 0.25% sodium deoxycholate and 2 U of RQ1 DNase (Promega). The lysates were centrifuged at 20,000g for 30 min at 4°C and polynucleotides-containing supernatants (20 A₂₆₀ nm absorbance units) were loaded onto the 12-ml 10–50% sucrose gradient buffer: 20 mM Tris-HCl, pH 7.5, 150 mM MgCl₂, 100 mM NH₄Cl, 2 mM β-mercaptoethanol. Polysomes were resolved by centrifugation in a SW-41 rotor (39,000 rpm, 3 h, 4°C). Gradients were fractionated using BioComp Instrument gradient fractionator and the fractions were collected in the wells of a 96-well plate using gradient fractionator (BioComp Instrument).

Probing the structure of the Ribo-T tethers. The structure of the tethers was probed by dimethylsulfate (DMS) modification following a published protocol32. In brief, 10 pmol of Ribo-T or wild-type ribosomes were activated by incubation for 5 min at 42°C in 50µl of buffer 80 mM HEPE-KOH, pH 7.6, 150 mM MgCl₂, 100 mM NH₄Cl containing 20 U of Ribolock RI RNase inhibitor (Thermo Fisher Scientific). Two microliters of DMS (SIGMA) diluted 1:10 in ethanol were added (2 µl of ethanol were added to the unmodified controls) and samples were incubated for 10 min at room temperature. The modification reaction was stopped and RNA extracted as described32. Primer extensions were carried out using the primers 5’-GACATGACGGGACTCCAACG-3’ and 5’-AAGTGTAAGCGTCAGCGG-3’ (for tether T1) or 5’-CCCTACGGTTACCTGGTTG-3’ for tether T2.
Additionally, the integrity of the tethers in the Ribo-T preparation was tested by extension of the primers annealing immediately 3′ to the tether. Primer 5′-GTACCGGTTAGCTCAAGGACATC-3′ was extended by reverse transcription across tether T1 in the presence of dATP, dTTP, dGTP and ddTTP, and primer 5′-CCACAAAATGGAAGGCGCCCTC-3′ was extended across tether T2 in the presence of dATP, dTTP, dGTP and ddTTP.

Testing Ribo-T activity in cell-free translation system. The DNA template containing the T7 promoter and the sf-GFP gene was PCR amplified from a pYT1-sfGFP plasmid using primers 5′-TAATACGACTCACTATAGGG-3′ and 5′-CTTCTCTTGCGGGTGTTT-3′. GFP mRNA was purified by in vitro transcription and purified by size-exclusion chromatography on a Sephadex G50 mini-column, phenol extraction and ethanol precipitation. The transcript was translated in the Δr(ribosome, amino acid, rRNA) PUREExpress system kit (New England Biolabs). A typical translation reaction was assembled in a total volume of 10 μl and contained 2 μl of the kit solution, 1 μl α-factor mixture, 1 μl amino acid mixture (3 mM each), 1 μl rRNA (20 μg/ml), 0.4 μl Ribolock RNase inhibitor (40 U μl⁻¹), 5 μg (~20 pmol) GFP transcript and 22 pmol of wild-type ribosomes or Ribo-Ts. Samples were placed in wells of a 384-well black wall/clear flat bottom tissue-culture plate (BD Biosciences) and covered with the lid. Reactions were incubated at 37°C in a microplate reader (Tecan), and fluorescence values were recorded every 20 min at λex = 488 nm and λem = 520 nm over 7 h. Protein synthesis rates were calculated by linear regression over the time points 0, 40 and 60 min (R² > 0.9) using the trendline function of Excel (Microsoft). Time point 20 min was not taken into consideration because the plate was switched from ice to 37°C at time 0.

Transcription/translation of the dihydrofolate reductase template supplied with the Δ(ribosome, amino acid, rRNA) PUREExpress kit (New England Biolabs) was carried in the presence of [35S]-methionine (1,175 Ci mm⁻¹) using manufacturers protocol. A typical 5 μl reaction, assembled as described above but using 50ng of the DNA template, was supplemented with 5 μCi [35S]-methionine and 10 pmol of wild-type or Ribo-T ribosomes. When needed, the reactions were supplemented with 50 μM ethyrythromycin. Reactions were incubated for 2 h at 37°C, and protein products were analysed by SDS–PAGE in 16.5% Bis-Tris gels (Biorad) using NuPAGE MES/SDS running buffer (Invitrogen). Gels were stained, dried and exposed to a phosphorimager screen overnight. The gel was imaged by autoradiography using a phosphoimager screen overnight. The gel was imaged by autoradiography using a phosphoimager screen (BioRad) using NuPAGE MES/SDS running buffer (Invitrogen). Gels were stained, dried and exposed to a phosphorimager screen overnight. The gel was imaged by autoradiography using a phosphoimager screen (BioRad) using NuPAGE MES/SDS running buffer (Invitrogen). Gels were stained, dried and exposed to a phosphorimager screen overnight. The gel was imaged by autoradiography using a phosphoimager screen.

Translation products were analysed in 16.5% Tricine SDS–PAGE with a 2 μl aliquot of the reaction mixture loaded onto the gel (λex 280 nm). The gel was stained with Coomassie blue, destained and dried. A phosphorimager screen (BioRad) was used to capture the images. The gel was imaged by autoradiography using a phosphoimager screen.
tetracycline. Then colonies were re-streaked on LB-agar plates containing 10 μg ml⁻¹ tetracycline, 200 μM IPTG and 80 μg ml⁻¹ X-Gal. The replacement of wild-type lacZ with the AlacZ58(M15) allele was verified by PCR using primers 5'-ATCTCATGACCAA AATCCCTTAACGTGAGT-3' and 5'-CCGGTTAGCTTTTACCCCTGCATCT TTGAG-3'. A 568-bp DNA fragment in which the ends overlapped with the amplified pACYC177 backbone and which contained T7 promoter, the orthogonal Shine–Dalgarno sequence CACCAC, the secM(121–166)-lacZ fusion from the plasmid pNH122 (ref. 18), was synthesized by Integrated DNA Technologies. The pACYC177 backbone and the secM-lacZ construct were combined using Gibson Assembly and introduced in the C41(DE3)/lacZ58(M15) cells.

Construction of the 2451/2452 mutant poRibo-T library and selecting mutants capable of alleviating SecM-mediated translation arrest. A library of A2451N/C2452N mutants was generated by inverse PCR using plasmid poRibo-T2 as a template, Phusion High Fidelity DNA polymerase, and primers 5'-AGGC TGATACGGGACAA TATCCCTTAACGTGAGT-3' and 5'-GGGTAGCTTTTACCCCTGCATCT TTGAG-3'. A 30 s; 72 °C, 120 s), followed by final extension 72 °C, 10 min. The PCR-amplified DNA band was purified by extraction from the agarose gel with an E.Z.N.A. gel extraction kit, and re-circularized by Gibson assembly for 1 h at 50 °C. Two micro-litres of the reaction were transformed into electropotent POP2136 cells plated on LB-agar plates containing 50 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ ampicillin and incubated overnight at 37 °C. Three colonies from each transformation were then streaked on LB-agar plates containing 50 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ ampicillin and supplemented with 0.5 mM IPTG, 40 μg ml⁻¹ X-Gal and 2 mM PETG. Plates were incubated at 37 °C for 22 h and photographed.

Extended Data Figure 1 | Key plasmids used in the study. a, The pAM552 plasmid is a derivative of pLK35 (ref. 27), from which the unessential segments of the pBR322 cloning vector have been removed. pAM552 contains the entire rrrB operon of E. coli under the control of the phage lambda P\textsubscript{L} promoter, which is constitutively active in the conventional E. coli strains but is silent at 30 °C in the strain POP2136 (30 °C) carrying the cl857 gene of the temperature-sensitive lambda repressor. The 16S rRNA gene is shown in orange, and the 16S rRNA processing stem sequences indicated in yellow. The 23S rRNA gene is blue, and the corresponding processing stem sequences are light blue. The intergenic tRNA\textsubscript{Glu} gene is shown in dark grey. b, The map of the pRibo-T8/9 plasmid derived from pAM552. The native 5' and 3' ends of the 23S rRNA were linked via a tetranculeotide sequence GAGA (connector C shown in green), and circularly permuted 23 rRNA gene, 'opened' in the apex loop of H101, was inserted in the apex loop of 16S rRNA helix h44 via an A\textsubscript{8} linker T1 and an A\textsubscript{9} linker T2 (red bars). c, The map of the backbone plasmid pT7wtK and the reporter plasmids pT7oGFP and pLpp5oGFP, expressing sf-gfp controlled by an orthogonal Shine–Dalgarno sequence (orange semi-circle) under T7 or lpp5 promoters (black triangles). d, The map of the pACYC177-derived plasmid containing the secM-lacZ\textsubscript{a} reporter gene controlled by the T7 promoter (black triangle) and alternative Shine–Dalgarno sequence (orange semi-circle). The sequence of the secM-lacZ\textsubscript{a} reporter matches that in the originally described plasmid pNH122 (ref. 18).
The experimental scheme of preparing and testing circularly permuted 23S rRNA gene library. 

**a. CP23S generation**
- The CP23S template is generated from pCP23S-EagI plasmid by EagI digestion and ligation. Each CP23S variant is generated by PCR using circularized 23S rRNA gene as a template and a unique primer pair, with added sequences overlapping the destination plasmid backbone.

**b. Plasmid backbone**
- The plasmid backbone is prepared by digestion of pAM552-Δ23S-AflII with the AflII restriction enzyme, which linearizes the backbone at the 23S processing stem site.

**c. Gibson assembly**
- Gibson assembly is used to incorporate each CP23S variant into the plasmid backbone to generate the 91 target circular permutants.

**d. Introduction of the CP23S construct in the Δ7rm strain**
- The pAM-CP23S plasmids are transformed into the SQ171 strain lacking chromosomal rRNA operons and carrying the pCSacB plasmid with the wild-type rRNA operon, and transformants resistant to ampicillin, erythromycin and sucrose are selected.

**e. Three primer PCR check on total plasmid extract**
- A complete replacement of pCSacB with pAM-CP23S is verified by a three-primer diagnostic PCR.

Extended Data Figure 2 | The experimental scheme of preparing and testing circularly permuted 23S rRNA gene library. a. The CP23S template is generated from pCP23S-EagI plasmid by EagI digestion and ligation. Each CP23S variant is generated by PCR using circularized 23S rRNA gene as a template and a unique primer pair, with added sequences overlapping the destination plasmid backbone. b. The plasmid backbone is prepared by digestion of pAM552-Δ23S-AflII with the AflII restriction enzyme, which linearizes the backbone at the 23S processing stem site. c. Gibson assembly is used to incorporate each CP23S variant into the plasmid backbone to generate the 91 target circular permutants. d. The pAM-CP23S plasmids are transformed into the SQ171 strain lacking chromosomal rRNA operons and carrying the pCSacB plasmid with the wild-type rRNA operon, and transformants resistant to ampicillin, erythromycin and sucrose are selected. e. A complete replacement of pCSacB with pAM-CP23S is verified by a three-primer diagnostic PCR.
Extended Data Figure 3 | The Ribo-T tethers allow for the ribosome ratcheting. Distance changes (Å) between the 16S rRNA and 23S rRNA residues h44 and H101 connected by the oligo(A) linkers in Ribo-T when the ribosome undergoes the transition from the classic to the rotated state. The distances between the 5’ phosphorus atoms of the corresponding nucleotides are shown. 16S and 23S rRNAs in the non-rotated state are tan and pale blue, and in the rotated state are gold and blue, respectively. The structures of the E. coli ribosomes used for measuring the distances and generating the figure have PDB accession numbers 3R8T and 4GD2 (non-rotated state) and 3R8S and 4GD1 (rotated state).
Extended Data Figure 4 | Chromosomal mutations enhance growth of SQ171 cells in which Ribo-T completely replace wild-type ribosomes.

a, Growth curves of the parental SQ171 cells transformed with the pAM552(G2058) plasmid (black curve) or pRibo-T8/9 plasmid (blue curve) or selected fast growing mutant (SQ171fg) transformed with pRibo-T8/9 (green curve). The cells express homogeneous populations of ribosomes (wt for pAM552 transformants or Ribo-T for the pRibo-T8/9 transformants, see panels b and c). b, PCR analysis of rDNA in the SQ171fg strain transformed with pRibo-T8/9 (the SQ110 strain that carries a single chromosomal copy of the rrn allele served as a wild-type control). The PCR primers amplify the 302-base-pair 23S rRNA gene segment 'across' the H101 hairpin in wild-type rDNA. In pRibo-T, the primer annealing sites are more than 4.8 kb apart (black dashed line), which prevents formation of the PCR product. Two additional primers designed to amplify a 467-bp fragment from the lacZ gene were included in the same PCR reaction as an internal control. The gel is representative of two independent biological experiments. c, Primer extension analysis of rRNA expressed in the SQ171fg cells transformed with pAM552 (WT), pAM552 with the A2058G mutation, or pRibo-T8/9, which carries the A2058G mutation. Primer extension was carried out in the presence of dTTP and ddCTP. Because Ribo-T contains the A2058G mutation in the 23S rRNA sequence, the generated cDNA is one nucleotide shorter than the one generated on the wild-type 23S rRNA template. The lack of the 20-nucleotide cDNA band in the Ribo-T sample demonstrates the absence of wild-type 23S rRNA in the SQ171fg cells transformed with pRibo-T8/9. The gel is representative of three independent biological experiments. d, e, Chromosomal mutations in SQ171fg: a nonsense mutation in the Leu codon 22 of the ybeX gene encoding a protein similar to Mg$^{2+}$/Co$^{2+}$ efflux transporter (d); and a missense mutation in codon 549 of the rpsA gene encoding ribosomal protein S1 (e).
Extended Data Figure 5 | Ribo-T composition and integrity of the linkers.

a, b, Analysis of rRNA extracted from the isolated wild-type ribosomes or Ribo-T in a denaturing 4% (a) or 8% (b) polyacrylamide gel. a, Ribo-T(1) and Ribo-T(2) represent two individual preparations with Ribo-T(2) isolated following the standard procedure (see Methods), and Ribo-T(1) isolated by immediate pelleting through the sucrose cushion after the cell lysis. The faint bands in the Ribo-T2 preparation indicated by the asterisks could be occasionally seen in some preparations; they probably represent rRNA fragments generated by cleavage of the linkers in a small fraction of Ribo-T either in the cell or during Ribo-T preparation. b, 5S rRNA is present in Ribo-T.

c, The relative abundance of small and large subunit proteins in Ribo-T in comparison with wild-type ribosome as determined by mass spectrometry (protein L26 could not be reliably quantified in Ribo-T and wild-type ribosomes). The data represent the average of three technical replicates, and error bars indicate the s.d.

d, Analysis of the integrity of the T1 and T2 linkers in a Ribo-T preparation by primer extension. The 22-nucleotide-long primer was extended across the T1 linker in the presence of ddCTP terminator and the 23S-nucleotide-long primer was extended across the T2 linker in the presence of ddGTP terminator. Control samples (−) represent the unextended primers. The gels are representative of two independent experiments.
Extended Data Figure 6 | Ribo-T can successfully translate most cellular polypeptides. **a**, Protein synthesis rate in SQ17Tfg cells expressing wild-type ribosomes or Ribo-T. Protein synthesis was measured by quantifying the incorporation of [\(^{35}\)S]-methionine into TCA-insoluble protein fraction during a 45-s incubation at 37 °C in minimal medium. The bar graphs represent the average values of experiments performed in two biological replicates each done in two technical duplicates. Error bars denote s.d. **b, c**, 2D gel electrophoresis analysis of the proteins expressed in exponentially growing SQ17Tfg transformed with pAM552 (A2058G) (b) or pRibo-T (c).
Extended Data Figure 7 | Chemical probing of the structure of the Ribo-T linkers. Ribo-T or wild-type ribosomes were modified by dimethylsulfate, and extracted rRNA was subjected to primer extension analysis. In each gel, the left two lanes ('C' and 'A') represent sequencing reactions followed by dimethylsulfate-modified sample and control (unmodified) RNA. The diagrams on the right represent the secondary structures of helices H101 and h44 in wild-type ribosomes (left) and Ribo-T (right), with the nucleotide residues modified strongly, moderately and weakly indicated by black, grey and white circles, respectively. The gels are representative of two independent experiments.
Extended Data Figure 8 | Translation of the orthogonal sf-gfp gene by oRibo-T in vivo and in vitro. a, Expression of an orthogonal sf-gfp reporter in the E. coli POP2136 cells transformed with pAM552 plasmid encoding wild-type rRNA (wt Rbs), pAM552 with an orthogonal Shine–Dalgarno sequence in 16S rRNA of a non-tethered ribosome (oRbs) or poRibo-T1 expressing an orthogonal Ribo-T (green bar). Cells lacking gfp reporter gene (wt Rbs Δgfp) were used as a background fluorescence control. The data represent the average value of six biological replicates in technical triplicates; error bars indicate the s.d. b, In vitro translation of the orthogonal sf-gfp reporter by non-tethered non-orthogonal wt ribosomes (pink lines), or oRibo-T(A2058G) (which also contained cellular wild-type ribosomes) (green lines). The dotted lines correspond to the translation reactions without antibiotic and solid lines represent reactions supplemented with 50 μM clindamycin (Cld). c, Same as in b, but oRibo-T contained a G693A mutation instead of A2058G and clindamycin was replaced with 100 μM pactamycin (Pct). The red stars indicate the ribosomal subunit carrying the antibiotic-resistance mutation. Graphs in b and c are each representative of two biological replicates each performed in technical triplicates, and error bars indicating the s.d.
Extended Data Figure 9 | Promoter mutation in oRibo-T improves transformation of the *E. coli* cells. **a, b,** Several *E. coli* strains, including BL21 shown in this figure, as well as JM109 and C41, produced slowly growing, heterogeneous colonies when transformed with poRibo-T1. **c,** Fortuitously, in the course of the experiments we isolated a spontaneous mutant plasmid, poRibo-T2, which showed improved transformation efficiency, producing evenly sized colonies after a single overnight incubation. Sequencing of poRibo-T2 revealed a single mutation in the P_L promoter controlling Ribo-T expression, altering the ‘-10’ box from GATACT to TATACT bringing it closer to the TATAAT consensus. It is unclear why the promoter mutation improves performance of poRibo-T (as well as of non-orthogonal pRibo-T) in ‘unselected’ *E. coli* cells. The plates show representative results of three independent biological experiments.
## Extended Data Table 1 | Characterization of the growth of *E. coli* SQ171 cells expressing a pure population of ribosomes with circularly permuted 23S rRNA

<table>
<thead>
<tr>
<th></th>
<th>Doubling time (min) *</th>
<th>Cell density (OD&lt;sub&gt;600&lt;/sub&gt;) at saturation †</th>
<th>n ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 °C</td>
<td>37 °C</td>
<td>30 °C</td>
</tr>
<tr>
<td>pAM552</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>61.0 ± 3.2</td>
<td>53.9 ± 1.0</td>
<td>1.04 ± 0.06</td>
</tr>
<tr>
<td>pAM552-AflII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>67.4 ± 1.0</td>
<td>53.3 ± 2.4</td>
<td>1.07 ± 0.01</td>
</tr>
<tr>
<td>CP67</td>
<td>106.4 ± 5.4</td>
<td>69.6 ± 2.1</td>
<td>0.83 ± 0.05</td>
</tr>
<tr>
<td>CP95</td>
<td>144.9 ± 35.9</td>
<td>82.4 ± 24.4</td>
<td>0.66 ± 0.31</td>
</tr>
<tr>
<td>CP104</td>
<td>90.8 ± 10.3</td>
<td>52.7 ± 3.2</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>CP166</td>
<td>123.8 ± 27.9</td>
<td>57.7 ± 1.9</td>
<td>0.70 ± 0.22</td>
</tr>
<tr>
<td>CP281</td>
<td>100.1 ± 11.0</td>
<td>54.6 ± 10.1</td>
<td>1.01 ± 0.04</td>
</tr>
<tr>
<td>CP549</td>
<td>101.7 ± 18.2</td>
<td>46.5 ± 3.9</td>
<td>1.00 ± 0.02</td>
</tr>
<tr>
<td>CP617</td>
<td>231.7 ± 20.5</td>
<td>91.5 ± 18.5</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>CP634</td>
<td>162.0 ± 34.2</td>
<td>212.5 ± 58.1</td>
<td>0.46 ± 0.19</td>
</tr>
<tr>
<td>CP879</td>
<td>106.6 ± 4.7</td>
<td>51.4 ± 4.6</td>
<td>1.03 ± 0.02</td>
</tr>
<tr>
<td>CP891</td>
<td>144.5 ± 41.8</td>
<td>60.7 ± 4.1</td>
<td>0.56 ± 0.43</td>
</tr>
<tr>
<td>CP1112</td>
<td>89.6 ± 6.0</td>
<td>57.8 ± 12.2</td>
<td>0.96 ± 0.02</td>
</tr>
<tr>
<td>CP1178</td>
<td>102.5 ± 11.0</td>
<td>48.2 ± 1.3</td>
<td>0.96 ± 0.02</td>
</tr>
<tr>
<td>CP1498</td>
<td>167.5 ± 17.5</td>
<td>118.0 ± 17.1</td>
<td>0.56 ± 0.32</td>
</tr>
<tr>
<td>CP1511</td>
<td>131.5 ± 4.2</td>
<td>76.7 ± 1.5</td>
<td>0.88 ± 0.01</td>
</tr>
<tr>
<td>CP1587</td>
<td>98.1 ± 12.4</td>
<td>55.1 ± 6.6</td>
<td>0.93 ± 0.05</td>
</tr>
<tr>
<td>CP1716</td>
<td>174.4 ± 31.9</td>
<td>117.8 ± 16.5</td>
<td>0.44 ± 0.16</td>
</tr>
<tr>
<td>CP1733</td>
<td>117.3 ± 8.2</td>
<td>83.8 ± 2.2</td>
<td>0.95 ± 0.01</td>
</tr>
<tr>
<td>CP1741</td>
<td>230.0 ± 14.7</td>
<td>269.0 ± 50.3</td>
<td>0.28 ± 0.00</td>
</tr>
<tr>
<td>CP1873</td>
<td>108.4 ± 6.5</td>
<td>52.9 ± 0.8</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>CP2148</td>
<td>83.0 ± 2.9</td>
<td>52.4 ± 3.9</td>
<td>0.73 ± 0.09</td>
</tr>
<tr>
<td>CP2800</td>
<td>85.9 ± 15.7</td>
<td>53.5 ± 9.7</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td>CP2861</td>
<td>138.4 ± 10.7</td>
<td>93.7 ± 4.5</td>
<td>0.88 ± 0.00</td>
</tr>
</tbody>
</table>

*Growth in 100 μl LB media supplemented with 50 μg ml⁻¹ carbencillin in 96-well plate with shaking.

†After 18 h of growth.

‡pAM552: wild-type rrnB operon.

§pAM552-AflII: rrnB operon with the 23S rRNA mutations G2C and C2901G used to introduce the AflII restriction sites.

||pAM552-AflII with 23S rRNA mutations G2C and C2901G used to introduce the AflII restriction sites.

†|CPx: rrnB with 23S circular permutations and G2C/C2901G mutations; x indicates the 5’ starting nucleotide of the circularly permuted 23S gene.

*Biological replicates are indicated in the ‘n’ column, which is the number of separate colonies that were used for each mean number and s.d.