Protein synthesis by ribosomes with tethered subunits

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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 initiation1. 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 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 (termed Ribo-T) by selecting otherwise dominantly lethal rRNA mutants within the loop of helix 101 (H101). However, the length of H101 varies among different species, and its terminal loop sequence can tolerate alterations, h44 was a promising site for grafting. The randomized nature of the 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 previously9, and a subsequent pilot study showed that three 23S rRNA circular permutation variants could assemble into a functional subunit in vivo6. 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 peptidyl transferase and other proteins required for translation become connected to 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 synthesis10–12 (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 RNA species corresponding to the 16S–23S rRNA hybrid (Supplementary Data Fig. 1a). 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.

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wild-type tRNA 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\(^{2+}\)/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 rRNA. Analysis of Ribo-T preparations in a denaturing gel showed only very faint 16S and 23S-like rRNA 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 rRNA. 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 rRNA (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 5S rRNA and the full complement of ribosomal proteins in quantities closely matching the composition of wild-type ribosome (Extended Data Fig. 5b, 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 disassociates 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).

Figure 1 | Global screening of circularly permutated 23S rRNAs identifies variants capable of replacing the natural 23S rRNA in a functional ribosome. a, The general scheme for constructing the tRNA operon in which the mature 23S rRNA gene sequence is replaced with the circularly permuted gene (CP23S). b, Secondary structure diagram of 23S rRNA\(^\ast\) showing circular permutation (CP) constructs tested for their ability to support cell growth in the absence of wild-type ribosomes, named according to the number of the new 5' position in the wild-type (WT) 23S rRNA structure (for example, CP104). Viable circular permutation variants are green and italicized, non-viable variants are red. To assess the viability of CP mutants, two independent attempts to replace wild-type ribosomes with the CP construct were carried out. For all viable CP constructs, the lack of wild-type rRNA genes was confirmed by PCR as shown in the Extended Data Fig. 2, and the identity of the constructs in the resulting clones was verified by sequencing. c, The location of the new 5' ends (spheres, viable in green, non-viable in red) of CP variants of the 23S rRNA in the crystallographic structure of the E. coli 70S ribosome\(^1\) (Protein Data Bank (PDB) accession code 4V9D). The loops of helices h44 and H101 in the small and large subunit rRNA, respectively, used for subsequent experiments, are indicated by arrows.
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...
The result was qualitatively verified in an independent experiment performed at MgCl₂ concentrations 1.5 mM and 10 mM. In vitro translation of proteins by isolated Ribo-T. Top, SDS-PAGE analysis of the dihydrofolate reductase (DHFR) protein synthesized in the Δribosome 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]L-methionine in the absence or presence of 50 μM 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 Δribosome PURExpress system supplemented with purified wild-type (black) or Ribo-T (grey) ribosomes. The kₐ₅₆ 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 (black arrowhead). The threonyl-tRNA synthetase inhibitor borrelidin (BOR) arrests translation at the fourth codon of RST1 mRNA (grey arrowhead). The position of the 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.

Figure 3 | Functional characterization of Ribo-T. a. Sucrose gradient analysis of wild-type ribosomes (top) and Ribo-T (bottom) under 15 mM MgCl₂ (solid line) or 1.5 mM MgCl₂ 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 MgCl₂ 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 Δribosome 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]L-methionine in the absence or presence of 50 μM 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 Δribosome PURExpress system supplemented with purified wild-type (black) or Ribo-T (grey) ribosomes. The kₐ₅₆ 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 (black arrowhead). The threonyl-tRNA synthetase inhibitor borrelidin (BOR) arrests translation at the fourth codon of RST1 mRNA (grey arrowhead). The position of the 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.
C2452 sequence (the A2451C mutation) in the PTC. By contrast, none of the 16 analysed ‘white’ colonies had this sequence, and instead exhibited a variety of dinucleotide combinations at positions 2451–2452 (Fig. 4c). We corroborated these results by individually testing all possible 2451–2452 mutants in poSML-transformed E. coli C41(DE3) cells. Importantly, all the mutants were viable, confirming that oRibo-T is suitable for expression of dominantly lethal 23S rRNA mutations in vivo, indicating a low degree of cross-association of oRibo-T with free wild-type 30S subunits. Consistent with our previous result (Fig. 4c), the A2451C mutation confers the most pronounced blue colour of the transformants, comparable to that seen in cells expressing oRibo-T with the tunnel mutation A2058G (Fig. 4d). The A2451U mutation also increased the blue hue of the cells although to a lesser extent. These results suggested that the A2451C (and A2451U) mutants were not only functional in cellular 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 gene was translated in the λribosome PURExpress cell-free translation system supplemented with wild-type non-tethered ribosomes or preparations of oRibo-T (A2451 or C2451). The Ribo-T constructs carried the pactamycin-resistance mutation G693A in 16S rRNA, and the reactions were carried out in the presence of pactamycin, which, in addition to the presence of an orthogonal Shine–Dalgarno sequence, ensured that the reporter is translated exclusively by oRibo-T (see the control wild-type lane with no translation products). Numbers on the left indicate the size (kDa) of molecular mass markers. The bar graph at the bottom shows the efficiency of bypass (ratio between the full-size and SecM-arrested translation products). A representative gel of two independent experiments is shown, with error bars indicating the s.d.
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, it was surprising that Ribo-T would be able 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 evolvable 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 up 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.

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

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**Author Contributions** M.C.J. and A.S.M. designed the study, analysed results, and wrote the paper. G.D. 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@uiuc.edu) or M.C.J. (m-jewett@northwestern.edu).
No statistical methods were used to predetermine sample size.

Preparation of circularly permuted variants of the 23S RNA. The A2058G mutation was introduced into the pAM552 plasmid (Extended Data Fig. 1a) by inverse PCR using primers 5'-GGCTCTTGCCGGCTGCAG-3' and 5'-GGTACCCCGCGGCGGCGA-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 reaction4 (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'-GAGAACGACAGCTGGTCTTCCGGCGGA-3' and 5'-CACTCGTCGAGATCTGCTCTTCCGGCGGCGGTTAC-3' (added homology to the T7-Flag 4 vector underlined) and Gibson-assembled with the T7-Flag 4 vector amplified with the primers 5'-GAATGACTGTCGAGGAGTG-3' and 5'-GAAAGGCCAAGTGTGTTC-3'. The cloned circularly permuted 23S rRNA gene in the resulting plasmid pcP23S-Eagl containing a pBR322 origin of replication and KanR selective marker (Extended Data Fig. 2) was fully sequenced.

The pCP23S-Eagl plasmid was then digested with Eagl (New England Biolabs) for 1 h at 37 °C, and the circularly permuted 23S rRNA (pCP23S) gene was isolated from a SYBR Safe-stained 0.7% agarose gel using a E.Z.N.A. Gel Extraction kit (Omega). The 23S rRNA was circularized by T4 DNA ligase (New England Biolabs) in a 50 μl reaction with 2.5 ng ml⁻¹ DNA for 14 h at 16 °C, followed by heat inactivation for 15 min at 72 °C. 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 pcP23S 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 RNA termini in the fully assembled 50S ribosomal subunit. Each pcP23S rRNA gene was PCR-amplified 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 circularization reaction as template. Each primer pair adds to the 5' and 3' ends of the amplified pcP23S gene 20-base-pair (bp) of homology to the 23S rRNA processing stem (position 1905 in wild-type 23S RNA) into T7-Flag-4 plasmid (Sigma Aldrich) as follows. The circularized 23S rRNA gene was amplified by inverse PCR using primers 5'-GAGACACAAAGGGTCTTCGCGGCGGCACTATATAACG-3' and 5'-CACCTCGGAGATCTGCTCTTCCGGCGGCGTTAC-3' (added homology to the T7-Flag 4 vector underlined) and Gibson-assembled with the T7-Flag 4 vector amplified with the primers 5'-GAATGACTGTCGAGGAGTG-3' and 5'-GAAAGGCCAAGTGTGTTC-3'. The cloned circularly permuted 23S rRNA gene in the resulting plasmid pcP23S-Eagl containing a pBR322 origin of replication and KanR selective marker (Extended Data Fig. 2) was fully sequenced.

The pcP23S-Eagl plasmid was then digested with Eagl (New England Biolabs) for 1 h at 37 °C, and the circularly permuted 23S rRNA (pCP23S) gene was isolated from a SYBR Safe-stained 0.7% agarose gel using a E.Z.N.A. Gel Extraction kit (Omega). The 23S rRNA was circularized by T4 DNA ligase (New England Biolabs) in a 50 μl reaction with 2.5 ng ml⁻¹ DNA for 14 h at 16 °C, followed by heat inactivation at 65 °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).

A 23S-AflII restricted plasmid was prepared. The introduced mutations preserved the integrity of the 23S rRNA gene by introducing a GAGA tetra-loop followed by re-circularization by Gibson assembly reaction4 (all primers used in this study were synthesized by Integrated DNA Technology). The 23S-AflII-digested purified backbone was added to threefold molar excess of the PCR-amplified and purified pcP23S insert. Gibson assembly mix4 (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 pcP23S insert was added to the negative control reaction. To check the efficiency of DNA assembly, 2 μl of selected assembly reactions were transformed into electrocompetent 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 pcP23S assembly reaction generated 30–120 POP2136 colonies with the control reaction generating only few colonies.

Testing pcP23S RNA constructs. Transformation of SQ171/pPCsacB rubidium chloride–competent cells was carried out in a 96-well plate. Two microtubes of the Gibson Assembly Reaction reactions were added to 20 μl competent cells in the pre-chilled plate. After a 45-min incubation in ice/water bath, 45 s at 42 °C and 2 min on ice, 130 μl of SOC medium was added to the wells and the plate was incubated 2 h at 37 °C with shaking at 600 rpm on a microplate shaker. Forty microliters 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 rpm. After overnight incubation, the plasmid backbone was digested using 96-well pin replicator 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 pcPcsacB plasmid with the plastids carrying circularly permuted 23S rRNA gene was verified by PCR using a mixture of three primers: primer 1 (5'-GGTGATTAGCCTGAGTCTTC-3') complementary to the 23S RNA segment 50-69, primer 2 (5'-CGTGTAGGCTAAAGGTACT-3') containing the sequence of the 23S RNA segment 2863-2882, and primer 3 (5'-GGGTTGAGTTGAGATATTC-3') corresponding to the sequence of the 16S/23S intergenic spacer 139-116 bp upstream from the 23S RNA gene in rrsB (Extended Data Fig. 2e). The combination of the primers 1 and 3 produces a 207-bp PCR band if the wild-type rnr operon is present; the combination of primers 1 and 2 produces a 112-bp PCR band on the templates with circularly permuted 23S rRNA gene (Extended Data Fig. 2e).

To reduce the number of false-negative pcP23S RNA variants, the experiment was repeated one more time using de novo assembled Gibson reactions with the pcP23S RNA constructs that failed to replace pcPcsacB in the first experiment. Two additional functional pcP23S RNA constructs were recovered from the second attempt. Altogether, 22 pcP23S RNA variants were able to replace pcPcsacB in the plasmid backbone, each with one rRNA operon present; the combination of the primers 1 and 3 produces a 207-bp PCR band if the wild-type rnr operon is present; the combination of primers 1 and 2 produces a 112-bp PCR band on the templates with circularly permuted 23S rRNA gene (Extended Data Fig. 2e).

Concentration of rPbO-T. To avoid generation of mutations in the 23S RNA gene during PCR amplification for Gibson assembly, the 23S RNA gene variant circularly permuted at H101 (corresponding to CP2861 from Fig. 1) was first cloned in the pUC18 vector. For that, the 23S RNA gene circularly permuted at H101 was PCR-amplified from circularized 23S RNA gene prepared in the circular permutation study (see above and Extended Data Fig. 2a) by using the high-fidelity polymerase to confirm the identity of the DNA construct 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 nm after 18 h are shown in Extended Data Table 1.

Construction of rPbO-T. To avoid generation of mutations in the 23S RNA gene during PCR amplification for Gibson assembly, the 23S RNA gene variant circularly permuted at H101 (corresponding to CP2861 from Fig. 1) was first cloned in the pUC18 vector. For that, the 23S RNA gene circularly permuted at H101 was PCR-amplified from circularized 23S RNA gene prepared in the circular permutation study (see above and Extended Data Fig. 2a) by using the high-fidelity polymerase to confirm the identity of the DNA construct 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 nm after 18 h are shown in Extended Data Table 1.

Preparation for rPbO-T (Extended Data Fig. 1b), pAM552-A23S-AllII plasmid (see above) served as a recipient for the CP2861 23S RNA gene. The CP2861 23S RNA gene was excised from the pUC23S plasmid by BamHI digestion and gel purified. To graft the CP2861 23S RNA gene into the 16S rRNA gene, the 16S rRNA gene was PCR amplified for 14 h. The 23S rRNA gene was PCR amplified for 14 h. Then, the PCR product was prepared by PCR-amplifying the plasmid pAM552-A23S-AllII (5 ng in 50 μl reaction) using primers introducing poly-A linkers and sequences corresponding to H101 of 23S RNA (underlined) and h44 in 16S RNA (italized) TTAATCAGCCGTTCGATCAACCGATCG(T)↓;5'-GAGA GGGTTAACTACACTTCCTTTG (reverse primer with tether T1) and TTG...
ATAGGCCGGGCTGTTAAGGCAG(A), uGGAGGCGCCTTACCCACCTTTGT (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 72 °C 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. 

### Functional placement of the wild-type ribosomes by Ribo-T

The SQ171 cells carrying the pSacB plasmid, which contains the wild-type rrn 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 rubidium-chloride competent cells. The cells were incubated for 45 min on ice, 45 s at 42 °C and then 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 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 colonies 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 pSacB plasmid with the pRibo-T plasmids carrying Ribo-T was verified by PCR using primers 5'-GAACCTTACCTGGTCTTGACATC-3' (corresponding to the 16S rRNA sequence 976–998) and 5'-ATATCGAGCGCGGCTTGTG-3' (corresponding to the 23S rRNA sequence 2476–2495) to verify the identity 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.

### Case study

**Preparation of Ribo-T and wild-type ribosomes and analysis of their RNA and protein content.** Ribosomes were prepared from the exponentially growing cells of the SQ171fg strain transformed with either pAM552 (wild-type) or pRibo-T8/9 as described. RNA was phenol extracted, precipitated as described previously and resolved by electrophoresis in a denaturing 6% acrylamide-acylamide ratio 1:19, w/w polyacrylamide gel (for the SS rRNA analysis) or 4% acrylamide-acrylamide ratio 1:29, w/w polyacrylamide gel (for the analysis of large rRNAs).

Ribo-T-associated ribosomal proteins were analysed by mass spectrometry at the Proteomics Center of Excellence, Northwestern University. Ribosomes were precipitated by incubation in 20% trichloroacetic acid at 4 °C overnight and centrifugation at 14,000 rpm for 10 min. Precipitated ribosomes were washed once with cold 10% trichloroacetic acid and twice with acetone. The pellet was air-dried for 10–20 min before resuspension in 20 μl 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, 89870) and lyophilized. Amino-reactive tandem mass tag (TMT) reagents (126/127, Thermo Scientific, 90065) were used for peptide labelling. The reagents were dissolved in 1 μl acetonitrile and added to the lyophilized peptides dissolved in 100 μl 1 mM triethylamine 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 using 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 ions 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.

**Sucrose gradient analysis of ribosomes and ribosomal subunits.** Wild-type 7OS ribosomes or Ribo-T isolated from SQ171 fg cells as described above were diluted approximately 70-fold in high Mg²⁺ buffer (20 mM Tris-HCl, pH 7.5, 15 mM MgCl₂) supplemented with 1 mg ml⁻¹ lysozyme 0.25% sodium deoxycholate and 2 U of RNase (Promega). The lysates were centrifuged at 20,000g for 30 min at 4 °C and polysomes-containing supernatants (20–200 mM absorbance units) were loaded onto the 12-10–50% sucrose gradient in a 10 mM HCl, 0.1 mM PMSF, 1 mM dithiothreitol, and 50 mM Tris–HCl (pH 7.5) buffer. Polyribosome subunits were resolved in a 10–40% 12 ml sucrose gradients prepared with the same buffers. Gradients were centrifuged in the SW 41 rotor at 38,000 r.p.m. for 3 h at 4 °C. Ribosome profiles were then analysed 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 protocol. In brief, 10 pmol of Ribo-T or wild-type ribosomes were activated by incubation for 5 min at 42 °C in 50 μl of buffer 80 mM HEPES-KOH, pH 7.6, 15 mM MgCl₂, 100 mM NH₄Cl containing 20 U of RiboLock R1 RNase inhibitor (Thermo Fisher Scientific). Two microlitres of DMS (SIGMA) diluted 1:10 in ethanol were added to modified samples to react for 5 min at room temperature. Samples were then incubated for 10 min at 37 °C. The modification reaction was stopped and RNA extracted as described. Primer extensions were carried out using the primers 5′-GACTGCGAGGGATCATCCACGG-3′ and 5′-AAGGTAAAGCTTCACGG-3′ (for tether T1) or 5′-CCTACGTTACCTTGTACG-3′ for tether T2.

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Additionally, the integrity of the tetters in the Ribo-T preparation was tested by extension of the primers annealing immediately 3’ to the primer 5’-GTACCCTGTTAGCTCAAGCATC-3’ extended by reverse transcriptase across tether T1 in the presence of dATP, dTTP, dGTP and dCTP, and primer 5’-CAGAAAATGTAGGCGCCCTC-3’ extended across tether T2 in the presence of dATP, dTTP, dGTP and dCTP.

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 pY71-sfGFP plasmid using primers 5’-TAATACGACTCACTATAGGG-3’ and 5’-CITCCTCTTTGCGGCTTGT-3’. GFP mRNA was prepared by in vitro transcription and purified by size-exclusion chromatography on a Sephadex G50 mini-column, phenol extraction and ethanol precipitation. The transcript was transcribed in the α(ribosome, amino acid, trna) PURExpress 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 A, 1.2 μl of mixture factor, 1 μl amino acid mixture (3 mM each), 1 μl trna (20 mg/ml), 0.4 μl Ribolock RNase inhibitor (40 U μl−1), 5 μl (±20 pmol) GFP transcript and 22 pmol of wild-type ribosomes or Ribo-T. Samples were placed in wells of a 384-well black/well/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 λmax = 488 nm and λmm = 520 nm over 7 h. Protein synthesis rates were calculated by linear regression over the time points 0, 40 and 60 min with a 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 α(ribosome, amino acid, trna) PURExpress system kit (New England Biolabs) was carried in the presence of [5′-32P]-methionine (1,175 Ci mmol−1) using manufacturers protocol. A typical 5 μl reaction was assembled as described above but using 50 ng of the DNA template, was supplemented with 5 μCi [5′-32P]-methionine and 10 pmol of wild-type or Ribo-T ribosomes. When needed, the reactions were supplemented with 50 μM erythromycin. 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. Radioactive bands were visualized by Typhoon phosphorimager (GE Healthcare).

Toepring analysis. Toepring was performed as previously described19,20. When the necessary trna-synthase inhibitor borrelia and the initiation inhibitor thiostrepton were added to the reactions to the final concentrations of 10 μM or 100 μg/ml, respectively.

Construction of the plasmids for testing oRibo-T activity in vivo. The backbone plasmid pT7wtK (Extended Data Fig. 1c) was first prepared from the commercial plasmid T7-Flag-4 (Sigma Aldrich) by introducing the following changes. First, the blu gene was deleted using inverse PCR with phosphorylated primers 5’-TAATACGACTCACTATAGGG-3’ and 5’-ACTCTTCCTCTTTATATATAATGGAAG-3’ and Phusion High Fidelity DNA polymerase. Following purification with E.Z.N.A. Cycle Pure kit, DNA was blunt-end ligated for 14 h at 16°C using T4 DNA ligase, and transformed into electrocompetent DH5α E. coli cells and plated on LB-agar supplemented with 30 μg ml−1 kanamycin. The pT7wtK digested vector was treated with alkaline phosphatase CIP (New England Biolabs) for 1 h at 37°C and contained 2 μg/ml of the kit solution A, 1.2 μg/ml of the kit solution B, 0.5 μg/ml of the kit solution C, 3 min followed by 25 cycles (98°C, 30 s; 55°C, 30 s; 72°C, 120 s), followed by final extension 72°C, 10 min. Correct size band was purified by agarose gel electrophoresis and extracted using the E.Z.N.A. Gel Extraction kit. It was circulated by blunt-end ligation and transformed into PO2136 electrocompetent cells. Cells were plated on LB/agar plates supplemented with 50 μg/ml carbenicillin and grown at 30°C overnight. Colonies were isolated and poRibo-T was fully sequenced.

Testing activity of oRibo-T in vivo. Electromagnetic PO2136 cells were transformed with the following plasmid combinations: (1) pAM552 and pT7wtK (no gfp control), (2) pAM552 and pLLpsGFP, (3) pAM552o and pLLpsGFP, and (4) poRibo-T and plpLpsGFP. Transformants were plated on LB plates supplemented with 50 μg/ml carbenicillin and 30 μg/ml kanamycin and incubated for 24 h at 30°C. Wells of a 96-well plate with low evaporation lid (Costar) was filled with 100 μl of LB media supplemented with 50 μg/ml carbenicillin and 30 μg/ml kanamycin. The wells were inoculated with colonies from each plasmid combination above (six colonies each), and incubated at 30°C for 18 h with shaking. Clear bottom chimney wells of another 96-well plate (Costar) were filled with 100 μl of LB media supplemented with 50 μg/ml carbenicillin and 30 μg/ml kanamycin, and 1 mM IPTG. The plate was inoculated with 2 μl of saturated initial inoculation plate, and incubated with linear shaking (731 cycles per min) for 16 h at 42°C on a Biotek Synergy H1 plate reader, with continuous monitoring of cell density (Abs650 nm) and sfGFP fluorescence (excitation 485 and emission 528 with sensitivity setting at 80).

Testing oRibo-T activity in cell-free translation system. Ribosomes (wild-type or oRibo-T) (mixed with wild-type ribosomes) were prepared from SQ171fg cells transformed with pAM552 or poRibo-T, respectively. An orthogonal sf-gfp gene was isolated and poRibo-T was fully sequenced.

Construction of the plasmids for testing oRibo-T activity in vivo. The backbone plasmid pT7wtK (Extended Data Fig. 1c) was first prepared from the commercial plasmid T7-Flag-4 (Sigma Aldrich) by introducing the following changes. First, the blu gene was deleted using inverse PCR with phosphorylated primers 5’-TAATACGACTCACTATAGGG-3’ and 5’-ACTCTTCCTTTATATATAATGGAAG-3’. The sf-GFP transcript was then introduced using phosphorylated primers 5’-AGATCTGTGGTACCTAGGGCG TGCCGGCGCTGCAATCTCTAGCGAGG-3’ and 5’-GGGCTTTGTTAGCAG-3’ and 5’-ACTCTTCCTTTATATATAATGGAAG-3’ and 5’-AGATCTGTGGTACCTAGGGCG TGCCGGCGCTGCAATCTCTAGCGAGG-3’. The anti-Shine–Dalgarno sequence of pRibo-T was mutated from 5′-ATGAGCAAAGGTGAAGAAC-3’ to 5′-ATGAGCAAAGGTGAAGAAC-3’. The translation-translation reaction was carried out in α(ribosome, amino acid, trna) PURExpress system kit (New England Biolabs) with 50 μg/ml carbenicillin and 1 mM IPTG. The plate was inoculated with 2 μl of saturated initial inoculation plate, and incubated with linear shaking (731 cycles per min) for 16 h at 42°C on a Biotek Synergy H1 plate reader, with continuous monitoring of cell density (Abs650 nm) and sfGFP fluorescence (excitation 485 and emission 528 with sensitivity setting at 80).

For in vitro translation of an orthogonal secM-lacZa2a template, it was PCR amplified from the poSML plasmid using a direct primer 5′-TATAGCTGACTCATTAGG-3′ corresponding to the T7 promoter and a reverse primer 5′-CTAGCTGTGGTACCTAGGGCGTGCCGGCGCTGCAATCTCTAGCGAGG-3′. The secM arrest sequence was introduced by PCR amplification from the plasmid pT7wtK using primers 5′-TTATTTTTTCA-3′ and 5′-ACACAAATTTTTATGGCATTATTTTTTTATGGCATTATTTTTTT-3′. The transcription-translation reaction was carried out in α(ribosome, amino acid, trna) PURExpress system as described above. The 7.5-μl reactions were supplemented with 18.75 ng DNA template and 7.5 pmol ribosomes, and when needed, clindamycin or pactamycin were added to the reactions to the final concentrations of 50 μM or 100 μM, respectively.

For in vitro translation of an orthogonal secM-lacZa2a template, it was PCR amplified from the poSML plasmid using a direct primer 5′-TATAGCTGACTCATTAGG-3′ corresponding to the T7 promoter and a reverse primer 5′-CTAGCTGTGGTACCTAGGGCGTGCCGGCGCTGCAATCTCTAGCGAGG-3′. The secM arrest sequence was introduced by PCR amplification from the plasmid pT7wtK using primers 5′-TTATTTTTTCA-3′ and 5′-ACACAAATTTTTATGGCATTATTTTTTTATGGCATTATTTTTTT-3′. The transcription-translation reaction was carried out in α(ribosome, amino acid, trna) PURExpress system as described above. The 7.5-μl reactions were supplemented with 18.75 ng DNA template and 7.5 pmol ribosomes, and when needed, clindamycin or pactamycin were added to the reactions to the final concentrations of 50 μM or 100 μM, respectively.

Construction of C41(DE3)/poRibo-T was achieved by transduction from the E. coli strain K1342 (E. coli Genetic Stock Center, Yale) into E. coli C41(DE3) strain by P1 phage transduction protocol24. Transductants were selected on LB agar supplemented with 10 μg/ml carbenicillin.

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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’-AAGCTTACGAGGATCCACAAATCCGTATACGAGGATC3’ and 5’-CCGCTTAACGTGAGTATCCGCTATCCTTGAG-3’ of the expected PCR products are 467 bp for wild-type and 374 bp for the mutant.

Construction of the orthogonal SecM-lacZ reporter poSML. The backbone of the pACYC177 vector was PCR-amplified using primers 5’-ATCTCATGACCAAATCCGTATACGAGGATC3’ and 5’-CCGCTTAACGTGAGTATCCGCTATCCTTGAG-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 CACCAC3, the pACYC177 vector was PCR-amplified using primers 5’-AATCCCTTAACGTGAGT-3’ and 5’-CCGCTTAACGTGAGTATCCGCTATCCTTGAG-3’, with added sequence (underlined) used for re-circularization with Gibson assembly. PCR reaction was carried out under the following conditions: 98 °C, 3 min followed by 25 cycles (98 °C, 30 s; 55 °C, 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 transformation experiment. The transformed cells were 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.

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’-AGGCCTCTTTTTATC3’ and 5’-CTCTTGGGCGGTATCAGCCT-3’, with added sequence (underlined) used for re-circularization with Gibson assembly. PCR reaction was carried out under the following conditions: 98 °C, 3 min followed by 25 cycles (98 °C, 30 s; 55 °C, 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 transformation experiment. The transformed cells were plated on LB-agar plates supplemented with 50 μg ml⁻¹ carbencillin and grown for 24 h at 30 °C. Individual colonies were picked and sequenced to identify all possible 16 variants of the library.

The C41(DE3)/AlacZ58(M15) cells were transformed with the poSML reporter plasmid (Extended Data Fig. 1d) and plated on LB-agar containing 50 μg ml⁻¹ kanamycin. One of the colonies, which appeared after overnight incubation at 37 °C, was inoculated into liquid culture, grown in the presence of 50 μg ml⁻¹ kanamycin and cells were rendered chemically competent. Cells were transformed with the pooled library of 16 2451/2452 mutants. Transformed cells were plated on LB-agar containing 50 μg ml⁻¹ kanamycin, 100 μg ml⁻¹ ampicillin, 0.5 mM IPTG, 40 μg ml⁻¹ X-Gal and 2 mM lacZ inhibitor phenylethyl-[β-d-thiogalactopyranoside (PETG). Plates were incubated at 37 °C for 24 h and photographed. Sixteen white colonies or fifteen blue colonies were inoculated in 5 ml of LB medium supplemented with 100 μg ml⁻¹ ampicillin and grown overnight. The plasmids were isolated and the identities of nucleotide residues at the position 2451 and 2452 of the 23S rRNA were analysed by sequencing. Alternatively, the poSML-transformed C41(DE3)/AlacZ58(M15) cells were transformed with individual plasmids representing all possible 16 variants of the nucleotide combinations at positions 2451 and 2452.

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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 rRNA operon of E. coli under the control of the phage lambda P_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 cI857 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^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 tetranucleotide 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_k linker T1 and an A_k 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-lacZa reporter gene controlled by the T7 promoter (black triangle) and alternative Shine–Dalgarno sequence (orange semi-circle). The sequence of the secM-lacZa reporter matches that in the originally described plasmid pNH122 (ref. 18).
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-D23S-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**, 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; and a missense mutation in codon 549 of the \( rpsA \) gene encoding ribosomal protein S1.

**e**, representative of two independent biological experiments.
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-T(2) 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 SQ171fg cells expressing wild-type ribosomes or Ribo-T. Protein synthesis was measured by quantifying the incorporation of [35S] l-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 SQ171fg 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 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

|          | Doubling time (min) | Cell density (OD₆₀₀) at saturation |
|----------|---------------------|-----------------------------------|
|          | 30 °C | 37 °C | 30 °C | 37 °C | n  |
| pAM552   | 61.0 ± 3.2 | 53.9 ± 1.0 | 1.04 ± 0.06 | 0.93 ± 0.03 | 4  |
| pAM552-AflII | 67.4 ± 1.0 | 53.3 ± 2.4 | 1.07 ± 0.01 | 0.97 ± 0.00 | 4  |
| CP67     | 106.4 ± 5.4 | 69.6 ± 2.1 | 0.83 ± 0.05 | 0.41 ± 0.07 | 3  |
| CP95     | 144.9 ± 35.9 | 82.4 ± 24.4 | 0.66 ± 0.31 | 0.51 ± 0.18 | 6  |
| CP104    | 90.8 ± 10.3 | 52.7 ± 3.2 | 0.98 ± 0.03 | 0.95 ± 0.02 | 3  |
| CP168    | 123.8 ± 27.9 | 57.7 ± 1.9 | 0.70 ± 0.22 | 0.88 ± 0.12 | 10 |
| CP281    | 100.1 ± 11.0 | 54.6 ± 10.1 | 1.01 ± 0.04 | 0.93 ± 0.13 | 3  |
| CP549    | 101.7 ± 18.2 | 46.5 ± 3.9 | 1.00 ± 0.02 | 0.98 ± 0.03 | 3  |
| CP617    | 231.7 ± 20.5 | 91.5 ± 18.5 | 0.16 ± 0.03 | 0.85 ± 0.05 | 4  |
| CP634    | 162.0 ± 34.2 | 212.5 ± 58.1 | 0.46 ± 0.19 | 0.50 ± 0.10 | 3  |
| CP879    | 106.6 ± 4.7 | 51.4 ± 4.6 | 1.03 ± 0.02 | 0.99 ± 0.04 | 3  |
| CP891    | 144.5 ± 41.8 | 60.7 ± 4.1 | 0.56 ± 0.43 | 0.76 ± 0.23 | 6  |
| CP1112   | 89.6 ± 6.0 | 57.8 ± 12.2 | 0.96 ± 0.02 | 0.91 ± 0.12 | 3  |
| CP1178   | 102.5 ± 11.0 | 46.2 ± 1.3 | 0.96 ± 0.02 | 0.99 ± 0.01 | 3  |
| CP1498   | 167.5 ± 17.5 | 118.0 ± 17.1 | 0.56 ± 0.32 | 0.52 ± 0.19 | 3  |
| CP1511   | 131.5 ± 4.2 | 76.7 ± 1.5 | 0.88 ± 0.01 | 0.88 ± 0.01 | 3  |
| CP1587   | 98.1 ± 12.4 | 55.1 ± 6.6 | 0.93 ± 0.05 | 0.92 ± 0.08 | 3  |
| CP1716   | 174.4 ± 31.9 | 117.8 ± 16.5 | 0.44 ± 0.16 | 0.62 ± 0.34 | 3  |
| CP1733   | 117.3 ± 8.2 | 83.8 ± 2.2 | 0.95 ± 0.01 | 0.80 ± 0.01 | 3  |
| CP1741   | 230.0 ± 14.7 | 269.0 ± 50.3 | 0.28 ± 0.00 | 0.66 ± 0.09 | 3  |
| CP1873   | 108.4 ± 6.5 | 52.9 ± 0.8 | 0.94 ± 0.01 | 0.91 ± 0.01 | 3  |
| CP2148   | 83.0 ± 2.9 | 52.4 ± 3.9 | 0.73 ± 0.09 | 0.82 ± 0.02 | 4  |
| CP2600   | 85.9 ± 15.7 | 53.5 ± 9.7 | 1.04 ± 0.03 | 0.91 ± 0.12 | 3  |
| CP2861   | 138.4 ± 10.7 | 93.7 ± 4.5 | 0.88 ± 0.00 | 0.83 ± 0.04 | 3  |

*Growth in 100 μl LB media supplemented with 50 μg ml⁻¹ carbenicillin 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.
∥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.