In vitro self-replication and multicistronic expression of large synthetic genomes

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The generation of a chemical system capable of replication and evolution is a key objective of synthetic biology. This could be achieved by in vitro reconstitution of a minimal self-sustaining central dogma consisting of DNA replication, transcription and translation. Here, we present an in vitro translation system, which enables self-encoded replication and expression of large DNA genomes under well-defined, cell-free conditions. In particular, we demonstrate self-replication of a multipartite genome of more than 116 kb encompassing the full set of *Escherichia coli* translation factors, all three ribosomal RNAs, an energy regeneration system, as well as RNA and DNA polymerases. Parallel to DNA replication, our system enables synthesis of at least 30 encoded translation factors, half of which are expressed in amounts equal to or greater than their respective input levels. Our optimized cell-free expression platform could provide a chassis for the generation of a partially self-replicating in vitro translation system.
Self-encoded reproduction is a major requirement for the creation of artificial life. In systems inspired by existing biochemistry, such as minimal protein-based cells (MPCs), self-replication demands a complete cell-free reconstitution of the central dogma of molecular biology, including DNA replication, transcription and translation. In vitro protein synthesis from DNA can be achieved in well-defined recombinant systems based on phage RNA polymerases, core parts of the Escherichia coli translation machinery and a minimal energy regeneration system (PURE—Protein synthesis Using Recombinant Elements). In contrast, transcription—translation-coupled DNA replication (TTCdR) of a genome encoding all macromolecular components of the PURE system by a self-encoded replisome remains difficult. DNA replication employing DNA polymerases (DNAP) from phages such as Phi29 are promising candidates for self-encoded TTCdR of minimal genomes. For example, partially self-encoded TTCdR inside liposomes was accomplished using small linear Phi29 genomes encoding a minimal two-gene replicon on three kilobases (kb). TTCdR of the Phi29 full-length genome (~19 kb) in a PURE-based system was also achieved, but only if sufficient amounts of replication factors were either supplied externally or co-expressed from an excess of non-replicative DNA templates. TTCdR of small circular DNAs (2 kb) encoding only the Phi29-DNAP was recently realised by coupling the reaction to Cre-Lox recombination. Despite these advances, a concurrent, self-encoded replication and expression of the up to 150 genes (113 kb) proposed for MPC self-replication is currently out of reach. Here, we describe a modified PURE reaction that enables direct co-expression and Phi29-DNAP-dependent TTCdR of large multicistronic DNA elements that reach the predicted genome size required to encode a minimal cell. In particular, we demonstrate self-replication of a multipartite genome larger than 116 kb encompassing the full set of Escherichia coli translation factors, all three ribosomal RNAs, an energy regeneration system, as well as RNA and DNA polymerases. Parallel to DNA replication, our system enables synthesis of at least 30 encoded translation factors, half of which are expressed in amounts equal to or greater than their respective input levels.

**Results**

**PURErep enables self-encoded DNA replication.** Initially, we tested self-encoded Phi29-DNAP-dependent TTCdR using the standard protocol of the commercially available PURExpress system. The Phi29-DNAP coding region flanked by a T7 promoter was first cloned into a pCR-Blunt TOPO vector (pREP, Fig. 1a). In principle, this construct should enable spontaneous RNA-primed rolling-circle replication by the self-encoded DNAP without additional replication proteins or externally supplied DNA primers as reported previously. However, using the standard PURExpress reaction supplied with dNTPs and 4 mM PreP, we were unable to detect de novo synthesis of DNA by either agarose gel electrophoresis or qPCR (Fig. 1b, c). This finding is in agreement with previous studies which reported that the high tRNA and rNTP concentrations in standard PURE systems impair DNA-polymerase (DNAP) activity and that optimised custom systems are required to achieve efficient TTCdR. In order to improve DNA replication without access to tailor-made PURE systems, we set out to optimise the PURExpress standard reaction protocol. To this end, we increased the relative amount of translation factors, ribosomes and reducing agent while decreasing tRNA and rNTP levels (Fig. 1d; Supplementary Table 1). Using this optimised PURE formulation (PURErep), we achieved, depending on the PreP input concentration, ~5~12-fold replication of PreP monomer units in overnight TTCdR reactions (Fig. 1b, e). Full-length de novo synthesis of pREP was confirmed by M13I digestion of the replication product (Fig. 1c). Taking superfolder green fluorescent protein (sGFP) expression as an overall measure for in vitro translation (IVT) activity, we found that the altered PURE formulation resulted in a batch-dependent reduction of protein synthesis yields of 20–40% compared with the TTCdR-incompetent PURExpress system (Supplementary Fig. 1A, B). Thus, the improved compatibility of the PURErep system with DNA replication is achieved at the expense of only a modest reduction in overall protein expression strength.

**TTCdR products can be transformed and propagated in E. coli.** A qPCR-based analysis of DNA replication revealed a robust doubling time of 1–2 h for different initial template concentrations with DNA replication proceeding even after 24 h at 30°C (Fig. 1e). TTCdR of pREP was also sustainable for more than five successive generations of serial dilution when 4% of an overnight PURErep/PreP reaction was directly transferred into a fresh PURErep mix (Fig. 1f). This result implies that TTCdR products can serve as templates for self-coded DNA replication over several generations. As expected from the rolling-circle-type replication, we observed a considerable amount of product with low electrophoretic mobility, likely representing large molecular weight concatamers and/or DNA-MgPPi, clusters as reported previously for similar reactions (Supplementary Fig. 1C). Unexpectedly, we also observed formation of ~5 kb products in unprocessed samples, suggesting that TTCdR reactions may produce considerable amounts of monomeric pREP copies (Supplementary Fig. 1C). We were also able to transform de novo synthesised products into E. coli after removal of parental plasmids (Supplementary Fig. 2A). Purified in vivo amplified products were identical in size to monomeric pREP (Supplementary Fig. 2B).

**PURErep enables TTCdR of large multipartite genomes.** Encouraged by the efficient TTCdR in PURErep, we set out to co-replicate a collection of genes coding for crucial components of the PURE reaction such as the 31 essential E. coli translation factors (TFs). To this end, we probed co-TTCdR of pREP (4.6 kb) together with each one of the three large plasmids pLD1 (30 kb, 13 translation factors – TFs), pLD2 (20 kb, 8 TFs), or pLD3 (23 kb, 9 TFs), which were recently cloned to enable recombinant expression of 30 of the 31 TFs. Indeed, the TTCdR products of all four plasmids (including pREP) showed identical M13I restriction patterns as clonal plasmids conventionally propagated in E. coli (Fig. 2a). Moreover, the pLD TTCdR products could be directly transformed into E. coli, from where they were maintained as monomeric plasmids (demonstrated for pLD3, Supplementary Fig. 2C, D). The optimised PURErep mix enabled even the complete replication of all three pLD plasmids together with PURErep in a one-pot reaction (Fig. 2b, Supplementary Fig. 3A, B).

Next we sought to further expand the genetic load of the TTCdR system by co-replicating plasmids encoding additional components of the PURE system such as EF-Tu (pEFTu), which is missing in the pLD system, and also the ribosomal RNA operon rrrB (prRNA), which encodes for 23S rRNA, 16S rRNA, 6S rRNA and rRNA(Glu2) (Fig. 2c). qPCR experiments targeting plasmid-specific amplicons confirmed that monomer units of all six plasmids (total DNA length 93 kb) were replicated about 2–8-fold relative to their respective input levels in the presence of pREP and dNTPs after overnight incubation (Fig. 2c). In support of complete co-replication of all plasmids, transformations of DpnI-treated PURErep reaction products into E. coli resulted in colonies resistant to either zeocin (pREP), kanamycin (pLD plasmids and prRNA) or carbenicillin (pEFTu) (Fig. 2d). DNA preparations of 26 randomly
picked clonal colonies followed by restriction pattern analysis indeed confirmed successful TTcDR of all six plasmids (Fig. 2e; Supplementary Fig. 3C–E). In contrast, almost no background colonies were obtained when samples from dNTP-free PURErep experiments were transformed into E. coli (Fig. 2d). Using the same approach (Fig. 3; Supplementary Fig. 4), we were able to demonstrate co-replication of five additional plasmids encoding all but one of the missing proteins of the PURE enzyme mix (Supplementary Table 2, except peptidylprolyl isomerase). The additional plasmids include the genes for a minimal nucleoside triphosphate regeneration system based on creatine kinase (pCKM), adenylate kinase (pAK1) and nucleoside diphosphate kinase (pNDK), as well as T7-RNA polymerase (T7RNAP) and pyrophosphatase (pIPP), which is added to more recent versions of the PURE system18. With a total size of 116.3 kb, this set of 11 plasmids contains the genes for a minimal nucleoside triphosphate regeneration system based on creatine kinase (pCKM), adenylate kinase (pAK1) and nucleoside diphosphate kinase (pNDK), as well as T7-RNA polymerase (T7RNAP) and pyrophosphatase (pIPP), which is added to more recent versions of the PURE system18. With a total size of 116.3 kb, this set of 11 plasmids.

**PURErep enables synthesis of 30 TFs during TTcDR.** Having shown combined TTcDR of the multicistronic plasmids that encode almost all proteins of the PURE enzyme mix, we explored whether the PURErep mix could also enable parallel expression of these genes during replication. A (partially) self-replicating system based on the central dogma needs to be able to regenerate at least some of its different protein components. As a first step in this direction, we focused on the multicistronic expression of the TFs encoded on the three pLD plasmids pLD1, pLD2 and pLD3 (not including pEFTU). To explore whether PURErep is generally capable of supporting multicistronic expression from these plasmids, we performed cell-free expression from each individual plasmid in presence of BODIPY-Lys-tRNALys, which enables the fluorescent labelling of translation products at lysine residue sites. Using the reported expression patterns for all TF translation factors, AAs amino acids, DT T dithiothreitol, 10-THF 10-Formyltetrahydrofolate, SP spermidine, CP creatine phosphate). π TTcDR of pREP at different input concentrations. Fold changes relative to the initial concentration (4 nM) were used to approximate the concentrations before and after each generation (mean ± 68% CI, biological triplicates using different PURErep batches for each concentration).

Fig. 1 PURErep enables efficient transcription-translation coupled DNA replication. a Map of pREP. The plasmid encodes the gene for Phi29-DNAP under the control of a T7 promoter (T7p) and a bidirectional T7-terminator (T7t). IVT of the DNAP gene is increased by a T7 g10-L leader sequence. The use of pUC origin allows selective propagation in E. coli. b Replication of pREP in PURExpress (grey) or PURErep (blue) after 6 h at 30 °C. Fold changes were determined by qPCR relative to the pREP input levels (4 nM). Bars show the means with their 68% confidence intervals (CI) from biological triplicates using different PURErep/PURExpress batches. c Image of representative agarose gels loaded with TTcDR samples of pREP (8 nM input DNA) after MluI treatment. Samples were tested in three biological replicates. d Relative changes in compound levels between PURErep and PURExpress (log2-scale). Estimated compound levels for PURExpress are based on the numbers from Kuruma and Ueda40 (TF translation factors, AAs amino acids, DTT dithiothreitol, 10-THF 10-Formyltetrahydrofolate, SP spermidine, CP creatine phosphate). e TTcDR of pREP at different input concentrations. Fold changes relative to input levels were measured by qPCR (means with 68% CI, biological triplicates using different PURErep batches for each concentration). f pREP propagation over repeated passages of serial transfer. After each overnight TTcDR reaction in PURErep, 4% of the volume was transferred into E. coli. colonies were obtained when samples from dNTP-free PURErep experiments were transformed into E. coli (Fig. 2d). Using the same approach (Fig. 3; Supplementary Fig. 4), we were able to demonstrate co-replication of five additional plasmids encoding all but one of the missing proteins of the PURE enzyme mix (Supplementary Table 2, except peptidylprolyl isomerase). The additional plasmids include the genes for a minimal nucleoside triphosphate regeneration system based on creatine kinase (pCKM), adenylate kinase (pAK1) and nucleoside diphosphate kinase (pNDK), as well as T7-RNA polymerase (T7RNAP) and pyrophosphatase (pIPP), which is added to more recent versions of the PURE system18. With a total size of 116.3 kb, this set of 11 plasmids encodes the gene for Phi29-DNAP under the control of a T7 promoter (T7p) and a bidirectional T7-terminator (T7t). IVT of the DNAP gene is increased by a T7 g10-L leader sequence. A zeocin-resistance gene and a pUC origin allow selective propagation in E. coli. colonies were obtained when samples from dNTP-free PURErep experiments were transformed into E. coli (Fig. 2d). Using the same approach (Fig. 3; Supplementary Fig. 4), we were able to demonstrate co-replication of five additional plasmids encoding all but one of the missing proteins of the PURE enzyme mix (Supplementary Table 2, except peptidylprolyl isomerase). The additional plasmids include the genes for a minimal nucleoside triphosphate regeneration system based on creatine kinase (pCKM), adenylate kinase (pAK1) and nucleoside diphosphate kinase (pNDK), as well as T7-RNA polymerase (T7RNAP) and pyrophosphatase (pIPP), which is added to more recent versions of the PURE system18. With a total size of 116.3 kb, this set of 11 plasmids encodes the gene for Phi29-DNAP under the control of a T7 promoter (T7p) and a bidirectional T7-terminator (T7t). IVT of the DNAP gene is increased by a T7 g10-L leader sequence. A zeocin-resistance gene and a pUC origin allow selective propagation in E. coli.
isotope-labelled peptides, we found strong evidence for the de novo synthesis of all pLD-encoded TF protein subunits in overnight reactions (Fig. 4a). In particular, we obtained H/L ratios close to or larger than 1 for 12 of the 13 TFs encoded on pLD1 implying full regeneration of most of the encoded proteins during IVT. Partial or even full regeneration was also observed for the encoded TF peptide chains in yields comparable or exceeding their initial PURErep input concentrations.

Discussion

We demonstrated that under optimised TTcDR conditions, synthetic multipartite DNA genomes approaching the size of a postulated MPC genome can self-replicate and express proteins under cell-free conditions. Surprisingly, primer-free TTcDR by Phi29-DNAP alone is already sufficient to generate a significant amount of monomeric replication products from circular plasmids without any enzymatic post-processing, suggesting that partially recursive genome replication in cell-free systems can be achieved with only a single DNA polymerase. Furthermore, both the monomeric and concatamer TTcDR products can be directly transformed into E. coli 10-betacells. Additionally, we showed that the monomeric and concatemer TTcDR products can be directly transformed into E. coli 10-beta cells transformed with overnight TTcDR reactions from c. TTcDR reactions were carried out in presence (+dNTPs) or, as negative control for background colonies, in absence (-dNTPs) of dNTPs. Cell-derived plasmid templates were digested with DpnI. Cells were grown under selective conditions for zeocin (Zeo, pREP), kanamycin (Kan, pLD1-3, prRNA) and carbenicillin (Cb, pEFTu). e Circle diagrams of the relative transformation frequencies for the six plasmid species isolated from 26 randomly picked colonies from the three +dNTP plates in d (7 for Zeo, 12 for Kan and 7 for Cb). For the restriction pattern analyses, see Supplementary Fig. 3C-E. Source data are available in the Source Data file.

Next, we probed multistratropic expression of all three pLD plasmids during parallel TTcDR induced by the addition of pREP. Despite the considerably increased synthetic burden (replication of a 78 kb genome and transcription/translation of 33 protein chains), we detected H/L ratios >0.73 for 16 of the 32 encoded protein subunits. The H/L ratios of remaining TF subunits indicated regeneration levels between 10–70% (N = 10) and 4–9% (N = 6) (Fig. 3b). Thus, even under non-optimised batch conditions, PURErep in combination with pREP enables both the complete replication of 32 pLD-encoded TF cistrons as well as expression of about half of the encoded TF peptide chains in yields comparable or exceeding their initial PURErep input concentrations.
multimpartite model genome is taken up by the plasmid backbones, which maintain compatibility with in vivo propagation. In future genome designs, these sections could be replaced by the ~110 genes that are currently missing for the encoding of a complete minimal replicator dependent only on small-molecule nutrients.

An additional core requirement for a future minimal replicator such as a MCP is the ability to regenerate its individual protein components. In proof-of-concept batch PURErep reactions, we found that de novo synthesis of 30 of the 31 essential E. coli TFs can be detected after TTCtDR of their encoding ~78 kb plasmid ensemble. The relative (apparent) regeneration of the TFs encoded on pLD1 was generally efficient and reached H/L ratios ≥0.8 for 10/13 TFs during co-TTCtDR of all pLD plasmids and >0.9 for 12/13 TFs when only pLD1 alone was added to PURErep. In comparison, much lower regeneration levels were achieved for the TFs encoded on pLD2 and pLD3. These results correlate well with the concentrations of the individual TFs in the PURErep starting solution (Supplementary Table 2): The proteins encoded on pLD1 are the lowest concentrated TFs in PURE (approximate concentrations of 15–480 nM) and therefore readily compatible with the protein expression yields that can be achieved with current recombinant IVT systems. In contrast, the initial concentrations of pLD2-encoded TFs, which performed worst in our co-expression experiments, are much higher (approximate concentration of 0.75–3.2 μM) and therefore cannot be efficiently regenerated in the current PURErep system.

While quantification using stable-isotope labelling is considered a reliable and robust methodology to determine relative expression levels (in particular in cell-free environments), several factors such as incomplete trypsin digestion, translational arrest, incomplete peptide labelling or low peptide counts in e.g., Arg/Lys-rich proteins may affect quantification and therefore obscure the achieved regeneration levels. Furthermore, the current MS-based approach provides no information on the correct folding of the synthesised polypeptide chain and, thus, the actual amount of functional protein obtained during expression. Therefore, a direct functional feedback of the synthesised TFs back into IVT will be required in future experiments to assess or improve the amount of active protein that can be generated during TTCtDR. Fortunately, E. coli tRNA synthetases, which are one of the major TF factors in IVT systems, can be very well expressed in their active soluble form in PURE. Furthermore, the current IVT activity of PURErep is sufficient to generate systems capable of regenerating self-coded proteins of which only low concentrations are required.

In addition to addressing the functional state of the in vitro expressed proteins, self-encoded regeneration of TF factors will require further optimisation of expression stoichiometries and yields. Balanced stoichiometries could be achieved through optimised ribosome-binding sites, cistron positioning, promotor optimisations or feedback regulation. Enhanced protein expression will most likely require continuous mode cell-free
protein synthesis setups, e.g., based on miniaturised fluid array devices, which greatly increase protein yields. Using this approach, the regeneration of the other pLD-TFs, EF-Tu and the other proteins of the PURE enzyme mix could be achieved. The construction a self-regenerating IVT-system that is completely independent from the external supply of external macromolecules will also require integrated ribosome synthesis, assembly and translation (iSAT) Recent non-commercial protocols for in-house PURE-production provide attractive starting points for the generation of improved PURErep formulations that may be compatible with these key activities.

In its current form, PURErep can achieve modular in vitro replication of large genome-sized plasmid ensembles that retain their compatibility with bacterial in vivo propagation. This direct transferability could improve design, evolution and prototyping of MPC modules, orthogonal central dogmas or synthetic gene circuits, which were before not amenable to TTcDR-based in vitro replication.

**Methods**

**DNA constructs.** All primers used for cloning, mutagenesis and/or qPCR are listed in Supplementary Table 3, and were either ordered from IDT or Eurofins. All plasmids used in this study are listed in Supplementary Table 4. The open-reading frame of the Phi29 DNA-Polymerase (Gene ID: 6446511) was ordered as synthetic gene (gblock, IDT) and cloned into a PCR-Blunt vector using the ZeroBlunt cloning kit (Thermo Fisher) according to the manufacturer’s instructions. All constructs were verified by sequencing (Eurofins Genomics).

**sfGFP expression.** The difference in protein synthesis yields between PURExpress and PureRep was estimated using fluorescence of de novo synthesised sfGFP. To this end, 25 µL PURExpress reactions were set up according to the manufacturer’s instructions. The resulting construct was further optimised for in vitro translation by adding a T7 promoter with T7 gene 10 translation-enhancer sequence and a downstream bidirectional transcription terminator using Q5 site-directed mutagenesis (NEB) according to the supplier’s instructions. The identity of the final construct pREP was verified by sequencing. All cloning procedures were performed with chemically competent E. coli DH5alpha. The plasmids pLD1, pLD2 and pLD3 were a generous gift from A. Forster (Uppsala University) and are described in more detail elsewhere The plasmid pEFTu, which for historic reasons also encodes a gene copy of IF-1, was constructed from respectively linearised genes and a pIVEX 2.3d backbone using the HiFi assembly kit (NEB). First, an intermediate version was assembled from linear overhang PCR products using the primer pairs 152, 152 (EF-Tu fragment) and 153, 154 (pIVEX backbone). Subsequently, three linear overhang PCR products created using the primer pairs 161, 162 (EF-Tu fragment) and 163, 164 (gene spacer fragment) and 158, 159 (intermediate backbone containing IF-1) were assembled into the final construct. For the generation of prRNA, the E. coli ribosomal operon was directly amplified from Top10 E. coli by colony PCR using Q5-DNA polymerase with the primer pairs 85, 86, and cloned into a PCR-Blunt vector using the ZeroBlunt cloning kit (Thermo Fisher) according to the manufacturer’s instructions. Plasmids encoding for nucleotide-diphosphate kinase (pNDK, ID:124136), T7-RNA polymerase (pT7RNAP, ID:124138), creatine kinase m-type (pCKM, ID:124134), inorganic pyrophosphatase (pIPP, ID:118978) and adenylate kinase 1 (pAK1, ID:118977) were obtained from Addgene. Ampicillin-resistance genes were deleted by PCR in pT7RNAP, pCKM and pNDK using primers 200 and 201 (Supplementary Table 3). dsDNA concentrations were measured using a NanoDrop One-c (Thermo Scientific) following the manufacturer’s instructions. All constructs were verified by sequencing (Eurofins Genomics).

**Fig. 4 A substantial number of TFs are efficiently expressed during co-replication of pLD1, pLD2, pLD3 and pREP.** a) H/L ratios of the 32 TF protein subunits after 15N,13C-lysine and 15N,13C-arginine incorporation during in vitro transcription/translation of pLD1 (green, n = 4), pLD2 (red, n = 3) or pLD3 (purple, n = 4) in PURErep overnight reactions. The line H/L = 1 indicates full regeneration of a protein to its respective input concentration. b) Expression levels during parallel TTcDR of pLD1 (green), pLD2 (red) and pLD3 (purple) during TTcDR induced by the addition of prREP. All H/L values are means ± s.d. (n = 3) from biological replicates triplicates using different PURErep batches. Source data are available in the Source Data file.
instructions using 150 ng of pIVEX-sfGFP plasmid. In total, 25 µL of PURErep reactions consisted of 2.5 µL 10× energy mix (EM, Supplementary Table 1), 1 µL solution A (PUREExpress, NEB), 15 µL solution B (PUREExpress, NEB), 0.6 µL 25 mM dNTPs (equimolar), 0.5 µL RNTP mix (18.75 mM ATP, 12.5 mM GTP, 6.25 mM UTP/CTP) and 150 ng pIVEX-sfGFP plasmid DNA. After 2 h of incubation at 37 °C, 2× SDS loading buffer was added to the respective mixtures, and they were incubated for 5 min at 55 °C to preserve sfGFP fluorescence. In all, 10 µL of each sample loaded on a 12% polyacrylamide SDS-Gel. Fluorescent bands were directly visualised using a Typhoon FLA 7000, and analysed via ImageQuant, GE Healthcare Life Sciences. To assess pL-primed encoded gene expression, plD plasmids (final concentration 4 nM) were added to PUREExpress reactions containing 1 µL of FluoroTect Green-Lys (Promega). Prior to the addition of 2× SDS loading buffer, the samples were incubated for 30 min at 37 °C with 1 µL of RNase Cocktail (Thermo Fisher Scientific). After denaturing PAGE, de novo synthesised proteins were visualised using a Typhoon FLA 7000 scanner.

**Transcription-translation-coupled DNA replication.** The reaction composition for a typical 25 µL TTcDR reaction was as follows: 2.5 µL 10× EM, 1 µL solution A (PUREExpress, NEB), 15 µL solution B (PUREExpress, NEB), 0.6 µL 25 mM dNTPs (equimolar), 0.5 µL RNTP mix (18.75 mM ATP, 12.5 mM GTP, 6.25 mM UTP/CTP) and plasmid DNA (as specified in the main text). If necessary, the reaction volumes were adjusted to 25 µL with ddH2O. TTcDR reactions in the conventional PURERep system were assembled according to the standard protocol for a 25 µL reaction: 10 µL solution A, 7.5 µL solution B, 0.6 µL 25 mM dNTPs, plasmid DNA as specified in the main text. The final reaction volume was adjusted to 25 µL with ddH2O. PUREExpress and PURERep samples were incubated at 30 °C for up to 16 h in a ProFlex thermocycler (Applied Biosystems) for the time indicated. Time point zero samples were aliquoted directly after mixing, flash-frozen in liquid nitrogen and stored at −80 °C until further use.

**Gel analysis of TTcDR products.** Untreated TTcDR samples were directly analysed by neutral agarose gel electrophoresis in 1× TAE (Tris-Acetate-EDTA, gels 0.8%). Gel analysis of TTcDR products was performed on a 12% polyacrylamide SDS-Gel. Of each sample was subsequently loaded on a 12% polyacrylamide SDS-Gel. Fluorescent bands were directly visualised using a Typhoon FLA 7000, and analysed via ImageQuant, GE Healthcare Life Sciences. To assess pL-primed encoded gene expression, plD plasmids (final concentration 4 nM) were added to PUREExpress reactions containing 1 µL of FluoroTect Green-Lys (Promega). Prior to the addition of 2× SDS loading buffer, the samples were incubated for 30 min at 37 °C with 1 µL of RNase Cocktail (Thermo Fisher Scientific). After denaturing PAGE, de novo synthesised proteins were visualised using a Typhoon FLA 7000 scanner.

**Stable-isotope labelling of co-expression products.** For stable-isotope labelling of de novo synthesised protein, TTcDR samples were mixed with an energy mix containing 15N213C6-lysine and 15N413C6-arginine, instead of the unlabelled amino acids. After incubating for 2 h at 37 °C, the samples were analysed via mass spectrometry. First, the reaction mixture was diluted with equal volumes of buffer containing 1% sodium deoxycholate, 10 mM TCEP and 40 mM chloroacetamide in 25 mM Tris-HCl at pH 8.5 to be incubated at 37 °C for 20 min. The reaction mixture was further diluted and incubated overnight with roughly 4 µg of trypsin. Digested peptides were acidified and purified through SCX (strong cation exchange) StageTips (Thermo Scientific). Liquid chromatography–mass spectrometry (LC-MS) analysis was performed on a Q-Exactive HF mass spectrometer (Thermo Scientific) operated in a data-dependent fashion. The raw data were processed using the MaxQuant19 computational platform (version 1.6.6.0), and all peptide and protein identifications were filtered at 1% false discovery rate. The derived peak list was searched using Andromeda search engine integrated in MaxQuant against the E. coli K12 proteome (Proteome ID: UP000000625/Genome accession: U00096), obtained from UniProt (4391 protein entries; last modified May 14, 2019). The obtained H/L values for each pL-encoded protein were analysed and plotted using Graphpad Prism 7.05.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The source data underlying Figs. 1b, 1c, 1f, 2a, 2b, 2c, 2h, 4a, 4b, Supplementary Figs. 5b, 5c and 5 are provided in a Source Data file. MaxQuant outputs are provided in two separate source files. Plasmids encoding for nucleotide-diphosphate kinase (ID:124136), T7-RNA polymerase (ID:124138), creatine kinase m-type (ID:124134), inorganic pyrophosphatase (ID:118978) and adenylate kinase 1 (ID:118977) were obtained from Addgene. Further data supporting the findings of this paper are available from the corresponding author upon reasonable request.

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Author contributions

H.M. conceived the project. K.L. and H.M. designed the experiments. M.H. contributed to plasmid sample preparation, discussed the results and commented on the paper. K.L. carried out all experiments. R.H. contributed to qPCR experiments. K.L. and H. M. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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