nature portfolio

https://doi.org/10.1038/s41589-022-01064-w

Supplementary information

Three-dimensional structure-guided evolution of a ribosome with tethered subunits

In the format provided by the authors and unedited

Supplementary Information

Title: 3D-structure-guided evolution of a ribosome with tethered subunits

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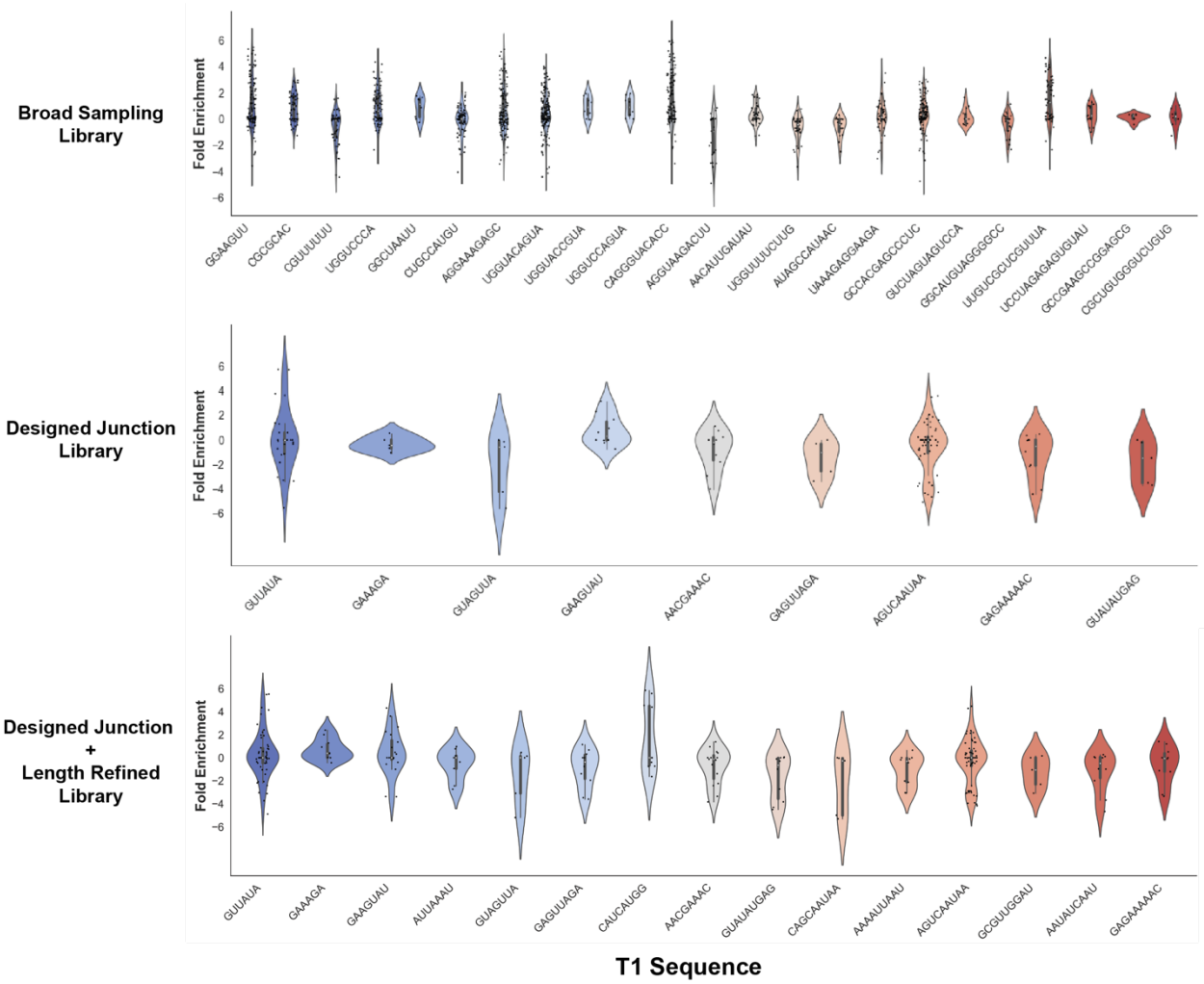
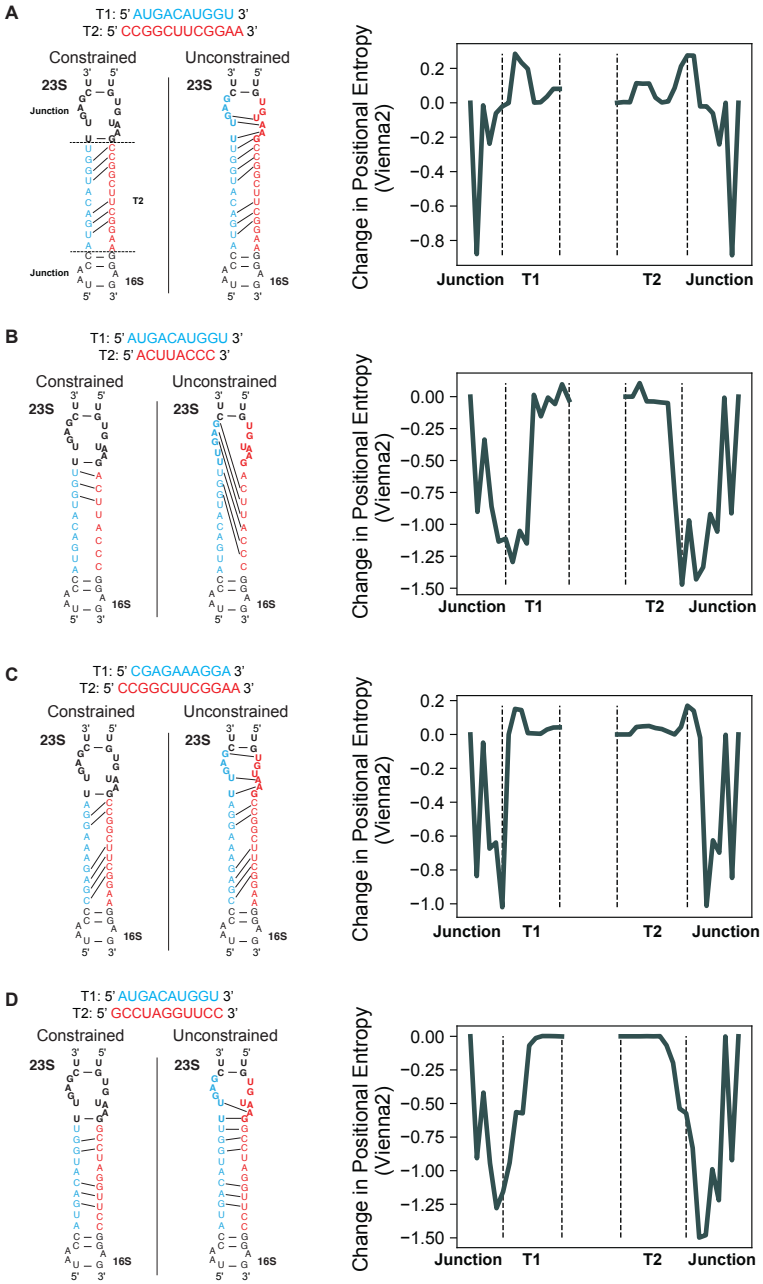
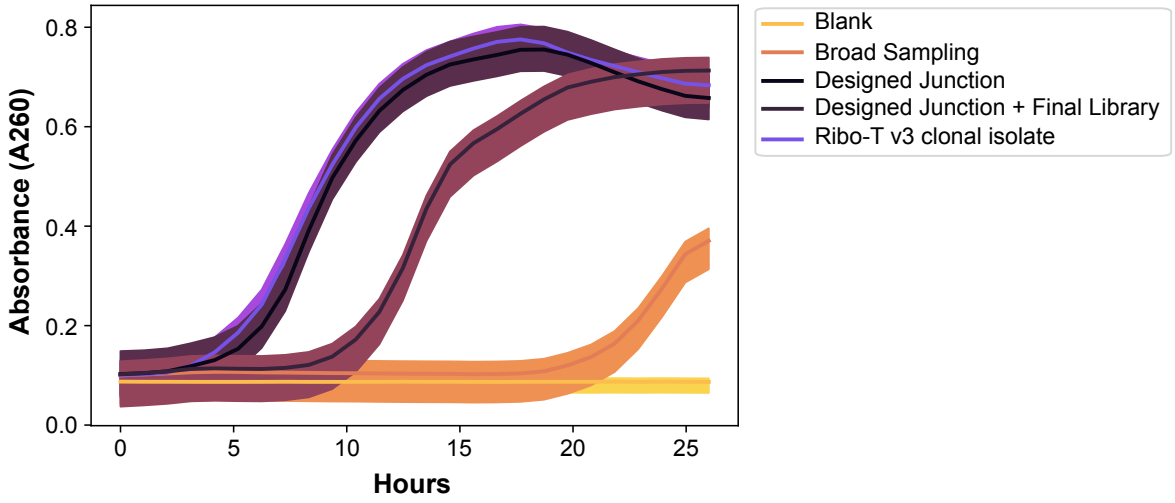
Supplementary Figures



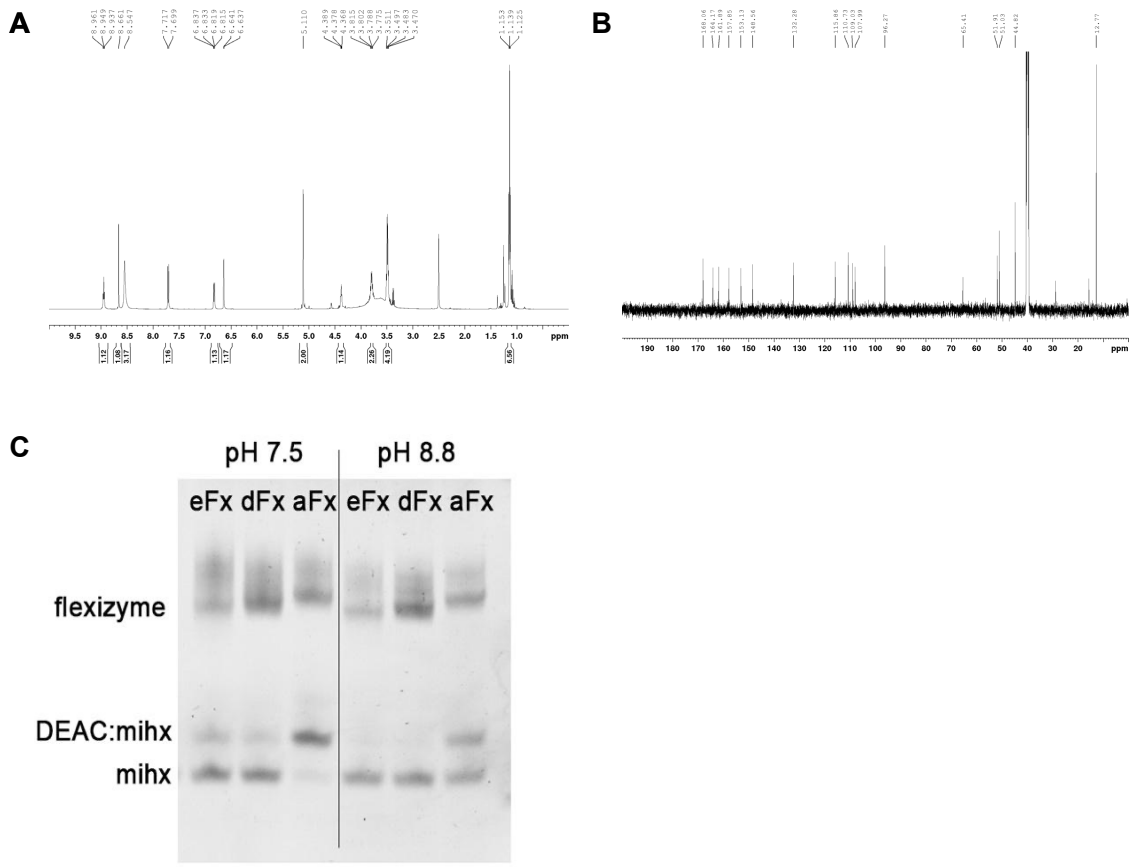
Supplementary Figure 1. Distribution of T2 sequences for most enriched T1 sequences. Distribution of T2 sequences for the most popular T1 sequences displayed for the three libraries tested (Broad Sampling Library, Designed Junction Library, Designed Junction + Length Sampling Library). Scatter plot represents unique T2 sequences for a given T1 sequence. Violin plot and scatter plot data representative of three independent experiments. Boxplots are centered at the 50th percentile, with minima and maxima representative of 25th and 75th percentiles, respectively. The bounds of whiskers extend to the range of values observed less the outliers.



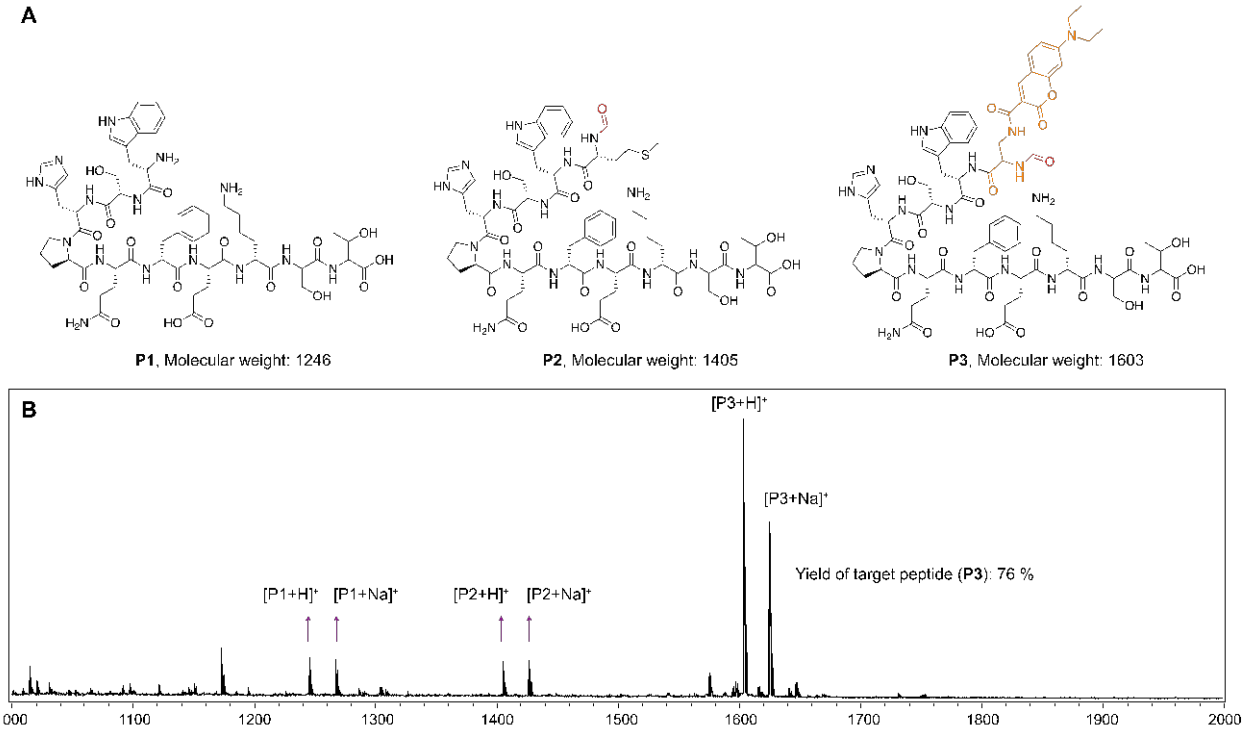
Supplementary Figure 2. Analysis of enriched genotypes from the Broad Sampling Library. **(A-D)** Each panel shows an enriched sequence modeled using RNAcofold. For three of the genotypes, **(A), (C),** and **(D)**, the same tether base pairs are formed in the constrained and unconstrained minimum free energy (MFE) structures. **(B)** For one of the genotypes, significant rearrangement is observed between the constrained vs. unconstrained MFE structures. On the right half of each panel, delta positional entropy is shown as calculated in Vienna2.



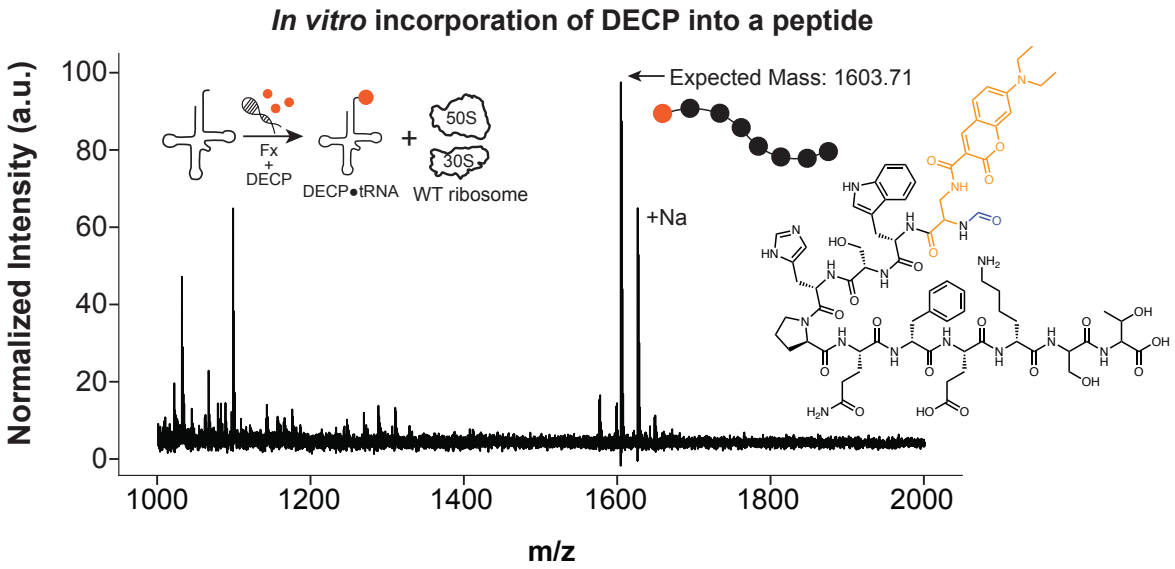
Supplementary Figure 3. Comparison of growth rates across libraries. Library growth characterization in LB media during liquid passaging for selection. Data are presented as mean values of 8 biological replicates (n=8), grown from initial inoculation from agar plates. Shaded area as shown are ± standard deviation at each time point.



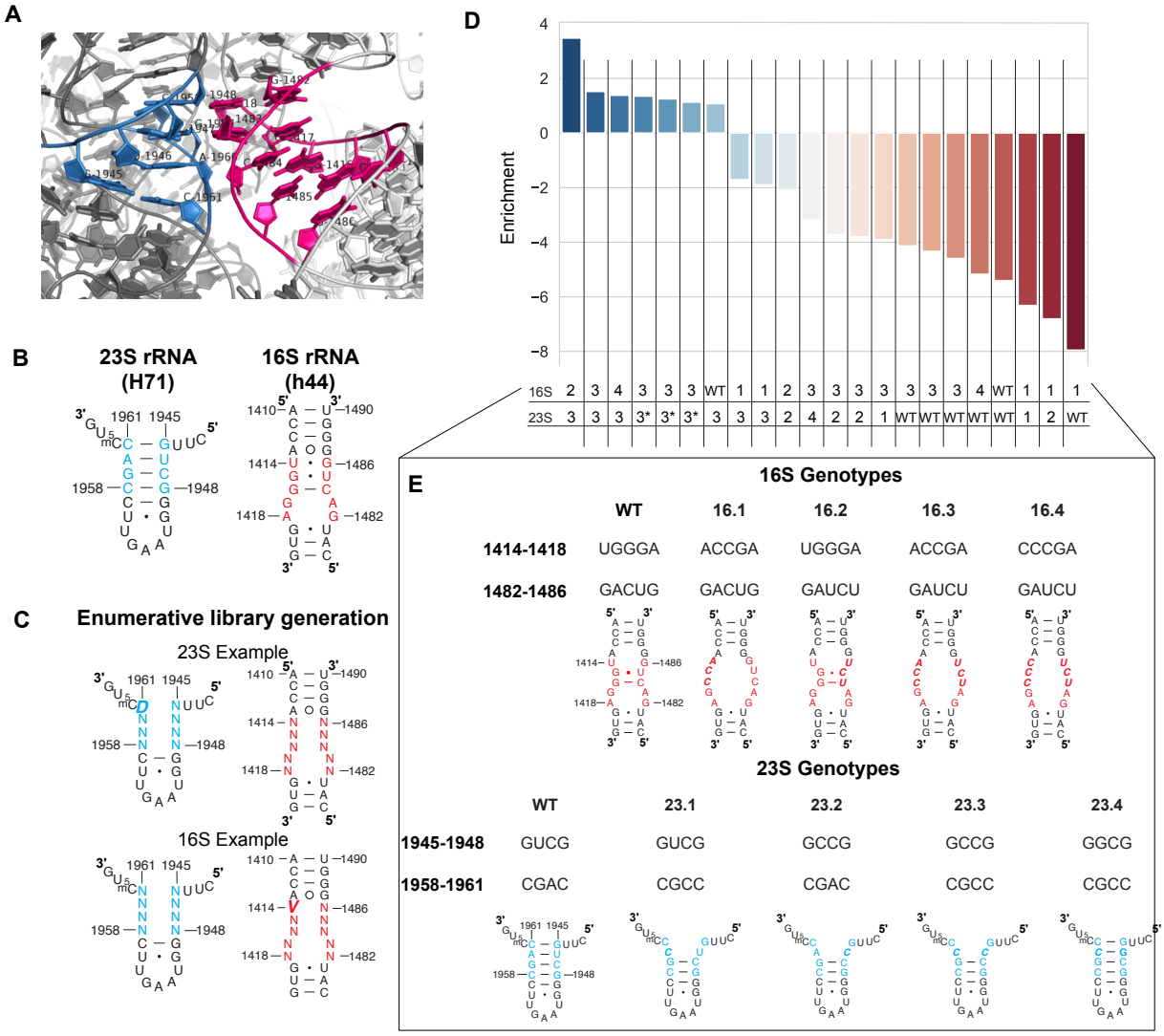
Supplementary Figure 4. Preparation of DECP for incorporation into polypeptide. (A) ^{1}H NMR spectra of DECP-CME (**5**) and **(B)** ^{13}C NMR spectra of DECP-CME **(5). (C)** Acylation of microhelix with DECP. The Fx-mediated acylation reaction was monitored using microhelix (a tRNA mimic) under the two different pH (7.5 and 8.8) over 16 h with three different flexizymes (eFx, dFx, and aFx) at 0° C. The highest acylation yield (86 %) was found when aFx was used in pH 7.5, which was used to charge the substrate into $tRNA^{fMet}$(CAU) and incorporate it into the N-terminus of a peptide *in vitro*. Gel representative of three independent experiments.



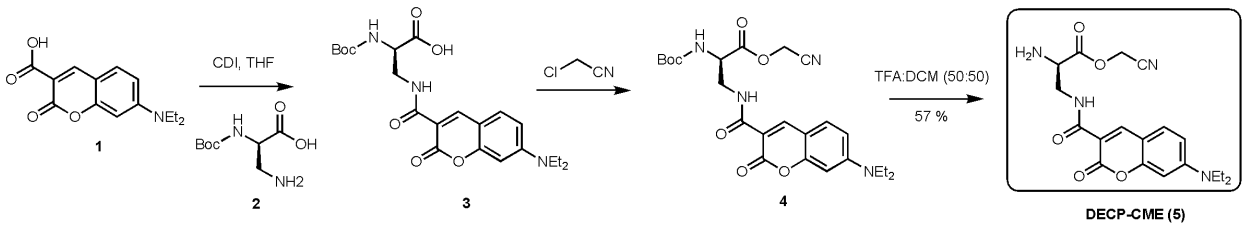
Supplementary Figure 5. Characterization of N-terminus functionalized peptide hybridized with 2-amino-3-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido) propanoate (DECP). (A) Structure and molecular weight of peptides that are produced in the *in vitro* translation reaction. **(B)** MALDI mass spectrometry data (**Fig. 5E**) obtained from attempt to incorporate DECP with Ribo-T v3. The truncated peptide (**P1**) was produced likely because Ribo-T v3 skipped the incorporation of DECP at the initiating codon (AUG) on mRNA. **P2** was produced presumably because of the contaminations of either amino acid or fMet-charged tRNA ($tRNA^{fMet}$) when Ribo-T v3 obtained from *E. coli* cell was supplemented into the *in vitro* translation reaction. The percent yield (76 %) of the target peptide (**P3**) was determined based on the relative peak area (PA) of **P3** over a total amount of the byproducts (**P1** and **P2**) and **P3** (i.e., relative yield (%) = Σ of PA (**P3**) / Σ of PA (**P1** + **P2** + **P3**) × 100). MALDI data representative of three independent experiments.



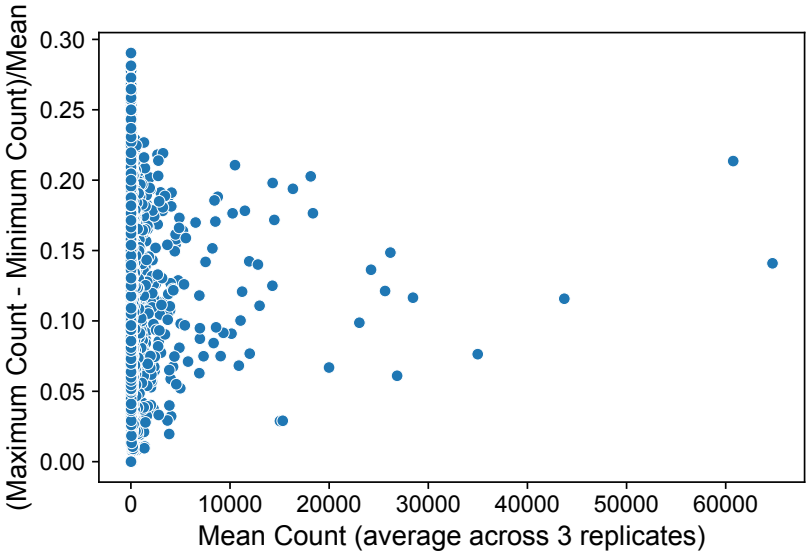
Supplementary Figure 6. Incorporation of DECP using a wild-type (WT) ribosome. Purified wild-type ribosomes and flexizymes were both used in an *in vitro* protein synthesis reaction to incorporate 2-amino-3-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido) propanoate (DECP) into a peptide. Arrow indicates expected peak and sodium adduct, while the typical side reaction products (misincorporated or truncated peptide, described further in **Fig. 5**) were not detected. Data are representative of three independent experiments.



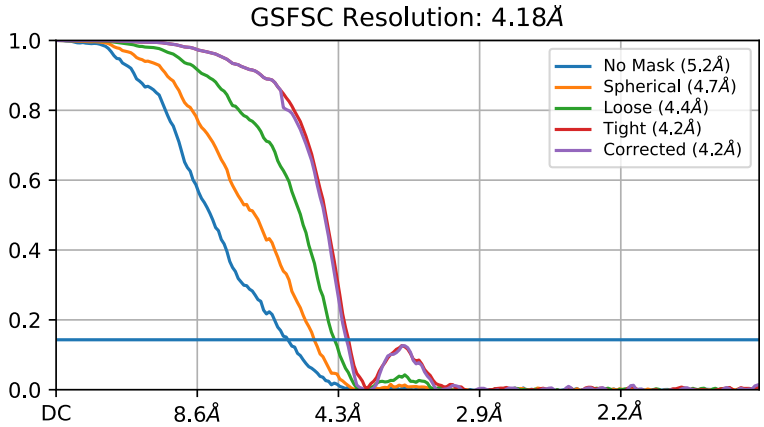
Supplementary Figure 7. Extension of Evolink to other ribosomal helices. (A) Ribosomal helices H71 (blue) of the 23S rRNA and h44 (red) of the 16S rRNA, which are proximal as shown in the structure of the *E. coli* ribosome (4YBB). **(B)** Bases in H71 and h44 selected for mutagenesis and Evolink. **(C)** Examples of the libraries constructed for Evolink. At least one nucleotide in the interested region is fixed to be not the wild-type residue while others are completely randomized. **(D)** Log2-fold enrichment values are obtained for combinations of genotypes in H71 and h44. **(E)** Legend depicting genotypes and likely secondary structures of 23S rRNA and 16S rRNA obtained from Evolink on H71 and h44 where bolded nucleotides reveal mutations away from the wild type residue. Data representative of three independent experiments. * indicates a genotype where a mutation observed in another part of the rRNA that was not intentionally mutated.



Supplemental Figure 8. Schematic for preparation of DECP-CME.



Supplemental Figure 9. Measurement of Evolink Replicability. Scatterplot showing the (maximum count – minimum count)/mean count vs. mean count for each genotype across three sequencing reads across all samples sequenced during the Evolink method.



Supplemental Figure 10. Gold Standard Fourier Shell Correlation (GSFSC) curve. GSFSC curve for the final Cryo-EM density of the RiboTv3 ribosome as calculated by the CryoSPARC 3D refinement step.

Supplementary Tables

Supplementary Table 1. DNA primers used in this study. Sequences are listed 5′ to 3′. For primers indicated with '\Phos\', Phosphorylation performed on oligos with polynucleotide kinase (PNK) prior to PCR for use in blunt end ligation. 'N' indicates degenerate oligonucleotides. All oligonucleotides purchased from Integrated DNA Technologies (IDT).

Use	Primer Name	Sequence (5′ → 3′)	Description
Broad Sampling Library construction, insert	RTv3_BroadSample_5N-f	AAGAAGTAGGTAGCTTAACCnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 5 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_5N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 5 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_6N-f	AAGAAGTAGGTAGCTTAACCnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 6 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_6N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 6 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_7N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 7 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_7N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 7 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_8N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 8 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_8N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 8 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_9N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 9 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_9N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 9 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_10N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 10 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_10N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 10 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_11N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 11 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_11N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 11 degenerate nucleotides in T2 region, Broad Sampling Library

	RTv3_BroadSample_12N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 12 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_12N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 12 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_13N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 13 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_13N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 13 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_14N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 14 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_14N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 14 degenerate nucleotides in T2 region, Broad Sampling Library
	RTv3_BroadSample_15N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnnnnnnnTTGAGCTAACCGGTACTAATGAACC	Forward primer used to install 15 degenerate nucleotides in T1 region, Broad Sampling Library
	RTv3_BroadSample_15N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnnnnnnnCTTACACACCCGGCCTATCAA	Reverse primer used to install 15 degenerate nucleotides in T2 region, Broad Sampling Library
Tether-H101 Junction Library construction, insert	d1_RTv2-f	GAAGTAGGTAGCTTAACCcaatgaacaattggaGCGTTGAGCTAACCGGTACTAATGAAC	Forward primer for 1 residue deletion in Tether-H101 junction
	d1_RTv2-r	AATCACAAAGTGGTAAGCGCCCTCCactagttatcGCGCTTACACACCCGGCCTATCAA	Reverse primer for 1 residue deletion in Tether-H101 junction
	d2_RTv2-f	GAAGTAGGTAGCTTAACCcaatgaacaattggaCGTTGAGCTAACCGGTACTAATGAACC	Forward primer for 2 residue deletion in Tether-H101 junction
	d2_RTv2-r	AATCACAAAGTGGTAAGCGCCCTCCactagttatcCGCTTACACACCCGGCCTATCAA	Reverse primer for 2 residue deletion in Tether-H101 junction
	d3_RTv2-f	GAAGTAGGTAGCTTAACCcaatgaacaattggaGTTGAGCTAACCGGTACTAATGAACC	Forward primer for 3 residue deletion in Tether-H101 junction
	d3_RTv2-f	AATCACAAAGTGGTAAGCGCCCTCCactagttatcGCTTACACACCCGGCCTATCAA	Reverse primer for 3 residue deletion in Tether-H101 junction
	d4_RTv2-f	AAGAAGTAGGTAGCTTAACCcaatgaacaattggaTTGAGCTAACCGGTACTAATGAACC	Forward primer for 4 residue deletion in Tether-H101 junction
	d4_RTv2-r	AATCACAAAGTGGTAAGCGCCCTCCactagttatcCTTACACACCCGGCCTATCA	Reverse primer for 4 residue deletion in Tether-H101 junction
	d5_RTv2-f	AAGAAGTAGGTAGCTTAACCcaatgaacaattggaTGAGCTAACCGGTACTAATGAACC	Forward primer for 5 residue deletion in Tether-H101 junction
	d5_RTv2-r	AATCACAAAGTGGTAAGCGCCCTCCactagttatcTTACACACCCGGCCTATCAA	Reverse primer for 5 residue deletion in Tether-H101 junction
Designed Junction Library	RTv3_DesignedJunc_5N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnGCCTTACACACCCGGCCTATCAA	Forward primer for 5 degenerate residues for Designed Junction Library

construction, insert	RTv3_DesignedJunc_5N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnGTCCTTACACACCCGGCCTATCAA	Reverse primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_6N-f	AAGAAGTAGGTAGCTTAACCnnnnnnGCTTGAGCTAACCGGTACTAATGAACC	Forward primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_6N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnGCCTTACACACCCGGCCTATCAA	Reverse primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_7N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnGCTTGAGCTAACCGGTACTAATGAACC	Forward primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_7N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnGCCTTACACACCCGGCCTATCAA	Reverse primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_8N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnGCTTGAGCTAACCGGTACTAATGAACC	Forward primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_8N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnGCCTTACACACCCGGCCTATCAA	Reverse primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_9N-f	AAGAAGTAGGTAGCTTAACCnnnnnnnnnGCTTGAGCTAACCGGTACTAATGAACC	Forward primer for 5 degenerate residues for Designed Junction Library
	RTv3_DesignedJunc_9N-r	AATCACAAAGTGGTAAGCGCCCTCCnnnnnnnnnGCCTTACACACCCGGCCTATCAA	Reverse primer for 5 degenerate residues for Designed Junction Library
Backbone amplification for Ribo-T v3 library construction	Ribo-T_lib_bb-f	GGAGGGCGCTTACCACTTTGTGATT	Forward primer for amplification of backbone with library inserts, assemble by isothermal assembly
	Ribo-T_lib_bb-r	GGTTAAGCTACCTACTTCTTTTGCA	Reverse primer for amplification of backbone with library inserts, assemble by isothermal assembly
Testing PCR1 compatibility with different ligation methods	T1-T2_PCR1_GA_-f	GGAACGTTGAAGACGACGACGTTGATAGG	Forward primer for PCR1, compatible for isothermal assembly
	T1-T2_PCR1_GA-r	CCTATCAACGTCGTCGTCTTCAACGTTCCACGGTTCATTAGTACCGGTTAGC	Reverse primer for PCR1, compatible for isothermal assembly
	\Phos\T1-T2_PCR1_blunt-f	GGAACGTTGAAGACGACGACGTTGATAGG	Forward primer for PCR1, compatible for blunt end ligation (phosphorylated prior to PCR)
	\Phos\T1-T2_PCR1_blunt-r	CACGGTTCATTAGTACCGGTTAGC	Reverse primer for PCR1, compatible for blunt end ligation (phosphorylated prior to PCR)
	T1-T2_PCR1_BamHI-for	agatggatccGGAACGTTGAAGACGACGACGTTGATAGG	Forward primer for PCR1, compatible for digestion with BamHI prior to ligation
	T1-T2_PCR1_BamHI-rev	ggatccatctCACGGTTCATTAGTACCGGTTAGC	Reverse primer for PCR1, compatible for digestion with BamHI prior to ligation
	T1-T2_PCR1_SapI-for	gctcttcagcgGGAACGTTGAAGACGACGACGTTGATAGG	Forward primer for PCR1, compatible for digestion with SapI prior to ligation
	T1-T2_PCR1_SapI-rev	ggctcttcacgcCACGGTTCATTAGTACCGGTTAGC	Reverse primer for PCR1, compatible for digestion with SapI prior to ligation
PCR2	T1-T2-PCR2-for	AGTGGGTTGCAAAAGAAGTAGGTAGC	Forward primer for PCR2
	T1-T2-PCR2-rev	CCAGTCATGAATCACAAAGTGGTAAGC	Reverse primer for PCR2
Targeted DMS-MaPseq	oVT628	acactctttccctacacgacgctcttccgatctCGCTAGTAATCGTGGATCAGAATGCCA	Forward PCR primer for tether 1 with iTru indexing primer overhang

of tether regions	oVT629	gtgactggagttcagacgtgtgctcttccgatcCTGACTGCCAGGGCATCCAC	Reverse transcription and reverse PCR primer for tether 1 with iTru indexing primer overhang
	oVT630	acactctttccctacacgacgctcttccgatctGAAACTTGCCCCGAGATGAGTTCTCC	Forward PCR primer for tether 2 with iTru indexing primer overhang
	oVT631	gtgactggagttcagacgtgtgctcttccgatcTGATCCAACCGCAGGTTCCCC	Reverse transcription and reverse PCR primer for tether 2 with iTru indexing primer overhang
	iTru501L	AATGATACGGCGACCACCGAGATCTACACACCGACAAACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru502L	AATGATACGGCGACCACCGAGATCTACACAGTGGCAAACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru503L	AATGATACGGCGACCACCGAGATCTACACCACAGACTACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru504L	AATGATACGGCGACCACCGAGATCTACACCGACACTTACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru505L	AATGATACGGCGACCACCGAGATCTACACGACTTGTGACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru506L	AATGATACGGCGACCACCGAGATCTACACGTGAGACTACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru507L	AATGATACGGCGACCACCGAGATCTACACGTTCCATGACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru508L	AATGATACGGCGACCACCGAGATCTACACTAGCTGAGACACTCTTTCCCTACACGAC	Forward indexing primer to add sample-specific barcode to 5' end of sequencing library
	iTru701L	CAAGCAGAAGACGGCATACGAGATGGTAACGTGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru702L	CAAGCAGAAGACGGCATACGAGATCAACACAGGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru703L	CAAGCAGAAGACGGCATACGAGATACACCTCAGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru704L	CAAGCAGAAGACGGCATACGAGATCATGGATCGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru705L	CAAGCAGAAGACGGCATACGAGATTTGATAGGCGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru706L	CAAGCAGAAGACGGCATACGAGATCGGTTGTTGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru707L	CAAGCAGAAGACGGCATACGAGATCAACGAGTGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library

	iTru708L	CAAGCAGAAGACGGCATACGAGATACCATAGGGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru709L	CAAGCAGAAGACGGCATACGAGATGGTGTACAGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru710L	CAAGCAGAAGACGGCATACGAGATCAGCATACGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru711L	CAAGCAGAAGACGGCATACGAGATGGACATCAGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
	iTru712L	CAAGCAGAAGACGGCATACGAGATAGAAGGACGTGACTGGAGTTCAGACGTG	Reverse indexing primer to add sample-specific barcode to 3' end of sequencing library
qPCR for rRNA amounts	RiboT_qPCR-f	GAGGCTTAACCGAGAGGTTAAGCG	Forward primer for probing Ribo-T ribosomal RNA in qPCR
	RiboT_qPCR-r	ATATTCAGACAGGATACCACGTGTCC	Reverse primer for probing Ribo-T ribosomal RNA in qPCR

Supplementary Table 2. Comparisons of orthogonal sfGFP production by multiple Ribo-T v3 candidates (Figure 5C) compared to Ribo-T v2. One-sided Welch's t-test performed to compare Ribo-T v3 candidates to Ribo-T v2. T1 and T2 sequences are shown 5′ to 3′. Data shown representative of three independent experiments, and within each experiment, data from three replicates per T1 and T2 sequence genotype used to calculate standard deviation and perform t-test. Experiment and analysis were performed to analyze which Ribo-T v3 candidates had greater orthogonal sfGFP synthesis ability. * marks the sequence chosen as Ribo-T v3.

T1 sequence	T2 sequence	Normalized sfGFP expression (fluorescence/A_{600})	Standard Deviation	P-value
GUUAUA	AUCCCAGG	13457	103	0.000145
GUUAUA	UCACAAC	15196	871	0.000362
GUUAUA	GACCUUCG	12628	733	0.002386
GUUAUA	ACAUAAUG	6998	233	0.000793
AGUCAAUAA	AUCCCAGG	12997	222	0.000300
AGUCAAUAA	UCACAAC	13597	834	0.001172
AGUCAAUAA*	GACCUUCG*	15097	682	0.000207
AGUCAAUAA	ACAUAAUG	12327	543	0.001885
CAUCAUGG	AUCCCAGG	10482	525	0.061960
CAUCAUGG	UCACAAC	12729	1559	0.015705
CAUCAUGG	GACCUUCG	10866	1221	0.092446
CAUCAUGG	ACAUAAUG	13455	979	0.002063
AUAUAAU	AUCCCAGG	14094	483	0.000227
AUAUAAU	UCACAAC	13501	1135	0.003005
AUAUAAU	GACCUUCG	13572	1896	0.012928
AUAUAAU	ACAUAAUG	6057	428	0.000444
Ribo-Tv2 (CAAUGAACAAUUGGA)	Ribo-Tv2 (GAUAACUAGU)	9629	550	1
WT (no tether)	WT (no tether)	673	44	-

Supplementary Table 3. Kinetic growth parameters comparing Ribo-T v3 and Ribo-Tv2 in minimal M9 media and LB media. Sigmoidal functions were fit to kinetic data (Figure 4D & 4E) to calculate parameters. Notably in Figure 4E, Ribo-T v2 does not reach full stationary phase in 24 hours while Ribo-T v3 grows to stationary phase between 18-20 hours. Standard deviation (s.d.) calculated for six replicates (n=6).

	Media	**Max OD_{600} ± s.d.**	**Slope (OD_{600}/hours) ± s.d.**	**Lag (hours) ± s.d.**
Ribo-T v3	M9	0.384 ± 0.031	0.037 ± 0.004	6.922 ± 1.465
	LB	0.872 ± 0.037	0.084 ± 0.010	6.227 ± 1.506
Ribo-T v2	M9	0.443 ± 0.052	0.019 ± 0.006	12.222 ± 4.465
	LB	0.588 ± 0.075	0.091 ± 0.019	9.941 ± 1.401

Supplementary Table 4. Quantification of relative orthogonal rRNA amounts. Cq values and ratios of rRNA amounts determined (via quantitative PCR) on RNA extractions from cells harboring orthogonal Ribo-Tv3 (oRibo-Tv3) and cells harboring orthogonal oRibo-Tv2 (oRibo-Tv2) ribosomes as seen in **Fig. 5c**.

Replicate	Cq values: oRibo-Tv3 cells	Cq values: oRibo-Tv2 cells	Ratio of Cq values (oRibo-Tv3/oRibo-Tv2)
1	7.427	5.898	1.259
2	5.297	4.877	1.086
3	5.013	3.067	1.635
4	6.685	5.378	1.243
5	4.233	4.245	0.997
6	3.782	2.313	1.635
		Median	1.251
		Mean	1.309
		Standard Deviation	0.271

Supplementary Table 5. Sequences of DNA template used for *in vitro* transcription.

fMet (CAU)	5′-GTAATACGACTCACTATAGGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTCATAACCCGAAGATCGTCGGTTCAAATCCGGCCCCCGCAACCA-3′
eFx	5′-GTAATACGACTCACTATAGGATCGAAAGATTTCCGCGGCCCCGAAAGGGGATTAGCGTTAGGT-3′
dFx	5′-GTAATACGACTCACTATAGGATCGAAAGATTTCCGCATCCCCGAAAGGGTACATGGCGTTAGGT-3′
aFx	5′-GTAATACGACTCACTATAGGATCGAAAGATTTCCGCACCCCCGAAAGGGGTAAGTGGCGTTAGGT-3′

**Underlined is the T7 promoter sequence.*

Supplementary Table 6. Kendall-Tau rank correlations of genotypes ranked within each Evolink experiment.

	Replicate 1	Replicate 2	Replicate 3
Replicate 1	1	0.907	0.905
Replicate 2	0.907	1	0.906
Replicate 3	0.905	0.906	1

Supplementary Table 7. Values for Cryo-EM data collection, refinement and validation statistics

	#1 RiboTv3-Combined (EMDB-EMD-26666) (PDB- 7UPH)
Data collection and processing	
Magnification	100,000X
Voltage (kV)	200
Electron exposure (e–/Å^2)	1.25
Defocus range (μm)	-1.2 μm to -2.2 μm
Pixel size (Å)	0.86 Å/pixel
Symmetry imposed	C1
Initial particle images (no.)	102,279
Final particle images (no.)	34,852
Map resolution (Å) FSC threshold	4.18 Å at FSC threshold = 0.143
Map resolution range (Å)	4.18 – 4.5 Å (FSC thresholds of 0.143, 0.5)
Refinement	
Initial model used (PDB code)	4YBB
Model resolution (Å) FSC threshold	4.8 at FSC threshold = 0.143
Model resolution range (Å)	5.5 – 4.8
Map sharpening *B* factor (Å^2)	N/A (insufficient resolution)
Model composition Non-hydrogen atoms Protein residues Ligands	2 RNA chains (4,556 total residues), 47 protein chains (5342 total residues)
B factors (Å^2) Protein Ligand	N/A (insufficient resolution)
R.m.s. deviations Bond lengths (Å) Bond angles (°)	N/A
Validation MolProbity score Clashscore Poor rotamers (%)	Molprobity clash score: 122.24 Rotamer Outliers : 0.02 % Cbeta Deviations : 0.02 % Peptide Plane: Cis-proline : 0.00 % Cis-general: 0.00 % Twisted Proline: 0.56 % Twisted General: 0.19 %
Ramachandran plot Favored (%) Allowed (%) Disallowed (%)	FAVORED : 86.50 % ALLOWED : 12.50 % OUTLIERS : 0.99 %