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Corresponding author(s): Floyd E. Romesberg

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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☒	☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No custom code used. Perkin Elmer Envision 2103 was controlled by Perkin Elmer Envision Manager (v. 1.13). TECAN Infinite M200 PRO was controlled by TECAN i-control I.II. Western blots and fluorescence scans were acquired using Typhoon Scanner Control (v. 5). HRMS data was acquired using Waters MassLynx (v.), hereunder deconvolution using Waters MaxEnt1.
Data analysis	No custom code used. Basic statistical analysis was performed using Microsoft Excel or R (v. 3.2.2). Figures and plots were made using ggplot2 and Adobe Illustrator (CC 2015). Fluorescence overlays between the anti-GFP western blots and TAMRA fluorescence scans were performed using Adobe Photoshop (CC 2015). Streptavidin-biotin shift gels were quantified using Image Studio Lite (v. 5.2.5, LI-COR Biosciences) or Fiji (ImageJ v. 2.0). Western blot processing, band quantification, and pseudocoloring, was done using Image Studio Lite (v. 5.2.5, LI-COR Biosciences).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Annotated plasmid sequences from this study are available via Genbank (accession numbers MN882182–MN882190) as detailed in Supplementary Table 4. All data

supporting the findings of this study is available within the paper and the supplementary information or from the corresponding author upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculation was used. Sample size of n=3 was used throughout the study, with only few exceptions where sample size was n>3. Sample size was restricted due to material constraints (i.e. especially for unnatural nucleoside triphosphates). We believe this sample size is sufficient for working with lab strains of E. coli.
Data exclusions	Due to failure in plasmid assembly/UBP replication, not all clones transformed with a UBP containing plasmid successfully become SSOs. SSOs were screened as described in methods and only SSOs that satisfied these specified criteria were included in the study.
Replication	The initial codon screens (Fig. 1d and Supplementary Fig. 1) were not replicated in full due to material constraints, owing to the amount of unnatural nucleoside triphosphates required for UBP media. Select codon/anticodon pairs from these experiments were replicated at least once and showed similar results. The data describing codon function in clonal SSOs (Fig. 2 and Supplementary Fig. 3) was replicated at least once but, in some cases, with slight variations in methods for preparation of SSOs or alternative expression schemes. Data for double or triple codons expression (Fig. 3b-f and Fig. 4b) were replicated in full three times and showed identical results. However, HRMS ESI-TOF analyses for these experiments were only replicated once.
Randomization	No randomization was done as there is no tradition for randomization in the field. Randomization would be impractical, and perhaps even prohibitive, for the conventional workflow of a molecular biology lab.
Blinding	No formal blinding was used as there is no tradition for blinding in the field. Blinding would be impractical, and perhaps even prohibitive, for the conventional workflow of a molecular biology lab.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Rabbit anti-GFP antibody (#G1544, lots 046M4871V, 077M4827V, 098M4868V, Sigma-Aldrich) at dilution 1;3,000. Goat anti-rabbit-Alexa Fluor 647-conjugated antibody (#A32733, lot #SD250298, ThermoFisher) at dilution 1;20,000.
Validation	All antibodies are validated for the application used in this study (Western blotting) according to the respective manufacturers' websites.