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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	none
Data analysis	Code used in creating the designed sequence and in analysis is described in the methods, where links to the Github repository are provided (http://github.com/TiongSun/genome_recoding)(http://github.com/TiongSun/recoding_landscape). These links will be made available upon publication. This code was made available to reviewers and editors during review. Publicly available software bowtie2 2.3.2, samtools 1.3.1, iSeq (http://github.com/TiongSun/iSeq), Integrative Genomics Viewer 2.4, ueye cockpit software 4.91.1, Nikon NIS elements 4.50.00, MaxQuant 1.5.5.1, Perseus 1.5.5.3, and Fiji were used for data analysis.

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

The sequences and genome design details used in this study are available in the Supplementary Data. Supplementary Data 1 provides the GenBank file of the E. coli MDS42 genome (NCBI accession number AP012306.1); Supplementary Data 2 provides the GenBank file of designed synthetic E. coli genome with codon replacements and refactorings; Supplementary Data 3 provides the table of target codons; Supplementary Data 4 provides the table of overlaps and refactoring;

Supplementary Data 5 provides the table of 10-kb stretches; Supplementary Data 6 provides the GenBank file of the BAC sacB-cat-rpsL; Supplementary Data 7 provides the GenBank file of BAC-rpsL-kanR-sacB; Supplementary Data 8 provides the GenBank file of the BAC rpsL-kanR-pheS?-HygR; Supplementary Data 9 provides the table of BAC construction; Supplementary Data 10 provides the table of BAC assembly; Supplementary Data 11 provides the table of REXER experiments; Supplementary Data 12 provides the GenBank file of spacer plasmids without trans-activating CRISPR RNA (tracrRNA) and annotation for linear spacers; Supplementary Data 13 provides the GenBank file of spacer plasmids with tracrRNA and annotation for linear spacers; Supplementary Data 14 provides the table of oligonucleotides used for recoding fixing experiments; Supplementary Data 15 provides the GenBank file of the gentamycin-resistance oriT cassette; Supplementary Data 16 provides the oligonucleotide primers used for conjugation; Supplementary Data 17 provides the GenBank file of the pJF146 F' plasmid that does not self-transfer; Supplementary Data 18 provides the GenBank file of the fully recoded genome of Syn61, verified by next-generation sequencing; Supplementary Data 19 provides the table of design optimizations and nonprogrammed mutations; Supplementary Data 20 provides a list of the proteins identified by tandem mass spectrometry; and Supplementary Data 21 provides a list of the primers used for deletion experiments. All other datasets generated and/ or analysed in this study are available from the corresponding author on reasonable request. All materials (Supplementary Data 9, 12, 13, 17, 18) from this study are available 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 performed. Because the variation in the assays used is small and we are interested in large effects, the sample sizes used, as indicated in the manuscript, were deemed appropriate.
Data exclusions	There are no data exclusions
Replication	The exact number of replicates is stated in the relevant legend. All attempts at replication were successful.
Randomization	No randomization. This is not relevant because the samples form defined groups.
Blinding	No blinding. Blinding is not relevant to the study. The groups were defined and studied by the investigator using standard protocols.

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