nature research

Corresponding author(s): Floyd E. Romesberg

☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

Describe how sample size was determined.

No sample size calculation was performed. Sample sizes were based on material constraints (i.e. the amount of unnatural triphosphates required to grow all the cultures used in the manuscript). As indicated in the text, all experiments were performed with n = 4 cultures (except one with n = 3) each inoculated from a single colony, which provides some limited information about the distribution of the measurements and is typical of similar experiments and studies in the field.

2. Data exclusions

Describe any data exclusions.

The plasmid cloning strategy described in the Methods results in a small percentage of plasmids that do not contain the UBP and/or contain other assembly errors during plasmid construction. Colonies transformed with such plasmids (as determined by the UBP retention assay described in the text) were excluded from analysis, as experiments conducted with cultures derived from such colonies are not relevant for assessing the ability of UBPs to be decoded in vivo. This exclusion criteria was pre-established in a previous study (see doi: 10.1073/pnas.1616443114).

3. Replication

Describe whether the experimental findings were reliably reproduced.

As indicated in the Methods, all experiments involving plated colonies were performed once due to material constraints. However, comparable experiments involving direct inoculation of electroporated cells into liquid media were also performed and all such experimental replicates were successful and reproduced the findings.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Bacterial colonies from transformations were selected randomly. Allocation into experimental groups is irrelevant; single colonies were propagated as bacterial cultures, which were then split and tested with various experimental conditions, where applicable.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Blinding is not relevant to our study because none of our data is based on qualitative scoring metrics nor does it involve animals or human research participants. As described in the above section for Randomization, blinding during group allocation is irrelevant because the samples of bacterial cultures that were split into different conditions were random samplings and there is no control over which cells will be selected and thus, no bias during group allocation.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☒	☐ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☒	☐ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☒	☐ The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☒	☐ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Basic statistical analysis was performed using Microsoft Excel. Chemiluminescent western blots were processed in UVP VisionWorks® LS (v 8.6.15114.8618) using the noise removal setting (Starfield Subtraction) and then further processed (black-white inverted) using Image Studio Lite (v. 5.2.5, LI-COR Biosciences). Fluorescent western blots were processed (pseudo-colored) and quantified using Image Studio Lite (v. 5.2.5, LI-COR Biosciences). Fluorescence overlays between the anti-GFP western blots and TAMRA fluorescence scans were performed with Paint.NET (v. 3.5.10). Biotin shift gels were quantified using Image Studio Lite (v. 5.2.5, LI-COR Biosciences). Peptide database searches were performed with MaxQuant (v. 1.5.8.0) using the default search parameters with minor changes, as described in Methods. Quantitation of LC/MS chromatographic peak intensities was performed by extracting MS1 precursor ion chromatograms with Skyline Software (v. 3.6.0.10167).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

As indicated in the Methods, the unnatural nucleotide triphosphates are available by custom chemical synthesis from commercial vendors (WuXi AppTec, Shanghai, China for nucleosides and TriLink BioTechnologies LLC, San Diego, USA or MyChem LLC, San Diego, USA for triphosphorylation of the nucleosides)

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Rabbit anti-GFP antibody (product #G1544, lot 046M4871V, Sigma-Aldrich)
Goat anti-rabbit-Alexa Fluor 647-conjugated antibody (product #A32733, lot #SD250298, ThermoFisher)
Goat anti-rabbit-HRP (product #170-6515, lot #64106148, BioRad)
All antibodies are validated for the application used in this study (Western blotting) according to the respective manufacturers' websites

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used. n/a

b. Describe the method of cell line authentication used. n/a

c. Report whether the cell lines were tested for mycoplasma contamination. n/a

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. n/a

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study. n/a

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants. n/a