nature portfolio

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Reporting Summary

Nature Portfolio 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 Portfolio policies, see our Editorial Policies 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	NCBI DATABASE, Time Tree of Life 4th edition;
Data analysis	AlphaFold2; PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC; SUPERPOSE software, Version 1.0; CCP4 program, Version 8.0; BEAST V1.10.4 package software; Figtree v1.4.3; MEGA11; PAML v4.9; ImageJ 1.48v; Igor Pro 6.32A; Geneious Prime 2020; Mosaic Finder; CrispRVariants R-software package v4.2; Jalview program v2.8.; HT-PAMDA Sequencing reads were analyzed via Python scripts (available at Zenodo: Sequence reads, https://zenodo.org/record/3710516#.YzqNb_78Jdd)

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 and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio 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 description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

We have made available sequencing data. Other data supporting the findings of this study are available from the corresponding authors upon reasonable request.

Plasmids for gene editing in human cells are available through Addgene (Supplementary Table 14; see also https://www.addgene.org/Raul_Perez-Jimenez/). Plasmids for HT-PAMDA experiments are available through Addgene (Supplementary Tables 8-10; see also www.addgene.org/Benjamin_Kleinstiver/). Sequencing data for PAM determination and gene editing experiments will be made available through the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under BioProject ID PRJNA832610. Sequencing data for HT-PAMDA experiments will be made available through the NCBI SRA under BioProject ID PRJNA832159.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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	Fifty-nine sequences were chosen for ancestral sequence resurrection of anCas. The selected sample size is a standard for the application of this methodology (Merkl and Sterner, Biol. Chem. 2016; 397(1): 1–21).
Data exclusions	No data was excluded.
Replication	Information on replication of the experiments is reported in the figure legends and methods section. In vitro and in vivo results are representative of least 2 independent experiments. All replication attempts showed similar results.
Randomization	No randomization was performed. Randomization is not relevant for our study that investigated the evolution of Streptococcus pyogenes Cas9 endonuclease based on ancestral sequence resurrection.
Blinding	No blinding was performed. Blinding is not relevant for our study that investigated the evolution of Streptococcus pyogenes Cas9 endonuclease based on ancestral sequence resurrection.

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 and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

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

Antibodies

Antibodies used	Anti-Cas9 rabbit antibody (Rockland, 600-401-GK0); Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (Invitrogen), ref. G21234, LOT 2321833
Validation	Anti-Cas9 rabbit antibody and HRP-labeled Goat Anti-Human IgG (H+L) were established commercial antibody. The validation of commercially available antibodies used in this work was described in technical data sheets provided by the manufactorers websites.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	HEK293T cells (ATCC, cat. no. CRL-3216)
Authentication	None of the cell lines used were authenticated
Mycoplasma contamination	Cells were confirmed to be free of mycoplasma by analyzing supernatant media monthly using MycoAlertTM PLUS (Lonza).
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.