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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 EPU (version 2.12), MultiNA, MiSeq (Illumina), Illumina NextSeq 550

Data analysis cryoSPARC (version 4.4.0), COOT (version 0.9), UCSF ChimeraX (version 1.7.1), servalcat (version 0.4.6)

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](#)

Plasmids used in this study, pcDNA3_SaCas9-NNG and pcDNA3_eSaCas9-NNG, are available from Addgene under accession numbers 252406 and 252407, respectively. The atomic models of eSaCas9-NNG-guide RNA-target DNA complexes have been deposited in the Protein Data Bank under the accession codes 8ZCY (interrogation state), 8ZCZ (intermediate state), 8ZD0 (translocation state), and 8ZDA (active state). The cryo-EM density maps have been deposited in the Electron Microscopy Data Bank under the accession codes EMD-39941 (interrogation state), EMD-39942 (intermediate state), EMD-39944 (translocation state), and

EMD-39954 (active state). The atomic models of SaCas9 and eSaCas9-NNG bound to the sgRNAs and target DNA containing a mismatch at position 21 have been deposited in the Protein Data Bank under the accession codes 9MB6 (SaCas9) and 9MB7 (eSaCas9-NNG). The cryo-EM density maps have been deposited in the Electron Microscopy Data Bank under the accession codes EMD-63767 (SaCas9) and EMD-63768 (eSaCas9-NNG). The NGS data have been deposited in the NCBI under accession code PRJNA1088532. Source data are provided as a Source Data file.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender	not applicable
Reporting on race, ethnicity, or other socially relevant groupings	not applicable
Population characteristics	not applicable
Recruitment	not applicable
Ethics oversight	not applicable

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](https://www.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 statistical method was used to determine the sample size. For cryo-EM analyses, sample sizes were determined by the availability of microscope time and the number of particles on electron microscopy grids enough to obtain a structure at the reported resolution. For biochemical analysis, sample size were determined based on the previous reports of this type of study and the reproducibility of results across independent experiments.
Data exclusions	For cryo-EM analyses, particles that did not contribute to improving map quality were excluded following the standard classification procedures in cryoSPARC. This is standard practice for structure determination by cryo-EM. For other biochemical experiments, no data was excluded.
Replication	Biochemical experiments were performed at least three times.
Randomization	For cryo-EM analyses, particles were randomly assigned to half-maps for resolution determination following the standard procedures in cryoSPARC. For biochemical analyses, randomization was not performed.
Blinding	Blinding is not applicable to this study, since neither structural nor functional experiments included subjective assignments.

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
☒	☐ Plants

Methods

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

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	HEK293 and TLR3 cell lines were obtained from JCRB cell bank(HEK293:JCRB9068; TLR3:IFO50680). HEK293T cell lines were obtained from ATCC #CRL-3216. AAVpro293T cell line was obtained from Takara Bio(632273).
Authentication	The HEK293, HEK293T, TLR3 and AAVpro293T cell lines were not authenticated as they were purchased commercially.
Mycoplasma contamination	The HEK293, HEK293T, TLR3 and AAVpro293T cell lines were not tested for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	The HEK293, HEK293T, TLR3 and AAVpro293T cell lines were not in the ICLAC database of misidentified cell lines.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	C57BL/6 mice were purchased from SLC Japan
Wild animals	This study did not involve wild animals.
Reporting on sex	Male
Field-collected samples	This study did not involve samples collected from the field.
Ethics oversight	All animal experimental procedures were approved by The Institutional Animal Care and Concern Committee of Jichi Medical University (permission number: 20051-10), and animal care was conducted in accordance with the committee's guidelines and ARRIVE guidelines. All mice were housed in isolators in the specific pathogen-free facility of Jichi Medical University under controlled environmental conditions (23 °C ± 3 °C, 50% ± 10% relative humidity) with a 12:12-h light/dark cycle.

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

Plants

Seed stocks	not relevant
Novel plant genotypes	not relevant
Authentication	not relevant