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Corresponding author(s): Xiaohua Wu

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Reporting Summary

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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	Data collection for cell viability assay was performed with Gene5 3.11 software; data collection for FACS was performed with BD Accuri C6 software; data collection for RT-qPCR was performed with iQ™5 Optical System software; data collection for PLA image was performed with Olympus Microscopy software; data collection for WB was performed with Biorad Gel Doc XR+ Imaging software.
Data analysis	CellProfiler 4.2.6 software, GraphPad Prism9 and Microsoft Excel

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

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

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 statistical method was used to predetermine sample sizes. For FACS analysis, sample size is 4/ 5. For PLA analysis, sample size is 300.
Data exclusions	No data exclusions.
Replication	To verify the reproducibility of our findings, experiments were performed using at least 3 biological replicates.
Randomization	N/A
Blinding	There was no blind setting, and the authors were aware of the status of the samples when conducting experiments.

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

Antibodies

Antibodies used

Antibodies used for PLA include:
FLAG (F1804, Sigma-Aldrich), FLAG (AE004, ABclonal), HA (E10176EF, Covance), PCNA (SC-56, Santa Cruz), PCNA (10205, Proteintech), PCNA K164Ub (13439, Cell Signaling Technology), γH2AX (05636, Upstate), γH2AX (07164, Upstate), RAD51 (05-530-I, Santa Cruz).
Antibodies used for WB include:
53BP1 (NB100-305, Novus Biologicals), FLAG (F1804, Sigma-Aldrich), PCNA (SC-56, Santa Cruz), PCNA-K164ub (13439, Cell Signaling Technology), HA (E10176EF, Covance), PRIM1 (10773-1-AP, Proteintech), RPA2 (NA19L, Calbiochem), SMARCAD1 (A5850, ABclonal), and KU70 (E-5, SC-17789, Santa Cruz Biotechnology)

Validation

FLAG (F1804, Sigma-Aldrich), **WB 1:1000**, **PLA 1:250**
https://www.sigmaaldrich.com/US/en/product/sigma/f1804
FLAG (AE004, ABclonal),**PLA 1:250**
https://abclonal.com/catalog-antibodies/RabbitantiDDDDKTagpAb/AE004
HA (E10176EF, Covance), **WB 1:1000**,**PLA 1:250**
https://www.biolegend.com/nl-be/products/anti-ha-11-epitope-tag-antibody-11071
PCNA (SC-56, Santa Cruz), **WB 1:1000**,**PLA 1:250**
https://www.scbt.com/p/pcna-antibody-pc10
PCNA (10205, Proteintech),**PLA 1:250**
https://www.ptglab.com/products/PCNA-Antibody-10205-2-AP.htm
PCNA K164Ub (13439, Cell Signaling Technology),**PLA 1:250**
https://www.cellsignal.com/products/primary-antibodies/ubiquityl-pcna-lys164-d5c7p-rabbit-mab/13439
γH2AX (05636, Upstate),**PLA 1:250**
https://www.sigmaaldrich.com/US/en/product/mm/05636
γH2AX (07164, Upstate),**PLA 1:250**
https://www.sigmaaldrich.com/US/en/product/mm/07164
RAD51 (05-530-I, Santa Cruz),**PLA 1:250**
https://www.scbt.com/p/rad51-antibody-3c10
53BP1 (NB100-305, Novus Biologicals), **WB 1:1000**
https://www.novusbio.com/products/53bp1-antibody_nb100-305
PRIM1 (10773-1-AP, Proteintech), **WB 1:1000**
https://www.ptglab.com/products/PRIM1-Antibody-10773-1-AP.htm
RPA2 (NA19L, Calbiochem), **WB 1:1000**
https://www.emdmillipore.com/US/en/product/Anti-Replication-Protein-A-Antibody-clone-RPA34-20,MM_NF-MABE285
SMARCAD1 (A5850, ABclonal), **WB 1:1000**
https://abclonal.com/catalog-antibodies/SMARCAD1RabbitpAb/A5850
KU70 (E-5, SC-17789, Santa Cruz Biotechnology), **WB 1:1000**
https://www.scbt.com/p/ku70-antibody-e-5

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	U2OS (human osteosarcoma) and HEK293T cells were obtained from the ATTC cell repository. RPE-1 cells sensitive to puromycin were received from Dr. Stephen P. Jackson's lab. UWB1 was received from Dr. Lee Zou 's lab.
Authentication	U2OS and HEK293T are authenticated by ATTC. RPE-1 is authenticated by Dr. Stephen P. Jackson's lab and we confirmed the sensitivity to puromycin. UWB1 is authenticated by Dr. Lee Zou's lab.
Mycoplasma contamination	All cells tested negative.
Commonly misidentified lines (See ICLAC register)	No

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	EGFP reporter cell lines were used for the FACS analysis. 5 days post cleavage by I-SceI or Cas9-gRNA, adherent cells were trypsinized and collected. Cells were then washed with 2ml of PBS and cell pellets were re-suspended with 500µl of PBS. Re-suspended cells were transferred to the 12 X 75mm polystyrene round bottom test tube (Falcon) for FACS analysis.
Instrument	BD Accuri C6 flow cytometer
Software	BD Accuri C6 Software
Cell population abundance	N/A
Gating strategy	P1: Alive cells were chosen for analysis after doublet discrimination by detection of disproportions between cell size (FSC-A) vs. cell signal (FSC-H). P2: Set the gate using nonfluorescent cells as a control. GFP-positive cells can be detected outside of the negative population of cells measured with a 488-530 nm laser.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.