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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

1. Cell depletion measurement via SRB assay was performed using FlexStation 3 (Molecular Devices) or Biotek Synergy Neo2 (Agilent) microplate readers and accompanying softwares.
2. Cell depletion measurement via phase confluence, cell counting, and GFP intensity was performed using the Incucyte® SX5 system (Sartorius) and Incucyte® Base Analysis Software (v2023A).
3. DNA damage foci and RNA FISH imaging was carried out using an Eclipse Ti-2 spinning disk confocal microscope (Nikon) equipped with Nikon NIS-Elements software (v6.02.03).
4. Flow cytometry was conducted using FACSCelesta (BD Biosciences) or MA900 (Sony) and accompanying softwares.
5. RNA-seq was perfomed using Hiseq 4000 (Illumina) or NovaSeq X series (Illumina).
6. qPCR was performed using QuantStudio 3 (ThermoFisher) and accompanying software.

Data analysis

1. Incucyte's standard basic analysis (AI confluence, GFP count per image, Total integrated intensity) and adherent cell-by-cell analysis (Phase object count per image) were used to quantify cell depletion over time and at endpoints.
2. Fiji ImageJ v2 was used to reduce background noise in the fluorescence images of DNA damage foci.
3. Ilastik v1 and built-in modules were used to segment cell bodies in fluorescence images of RNA FISH.
4. CellProfiler v4 and built-in modules were employed to automatically segment and quantify cell nuclei, DNA damage foci, and RNA FISH transcripts.
5. FlowJo v10.10 was utilized to gate and analyze flow cytometry data.
6. RStudio v4 was used to execute differential gene expression analysis and hallmark pathway. Sequencing reads were aligned to hg38 with Bowtie2 (v2.2.9). Read counts for each GENCODE gene were quantified with RSEM (v1.2.28). DESeq2 (v1.44.0) was used to normalize the read counts and perform differential expression analysis. Gene set enrichment analysis (GSEA v4.3.3) was run to determine the enriched gene sets.

The R package fgsea (v1.30.0) was used to calculate the normalized enrichment statistics for each gene set in the Hallmark Collection from the Molecular Signatures Database (PMID: 26771021).

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

All relevant sequencing data are publicly available at NCBI GEO under accession number GSE287139. The human Hallmark Gene Sets collection from the Molecular Signatures Database is available for public download at gsea-msigdb.org. All relevant gRNA and target sequences supporting this study are available within this article and the Supplementary Information section.

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	N/A
Reporting on race, ethnicity, or other socially relevant groupings	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	For cell imaging of 53BP1 foci, samples sizes were chosen to meet or exceed those of existing publications with similar analyses (PMIDs: 31645724; 32527834; 37024653). For all other studies, we conducted at least three biological replicates as the minimum number of independent replicates necessary to calculate standard deviation, perform statistical comparisons, and ensure reproducibility.
Data exclusions	During flow cytomtery analysis of GFP and RFP positive cells within a co-culture, cells which were gated as negative for either fluorescent reporter were excluded from quantification of total events. This exclusion criterion was pre-established given that negative cells contain neither the on-target nor off-target transcript necessary for analysis of Cas12a2 nuclease activation, and therefore represent sample impurities. This exclusion does not change the qualitative conclusion drawn from the dataset.
Replication	In vivo experiments were performed with five animals per treatment group. All cell assays were performed with three to eight biological replicates. In vitro cleavage assays were performed with three independent replicates, although only one representative gel image is shown. All attempts at replication were successful.
Randomization	For all cell-based experiments, cells were randomly allocated from growth cultures into experimental groups. All growth cultures were maintained under identical environmental conditions (save respective media) to mitigate covariates. For instrument-based assays, cells from experimental groups were randomly sampled to counter instrument measurement bias. Mice were randomized into experimental groups to counter possible selection bias. Randomization was not required for in vitro biochemical assays due to the lack of biological covariates.
Blinding	Data were not collected in a blinded manner. However, we applied the same analysis pipelines and gating strategies to all control and experimental samples. For instance, to measure average fluorescent 53BP1 foci in given cell populations, we used DAPI signal to segment all

cell nuclei in the field of view and quantified 53BP1 foci in those nuclei.

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

All antibodies are commercially available and validated.

For DNA damage foci imaging:
1. Rabbit anti-53BP1 antibody (Novus Biologicals, NB100-306)
2. Goat anti-Rabbit Alexa Fluor Plus 647 secondary antibody (Invitrogen, A32733)

For apoptosis analysis:
3. Alexa Fluor 647 Annexin-V antibody (BD Pharmingen, 567356)

Validation

1. https://www.bio-techne.com/p/antibodies/53bp1-antibody_nb100-305
2. https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A32733
3. https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/alexa-fluor-647-annexin-v.567356?tab=product_details

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

We included a detailed Table S11 in the Supplementary Information, describing all yeast and bacterial strains, as well as the mammalian cell lines used in this study, along with their sources.

Authentication

We primarily used commercially sourced cell lines and therefore did not perform independent authentication. However, we performed RNA-seq to verify the expression levels of our RNA targets, and these data are detailed in Table S2.

Mycoplasma contamination

All cultured mammalian cell lines were tested monthly for mycoplasma contamination using the PlasmoTest kit (InvivoGen, rep-pt1), and were consistently negative.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified lines were used.

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

5-7 weeks old NOG mice weighing 20.2 g ± 1.67 g were used for this study

Wild animals

No wild animals were used for this study.

Reporting on sex

Immunodeficient female NOG mice were used for this study.

Field-collected samples

The study did not involve samples collected from the field.

Ethics oversight

The work conducted in living mice, at Experimental Pharmacology and Oncology GmbH (Berlin, Germany), is in accordance with the German Animal Welfare Act as well as the UKCCCR (United Kingdom Coordinating Committee on Cancer Research) and all

procedures were approved by local authorities (Landesamt für Gesundheit und Soziales, LaGeSo Berlin, Germany) under approval number E0023/23.

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

Plants

Seed stocks	N/A
Novel plant genotypes	N/A
Authentication	N/A

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	For apoptosis analysis: 48 hours post electroporation, cells were treated with 0.05% Trypsin, centrifuged, and resuspended in Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). Resuspended cells were mixed with Alexa Fluor 647 Annexin-V antibody (BD Pharmingen, 567356) at a volumetric ratio of 1:40 and incubated at room temperature for 15-30 minutes. Immediately preceding data acquisition, samples were mixed with 10 µg/mL DAPI stain (BD Pharmingen, 564907) at a volumetric ratio of 1:80. For Caspase-3/7 analysis, resuspended cells were mixed with 0.2 mM NucView 405 Caspase-3/7 Substrate (Biotium, 10407) at a volumetric ratio of 1:200 and incubated for 30 minutes at room temperature. For cell cycle analysis: 48 hours post electroporation, cells were treated with 0.05% Trypsin, centrifuged, and resuspended in 200 µL phenol red-free Leibovitz's L-15 Medium followed by 200 µL of 4% PFA fixative and incubated on ice for 25 minutes. To remove fixative, cells were pelleted by centrifugation at 250 RCF for 5 minutes and washed with 500 µL L-15 twice. To stain genomic DNA, cells were pelleted again and resuspended in 500 µL of a 1x PBS solution with 10 µg/mL DAPI stain (BD Pharmingen, 564907) and 0.1% Triton-X for 15 minutes at room temperature followed by 10 minutes on ice. Cells were strained through a 35 µm mesh cap before analysis to ensure single cell suspension. For co-culture depletion analysis: Four days post electroporation, HeLa-GFP and HeLa-RFP cell co-cultures were treated with 0.05% Trypsin, centrifuged, and resuspended in 400 uL phenol red-free Leibovitz's L15 Medium and analyzed by flow cytometry using the Sony MA900 system with 488 nm and 561 nm laser excitation.
Instrument	BD FACSCelesta or Sony MA900
Software	FlowJo V10.10
Cell population abundance	10,000 events per condition/sample
Gating strategy	Viable and dying single cells were gated using SSC area versus FSC area (SSC-A x FSC-A). For assessment of fluorescent apoptosis markers, cells were gated using DAPI area versus Alexa Fluor 647 Annexin-V area (BV421-A x APC-A; “Annexin-V”) or cell count versus NucView 405 area (Count x BV421-A, “Caspase-3/7”). Boundaries separating positive from negative cell populations were set between 10^2 and 10^3 arbitrary intensities. For discrimination between GFP-positive and RFP-positive cell populations, cells were gated using RFP area versus GFP area (mCherry-A x EGFP-A). Boundaries separating positive from negative cell populations were set between 10^2 and 10^3 arbitrary intensities. Cells marked as double negatives (population impurities) were excluded from quantification of total events. For cell cycle assessment, cells were gated using cell count versus DAPI area (Count x BV421-A).

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