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<br>*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<br>*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. FACS DIVA Software 5.0.3 (flow cytometry)<br>2. NovoExpress 1.6.1 (flow cytometry)<br>3. LI-COR ImageStudio V5.2 (western blot)<br>4. ZEN 2012 (blue edition, version 1.1.0.0) microscopy software (microscopy) |
| Data analysis | 1. FlowJo 10.8.0 (flow cytometry)<br>2. LI-COR ImageStudio Lite 5.2.5 (western blot)<br>3. BioRad CFX Maestro 2.0 (qPCR)<br>4. GraphPad Prism 9.5.1 or 10.0.2 (plotting and statistics)<br>5. SnapGene 7.02 (DNA cloning design and sequence alignment)<br>6. ImageJ 2.0.0 (microscopy)<br>7. Foci analyzer ImageJ macro (https://github.com/BioImaging-NKI/Foci-analyzer; foci analysis microscopy)<br>8. Olympus ScanR Image Analysis Software 3.3.0 (Rad51 foci)<br>9. BasDAS screen analysis at http://barcode.appex.kr/barcode/0_main.php (screen analysis) |

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

Source data are provided with this paper. Sequencing files from the CRISPR/Cas9 screen are available through the Sequence Read Archive of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/sra), with project number PRJNA1013929, accessions SRX21658367, SRX21658368 and SRX21658369. Note that the sequencing files contain data obtained from two independent HEK 293T DSB-Spectrum_V2 clones (D9 and E5). The representative data presented in this manuscript are from clone D9.

## 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.

| | |
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| Reporting on sex and gender | *Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design; whether sex and/or gender was determined based on self-reporting or assigned and methods used.*<br>*Provide in the source data disaggregated sex and gender data, where this information has been collected, and if consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected.*<br>*Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.* |
| Reporting on race, ethnicity, or other socially relevant groupings | *Please specify the socially constructed or socially relevant categorization variable(s) used in your manuscript and explain why they were used. Please note that such variables should not be used as proxies for other socially constructed/relevant variables (for example, race or ethnicity should not be used as a proxy for socioeconomic status).*<br>*Provide clear definitions of the relevant terms used, how they were provided (by the participants/respondents, the researchers, or third parties), and the method(s) used to classify people into the different categories (e.g. self-report, census or administrative data, social media data, etc.)*<br>*Please provide details about how you controlled for confounding variables in your analyses.* |
| Population characteristics | *Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."* |
| Recruitment | *Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.* |
| Ethics oversight | *Identify the organization(s) that approved the study protocol.* |

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.

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| Sample size | No statistical method was used to predetermine sample size. Sample size was determined based on previous experience and common practice in the field. For flow cytometry experiments, at least 10,000 live and transfected (if applicable) events were measured per sample. For microscopy experiments, sample size was generally 50-100 cells per sample per experiment. For the CRISPR screen, sample size was constantly maintained at 250x the number of sgRNAs in the library to ensure representation of all sgRNAs. |
| Data exclusions | No data were excluded. |

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| Replication | For the CRISPR screen, three biological replicates were performed. For flow cytometry, at least three biological replicates were performed, each biological replicate containing two technical replicates. For qPCR analysis of end-resection, at least three biological replicates were performed, each biological replicate containing three technical replicates. For microscopy, at least two biological replicates were performed. For each replicate, in general, >50 individual nuclei were imaged, with some exceptions. For all experiments included in the manuscript, all attempts at replication were successful. |
| Randomization | No randomization was performed. These were studies using cell lines that can be simply split into multiple populations to subsequerntly study the phenotype upon drug treatment/genetic interference. |
| Blinding | Blinding was not relevant to the study. Unbiased quantification was guaranteed by software-based analysis using identical parameters between conditions. |

# 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 |
|---|---|
| [ ] | [x] Antibodies |
| [ ] | [x] Eukaryotic cell lines |
| [x] | [ ] Palaeontology and archaeology |
| [x] | [ ] Animals and other organisms |
| [x] | [ ] Clinical data |
| [x] | [ ] Dual use research of concern |
| [x] | [ ] Plants |

## Methods

| n/a | Involved in the study |
|---|---|
| [x] | [ ] ChIP-seq |
| [ ] | [x] Flow cytometry |
| [x] | [ ] MRI-based neuroimaging |

## Antibodies

Antibodies used

Target ; Company ; Cat. No., Clone ; Lot No. ; Application and dilution
1 . Ube2T ; Abcam ; ab140611 ; YJ062105CS ; WB (1:1000)
2 . FANCD2 ; Santa Cruz ; sc-20022 ; D1221 ; IF (1:100) and WB (1:1000)
3 . FANCD2 ; Novus ; NB100-182 ; L3 ; WB (1:10000)
4 . HSP90 ; Santa-Cruz ; Sc-13119 ; G2420 ; WB (1:1000)
5 . Tubulin ; Sigma Aldrich ; T6199, clone DM1A ; 127611 ; WB (1:5000)
6 . gamma-H2AX ; Millipore ; #05-636, clone JBW301 ; 4047974 ; IF (1:2000)
7 . MDC1 ; Abcam ; ab11171-50 ; GR252252-10 ; WB (1:1000)
8 . pRPA (S4/S8) ; Bethyl ; A300-245A ; 9 ; IF (1:1000)
9 . Rad51 ; Genetex ; GTX70230, Clone 14B4 ; 42590 ; IF (1:500)
10 . CtIP ; Sigma Aldrich ; MABE1060, Clone 14-1 ; 3780584 ; WB (1:1000) and IF (1:1000)
11 . CtIP ; Santa Cruz ; sc-271339, Clone D-4 ; F1510 ; WB (1:500)
12 . Actin ; MpBiomedicals ; 869100, Clone C4 ; SR05927-1 ; WB (1:1000)
13 . BrdU ; Roche ; 11170376001, Clone BMC9318 ; 10875400 ; FC (1:200)
14 . Mre11 ; Cell Signaling ; 4895 ; 3 ; WB (1:1000)
15 . GFP ; Abcam ; Ab290 ; GR3321575-1 ; WB (1:5000)

Validation

1. Ube2T: KO-validated in manuscript, Fig. 2a
2. FANCD2: KO-validated in manuscript, Fig. 3h
3. FANCD2: validated by knockdown in manuscript, Fig, S3e
4. HSP90: validated by supplier, cited in 710 publications (https://www.scbt.com/p/hsp-90alpha-beta-antibody-f-8)
5. Tubulin: Validated by supplier using its Antibody Enhanced validation method (https://www.sigmaaldrich.com/NL/en/product/sigma/t6199)
6. gamma-H2AX: Validated by suplier (https://www.sigmaaldrich.com/NL/en/product/mm/05636)
7. MDC1: Validated by supplier, 31 references on website (https://www.abcam.com/en-nl/products/primary-antibodies/mdc1-antibody-ab11171)
8. pRPA (S4/S8): Validated by suplier (https://www.thermofisher.com/antibody/product/Phospho-RPA32-Ser4-Ser8-Antibody-Polyclonal/A300-245A)
9. Rad51: Validated by suplier, cited in 113 publications (https://www.genetex.com/Product/Detail/Rad51-antibody-14B4/GTX70230)
10. CtIP: Validated by supplier for western blot, (https://www.sigmaaldrich.com/NL/en/product/mm/mabe1060)
11. CtIP: Validated by supplier, cited in 46 publications (https://www.scbt.com/p/ctip-antibody-d-4).
12. Actin: Validated by supplier, cited in 127 publications according to CiteAb.com (https://www.mpbio.com/eu/anti-actin-mouse-monoclonal-antibody-clone-c4).
13. BrdU: Validated by supplier (https://www.sigmaaldrich.com/NL/en/product/roche/11170376001)
14: Mre11: validated by knockdown in manuscript, Fig. S4e

15: GFP: validated by supplier, cited in 2582 publications (https://www.abcam.com/en-gb/products/primary-antibodies/gfp-antibody-ab290)

In addition, for all DNA damage respsonse genes, a clear increase in signal was observed at damaged DNA, further validating specificity of the antibodies.

## Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

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|---|---|
| Cell line source(s) | U2OS and HEK 293T cells were originally obtained from ATCC (HTB-96 and Crl-3216 respectively). Acquisition date and passage number was not indicated. U2OS 2-6-3 cells expressing ER-mCherry-LacI-FokI-DD were a kind gift from Roger Greenberg (University of Pennsylvania, Philadelphia, Pennsylvania, USA). The U2OS AsiSI cells were a kind gift from Gaelle Legube (CBI, Toulouse, France). |
| Authentication | Human U2OS and HEK 293T cells were authenticated using Short Tandem Repeat (STR) analysis by ATCC services (100% match). No commonly misidentified cell lines were used. |
| Mycoplasma contamination | All cell lines were routinely tested for mycoplasm infection and have always tested negative. |
| Commonly misidentified lines (See ICLAC register) | 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

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| Sample preparation | Cells were cultured as a monolayer and harvested by trypsinization. For harvesting of populations during the CRISPR screen, trypsin was quenched with medium+FCS, cells were pelleted by centrifugation, pellet was resuspended in medium+FCS and filtered using 35 micrometer strainer cap tubes. For DSB-Spectrum assays, trypsin was quenched and diluted by addition of a 4x volume PBS+1% BSA, resuspended and kept on ice. Immediately before sample acquisition, cells were resuspended and filtered using a 35 micrometer strainer cap tubes. For analysis of cell-cycle distribution, cells were fixed in 70% ethanol and subsequently denatured in 2M HCl. |
| Instrument | FACS was done on a BD FACSAria III, analysis was done on the BD LSRFortessa™ X-20 Cell Analyzer or the Agilent Novocyte Quanteon |
| Software | The FACSAria III and Fortessa were running FACS Diva software, continuously updated. The Novocyte Quanteon was running NovoExpress 1.6.1. Data was analyzed using FlowJo 10.8.0 |
| Cell population abundance | For the CRISPR screen, between 4*10^6 and 12*10^6 cells were sorted per population. For DSB-Spectrum assays, at least 10,000 transfected (iRFP(670)-positive) cells were analyzed. |
| Gating strategy | 1. Gating on FCS-A vs SSC-A to gate on live cells. 2. Gating on SSC-H vs SSC-W to exclude doublets. 3. Gating on FSC-H vs FSC-W to exclude doublets. (in some cases, single gating using FSC-A vs FSC-H was used to select single cells) 4. Gating on iRFP (670) positive (640 nm laser, APC filter settings) to select for iRFP(670) cells. 4. Gating on mut-EJ, SSA or HR populations as detailed in the manuscript. |

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