Supplementary information

Materials and Methods

Cell lines and culture conditions

HT1080 human cells were grown in low glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Sigma-Aldrich), 1% non-essential amino acids (NEAA; Sigma), G418 (25 μg/ml, Sigma Aldrich, A1720-5G) and puromycin (0.5 μg/ml, Sigma Aldrich, P9620). hTERT-immortalized human RPE-1 cells stably expressing Cas9 and hTERT-immortalized human RPE-1 with stable expression of GFP-H2B RFP-NLS SNAP-MDC1[1] were grown in DMEM/F12 medium (Sigma Aldrich) supplemented with 10% FBS and 1% penicillin-streptomycin. Shield1-inducible Dam-Lamin B1 HT1080 clone11 and hTERT RPE-1 Cas9 cell lines were generous gifts from B. van Steensel and D. Durocher, respectively. Cell lines were cultured at 37°C in a 5% CO_2 atmosphere.

All cell lines were regularly tested for mycoplasma contamination. All cell lines tested negative for mycoplasma contamination. Summarised information on cell lines used in this study can be found in Supplementary Table 1.

Plasmid generation

PSA05-AID-ER*-DPN1 and PSA11-GFP-m^{6}A-Tracer-APEX2-NES plasmids were assembled using golden gate cloning [2]. To generate PSA13-AID-ER*-DPN1, cassette AID-ER*-DPN1/TIR1 was PCR amplified (Primer 1 and Primer 2) from PSA05-AID-ER*-DPN1 and ligated into PLKU06-Hygro digested with ApaI and BamHI.

Plasmids for PRR14 tethering were adapted from [3]. The pMito-mCherry-FRB_IRES_FKBP(III)-GBPen plasmid was a kind gift from Professor Chris Chan (Addgene #128268). pMito-mCherry was removed using restriction sites AgeI and BsrGI. mCherry was amplified using primers (Primer 3, and Primer 4) and fused by PCR with a custom insert of codon-optimised PRR14 gBlock (IDT) using primers (Primer 5 and Primer 6). NEBuilder HiFi DNA assembly cloning kit (New England Biolabs, E5520S) was used to assemble PRR14-mCherry-FRB_IRES_FKBP(III)-GBPen. All oligonucleotide sequences can be found in Supplementary Table 2.

Cell lines generation

The Shield1-inducible Dam-Lamin B1 clonal line clone11 (HT1080) was a kind gift from Bas van Steensel and are previously described [4]. To generate a DpnI$^-$ cell line, clone11 cells were transfected with PSA11 carrying NES-m^{6}A-GFP-APEX2 containing a puromycin resistance gene using Lipofectamine 2000 (Invitrogen, 11668-019), according to manufacturers instructions. Cells were treated with 10 μg/ml Puromycin (Sigma Aldrich, P9620-10ML) for 5 days. Single colonies were picked and expanded, and cells were treated with 0.5 μM Shield1 (Takara, 632189) for 18 h. Clones were seeded for GFP immunofluorescence, as described below. The clone with best GFP ring induction was identified and used for subsequent experiments.

To generate a DpnI+ cell line, DpnI$^-$ cell line was transduced with the lentiviral vector PSA13-AID-ER*-DPN1 containing a BFP expression cassette. The lentivirus was prepared by transfection of HEK293T cells with third generation lentiviral plasmids, packaging plasmid 17.5 µg psPAX2 (packaging plasmid), 7.5 µg pMD2.G (envelope plasmid) and 25 µg of PSA13-AID-ER*-DPN1 using Lipofectamine 2000, according to manufacturers instructions. Viral particles were collected after 48 h, filtered (0.45 µm), and then used to infect 1x10^6 Dpn1$^-$ cells per 1 ml of virus. 48 hours after seeding, cells were FACS sorted for BFP expression. Single clones were expanded and tested for induction of 53BP1 and γH2AX foci formation at the nuclear periphery following 0.5 µM Shield1 (18 h) and 300 nM (Z)-4-Hydroxytamoxifen (4OHT; Sigma Aldrich, H7904) (2 h) treatment. The clone with the highest induction of damage was selected for further use.

Cas9 RNP transfection

2x10^4 RPE-1 GFP-H2B RFP-NLS SNAP-MDC1 cells were seeded onto a µ-Dish 35 mm, high (ibidi). 24 h later, cells were transfected with 250 ng TrueGuide Synthetic guide RNA C*A*T*TGACGATCATCCCACTG (asterisks indicate modified 2′-O-methyl bases with phosphorothioate linkages) and 750 ng TrueCut™ Cas9 Protein v2 (Thermo Fisher Scientific) using Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent (Thermo Fisher Scientific) according to manufacturer's protocol.

TracrRNA:crRNA transfection

Alt-R™ CRISPR-Cas9 tracrRNA (cat. number 1073191) and crRNAs (Alt-R™ Cas9 Negative Control crRNA #1 (cat. number 1079138), and CATTGACGATCATCCCACTG (Chr7 sgLAD; gRNA #1, Supplementary Table 2) [5] and AGGTCAGCCCACCCCCAGTT (Chr9 sgiLAD; gRNA #2, Supplementary Table 2) [6] were purchased from Integrated DNA Technologies (IDT) and transfected using Lipofectamine RNAiMAX (Invitrogen), following manufacturer's instructions. Briefly, tracrRNA and crRNA were mixed in a 1:1 molar ratio and annealed at 95°C for 5 min, followed by gradual cooling to 20°C in a thermocycler. The resulting tracrRNA:crRNA duplex was diluted in Opti-MEM Medium (Gibco) and mixed with RNAiMAX (Invitrogen), also diluted in Opti-MEM. After a 15 min incubation at RT, the sgRNA-lipid transfection complexes were added dropwise to the culture plate.

siRNA transfection

Dharmacon ON-TARGETplus Non-targeting pool (D-001810-10-20), SUN1 (L-025277-00-0005) and SUN2 (L-009959-01-0005) siRNA SMARTpools were transfected for 48 h using Lipofectamine RNAiMAX, according to manufacturer's instructions. Briefly, siRNAs were diluted in Opti-MEM Medium and mixed with RNAiMAX, also diluted in Opti-MEM. After a 15 min incubation at RT, the siRNA-lipid transfection complexes were added dropwise to the culture plate.

Immunofluorescence (IF)

Cells were grown on glass coverslips and treated as indicated. Cells were pre-extracted (2 min on ice in 0.2% Triton X-100/PBS), and then fixed (10 min in 4% paraformaldehyde/PBS), permeabilized (5 min in 0.2% Triton X-100/PBS), blocked 1 h in 5% bovine serum albumin (BSA/PBS; Sigma Aldrich, A7030-100G), and incubated with the indicated primary antibodies (1 h at RT or overnight at 4ºC in 1% BSA/PBS), followed by washing (3 × 5 min in 0.1% Tween20-PBS (PBS-T) and incubation with the corresponding AlexaFluor-conjugated secondary antibody (1 h at RT in 1% BSA/PBS) and washed again as described above. Finally, cells were counterstained with DAPI in antifade mounting medium for fluorescence (Vectashield, Vector Labs, H-1200). Primary antibodies: anti-γH2AX (Millipore, 05-636) 1:1000, anti-γH2AX (abcam, ab22551) 1:1000, anti-53BP1 (Novus Biologicals, NB100-904) 1:2500, anti-53BP1 (Millipore, MAB3802) 1:500, anti-Emerin (abcam, ab156871) 1:200, anti-GFP (abcam, ab290) 1:1000, anti-Lamin A (abcam, ab26300) 1:2000, anti-Lamin B1 (abcam, ab16048) 1:2000, anti-Lamin B1 (Sangon Biotech, D194916-0100) 1:200, anti-LAP2 (ThermoFisher Scientific, MA1-25323) 1:200, anti-LBR (abcam, ab32535) 1:200, anti-Nup153 (abcam, ab24700) 1:500, anti-SUN1 (Novus Biologicals, NBP1-87396) 1:500, anti-SUN2 (abcam, ab124916) 1:500. Secondary antibodies: Alexa Fluor 488-donkey anti-rabbit IgG (H+L), Alexa Fluor 546-donkey anti-rabbit IgG (H+L), Alexa Fluor 546-goat anti-mouse IgG (H+L), Alexa Fluor 568-donkey anti-rabbit IgG (H+L), Alexa Fluor 568-donkey anti-mouse IgG (H+L), Alexa Fluor 647-donkey anti-goat IgG (H+L), Alexa Fluor 647-donkey anti-mouse IgG (H+L), Alexa Fluor 647-goat anti-rabbit IgG (H+L) (ThermoFisher Scientific A21206, A10040, A11003, A10042, A10037, A21447, A31571, A21244, respectively, at 1:500 dilution). A compiled summary of all antibodies used in this study can be found in Supplementary Table 4.

53BP1 foci induced by Cas9

Cells with two 53BP1 foci were scored at various time points post-transfection. Values are shown as the percentage of cells with two 53BP1 foci. For each data point in each independent experiment, at least 200 cells were quantified. Scoring was performed manually under double-blind conditions.

Metaphase spreads

For metaphase spreads, cells were incubated with 0.2 μg/ml demecolcine (Sigma Aldrich, D1925) for 13 h and then harvested. Cells were collected using standard cytogenetic techniques, subject to hypotonic shock for 30 min at 37ºC in 0.03 M sodium citrate and fixed in 3:1 methanol:acetic acid solution. Fixed cells were dropped onto acetic acid-humidified slides before dehydration and FISH.

Fluorescence *in situ* hybridization (FISH)

Whole chromosome FISH was performed according to manufacturer's protocol (MetaSystems probes, Whole Chromosome Paint, D-0307-050-FI & D-0309-050-OR). Briefly, sample and probe were simultaneously denatured (2 min at 75ºC) and incubated for hybridization (overnight at 37ºC). Post-hybridization washes included 0.4X SSC (2 min at 72ºC), followed by 2X SSC, 0.05%

Tween-20 (30 seconds at RT). Slides were then counterstained with DAPI in antifade mounting medium for fluorescence. When indicated, IF of anti-Lamin B1 (abcam, ab16048) was performed followed by FISH according to manufacturer's protocol (Empire Genomics probes, RP11-460J13 BAC (LAD) & RP11-118N16 BAC (iLAD)). A compiled summary of all antibodies used in this study can be found in Supplementary Table 4.

Intensity measurements of proteins at the nuclear envelope

The intensity of nuclear envelope proteins under different experimental conditions was quantified using a custom macro plug-in designed for FIJI (ImageJ). Nuclei were first segmented based on a DAPI to generate masks for individual cells. To isolate the nuclear periphery, the masks were eroded (4 pixels total) to exclude the periphery of the nucleus. The intensity at the nuclear periphery was then calculated by subtracting the mean intensity of the internal (eroded) nuclear region from the total nuclear intensity for each cell. Intensities were quantified from at least 50 cells per replicate per condition.

Chromosomal breaks

Chromosomal breaks were quantified as chromosome 7 (containing the LAD-targeting site) broken per 100 metaphases. For each condition in each independent experiment, at least 50 metaphases were quantified. Scoring was performed manually under double-blind conditions.

Chromosomal translocations

Translocation frequencies were calculated as translocations per 100 metaphases in chromosomes 7 and 9, and plotted together. For each condition in each independent experiment, at least 150 metaphases were quantified. Scoring was performed manually under double-blind conditions.

Micronuclei/blebs

Micronuclei/blebs were analysed in HT1080 DpnI^{+} cells and RPE-1 cells previously seeded onto coverslips. For HT1080 DpnI^{+} cells, 10 μM ATM inhibitor (ATMi; KU-55933, Sigma Aldrich), or DMSO, was added for the last 30 min of the -Shield1/-4OHT or +Shield1 (0.5 μM)/+4OHT (300 nM)treatments. After treatment, the media was changed and Auxin (500 μg/ml) was added with 10 μM ATM or DMSO, and Shield1 (0.5 μM). A summary of all chemical treatments can be found in Supplementary Table 3. 24 h post-treatment, cells were fixed and subject to IF using anti-GFP (abcam, ab290) and anti-H3K9me2 (abcam, ab1220) antibodies, as described above (Supplementary Table 4). For RPE-1 cells, 10 μM ATMi (or DMSO) was added at the moment of transfection. 24 h post-transfection, cells were fixed and subject to IF-FISH as described above. For each condition, in each independent experiment, at least 500 cells were quantified. Scoring was performed manually under double-blind conditions.

PRR14 LAD tethering induction

Cells were seeded and transfected using Lipofectamine 2000 following manufacturers instructions. 24 h post-transfection cells were re-seeded onto glass coverslips. 6 h post reseeding, cells were treated with 0.5 μM Shield1 for 18 h. Following this, cells were treated with 200nM Ramapycin (553210, Sigma Aldrich) or DMSO control for 30 min, before addition of 300 nM 4OHT for 2 h. Cells were collected for 0, 4 and 8 h timepoints. For the 4 and 8 h samples, the cells were washed three times in PBS at 0h, and supplemented with media containing 500 μg/ml Auxin (I5148, Sigma Aldrich) +/- Rapamycin or DMSO control. For IF, cells were pre-extracted (30s on ice in 0.5% Triton X-100/PBS) and then fixed (10 min in 4% paraformaldehyde/PBS, on ice). An additional fixation with 4% paraformaldehyde/PBS at RT was then performed for 10 min. Coverslips were washed twice in PBS and then stored in PBS for future immunostaining alongside later timepoints. Cells were blocked for 1 h in 5% BSA/PBS and immunostaining conducted using primary antibodies against mCherry, 53BP1 and GFP (1 h at RT in 1% BSA/PBS). Antibody details are provided in Supplementary Table 4. Coverslips were then washed three times, for 5 min each, in PBS-T and incubated in the appropriate secondary antibody (1 h at RT in 1% BSA/PBS; Supplementary Table 4). Coverslips were washed again for three 5 min washed in PBS-T and counterstained with DAPI in antifade mounting medium for fluorescence (Vectashield, Vector Labs (H-1200)).

Microscopy

Confocal images were taken using a LSM880 AxioObserver confocal microscope (Zeiss) with a C Plan-Apochromat 63x.1.4 Oil DIC UV-VIS_IR M27 lens. Microscopy images for Cas9 expressing cells were taken using Apotome Axio Observer Z1/7 microscope (Zeiss) using a 100x/1.40 Oil DIC M27 lens. For IF-FISH to analyze if the LAD foci was attached to the lamina, Z-stacks were taken at 0.2μm intervals. These images were deconvolved using Huygens Professional 25.4 (Scientific Imaging B.V). Images were analyzed using Fiji imageJ.

Prior to live-cell microscopy, 250 nM JF646-SNAP-tag ligand (Janelia Materials) was added to the Cas9 RNP-transfected RPE-1 GFP-H2B RFP-NLS SNAP-MDC1, alongside 10μM ATMi KU-55933 (Sigma-Aldrich) or DMSO vehicle. Live cell imaging was then performed with the Visitron Spinning Disc Confocal System VisiScope 5-Elements in an environmental enclosure to maintain cell culture conditions (37 °C and humidified 5% CO_2). Images were collected for 24 h with a 20x/0.75 Air objective, at intervals of 10 min, with 2-by-2 tile stitching. Live cell imaging was analyzed with Fiji imageJ. 100 random mitoses were considered for each condition and scored based on whether one or both daughter cells present nuclear deformations, blebs, or MNi formation.

PCR and Sanger Sequencing for Tracking of Indels by Decomposition (TIDE)

Briefly, genomic DNA (gDNA) from cells transfected with respective gRNAs was extracted with DNeasy Blood and Tissue Kit (QIAGEN, 69504), following manufacturer's instructions. PCR of the target region was carried out using 100 ng of gDNA and PrimeSTAR Max DNA Polymerase (Takara, R045A) according to manufacturer's instructions with forward (Primer 7, Supplementary Table 2) and reverse primers (Primer 8, Supplementary Table 2). PCR conditions were: 1 min at 98°C once, then 10 seconds at 98°C, 10 seconds at 60°C, and 10 seconds at 72°C for 34 cycles, then 2 min at 72°C once. PCR products were inspected for quality and yield by

1.2% agarose gel electrophoresis in 1X TBE, and were purified by 0.9X ratio of AMPure XP Beads (Beckman Coulter, A63881), according to the manufacturer's protocol. Samples were sequenced by Eurofins Genomics TubeSeq Supreme, using primer 7 (Supplementary Table 2).

TIDE

Analysis of sequenced products were performed as described by[7], with TIDE version 5.0.0. Briefly, raw ab1 files were inspected visually for quality control. Next, using guide sequence (CATTGACGATCATCCCACTG, gRNA #1 Supplementary Table 2) and ab1 files from the uncut control and cut control samples, samples were deconvoluted with TIDE using a 300 bp (1-300 bp) alignment window, 150 bp (500-650 bp) decomposition window, and 20 bp indel size range. To calculate the proportion of repair events, indels detected with non-significant p-values ($p > 0.001$) were disregarded from analysis and all significant events were first summed. The proportion of NHEJ events was the summed incidence of indels between -4 and +2. Similarly, the proportion of MMEJ events was calculated as the summed incidence of deletions greater than 5bp. The incidence of '0' indels were ascribed as uncut events. Code used to analyse raw TIDE data and generate plots can be found at Zenodo [8].

APEX2 and Tandem mass tag (TMT) labelling

15-20 million cells per condition were treated with Shield1 (0.5 µM, Takara 632189) for 20 h prior to labelling. Apex labelling was performed similar to [9]. Briefly, cells were incubated with Biotin-Phenol (500 µM, Iris Biotech, LS-3500) for 30 min before addition of 1 mM H_2O_2 for 1 min. Cells were washed three times with quencher solution (10 mM Sodium Ascorbate, 5 mM TROLOX, 10 mM Sodium Azide), harvested by scraping on ice and pelleted. To isolate nuclei, pellets were washed in PBS, resuspended in Buffer A (10 mM HEPES/KOH pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and incubated for 10 min on ice, before centrifugation. Cell pellets were resuspended in Buffer A + 0.2% NP-40, 1 mM DTT, 1 mM PMSF, EDTA-free protease inhibitors (Roche, 11836170001). Cells were homogenised (Dounce homogeniser) and the suspension centrifuged. Nuclei pellets were resuspended in Buffer A + 150 mM NaCl, 1 mM DTT, centrifuged and incubated on ice with regular agitation by vortex. The suspension was centrifuged and the pellet retained. Nuclei were resuspended at RT in 4% SDS, 100 mM Tris pH 7.5, for 3 min. 350 µl SDS-free RIPA buffer (1 mM DTT, 1% NP40, 50 mM Tris pH7.5, 150 mM NaCl, 0.5% Na-Deoxycholate, 10 mM Sodium Ascorbate, 5 mM Sodium Butyrate, EDTA-free Protease Inhibitor Cocktail, Phosphatase Inhibitor (PhosStop, Roche, 4906845001)) was added for lysis, reducing SDS concentration to <1%. The suspension was centrifuged to extract chromatin and the supernatant (SN1) was retained. Chromatin pellets were resuspended in 300 µl SDS-free RIPA and sonicated (Qsonica, 70% Amp, 10 s on 20 s off, 6.5 min). Suspensions were centrifuged at maximum speed (12,000 RPM) for 20 min. Supernatants were collected (SN2) and any pellets resuspended and incubated for 20 min at RT in 20 µl of 8 M Urea. The urea concentration was gradually diluted to 0.4M using nuclease-free H_2O, and the suspension centrifuged, retaining supernatant (SN3). SN1, SN2 and SN3 were combined, and quantified (Bradford Plus protein assay reagent, Thermo Scientific, PI23238) to equalize protein loading for immunoprecipitation (IP). Dynabeads™ MyOne™ Streptavidin T1 (Thermo Fisher Scientific, 65601) were equilibrated in SDS-free RIPA, and equalized protein extract loaded for overnight incubation (at 4ºC, rotating), retaining 0.1% total extract for input immunoblot. Following incubation, beads were washed twice with SDS-free RIPA, once with 1 M KCl, once with

Na_2CO_3, once with 2 M Urea 10 mM Tris-HCl pH 7.5, and three times with 100 mM triethylammonium bicarbonate (TEAB, Thermo Fisher Scientific, 90114). Beads were then transferred to a new low protein binding vial (Thermo Fisher Scientific, 90410) and washed twice with 100 mM TEAB. All washes were conducted for 5 min at 4ºC, with rotation. Beads were centrifuged and resuspended in 100 µl TEAB + 5 mM TCEP (Sigma Aldrich, C4706-2G) + 10 mM Auxin (IAA; Merck, 8.04744) for 30 min at RT. 10 mM DTT was added to quench IAA, and beads incubated with 5 µg Trypsin (Promega, V5280) for 18 h at RT. Peptides were transferred to a new low protein binding vial and dried (SpeedVac). Peptides were resuspended in 15 µl TEAB and pH checked to be pH8. Peptides were incubated with 5 µl TMT (Thermo Fisher Scientific, A52047) at RT for 2 h. 0.5 µl of each sample was added to 100 µl 0.1% TFA (Thermo Fisher Scientific, 400003). A preliminary Mass spectrometry (see experimental details in next section) was conducted to ensure labelling efficiency, which was confirmed to be 99.70%. Once confirmed, samples were combined and dried (SpeedVac). Pellets were resuspended in 200 µl of 0.1% TFA. Peptides were fractionated using Pierce™ High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, 84868), according to manufacturers instructions for TMT labelled peptides. Samples were resuspended in 45 µl of 0.1% formic acid and 20 µl was injected into the mass spectrometer.

(TMT)-based mass spectrometry (MS)

LC-MS/MS analysis was performed using a Vanquish Neo UHPLC system coupled to an Orbitrap Exploris 480 mass spectrometer equipped with a FAIMS Pro Duo interface (Thermo Fisher Scientific), operated in data-dependent acquisition (DDA) mode.

In summary, samples were loaded onto a trap column (PepMap NEO C18 5µ, 300µm x 5mm, 100 A°) at a flow rate of 20 µl/min using 0.1% formic acid in H_2O and separated on an analytical column (Easy-Spray PepMap Neo 2 µm C18 75 µm X 500 mm, 100 A°, Thermo Scientific) with a flow rate of 350 nl/min with a linear gradient of 1 to 35% solvent B (80% AcN, 20% H_2O, 0.1% formic acid) over a 90 min gradient. Ion source conditions were maintained at 1.75kV positive voltage, 275°C ion transfer tube temperature and standard resolution with 3.8 L/min FAIMS carrier gas flow throughout the acquisition. Two compensation voltages (CV), -45 and – 65 V with 2 and 1 sec cycle time respectively for each CV were used with RF lens of 50% in both cases. Mass spectrometric data was acquired using a DDA MS2 fragmentation, with RunStart internal EasyIC, i.e. fluoranthene (*m/z* 202.0777), as lock mass. Monoisotopic Precursor Selection MIPS (peptide mode), intensity (1.0E4), precursor fit (50% and 0.7 amu fit window), charge state (2-7) and dynamic exclusion (30 sec) mass filters were used. Details are as follows: MS^1 Orbitrap resolution was set at 120,000 (*m/z* 200) over a mass range of *m/z* 375 to 1550 using a normalized automatic gain control (AGC) target of 300% and maximum injection time (maxIT) of 20 ms; MS^2 Orbitrap resolution was set at 60,000 (*m/z* 200) with the first mass set at *m/z* 110, an isolation window of 0.7 amu was used, with AGC was set at 200% with 105 msec IT, fragmentation was carried out using higher-energy collisional dissociation (HCD) with a normalized collision energy (NCE) of 34.

Data was analysed using Proteome Discoverer 3.0 (Thermo Fisher Scientific) with Sequest HT and Comet as the search engines. A modified *Homo Sapiens* (TaxID=9606) FASTA file (with Streptavidin and GFP-m^6a-Tracer-APEX2 peptide sequence), was used for database searching. For the Sequest HT engine precursor and fragment mass limits were fixed at 20 ppm and 0.02 Da accordingly and Trypsin protease was used to generate peptides. For database search, the

following dynamic modifications were used: oxidation +15.995 Da (M), Deamidated / +0.984 Da (N, Q), TMTpro, +304.207 Da (K), Carbamidomethyl, +57.021 Da (C) with a Dynamic Modifications (peptide terminus) N-Terminal Modification: TMTpro, +304.207 Da (N-Terminus. The protein FDR and the peptide spectrum match were both set to 0.01%. For the Comet engine the following variable (oxidation / +15.995 Da (M), Deamidated / +0.984 Da (N, Q)) and static (TMTpro / +304.207 Da (Any N-terminus), TMTpro / +304.207 Da (K) and Carbamidomethyl / +57.021 Da (C)) modifications were selected. FDR was set to the same value (0.01) for both search engines.

Proteomics Data Processing and Statistical Analysis

Raw protein abundances were first median-normalized within each sample (column-wise) to account for variation in protein loading. To reduce potential batch effects, $\log_2$ transformation and centring were performed within each biological group. Statistical analysis was conducted in *Perseus* (version 2.0.11.0,[10], where two-sample *t*-tests were applied to assess differential protein abundance between conditions. Proteins with $p < 0.05$ and absolute $\log_2$ fold change > 0.5 were considered significantly regulated and used as input for functional enrichment analysis using Fisher's exact test. Principal component analysis was performed in Python 3 using pandas and scikit-learn libraries. Expression values were standardized using StandardScaler from scikit-learn before dimensionality reduction with PCA. The data was visualised using matplotlib and seaborn libraries.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [11]partner repository with the dataset identifier PXD066954 and 10.6019/PXD066954

Western blot analysis

Cells were cultured and treated as indicated. Cells were lysed in 30 µl RIPA buffer supplemented with Protease and Phosphatase inhibitors. Protein concentration was determined using the BCA assay (Thermo Fisher Scientific, 23225). Equal volumes of protein were denatured using 4x Laemmli buffer (Thermo Scientific, J60015.AD) and heated at 95ºC for 10 min. Proteins were separated by gel electrophoresis on SDS-Page gels (Invitrogen, 4-12% NuPAGE Bis-Tris) in Tris/Glycine/SDS buffer at 80-140V, and then transferred onto Nitrocellulose membranes (0.45 µm, GE Healthcare, 10600062) for 90 min at 400 mA, using ice cold Tris/Glycine buffer + 10% methanol. Membranes were blocked for >30 min in 5% milk/PBS-T at RT. Membranes were incubated with primary antibodies p-ATM (Abcam, ab81292) 1:1000, γH2AX (Abcam, ab22551) 1:1000, GAPDH (Millipore, MAB374) 1:30000 (Supplementary Table 4), overnight at 4ºC with agitation. Three PBS-T washes were conducted prior to incubation for 1 h at RT with the appropriate secondary antibody: Goat Anti-Mouse IgG (H+L), HRP (Thermo Fisher Scientific, 31430) 1:5000 or Goat and-Rabbit IgG (H+L), HRP (Thermo Fisher Scientific, 31460) 1:5000 (Supplementary Table 4), diluted in 5% milk/PBS-T. Membranes were then washed three times, for 5 min each, in PBS-T. Western blots were developed using chemiluminescent reagent (Pierce ECL western blotting substrate, Thermo Fisher Scientific, 32109) and imaged using ImageQuant LAS 4000.

CUT&RUN

Cut & Run was carried out using reagents from the CUT&RUN Assay Kit (Cell signalling, 86652) unless specified otherwise. $1x10^5$ RPE-1 cells per input or reaction were collected 6 hr post-transfection with guide RNA and treatment with DMSO or 10 μM ATM inhibitor. Inputs were processed following the protocol of the CUT&RUN Assay Kit and sonication was carried out using a Qsonica Q800R2 instrument at 70% amplitude for 6 min with cycles of 10 seconds on and 20 seconds off, at 4°C. The remaining cells were processed following the protocol of the CUT&RUN Assay Kit. Briefly, cells were washed and bound to Concanavalin A magnetic beads. Then, incubation with primary antibodies (Supplementary Table 4) was then carried out at 4°C overnight, using rabbit IgG isotype control (1:20, Cell signalling 66362), mouse IgG1k isotype control (1:100, Invitrogen 14-4714-82), Lamin B1 (1:50, Santa Cruz sc56144), 53BP1 (1:50, Novis Bio NB100-904) and H3K9me3 (1:100, Abcam ab8898). Beads were then washed and pAG-MNase binding was carried out for 1 hr at 4°C. Beads were then washed twice more and MNase digestion was carried out by adding calcium chloride and incubating samples for 30 min at 4°C. To release digested DNA fragments into solution, beads were incubated with 1X Stop Buffer containing 50pg spike-in DNA per reaction for 10 min at 37°C. The solution containing DNA fragments was then collected and purified using QIAquick PCR Purification Kit (Qiagen), following the manufacturer's protocol. For analysis by qPCR, Luna qPCR master mix (NEB, M3003E) was used per the manufacturer's instructions, and the reactions were carried out in triplicate using an AriaMx Real-time system (Agilent). Primers used for qPCR are listed in Supplementary Table 2. The data was then analysed firstly by subtracting the average Ct of each antibody condition from the average Ct of the corresponding input to calculate the ΔCt. The % enrichment compared to input was then calculated with $100 \times 2^{\Delta Ct}$. To account for non-specific signals, the % of input values for the corresponding isotype controls were subtracted.

Statistical analysis

Statistical analysis is included in figure legends. In all cases, comparison tests were performed using GraphPad Prism version 10.5.0 for macOS.

Supplementary tables

Supplementary Table 1: Experimental systems/cell lines used in this study

Cell line	Construct	Reference	Source/lineage
HT1080 Shield1-inducible Dam-Lamin B1 clone11 (Clone 11)	LaminB1-Dam	[4]	Kindly provided by B. van Steensel
DpnI$^-$	LaminB1-Dam-DD-Shield1, APEX2-m^{6}A-Tracer-GFP	[12,13, 4]	Derived from clone11. This study. (A HT1080 cell line)
DpnI$^+$	LaminB1-Dam-DD-Shield1, APEX2-m^{6}A-Tracer-GFP, ER-Dpn1-AID	[12,13,4,14]	This study. (A HT1080 cell line)
hTERT RPE-1 Cas9	RPE-1-hTERT Cas9	[15]	Kindly provided by D. Durocher
RPE-1 GFP-H2B RFP-NLS SNAP-MDC1	GFP-H2B RFP–NLS SNAP–MDC1	[1]	Stamatis Papathanasiou Laboratory

Supplementary Table 2: Oligonucleotides used in this study

Oligo name	Sequence	Application
Primer 1	GGACAGCAGAGATCCAGTTTG GTTAGTACCGGGCCCGAAGAC AAATCCCTGTGGAATGTG	PSA13-AID-ER*-DPN1 Fwd primer targeting AID-ER*-DPN1/TIR1 in PSA05-AID-ER*-DPN
Primer 2	GGAAAAGCGCCTCCCCTACCC GGTAGAATTGGATCCGAAGCT CATAGGATTTTAAC	PSA13-AID-ER*-DPN1 Rv primer targeting AID-ER*-DPN1/TIR1 in PSA05-AID-ER*-DPN

Primer 3	CCACTGGACCGTGAGCAAGGG CGAGGAGGATAA	Fwd primer targeting mCherry
Primer 4	CAGAGGATTCCCCCTGTGTAC	Rv primer targeting mCherry
Primer 5	GATCCGCTAGCGCTACCGGT	PRR14 gBloc fwd fusion PCR
Primer 6	CTCGCCCTTGCTCACGGTC	PRR14 gBlo rv fusion PCR
crRNA #1	IDT, cat number 1079138	Alt-R™ Cas9 Negative Control crRNA #1
tracrRNA #1	IDT, cat number 1073191	tracrRNA
Primer 7	GTCCTTGGTTGCAGTGACAA	TIDE PCR/Sanger sequencing fwd primer
Primer 8	TTCCAGCTACACATCAAACTCT	TIDE PCR rv primer
Chr7 LAD Fw	AAACCTGCCAGTCCTGTTGG	CUT&RUN-qPCR
Chr7 LAD Rv	AGACGCCCTTTCCCCAGTTA	CUT&RUN-qPCR
Chr9 iLAD Fw	TGGCAAACTTTTGGCTGAAC	CUT&RUN-qPCR
Chr9 iLAD Rv	TCCTAAGGCTGGAGAGAAAC	CUT&RUN-qPCR
gRNA #1	CATTGACGATCATCCCACTG	Chr7 sgLAD
gRNA #2	AGGTCAGCCCACCCCCAGTT	Chr9 sgiLAD
TrueGuide gRNA	C*A*T*TGACGATCATCCCACTG	Synthetic guide RNA including 2' O-Methyl analogs and 3' phosphorothioate linkages (*)

Supplementary Table 3: Chemicals used in this study

Chemicals	Concentration	Manufacturer	Cat no
Shield1	0.5 μM	Takara	632189
(Z)-4-Hydroxytamoxifen (4OHT)	300 nM	Sigma Aldrich	H7904
IAA	10 mM	Merck	8.04744
KU-55933 (ATMi)	10μM	Sigma Aldrich	Sml1109
G418	25 μg/ml	Sigma Aldrich	A1720-5G
Puromycin	0.5 μg/ml	Sigma Aldrich	P9620
Ramapycin	200 nM	Sigma Aldrich	553210
NU7441(KU-5778) (DNAPKi)	10 μM	Selleckchem	S2638
ART558 (PolQi)	10 μM	Artios	gift
B02(RAD51i)	20 μM	Sigma Aldrich	SML0364
Demecolcine	0.2 μg/ml	Sigma Aldrich	D1925

Supplementary Table 4: Antibodies used in this study with their respective applications and dilutions

Antibody	Dilution	Manufacturer	Cat no	Application
anti-γH2AX	1:1000	Millipore	05-636	Immunofluorescence
anti-γH2AX	1:1000	Abcam	ab22551	Immunofluorescence and immunoblotting
anti-53BP1	1:2500	Novus Biologicals	NB100-904	Immunofluorescence
anti-53BP1	1:1000	Novus Biologicals	NB100-904	PRR14 immunostaining
anti-53BP1	1:50	Novus Biologicals	NB100-904	CUT&RUN
anti-53BP1	1:500	Millipore	MAB3802	Immunofluorescence
anti-Emerin	1:200	Abcam	ab156871	Immunofluorescence
anti-GFP	1:1000	Abcam	ab290	Immunofluorescence

anti-GFP	1:500	Novus Biologicals	NB100-1770	PRR14 immunostaining
anti-mCherry	1:2000	Abcam	ab167453	PRR14 immunostaining
anti-Lamin B1	1:2000	Abcam	ab26300	Immunofluorescence
anti-Lamin B1	1:2000	Abcam	ab16048	Immunofluorescence
anti-Lamin B1	1:50	Santa Cruz	sc56144	CUT&RUN
anti-LAP2	1:200	ThermoFisher Scientific	MA1-25323	Immunofluorescence
anti-LBR	1:200	Abcam	ab32535	Immunofluorescence
anti-Nup153	1:500	Abcam	ab24700	Immunofluorescence
anti-SUN1	1:500	Novus Biologicals	NBP1-87396	Immunofluorescence
anti-SUN2	1:500	Abcam	ab124916	Immunofluorescence
anti-p-ATM	1:1000	Abcam	ab81292	immunoblotting
anti-GAPDH	1:30000	Millipore	MAB374	immunoblotting
anti-H3K9me2	1:200	Abcam	ab1220	MNi Immunofluorescence
anti-H3K9me3	1:100	Abcam	ab8898	CUT&RUN
Alexa Fluor 488-donkey anti-rabbit IgG (H+L)	1:500	ThermoFisher Scientific	A21206	Immunofluorescence
Alexa Fluor 546-donkey anti-rabbit IgG (H+L)	1:500	ThermoFisher Scientific	A10040	Immunofluorescence
Alexa Fluor 546-goat anti-mouse IgG (H+L)	1:500	ThermoFisher Scientific	A11003	Immunofluorescence
Alexa Fluor 568-donkey	1:500	ThermoFisher Scientific	A10042	Immunofluorescence

anti-rabbit IgG (H+L)				
Alexa Fluor 568-donkey anti-mouse IgG (H+L)	1:500	ThermoFisher Scientific	A10037	Immunofluorescence
Alexa Fluor 568-donkey anti-mouse IgG (H+L)	1:250	ThermoFisher Scientific	A10037	PRR14 immunostaining
Alexa Fluor 647-donkey anti-goat IgG (H+L)	1:500	ThermoFisher Scientific	A21447	Immunofluorescence
Alexa Fluor 647-donkey anti-mouse IgG (H+L)	1:500	ThermoFisher Scientific	A31571	Immunofluorescence
Alexa Fluor 647-goat anti-rabbit IgG (H+L)	1:500	ThermoFisher Scientific	A21244	Immunofluorescence
Alexa Fluor 647-goat anti-rabbit IgG (H&L)	1:250	ThermoFisher Scientific	A21244	PRR14 immunostaining
Alexa Fluor 488-donkey anti-goat IgG (H+L)	1:250	ThermoFisher Scientific	A11055	PRR14 immunostaining
Goat Anti-Mouse IgG (H+L), HRP	1:5000	Thermo Fisher Scientific	31430	immunoblotting
Goat and-Rabbit IgG (H+L), HRP	1:5000	Thermo Fisher Scientific	31460	immunoblotting

Rabbit IgG isotype control	1:20	Cell signalling	66362	CUT&RUN
Mouse IgG1k isotype control	1:100	Invitrogen	14-4714-82	CUT&RUN

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