was washed three times with TBST, each for 10 min, followed by incubation with horseradish-peroxidase-conjugated or fluorophore-conjugated secondary antibodies (LI-COR) at RT for 1 h, in 5% (w/v) milk in TBST. The membrane was washed again three times with TBST, and incubated with Amersham ECL prime detection reagent (GE Healthcare) or imaged using LI-COR Odyssey (ImageStudio v5.2). The membrane was then exposed to X-ray film and the film developed in a darkroom.

For detection of Polθ, cells grown in 6-well plates were washed in PBS, lysed directly in Laemmli buffer (2% SDS, 10% Glycerol, 62.5 mM Tris-HCL pH 6.8) and boiled for 10 min. Samples were syringed five times through a 27G needle. Protein extracts were quantitated using the Bicinchoninic acid assay (ThermoFisher) against a BSA standard curve and made up in 4× NuPAGE LDS sample loading buffer (Invitrogen) supplemented with β-mercaptoethanol. lysates (40 μg) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on NuPAGE 3–8% Tris-Acetate gels (Invitrogen) in NuPAGE Tris-Acetate running buffer (Invitrogen) and wet-transferred in 1× NuPAGE Transfer Buffer (Invitrogen), 20% ethanol and 0.05% SDS to nitrocellulose membranes (Millipore). 5% (w/v) BSA/Tris-buffered saline + 0.01% (v/v) Tween-20 (TBST) was used for all blocking and incubation steps. Polθ protein was detected by probing the blot overnight at 4 °C with mouse monoclonal anti-Polθ antibody (1:5000, kind gift of Jean-Sébastien Hoffman, CRCT Toulouse) diluted in blocking buffer (5% BSA/TBST). As a loading control, levels of vinculin were determined by probing the membrane with mouse monoclonal anti-vinculin antibody (1:1000, SCBT sc-73614). The membrane was washed thrice for 5 min with TBST and incubated with HRP-conjugated goat anti-mouse IgG (Invitrogen 31430, 1:10,000) for 1 h at room temperature. After five 5 min washes with TBST, signals were detected with ECL detection reagent (GE Healthcare) and imaged on an Amersham Imager 600RGB.

All western blots were repeated independently at least two times with similar results.

For antibody details, see Supplementary Methods.

Immunofluorescence and image analysis. For nuclear γH2Ax and RPA foci quantification, cells were seeded in 96-well plates. Cells were fixed in 4% (v/v) paraformaldehyde (PFA) in PBS for 10 min at room temperature (RT), washed twice with PBS, and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min. After two additional washes, cells were blocked with 2% (w/v) BSA, 1% (v/v) FBS in PBS (IFF) for 1 h at RT. Cells were then incubated with primary antibodies in IFF at 4 °C overnight. The cells were then washed three times with PBS, each for 10 min, followed by incubation with Alexa Fluor 555-conjugated mouse and Alexa Fluor 488-conjugated rabbit secondary antibodies (Thermo Fisher Scientific), 1 μg/ml DAPI in IFF for 1 h at RT. Cells were then washed three times with PBS, and 100 μl PBS was finally added to each well prior to imaging. Plates were imaged using an Image Express high-content imaging system. Quantification of the number of γH2Ax foci and RPA foci (only PCNA-positive cells were used in the analysis) was performed under identical microscopy settings between samples, using the MetaExpress image analysis system (MolDev).

For nuclear pRPA foci quantification cells were seeded on 13 mm coverslips. Cells were fixed in 4% (v/v) paraformaldehyde (PFA) in PBS for 10 min at room temperature (RT), washed twice with PBS, and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min. After two additional washes, cells were blocked with 2% (w/v) BSA, 1% (v/v) FBS in PBS (IFF) for 1 h at RT. Cells were then incubated with primary antibodies in IFF at 4 °C overnight. The cells were then washed three times with PBS, each for 10 min, followed by incubation with Alexa Fluor 488–conjugated rabbit secondary antibodies (Thermo Fisher Scientific) in IFF for 1 h at RT. Cells were then washed three times with PBS, dried and mounted in Vectashield containing DAPI and imaged at ×60 on a Zeiss LSM 780.

Detection of incorporated BrdU in ssDNA by nondenaturing immuno-fluorescence staining. To measure levels of ssDNA using a nondenaturing BrdU IF staining procedure, cells cultured on coverslips were first incubated with BrdU (30 μM) for 24 h. As a control, cells were exposed to 2 mM hydroxyurea for 4 h prior to harvesting. Cells were then incubated with extraction buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 1.5 mM MgCl₂ and 0.5% (v/v) Triton X-100) for 2 min on ice. Subsequently, cells were fixed with 4% (v/v) paraformaldehyde in PBS at room temperature for 10 min. After washing with PBS, cells were blocked in 2% (w/v) BSA, 1% (v/v) FBS in PBS (IFF) 1 h at RT. Cells were then incubated for 2 h with anti-BrdU antibody diluted in IFF at room temperature. Subsequently, cells were washed three times with PBS containing 0.05% (v/v) Tween-20 before incubation with secondary antibody. After washing three times with PBS containing 0.05% (v/v) Tween-20, cells were mounted in Vectashield containing DAPI and imaged at ×63 on a Zeiss LSM 780 confocal microscope. The BrdU signal in individual nuclei (defined by the DAPI-stained area) was determined using ImageJ. Images of randomly selected cells for each sample were analysed.

Mitotic spreads. Following exposure to the indicated treatment, cells were incubated with 0.5% (w/v) colchicine for 4 h. Cells were harvested, washed in PBS and incubated in 0.56% (w/v) KCl at 37 °C for 15 min. Samples were then fixed (3:1 methanol:acetic acid). Cell solutions were dropped onto clean coverslips and mounted in Vectashield containing DAPI and mitotic spreads imaged at ×60 on a Zeiss LSM 780 confocal microscope.

Measurement of resection. ER-AsiSI U2OS cells were reverse-transfected with the mentioned siRNAs and after 24 h exposed to 10μM ART558 or DMSO for an additional 48 h. Cells were trypsinized, centrifuged and resuspended with 37 °C 0.6% low-gelling point agarose (BD Biosciences) in PBS (Gibco) at a concentration of $6 × 10^6$ cells/ml. A 50-μl cell suspension was dropped on a piece of Parafilm (Pechiney) to generate a solidified agar ball, which was then transferred to a 1.5-ml Eppendorf tube. The agar ball was treated with 1 ml of ESP buffer (0.5 M EDTA, 2% N-lauroylsarcosine, 1 mg/ml proteinase-K, 1 mM CaCl₂, pH 8.0) for 20 h at 16 °C while shaking, followed by treatment with 1 ml of HS buffer (1.85 M NaCl, 0.15 M KCl, 5 mM MgCl₂, 2 mM EDTA, 4 mM Tris, 0.5% Triton X-100, pH 7.5) for 20 h at 16 °C while shaking. After washing with 1 ml of PBS for 5 × 1 h at 4 °C with rotation, the agar ball was melted by placing the tube in a 70 °C heat block for 10 min. The melted sample was diluted 7-fold with 70 °C ddH₂O, mixed with equal volume of appropriate 2× NEB restriction enzyme buffer and stored at 4 °C for future use.

The level of resection adjacent to specific DSBs was measured by quantitative polymerase chain reaction (qPCR) using a modification of the method[43]. The sequences of qPCR primers are shown in Supplementary Table 3. Twenty μL of genomic DNA sample (~140 ng in 1× NEB restriction enzyme buffer 4) was digested or mock digested with 20 units of restriction enzymes (BsrGI, or HindIII-HF; New England Biolabs) at 37 °C overnight. Two μL of digested or mock-digested samples (~20 ng) were used as templates in 20 μL of qPCR reaction containing 10 μl of 2× Sybr Green PCR Master Mix (Thermo), 0.5 μM of each primer on an Applied Biosystems® QuantStudio™ 6 Flex. The percentage of ssDNA (ssDNA%) generated by resection at selected sites was determined. Briefly, for each sample, a ΔCt was calculated by subtracting the Ct value of the mock-digested sample from the Ct value of the digested sample. The ssDNA% was calculated with the following equation: '% digested-resistant' = $1/(2^{(\Delta Ct-1)} + 0.5)*100$.

Statistics and reproducibility. Numbers of independent replicates are included in each figure legend as are details of numbers of events counted.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data and materials used in the analysis are provided within the manuscript. Source data are provided with this paper.

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