Supplemental Materials and Methods

Cell lines

For generation of ZPET KO cell lines, U2OS cells were transfected with px458 derivative plasmids encoding gRNAs for ZNF280C using X-tremegene9 purchased from Promega. The transfection was done with pooled guides. The following guide sequences were selected from (http://crispr.mit.edu), reduced to 18-bp, and cloned into the px458 plasmid (Fu et al. 2014). One day following transfection, cells were transfected again and incubated for an additional 24 hr. The cells were then FACS sorted to isolate GFP+ and GPP- cells. Cells were plated in a 96-well plate at <1 cell per well and incubated until confluent. ZPET KO was confirmed by western blot.

Guide 1 CGATGAACTGATCTTTGT
Guide 2 CAAGATGAAATCTAGGCG
Guide 3 TACTGTTTGACTCGACCC
Guide 4 TGCAGTCCTGAATATTTC
Guide 5 GAGTGAGCCACACAGTCC

For the generation of reconstituted ZPET KO cell lines, Wild-type ZPET was HA tagged at both the N and C terminus by PCR amplifying with primers containing the HA sequence. The PCR product was then introduced into the pENTR D-TOPO vector (ThermoFisher) using the directional TOPO cloning kit (K240020 ThermoFisher). The sequences were validated and then shuttled into pInducer20, a Dox-inducible lentiviral vector, using the Gateway LR Clonase II Enzyme Mix (ThermoFisher 11791-020). Lentivirus particles were produced by co-transfecting 293T cells with the pInducer20 vector encoding ZPET, psPax2, and VSV-G using Lipofectamine 3000 (ThermoFisher). Viral particles were harvested and used to transduce either Wild-type U2OS of ZPET KO U2OS cells. Cells were selected with 600µg/ml G418 for 24 hr following transfection. ZPET expression was validated by western blot.

HR assay

One day prior to transfection, the indicated U2OS or derivative cells ($3.5x10^5$ cells) were plated on coverslips. On the following day, medium was replaced with antibiotic-free DMEM containing L-glutamine and 10% FBS. Cells were co-transfected with pX459-sgLMNA and Puc19-mClovr-LMNA using Fugene6. One day after transfection, medium was replaced with complete DMEM containing 1 μg/ml puromycin. After a 3-day selection, cells were washed 3 times with 2 ml of cold PBS followed by fixation with 3% PFA at room temperature for 20 min. Cells were then extracted for 5 min at room temperature in 0.25% Triton X-100 + 1 μg/ml DAPI. Finally, slides were rinsed 2 times with 1 ml of PBS-0.1% Tween-20 and then with water prior to mounting with VectaShield.

RNA interference

Silencer Select siRNAs were purchased from Invitrogen (5-20 nM) and transfected with Lipofectamine RNAiMAX from Invitrogen.

siZNF280C #1 (Silencer Select): GGCAGAACUCUUUAUGGAAtt
siZNF280C #2 (Silencer Select): GCUGGAGCAGAUUACCUAAtt
siZNF280C #3 (Silencer Select): CAAGUACAAUACUAACUGUtt
siHELB (Silencer Select): GGGUAAAGGAGGUAUCAAAtt
siCtIP (Silencer Select): GGGUCUGAAGUGAACAAGAtt
siBRCA1 (Silencer Select): CAGCUACCCUUCCAUCAUAtt

Cell viability assay

Cells (400 cells per well of a 96-well plate) were plated one day prior to treatment with DNA damaging agents. Cells were incubated for 6-7 days and then cell viability was determined using Cell Titer Glo from Promega. For the viability assays of BRCA1 knockdown and control cells, cells were plated one day prior to siRNA transfection. On the following day, cells were transfected and incubated for 24 hr.

The cells were plated in a 96-well plate and incubated for an additional 24 hr. The cells were then treated with Olaparib and incubated for 5 days.

Immunofluorescence

Cells expressing BirA^{R118G}-RAD18, BirA^{R118G}-GFP, or HA-ZPET were plated one day prior to analysis. For microirradiation with UV laser, cells were cultured in 10 uM BrdU for 24 hr. Cells were irradiated with a 355 nM UV laser or 10 Gy of IR on the next day. After 1hr, 0.5-1ug/ml exogenous biotin was added to the appropriate samples and incubated for 4 hr. The cells were then washed with PBS, pre-extracted for 5 min at 4 ºC, fixed for 20 min with 3% PFA sucrose, and then fixed with 100% methanol for 5 min at -20 ºC. The cells were then blocked in 3% BSA 0.1% Tween-20 for 1 hr at room temperature. Slides were incubated with primary antibody for 2 hr at room temperature followed by washing steps in 0.1% Tween-20. The cells were then incubated with secondary antibodies for 1 hr at room temperature followed by washing in 0.1% Tween-20 PBS. The cells were then incubated with 1 μg/ml DAPI in 0.1% Tween-20 PBS for 10 min followed by rinses and mounting using VectaShield. For immunofluorescence analysis of RPA phosphorylation, blocking buffer of 10% milk, 2% BSA, and 0.1% Tween-20 was used. Cells were imaged on a Nikon 90i microscope.

Proximity ligation assay

Expression of HA-tagged ZPET was induced by 500 ng/ml doxycycline in ZPET KO U2OS cells integrated with pInducer20-HA-ZPET. After 48 hours, cells were exposed to 10 Gy of IR, and further cultured for 1 hour. After 1 wash with PBS, cells were treated with Triton X-100 pre-extraction buffer (0.2% Triton X-100, 20 mM HEPES-KOH pH 7.9, 100 mM NaCl, 3 mM MgCl2, 300 mM sucrose, 1 mM EGTA) for 3 minutes on ice. Cells were fixed with PFA for 15 minutes on ice, and then with ice-cold methanol for 10 minutes at -20°C. Subsequently, cells were permeabilized with 1x PBS +0.5% Triton-x100 for 4 minutes and blocked with 3% BSA in PBST buffer (1xPBS with 0.05% Tween) for

1 hour at RT. Cells were washed with 1x PBS between each step. Next, cells were incubated with primary antibody overnight at 4°C (1:1000 HA mouse antibody alone; 1:1000 γ-H2AX rabbit antibody alone; or 1:1000 HA antibody with 1:1000 γ-H2AX antibody). After 3 washes with 1x PBST, cells were incubated in a pre-mixed solution of PLA probe anti-mouse minus and PLA probe anti-rabbit plus (Sigma) for 1 hour at 37˚C. The PLA reaction was performed with the Duolink In Situ Detection Reagents (Red) based on the manufacturer's instructions. After a final wash with buffer B (from PLA kit), cells were blocked with 3% BSA in PBST buffer (1xPBS with 0.05% Tween) again, and incubated with secondary antibody (conjugated with fluorescence 488) for 1h at RT. After 3 washes, including the DAPI staining during the second wash, slides were mounted with Prolong Gold and sealed with nail polish. Images were captured on Nikon 90i microscope at 20x and 60x. The number of PLA foci was quantified using Image J.

Imaging in the U2OS TRE/I-SceI cell line

pBROAD3/TA-KR, pCMV-NLS-I-SceI and the U2OS TRE/I-SceI cell line have been previously described (Wei et al. 2015). U2OS TRE/I-SceI cells were cultured in 35 mm glass-bottom dishes (MatTek, P35GC-1.5-14-C) to 60% confluence 24-36 h before the transfection. Cells were transfected with pCMV-I-SceI plasmid and incubated for 24 h before staining. γ-H2AX immunofluorescence staining was performed as previously described (Teng et al. 2018). Briefly, cells in a 35 mm dish were fixed in 4% paraformaldehyde (PFA; Affymetrix, 19943 1 LT) for 15 min at room temperature. They were permeabilized with 0.2% Triton X-100 in PBS for 10 min after washing three times with PBS. Then they were blocked with 5% bovine serum albumin (BSA) (SIGMA, A-7030) in PBS for 1 hour at room temperature. Primary antibodies (γ-H2AX ser139: JBW301, 05–636, EMD Millipore, 1:200) were diluted in blocking buffer and incubated with cells at 4 °C overnight. Then the cells were washed three times with 0.05% PBST and incubated with secondary antibodies (Fluor 405 anti-mouse IgG

conjugate 1: 400) for 1 h at room temperature. Fluorescence signals of GFP and Cherry fusion proteins were directly analyzed. The images were acquired using the Olympus FV1000 confocal microscopy system (Olympus) and FV1000 software.

Cell cycle analysis

Cells were incubated with 10 μM EdU for 30 min at 37 ºC. Cells were harvested and fixed in 100% ethanol. Cells were resuspended in PBS containing 1% BSA, Saponin and Click-iT EdU buffer and incubated for 30 min at room temperature. Cells were then washed with PBS containing 0.1 mM EDTA, 1% BSA, and 0.25% Tween-20. Cells were spun down and subsequently resuspended in 300-500 ul PI staining buffer (PBS, 0.1mM EDTA, 1% BSA, 0.25% Tween-20, 10 μg/ml Propidium Iodide, 50 μg/ml RNaseA), and incubated at 37 ºC for 20 min. Samples were analyzed with a BD™ LSRII flow cytometer.

Chromatin fractionation

U2OS cells (4.8 x 10^6) were plated in 15-cm Tissue Culture dish one day prior to harvesting. The following day cells were harvested with trypsin and lysed in 0.5 ml of Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34M sucrose, 0.1% Triton X-100, 10% glycerol) supplemented with 1mM DTT, complete protease inhibitors (Sigma), and phosphatase inhibitor cocktail 2 (Sigma) for 5 min on ice followed by centrifugation at 1,300x g for 5 minutes at 4 ºC. The supernatant was saved as the cytoplasmic fraction and the pellet washed once in Buffer A. The pellet was then lysed in 0.5 ml Buffer B (3 mM EDTA, 0.2 mM EGTA) supplemented with 1mM DTT, complete protease inhibitors (Sigma), and phosphatase inhibitor cocktail 2 (Sigma) for 40 minutes on ice. Lysates were centrifuged at 1,700 xg 4 ºC for 5 min. The supernatant was saved as the nuclear soluble fraction, and the pellet was washed once with Buffer B. The pellet was then lysed in 0.2 ml Buffer C (10 mM

HEPES pH 7.9, 100 mM NaCl, 2 mM MgCl2, 0.5% NP-40) supplemented with complete protease inhibitor cocktail, and phosphatase inhibitor cocktail 2 and 0.15 unit/µl Benzonase. The lysates were incubated overnight at 4 ^{o}C with rotation. Salt concentration was then increased by 200 mM and lysates were incubated for an additional 2 hours at 4 ^{o}C with rotation. Lysates were centrifuged at 13,200 RPM at 4 ^{o}C for 15 minutes and the supernatant was saved as the chromatin fraction. For the ssDNA pulldowns in Fig. 3A, 3C, 6B, S3C, chromatin and soluble fractions were combined. In Fig. 5D and S3A, cell fractionation was conducted using the Subcellular Protein Fractionation Kit for Cultured Cells (78840) from ThermoFisher. The buffers were supplemented with phosphatase inhibitor cocktail 2 (Sigma).

Chromatin immunoprecipitation

ChIP was carried out as previously described (Ouyang et al., 2009) with some modifications. In brief, $8x10^{6}$ U2OS-DiVA-GFP-ZPET cells (with or without 300nM 4OHT treatment) were used for anti-GFP or control IgG ChIP. After cross-linking with 1% formaldehyde for 10 minutes at RT and quenched with 0.125 M glycine for 5 minutes at RT, cells were washed with cold PBS and collected by scraping in ChIP Collection Buffer (5mM HEPES pH8.0, 85mM KCl, 0.5% NP-40, 1x EDTA-free protease inhibitor cocktail). Cells were re-suspended in ChIP Lysis Buffer (50 mM Tris pH8.0, 10 mM EDTA, 1% SDS) supplemented with Protease Inhibitor Cocktail and subjected to sonication to shear chromatin DNA an average 200-600bp using Q800R2 sonicator (Qsonica). After centrifugation, the supernatant was diluted 10 times with ChIP Dilution Buffer (16.7 mM Tris pH8.0, 250 mM NaCl, 0.01% SDS, 1% Triton X-100), pre-cleared with BSA-pretreated Protein A Dynabeads (Invitrogen) and subjected to immunoprecipitation using 5 µg antibody. The immunocomplex was captured by BSA-pretreated Protein A Dynabeads and washed with ChIP Dilution Buffer, LiCl Wash Buffer (100mM Tris pH 7.5, 500mM LiCl, 1% NP-40, 1% SDC) and TE Buffer (10mM Tris, pH 8.0, 1mM

EDTA). The bound immunocomplex was eluted with 150 μL Elution Buffer (1% SDS, 0.1M NaHCO3) twice at 65°C. After adding NaCl to eluted immunocomplex to a final concentration of 300 mM, the cross-link was reversed at 65°C overnight. The de-crosslinked samples were then subjected to DNA isolation using QIAquick PCR purification kit (QIAGEN) and the DNA was eluted with Elution Buffer (10mM Tris pH8.5). Quantitative PCRs were performed using primers listed below (Iacovoni et al. 2010; Aymard et al. 2014).

DSB-1: Fwd: CCTTCTTTCCCAGTGGTTCA; Rev: GTGGTCTGACCCAGAGTGGT
DSB-2: Fwd: GGAGAAGTGGCAGGACAATG; Rev: CAAGGCAAATTTGGGGACTA
DSB-3: Fwd: TATGGGACCAAGCGAGTAGG; Rev: GCCTCACACACACACCCATA
3.7Kb from DSB-1: Fwd: CCTTCTTTCCCAGTGGTTCA; Rev: GTGGTCTGACCCAG-AGTGGT
2Mb from DSB-1: Fwd: CCCATCTCAACCTCCACACT; Rev: CTTGTCCAGATTCG-CTGTGA
275Kb from DSB-4: Fwd: CAGCAAGTGGGAAGGTGTAATCC; Rev: CCCATTCTATCATC-AACGGGTACAA

iPOND

iPOND was essentially performed as described previously with minor modifications (Sirbu et al. 2011). One hundred million HEK293T cells were labeled with 10 μM EdU for 10 or 15 min and treated as indicated in the figures. For the pulse-chase condition, cells were washed with pre-equilibrated cell culture medium and incubated as indicated in the figures with medium containing 10 μM thymidine. Cells were crosslinked with 1 % formaldehyde for 20 min, quenched with 0.125 M glycine and washed three times with PBS. Cells were then permeabilized with 0.25% Triton X-100 in PBS for 30 min at room temperature and washed twice with PBS. For the conjugation of EdU with biotin azide, cells were incubated in click reaction buffer (20 μM biotin azide, 10 mM sodium ascorbate and 2 mM $CuSO_4$ in PBS) for 2 hr at room temperature. Cells were washed twice with PBS, and resuspended in

lysis buffer (50 mM Tris-HCl, pH 8, and 1% SDS) supplemented with protease inhibitors. Chromatin was solubilized by sonication using a microtip sonicator 550 Sonic Dismembrator (Fisher Scientific) for 1 min 30 sec at 4°C followed by centrifugation at 13,000 rpm for 10 min. Supernatants were diluted with 1:1 PBS (vol/vol) containing protease inhibitors and incubated overnight with streptavidin-agarose beads. Beads were washed once with lysis buffer, once with low salt buffer (1% Triton, 20 mM Tris pH8, 2 mM EDTA, 150 mM NaCl), once with high salt buffer (1% Triton, 20 mM Tris pH8, 2 mM EDTA, 500 mM NaCl), and once with lysis buffer. Captured proteins were eluted by boiling beads for 45 min in SDS sample buffer and resolved by electrophoresis using NuPAGE Novex 4-12% Bis-Tris gels. Standard immunoblotting procedures with the indicated antibodies were used to detect proteins.

DNA fiber assay

U2OS cells were first pulse labeled with 100 μM CldU for 30 min, and then washed twice with equilibrated PBS. The second pulse labeling was performed with 250 μM IdU under the conditions specified in the figure. Collected cells were resuspended in cold PBS ($1x10^6$ cell per milliliter), and 2.5 μL was stretched on glass slide after mixing with spreading buffer (7.5 μL) (0.5% SDS, 200 mM Tris-HCl pH 7.4, 50 mM EDTA). DNA fibers were fixed in methanol:acetic acid (3:1), denatured in 2.5 N HCl for 30 min, and blocked in 3% BSA/0.05% Tween-20 for 60 min at room temperature. CldU and IdU detection were done using rat anti-BrdU (1:100; Novus NB500-169) and mouse anti-BrdU (1:20; BD Biosciences) for 60 min at 37°C followed by Alexa-488 anti-mouse (1:100) and Cy3 anti-rat (1:100; Jackson ImmunoResearch) for 30 min at room temperature. Slides were mounted with Prolong Gold and dried overnight. Fibers were imaged with a 60X objective on a Nikon 90i microscope and quantified using ImageJ software.

HA-ZPET purification

U2OS cells harboring inducible HA-ZPET were used for protein purification. One gram of Dox induced cell mass was lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 500 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.1 % NP40, 100 μg/ml PMSF, 20 % Sucrose and protease inhibitor cocktail] with homogenizer and sonication. Lysed cells were centrifuged at 16,000 RPM for 1 hour and supernatant was loaded on anti-HA agarose beads and incubated for 2 hours. Beads were washed with buffer K [20 mM KH_2PO_4 (pH 7.5), 0.5 mM EDTA, 1 mM DTT and 10 % glycerol] containing 500 mM KCl. Beads were further washed with buffer K with 1 M KCl and buffer K with 200 mM KCl. HA-ZPET protein was eluted by buffer K with 200 mM KCl and 1 mg/ml HA peptides. Eluted fraction was dialyzed against buffer T [25 mM Tris-HCL (pH 7.5), 0.5 mM EDTA, 1 mM DTT and 10 % glycerol] with 300 mM KCl, concentrated and then stored at -80 ^{0}C.

Electro Mobility Shift Assay (EMSA)

Increasing concentrations of HA-ZPET were incubated with 5' end fluorophore-labeled ssDNA or dsDNA in buffer [35 mM Tris-HCL (pH 7.5), 1 mM DTT, 10 mM EDTA, 50 μg/ml BSA and 50 mM KCl] at 37 ^{0}C for 15 minutes. DNA-protein complexes were separated using 6% acrylamide gel and imaged using Odyssey scanner (LI-COR Biosciences).

ssDNA and dsDNA pull-down

M-280 streptavidin magnetic beads were washed and resusupended in DNA binding buffer (10 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol, 0.01% NP-40, and 10 μg/ml BSA). Beads were incubated in the absence or presence of biotinylated ssDNA or dsDNA (100 pmole per 100 μl of beads) for 30 min at room temperature. Beads were then washed with binding buffer and mixed with nuclear extracts comprised of both the nuclear soluble and chromatin fractions for Fig. 3A, 3C, 6B, S3C or chromatin

fraction alone for Fig. S3A. The NaCl concentration of the combined extracts for the binding assays was 100 mM, except 150 mM was used in Fig. 3C. The pull-downs were incubated for 30 min at room temperature with rotation. Beads were washed 3 times with 1 ml of binding buffer for each sample and then boiled for 5 min in 1X sample buffer.

References

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