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Supplemental Information

UBC13-Mediated Ubiquitin Signaling Promotes Removal of Blocking Adducts from DNA Double-Strand Breaks

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SUPPLEMENTAL INFORMATION

TRANSPARENT METHODS

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Figure S1 (related to Figure 1), UBC13 contributes to DSB repair by recruiting BRCA1 and RAP80 onto DSB sites during the G_1 phase.

Figure S2 (related to Figure 2), RAP80 and BRCA1 contribute to DSB repair during the G_1 phase.

Figure S3 (related to Figure 3), UBC13, RAP80, and BRCA1 promote the removal of etoposide-induced TOP2 adducts independent of TDP2.

Figure S4 (related to Figure 4), K63 ubiquitin signaling involving UBC13, RAP80, and BRCA1 is required for efficient recruitment of MRE11 nuclease onto DSB sites in G_1 cells.

Figure S5 (related to Figure 5), Repair kinetics of *Asi*SI-induced DSBs and IR-induced DSBs in TK6 cells.

Figure S6 (related to Figure 5), Repair kinetics of IR-induced DSBs in *MRE11*$^{AID/AID}$ HCT116 cells.

Figure S7 (related to Figure 5), Disruption of the genes encoding *POLβ*, *POLθ*, *TDP1*, *PARP1*, *ARTEMIS*, *EXD2*, and *EXO1* in TK6 cells and radiosensitivity of the resultant mutants.

TRANSPARENT METHODS

Key resource table

Reagent or Resource	Source	Identifier
Antibodies		
Mouse monoclonal α-Multi Ubiquitin mAb (clone FK2)	MBL	Cat# D058-3
Rabbit polyclonal α-53BP1	Calbiochem	Cat# PC712-100UL
Mouse monoclonal α-γH2AX (clone JBW301)	Millipore	Cat# 05-636, RRID:AB_309864
Rabbit monoclonal α-γH2AX	Cell Signaling Technology	Cat# 9718, RRID:AB_2118009
Rabbit polyclonal α-CyclinA (clone C19)	Santa Cruz	Cat# sc-596, RRID:AB_631330
Rabbit polyclonal α-RAP80	Novus Biologicals	Cat# NBP1-87156
Rabbit polyclonal α-RAP80	Bethyl Laboratories	Cat# A300-763A
Mouse monoclonal α-BRCA1 (clone D9)	Santa Cruz	Cat# sc-6954, RRID:AB_626761
Rabbit polyclonal α-BRCA1	Abcam	Cat# ab9141 RRID:AB 307041
Mouse monoclonal α-TOP2β	(Tsutsui et al., 2001)	N/A

Mouse monoclonal α-MRE11 (clone 12D7)	Gene Tex	Cat# GTX70212, RRID:AB_372398
Mouse monoclonal α-LIG4 (clone D-8)	Santa Cruz	Cat# sc-271299
Mouse monoclonal α-PARP1 (clone H-250)	Santa Cruz	Cat# sc-7150
Rabbit polyclonal α-TDP1	Bethyl Laboratories	Cat# A301-618A
Rabbit polyclonal α-TDP2	Bethyl Laboratories	Cat# A302-737A
Mouse monoclonal α-β-actin	Sigma	Cat# A5411
Mouse monoclonal α-DNA-PKcs (clone 18-2)	Abcam	Cat# ab1832
Mouse monoclonal α-UBC13 (clone F-10)	Santa Cruz	Cat# sc-376470
Mouse monoclonal α-RNF8 (clone B-2)	Santa Cruz	Cat# sc-271462
α-BrdU	Becton, Dickinson and Company	Cat# 555627
Goat polyclonal α-mouse AlexaFluor 488	ThermoFisher	Cat# A-11029, RRID:AB_2534088
Goat polyclonal α-rabbit AlexaFluor 488	ThermoFisher	Cat# A-11034, RRID:AB_2576217

Goat polyclonal α-mouse AlexaFluor 594	ThermoFisher	Cat# A-11032, RRID: AB_2534091
Goat polyclonal α-rabbit AlexaFluor 594	ThermoFisher	Cat# A11037, RRID:AB_2534095
Goat polyclonal α-mouse HRP	ThermoFisher	Cat# 32430
Donkey polyclonal α-rabbit HRP	Santa Cruz	Cat# sc-2313, RRID:AB_641181
α-mouse Ig, FITC conjugate	SBA	Cat# 1031-02
Chemicals, Peptides, and Recombinant Proteins		
Charcoal/Dextran treated FBS	Hyclone Laboratories	Cat#SH30068.03
Albumin, Bovine, F-V, pH5.2	Nacalai Tesque	Cat# 01863-48
Skim Milk for immunoassay	Nacalai Tesque	Cat# 31149-75
Paraformaldehyde	Wako	Cat# 16320145
FuGENE HD Transfection Reagent	Promega	Cat# E2312
Doxycycline Hydrochloride	MP Biomedicals, Inc.	Cat# 195044
Fluoro-KEEPER Antifade Reagent	Nacalai Tesque	Cat# 12745-74
Etoposide	Trevigen	Cat# 4886-400-01
KU7441	Selleckchem.com	Cat# S2638
3'-Indoleacetic Acid	Nacalai Tesque	Cat# 19119-61
2.5 g/l-Tripsin/1 mmol/l-EDTA Solution	Nacalai Tesque	Cat# 35554-64
Propidium iodine (PI)	DOJINDO	Cat# P378
BrdU	Nacalai Tesque	Cat# 05650-66
Critical Commercial Assays		

GeneArt Seamless Cloning Enzyme Mix	ThermoFisher	Cat# A14606
Stbl3	ThermoFisher	Cat# C737303
X10-gold Ultracompetent cells	Ajilent	Cat# 200314
Experimental Models: Cell Lines		
Human: MCF7, *wild-type*	ATCC	Cat# HTB-22
Human: TK6 (TSCER2), *wild-type*	A gift from Dr. Masamitsu Honma (Honma et al., 2003)	N/A
Human: HCT116 WT, *wild-type*	A gift from Dr. Masato Kanemaki	N/A
Human: Lenti-X^{TM} 293T	TAKARA	Cat# 632180
Experimental Models: Organisms/Strains		
The mutant genotypes of TK6, MCF7, and HCT116 are listed in Table S1	This work	
Oligonucleotides		
The primers are listed in Table S2	This work	
Recombinant DNA		
Plasmid: px330-U6-Chimeric_BB-CBh-hSpCas9	Addgene	Cat# 42230
Plasmid: *DT-ApA/MARKER^{R}*	CDB, RIKEN, Kobe	N/A
Plasmid: pSpCas9(BB)-2A-Puro(pX459)	(Ran et al., 2013)	Cat# 48139
Plasmid: lentiCRISPRv2-neo	Addgene	Addgene#98292
Plasmid: pLKO.1	(Hoa et al., 2016)	Cat#8453
Plasmid: pMD2.G	Addgene	Cat #12259
Plasmid: pMDLg/pRRE		Cat#8453

Plasmid: pRSV-Rev		Cat#12253
Plasmid: lentiCRISPRv2-*Asi*SI	A gift from Tanya Paull	N/A

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hiroyuki Sasanuma (hiroysasa@rg.med.kyoto-u.ac.jp)

Experimental model and subject details

Mutant cells and primer sequences used in this paper are described in Tables S1 and S2, respectively.

Method details

Cell culture

Human TK6 B cells were incubated in RPMI1640 medium (Cat# 3026456, Nacalai Tesque, Japan) supplemented with horse serum (5%) (Gibco, US), penicillin (100 U/ml), streptomycin (100 μg/ml) (Nacalai, Japan), and sodium pyruvate (200 mg/ml) (ThermoFisher, US). MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Cat# 0845964, Gibco, US) containing fetal bovine serum (10%) (Gibco, US), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Nacalai, Japan). HCT116 cells were maintained in McCoy's 5A medium (Cat# SH30200.01, GE

Healthcare, US) containing fetal bovine serum (10%) (Gibco, US), penicillin (100 U/ml), streptomycin (100 μg/ml) (Nacalai, Japan), and L-Glutamine (Nacalai, Japan). Lenti-X^{TM} 293T cells (Cat# 632180, TAKARA, Japan) were maintained in DMEM supplemented with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) (Nacalai, Japan), sodium pyruvate (200 mg/ml) and L-glutamine (Nacalai, Japan). TK6, MCF-7, HCT116 and Lenti-X^{TM} 293T cells were maintained at 37℃ under a humidified atmosphere and CO_2 (5%).

Designing gene-targeting constructs and transfection of them into TK6 cells

We designed gene-targeting constructs based on the manual provided by GeneArt Seamless Cloning Enzyme Mix (ThermoFischer, US)(Hoa et al., 2016). Table S1 shows the list of mutants and the antibiotic resistance markers (*MARKER^R*) used to generate the mutants in this study. To generate gene-targeting constructs, we amplified left and right arms (~1 kb each) from genomic DNA. The amplified arms were assembled with the *DT-ApA*/*MARKER^R vector digested with ApaI* and the *AflII* using GeneArt Seamless Cloning Enzyme Mix (ThermoFischer, US). Primer information about the left and right arms (~1 kb each) is described in Table S2. To generate the left arm, we needed to add the upstream and downstream sequences derived from the *ApaI* site *to the 5' and 3' ends, respectively, of* the PCR-amplified left arm. For this purpose, we added "5'-GCGAATTGGGTACCGGGCC" to 5' of the upstream primer and added "5'-CTGGGCTCGAGGGGGGGCC" to 5' of the downstream primer. We added the upstream and downstream sequences from the *AflII* site *to the 5' and 3' ends, respectively, of* the right arm. We added "5'-TGGGAAGCTTGTCGACTTAA" to 5' of the upstream primer of the PCR-amplified right arm and added "5'-

CACTAGTAGGCGCGCCTTAA" to 5' of the downstream primer of the PCR-amplified right arm. The *DT-ApA/MARKER*^{R} was provided by the Laboratory for Animal Resources and Genetic Engineering, Center for Developmental Biology, RIKEN Kobe (http://www.clst.riken.jp/arg/cassette.html).

The gRNAs were inserted into the *BbsI* site of pX330 vector (Cat# 42230, Addgene, US). The two resulting targeting vectors containing different antibiotic markers were transfected with pX330-gRNA into six million TK6 cells. The transfected pX330 expressed the Cas9-gRNA complex, which induced DSBs at the specific locus of the genomic DNA and thus facilitated HR between the genomic locus and the arms of the targeting vectors.

Generation of TK6 mutant cells

Schematic diagrams of *RAP80*, *RNF8, POLβ, POLQ, TDP1*, *PARP1, ARTEMIS*, *EXD2*, and *EXO1* target locations are depicted in Figures S2A, S3C, S7A-D and S7F-H. Generation of *BRCA1*$^{AID/AID}$, *BRCA1*$^{AID/AID}$*/RNF168*$^{-/-}$, *XRCC1*$^{-/-}$, and *MRE11*$^{-/H129N}$ cells was as described previously (Hoa et al., 2016, 2015; Keka et al., 2015; Saha et al., 2018; Sasanuma et al., 2018)(Zong et al., 2019). To generate *TDP1*$^{-/-}$*/TDP2*$^{-/-}$ and *LIG4*$^{-/-}$*/MRE11*$^{-/H129N}$ mutant cells, we disrupted *TDP2* genes in *TDP1*$^{-/-}$ cells and *LIG4* genes in *MRE11*$^{loxP/H129N}$ cells (Figures S7C and S5E). PCR genotyping using the primers shown in Table S2 was performed as a primary screening. The gene-disruption events were confirmed by western blotting, reverse transcription-PCR (RT-PCR), or Southern blotting analysis. Disruption of *EXO1* alleles was confirmed by genomic Southern blotting using a ^{32}P-labeled probe, which was amplified by the following primer pair:

5’-CCATGCTAGTGAAAATTGAGAACAACTTTT and 5’-CTCCTTACTTTATACATCAGCATTACTGAA (Figure S7H). Genomic DNA was digested with *Hind*III for Southern blotting analysis.

CRISPR/Cas9-mediated genome-editing in human MCF-7 cells

The gRNA sequences are shown in Table S2. gRNAs were inserted into the *BbsI* site of pX459 (Cat# 48139, Addgene, US). pX459 expresses both a gRNA under the control of the U6 promoter and Cas9 driven by the chicken β-actin promoter. Schematic diagrams of the *DNA-PKcs* and *RAP80* target locations are presented in Figures S1G and S2A, respectively. pX459-gRNA was transfected into MCF-7 cells with FuGENE HD (Promega, US). Following transfection, cells were incubated with puromycin-containing medium for 48 h, after which we removed the puromycin and further incubated the cells (for approximately 2 weeks) to isolate the clones. Gene-disruption events were confirmed by western blotting.

CRISPR/Cas9-mediated genome-editing in human HCT116 cells

The C-terminus of endogenous *MRE11* was fused with mAID-GFP in HCT116 cells constitutively expressing OsTIR1 (Natsume et al., 2016). We inserted gRNA into pX330-U3-Chimeric_BB-CBh-hSpCas9 (Addgene #42230) to cut 31bp downstream of the stop codon (Figure S7A). We constructed two donor plasmids, each containing a knock-in cassette that encodes mAID-GFP with a neomycin or hygromycin resistance marker. The donors harbor homology arms (about 1 kb) at both ends of the knock-in cassette for integration by homology-directed repair. CRISPR and donor plasmids were co-transfected into HCT116 CMV-OsTIR1 cells. Cells were selected in the presence of

G418 (700 μg/mL) and HygroGold (100 μg/mL) following a publicly available protocol (Yesbolatova et al., 2019). Isolated clones were evaluated by genomic PCR to verify the bi-allelic integration, and the expression of the MRE11-mAID-GFP protein was confirmed by western blotting.

Cell-cycle analysis

Cells were labeled for 15 min with 50 μM BrdU. They were then harvested and fixed at 4°C overnight with 70% ethanol, and successively incubated as follows: (i) in 2N HCl, 0.5% Triton X-100 for 30 min at room temperature; (ii) in FITC-conjugated anti-BrdU antibody (Becton, Dickinson and Company, Franklin Lakes, NJ) for 30 min at room temperature; (iii) in FITC- conjugated anti - mouse antibody (Southern Biotech, Birmingham, AL) for 30 min at room temperature; (iv) in 5 μg/ml PI in PBS. Subsequent flow cytometric analysis was performed on an LSRFortessa (Becton, Dickinson and Company). Fluorescence data were displayed as dot plots using the Cell Quest software (Becton, Dickinson and Company).

Measurement of cellular sensitivity to DNA damaging agents

Cellular sensitivity of an asynchronous population of TK6 cells to etoposide and IR was measured by clonogenic cell-survival analysis. Cells were grown in the respective medium described above, containing methylcellulose, for 10 days. Cellular sensitivity of an asynchronous population of MCF-7 cells to etoposide was measured by clonogenic cell-survival analysis. Cells were grown in the respective medium described above for 10 days.

Measurement of TOP2 trapped on genomic DNA DSBs

The protocol for this assay was as described by Hoa *et al*. 2016. TOP2ccs that migrated into the CsCl gradient during ultra-centrifugation were detected by slot blot.

Lentivirus-mediated gene silencing and *Asi*SI expression

Lentiviral vectors were simultaneously transfected with virus-packaging plasmids (pMD2.G, pMDLg/pRRE, and pRSV-Rev) into LentiX-293T cells (Cat# 632180, Takara, Japan). Lentiviruses were harvested at 48 h post-transfection. MCF-7 cells were infected with the virus for 48 h. Puromycin was added to enrich the infected cells at 24 h after infection. For gene silencing, shRNA sequences targeting MRE11 (5'-CGACTGCGAGTGGACTATAGC), UBC13 (5'-CTAGGCTATATGCCATGAATA) and BRCA1 (5'-CCCTAAGTTTACTTCTCTAAA) were cloned into a pLKO.1 lentiviral vector (Table S2). Downregulation of MRE11, UBC13, and BRCA1 was confirmed by western blotting (Figures S1C, S2G, S5B, S5C, and S6C). To obtain TK6 cells stably expressing regulatable *Asi*SI RE coupled to an estrogen receptor (ER-*Asi*SI) in cells (Caron et al., 2015)(Aymard et al., 2014), the lentiviral lentiCRISPRv2 vector containing both ER-*AsiS*I and puromycin-resistance genes (a gift from Gaëlle Legube and Tanya Paull) was transfected into the LentiX-293T cells. The lentiviral particles were harvested at 48 h post transfection and were infected into the TK6 cells.

To express ER-*Asi*SI in *MRE11*$^{-/H129N}$ TK6 cells (Figure 5A), we treated *MRE11*$^{+/H129N}$ cells with 4-OHT (200 nM) for 24 h to inactivate the *wild-type MRE11* gene, generating *MRE11*$^{-/H129N}$ cells. After 4-OHT was washed out from the medium, lentiviral particles expressing ER-*Asi*SI were infected into the 4-OHT-treated *MRE11*$^{-/H129N}$ cells. After

incubating the infected cells for two days, we analyzed the ER-*Asi*SI-induced γH2AX foci by addition of 4-OHT into the medium.

Immuno-staining of γH2AX in TK6 cells

Cells were treated with etoposide (10 μM) for 30 minutes and IR, and subsequently washed twice with warm PBS and cultured in drug-free media. Cells were collected using a cytospin (ThermoFisher, US) and subjected to fixation by formaldehyde (4%) (Wako, Japan) in PBS, permeabilization by Tween-20 (0.1%) (23926-35, Nacalai Tesque) in PBS, and blocking by BSA (5%) (01683-48, Nacalai Tesque) in PBS. We used α-phospho (Ser139) -histone H2AX (anti-γH2AX) antibody (1/1000 dilution, JBW301, Millipore, US). To distinguish cells in the G_1 phase from other phases, we used a α-cyclinA antibody (1/100 dilution, G0811, Santa Cruz Biotechnology, US). The slides were mounted in Fluoro-KEEPER containing 4', 6-diamidino-2-phenylindole (DAPI) (12745-74, Nacalai Tesque, Japan).

Immuno-staining in MCF-7 cells

We synchronized MCF-7 cells in the G_1 phase using serum-starvation for 24 h. We then treated cells with etoposide (10 μM) for 30 min and IR, followed by washing and incubation with serum-free media. For the detection of FK2 and MRE11 foci, MCF-7 cells were permeabilized with TritonX-100 (0.5%) on ice for 10 min and then fixed with formaldehyde (4%) on ice for 15 min. For detection of RAP80 and γH2AX foci, the cells were fixed with paraformaldehyde (4%) on ice for 20 min and permeabilized with TritonX-100 (0.2%) on ice for 7 min. After incubation in blocking solution (5%) (BSA in PBS), the cells were incubated with the following primary antibodies for 1 h: α-FK2

(1/1000, MBL Life Science, Japan), α-BRCA1 (1/500, D-9, mouse monoclonal, Santa Cruz Biotechnology, US), α-RAP80 (1/1000, A300-763A, rabbit polyclonal, Bethyl Laboratories, US), α-γH2AX (1/1000, JBW301, mouse monoclonal, Millipore, US), α-γH2AX (1/500, 20E3, rabbit polyclonal, Cell Signaling Technology, US), α-CyclinA (1/500, EPR17351, rabbit polyclonal, Abcam, UK), α-53BP1 (1/1000, PC712, rabbit polyclonal, Merck, US) and α-MRE11 (1/500, 12D7, mouse monoclonal, GeneTex, US). After washing three times with PBS, cells were stained with α-mouse (Alexa Fluor 488), α-rabbit (Alexa Fluor 488), α-mouse (Alexa Fluor 594) or α-rabbit (Alexa Fluor 594) secondary antibodies. The slides were mounted in Fluoro-KEEPER containing 4', 6-diamidino-2-phenylindole (DAPI) (12745-74, Nacalai Tesque, Japan).

Focus counting and statistical analysis

For MCF-7 cells, foci images were captured using a BZ-9000 fluorescence microscope (Keyence). The number of the nuclear foci signals was automatically counted using Hybrid cell count software (Keyence). In TK6 cells, the number of nuclear foci counted by Hybrid cell count software was underestimated, because nuclei in TK6 cells are more round and thicker (~5 μm in diameter) in TK6 cell than that in adherent MCF-7 cells. To count all nuclear foci, 5 images at different focal planes through the entire thickness of the cellular nuclei were captured at 1 μm intervals along the Z-axis using confocal microscope (SP8, Leica, Germany). Image Z-stacks were projected using the maximal intensity method using LAS AF software (Leica, Germany). The number of γH2AX foci were counted blind. We performed all foci experiments independently at least three times for calculation of standard deviation and counted more than 50 cells at each experiment. The average foci numbers were counted from each experiment. The

bars in foci graphs indicate the average of average foci number from each experiment. The error bars indicate standard deviation calculated from average number of each experiment. The box plots in supplemental figures indicate the median (the line inside the box) and distribution of all foci (more than 150 dots in total) including more than three experiments.

Immunoprecipitation and western blotting

Whole-cell extracts were prepared from 5×10^6 cells lysed in IP buffer containing HEPES pH 7.5 (50 mM), KCl (150 mM), Tween 20 (0.05%), NP-40 (0.05%), Glycerol (10 %), NaF (2μM), Na_3VO_4 (0.4μM), Na-pyrophosphate (0.5 μM), 2 × protease inhibitor cocktail (5056489001, Complete, Roche, Switzerland), and β-glycerophosphate (2 μM). BRCA1 was immunoprecipitated with α-BRCA1 antibody (1/500, D-9, mouse monoclonal, Santa Cruz Biotechnology, US) pretreated with protein G magnetic beads (10004D, ThermoFisher, US). The magnetic beads were washed three times with IP buffer containing KCl (200 mM). The immunoprecipitates were subjected to SDS-PAGE and immunoblotting with α-BRCA1 and α-MRE11 (1/1000, 12D7, GeneTex, US). For signal detection, membranes were incubated with the appropriate HRP-linked secondary antibodies at room temperature for 1 h and developed by chemiluminescence using ECL reagent.

Trichloroacetic acid (TCA) precipitation

Cells (0.5×10^6 cells) were lysed in RIPA buffer containing Tris-HCl pH 7.6 (50 mM), NaCl (150 mM), NP-40 (1%), Sodium Deoxycholate (0.5%), and Sodium Dodecyl Sulfate (0.1%). The cellular lysates were precipitated with 20% TCA on ice for 30 min.

Centrifuge the samples at 13,000 g at 4 degree for 10min. Aspirate the supernatant and wash the pellets (ppts) with ice-cold acetone several times to completely remove the remaining TCA. The protein ppts were dissolved in 2 × Laemmli sample buffer and bailed for 10 minutes.

Construction of lentiviral plasmids expressing RAP80-WT and RAP80-UIMΔ

Wild-type RAP80 (RAP80-WT) gene and RAP80 (RAP80-UIMΔ) gene lacking UIM domain were amplified by PCR using the following primers from pSNAPf-RAP80-FLAG and pSNAPf-RAP80r-UIM-deletion (gifts from Dr. Shibata); the forward primer, 5'-CGGGTTTGCCGCCAGAACACAGGACCGGTATGGACTACAAAGACCATGA-3'; the reverse primer, 5'-TGTTTCAGCAGAGAGAAGTTTGTTGCGCCGGTGAATTCGAATTTTCTCC-3'. The amplified products were cloned into lentiCRISPRv2-Neo (Addgene#98292) digested with *Xba*I and *BamH*I by seamless reaction.

Construction of lentiviral plasmids expressing UBC13-WT and UBC13-C87A

UBC13 gene was amplified by PCR from a cDNA library using the following primers; the forward primer, 5'-GGGTTTGCCGCCAGAACACAGGACCGGTTATGGCCGGGCTGCCCCGCAG-3'; the reverse primer, 5'-TGTTTCAGCAGAGAGAAGTTTGTTGCGCCAATATTATTCATGGCATATAGC-3'. To generate shRNA-resistant UBC13 gene, we introduced silent mutations into UBC13 gene by PCR using the two primers, 5'-

GAAAGTAATGCTCGATACTTTTCATGTGGTCATTGCTGGCC-3’ and 5’-GATAAATTAGGTAGGATCTGCTTAGATATTTTGAAAGATAAG -3’.

To introduce the catalytic inactive mutation (C87A) of UBC13 gene, we used the primer, 5’-GATAAATTAGGTAGGATCGCCTTAGATATTTTGAAAGATAAG-3’. The amplified products were cloned into lentiCRISPRv2-Neo (Addgene#98292) digested with *Xba*I and *BamH*I by seamless reaction.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (related to Figure 1)

UBC13 contributes to DSB repair by recruiting BRCA1 and RAP80 onto DSB sites during the G_1 phase.

(A) Representative images of etoposide-induced FK2 and 53BP1 foci in asynchronous and serum-starved MCF-7 cells. Green specks indicate FK2 signal. The nuclei are outlined.

(B) Cell cycle analysis of serum-starved cells. Cells were incubated with serum-free medium for 24 h and stained with FITC-conjugated anti-BrdU antibody to measure BrdU incorporation into genomic DNA (y-axis, logarithmic scale) and with propidium iodide (PI) to measure the total DNA (x-axis, linear scale).

(C) Western blot analysis of *UBC13*-shRNA-treated (shUBC13) MCF-7 cells. Lanes 1 and 2 show *wild-type* MCF-7 and control-shRNA-treated cells, respectively, representing positive controls. Lanes 3-5 show a successful depletion of UBC13 by shRNA. β-Actin was a loading control.

(D) Distribution of etoposide-induced FK2 foci in shControl- and shUBC13-treated MCF-7 cells. Box plots display the median (black bars) and 25-75th percentiles (box ranges). A representative experiment (n=3, 150 cells) is shown. Single asterisk indicates $p < 2.2 \times 10^{-16}$, calculated by a Wilcoxon rank sum test.

(E) Western blotting analysis of γH2AX upon etoposide treatment. Serum-starved cells were incubated with etoposide (10 μM) for 30 min.

(F) Distribution of etoposide-induced γH2AX foci per cells in the indicated genotypes. Box plots indicate the median (bars), 25th, and 75th percentiles (boxes ranges). Single asterisks indicate $p < 2.2 \times 10^{-16}$, calculated by a Wilcoxon rank sum test.

(G) Schematic diagram of *DNA-PKcs* genomic locus, target location, and guide RNA (gRNA) sequence in MCF-7 cells.

(H) Western blots of the whole-cell extracts prepared from *wild-type* and *DNA-PKcs*$^{-/-}$ MCF-7 clones. β-Actin was a loading control.

(I and J) Distribution of BRCA1 (I) and RAP80 (J) foci per cell following 0.5-h pulse exposure to etoposide (10 μM) in individual MCF-7 cells (upper). Box plots display the median (black bars) and 25-75th percentiles (box ranges). Single and double asterisks indicate $p < 2.2 \times 10^{-16}$ and $p=5.0 \times 10^{-11}$, respectively calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown. Green and red specks indicate BRCA1/RAP80 and γH2AX signals, respectively (lower). The nuclei are outlined.

(K) Western blotting analysis of RAP80 expression. The lenti-virus plasmids harboring *wild-type* RAP80-3FLAG and RAP80-ΔUIM-3FLAG (lacking UIM domain) were infected into *RAP80*$^{-/-}$ MCF-7 cells. The infected cells were selected in neomycin-containing medium. β-Actin was a loading control.

(L) Distribution (left graph) and representative images (right panels) of RAP80 foci per cell following 0.5-h pulse exposure to etoposide (10 μM) in individual MCF-7 cells. Box plots display the median (black bars) and 25-75th percentiles (box ranges). Single asterisk indicates $p < 2.2 \times 10^{-16}$ calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown. Green and red specks indicate RAP80 and γH2AX signals, respectively. The nuclei are outlined.

Figure S2 (related to Figure 2)

RAP80 and BRCA1 contribute to DSB repair during the G_1 phase.

(A) Schematic diagram of *RAP80* genomic locus, target location, gene-targeting construct and guide RNA (gRNA) sequence in TK6 and MCF-7 cells. Targeting vectors having neomycin and puromycin were used only for the disruption of *RAP80* in TK6 cells.

(B) Western blotting analysis with α-RAP80 antibody to confirm the gene disruption events in the indicated genotypes. β-Actin was a loading control.

(C and D) The sensitivity of TK6 (C) and MCF-7 (D) cells to etoposide was measured by clonogenic cell-survival analysis. The dose for etoposide is displayed by the x-axis on a linear scale. The percentage of cell survival relative to that of untreated cells is shown by Y-axis on a logarithmic scale. Error bars show standard deviation (SD) calculated from three independent experiments.

(E and F) Distribution of γH2AX (E) and BRCA1 (F) foci per cell following pulse exposure (0.5 h) to etoposide (10 μM) in individual MCF-7 cells (upper). Box plots display the median (bars) and 25-75th percentiles (box ranges). Single asterisks indicate $p < 2.2 \times 10^{-16}$, calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown. Representative images of etoposide-induced γH2AX (E) and BRCA1 (F) foci are shown (lower). Green specks indicate γH2AX (E) and BRCA1 (F) signals. The nuclei are outlined.

(G) Western blot analysis of BRCA1-shRNA-treated MCF-7 cells. Lanes 1 and 2 show *wild-type* MCF-7 and control-shRNA-treated cells, respectively. Lanes 3-5 show a successful depletion by shRNA in the indicated genotypes. β-Actin is a loading control.

(H) Distribution of γH2AX foci per cell following pulse exposure (0.5 h) to etoposide (10 μM) in individual MCF-7 cells (upper). A representative experiment (n=3, 150 cells) is shown. Box plots display the median (bars) and 25-75th percentiles (box

ranges). Single asterisks indicate $p < 2.2 \times 10^{-16}$, calculated by a Wilcoxon rank sum test. Representative images of etoposide-induced γH2AX foci are shown (lower). Green specks indicate γH2AX signals. The nuclei are outlined.

Figure S3 (related to Figure 3)

UBC13, RAP80, and BRCA1 promote the removal of etoposide-induced TOP2 adducts independent of TDP2.

(A) Schematic of *in vivo* TOP2cc measurement by immunodetection with α-TOP2 antibody.

(B) Degradation of BRCA1-mAID-GFP proteins two hours after exposure of *BRCA1*$^{AID/AID}$ cells to auxin (250 nM). The whole-cell extracts were analyzed by western blot with α-BRCA1 antibody. β-Actin is a loading control.

(C) Schematic diagram of *RNF8* genomic locus, target location, and guide RNA (gRNA) sequence in TK6 cells (upper). Western blots of the whole-cell extracts prepared from *wild-type* and *RNF8*$^{-/-}$ TK6 clones (lower). β-Actin was a loading control.

(D) Western blotting analysis of UBC13 expression. The lenti-virus plasmids harboring *wild-type* UBC13 (shUBC13-WT) and UBC13C87A (shUBC13-C87A, catalytic inactive form) were infected into UBC13-depleted MCF-7 cells. The infected cells were selected in puromycin-containing medium. β-Actin was a loading control.

(E, F, and G) Western blotting analysis of TOP2β in TK6 and serum-starved MCF-7 cells treated with etoposide (10 μM) ("+") or DMSO ("-") for 2 h.

Figure S4 (related to Figure 4)

K63 ubiquitin signaling involving UBC13, RAP80, and BRCA1 is required for efficient recruitment of MRE11 nuclease onto DSB sites in G_1 cells.

Representative images of MRE11 and 53BP1 foci following pulse-exposure (0.5 h) to etoposide. Green and red specks indicate MRE11 and 53BP1 signals, respectively. The nuclei are outlined.

Figure S5 (related to Figure 5)

Repair kinetics of *Asi*SI-induced DSBs and IR-induced DSBs in TK6 cells.

(A) Distribution of *Asi*SI-induced γH2AX foci in the indicated genotypes. Box plots display the median (bars) and 25-75th percentiles (box ranges). Single asterisks indicate $p < 2.2 \times 10^{-16}$, calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown.

(B and C) Western blot analysis of UBC13-shRNA-treated (shUBC13) (B) and BRCA1-shRNA-treated (shBRCA1) (C) TK6 cells. Lanes 1 and 2 show *wild-type* and control-shRNA-treated (shControl) TK6 cells, respectively, representing positive controls. Lane 3 shows a successful depletion of UBC13 or BRCA1 by shRNA. β-Actin was a loading control.

(D) Distribution of IR-induced γH2AX foci in the indicated genotypes. Box plots display the median (bars) and 25-75th percentiles (box ranges). Single asterisks indicate $p < 2.2 \times 10^{-16}$, calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown.

(E) Schematic diagram of *LIG4* genomic locus, gene-targeting construct, and gRNA sequence in TK6 cells (upper). Targeting events were confirmed by western blotting analysis (lower). β-Actin was a loading control.

(F) Distribution of IR-induced γH2AX foci in the indicated genotypes. Box plots display the median (bars) and 25-75th percentiles (box ranges). Single and double asterisks indicate $p = 1.9 \times 10^{-9}$ and $p=5.1 \times 10^{-8}$, respectively calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown.

(G) Western blot analysis of BRCA1-shRNA-treated (shBRCA1) TK6 cells. β-Actin was a loading control.

(H) Distribution of IR-induced γH2AX foci in the indicated genotypes. Box plots display the median (bars) and 25-75th percentiles (box ranges). Single and double asterisks indicate $p = 2.1 \times 10^{-12}$ and $p < 2.2 \times 10^{-16}$, respectively calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown.

Figure S6 (related to Figure 5)

Repair kinetics of IR-induced DSBs in *MRE11*$^{AID/AID}$ HCT116 cells.

(A) Schematic diagram of *MRE11* genomic locus, gene-targeting construct, and gRNA sequence in HCT116 cells. "mAID" indicates the minimized sequence of the Auxin-induced-degron-tag. gRNA binds genomic sequences at the downstream of the termination codon of *MRE11* gene.

(B) Degradation of MRE11-mAID-GFP proteins two hours after exposure of *BRCA1*$^{AID/AID}$ cells to auxin (250 nM). The whole-cell extracts were analyzed by western blot with α-MRE11 antibody. Ponceau staining was used to show equal loading.

(C) Western blotting analysis of MRE11-shRNA-treated (shMRE11) HCT116 cells. Lane 3 shows a successful depletion of UBC13 by shRNA. β-Actin was a loading control.

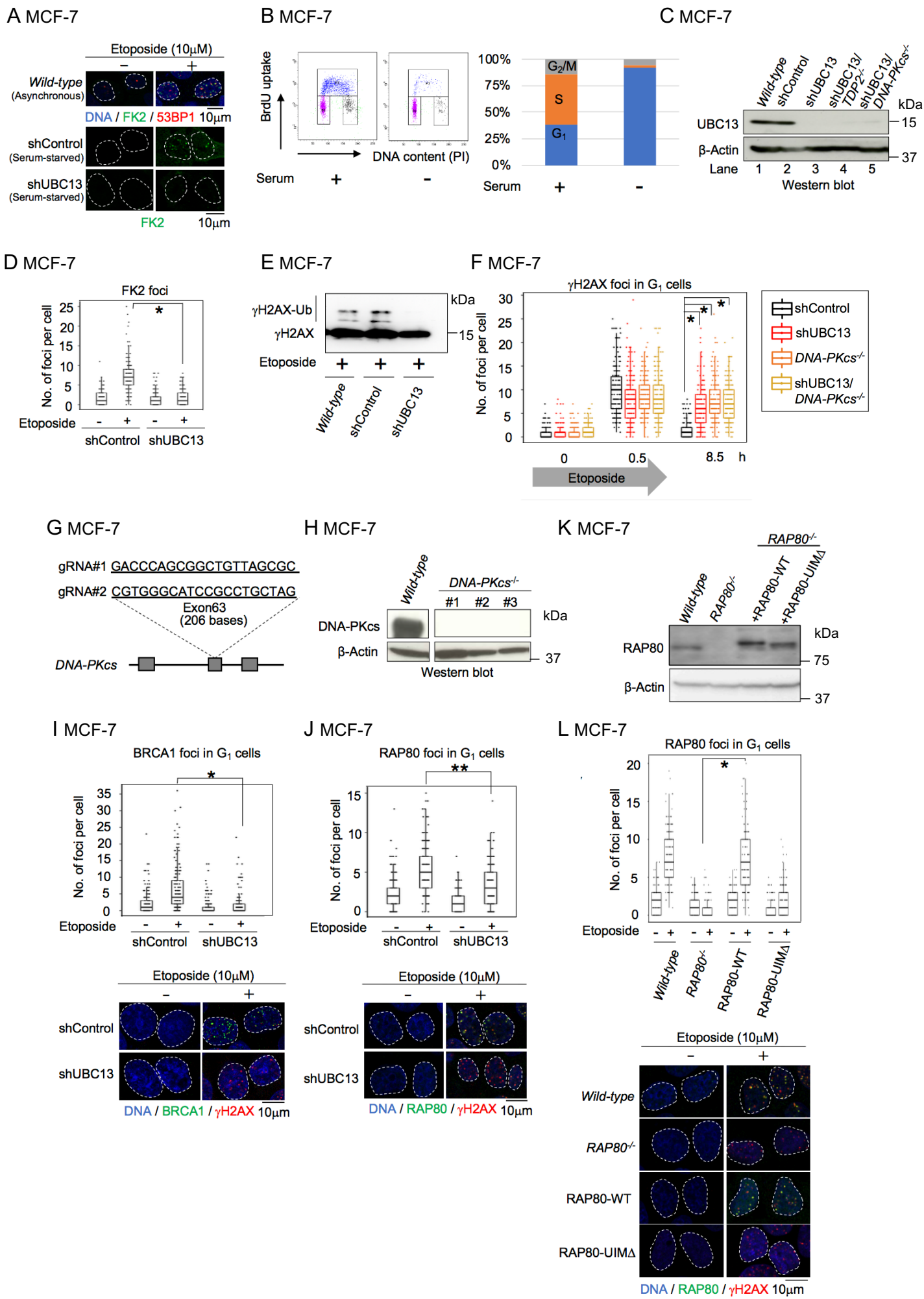
(D) Distribution of IR-induced γH2AX foci in the indicated cells. Box plots display the median (bars) and 25-75th percentiles (box ranges). Single asterisks indicate $p < 2.2 \times 10^{-16}$, calculated by a Wilcoxon rank sum test. A representative experiment (n=3, 150 cells) is shown.

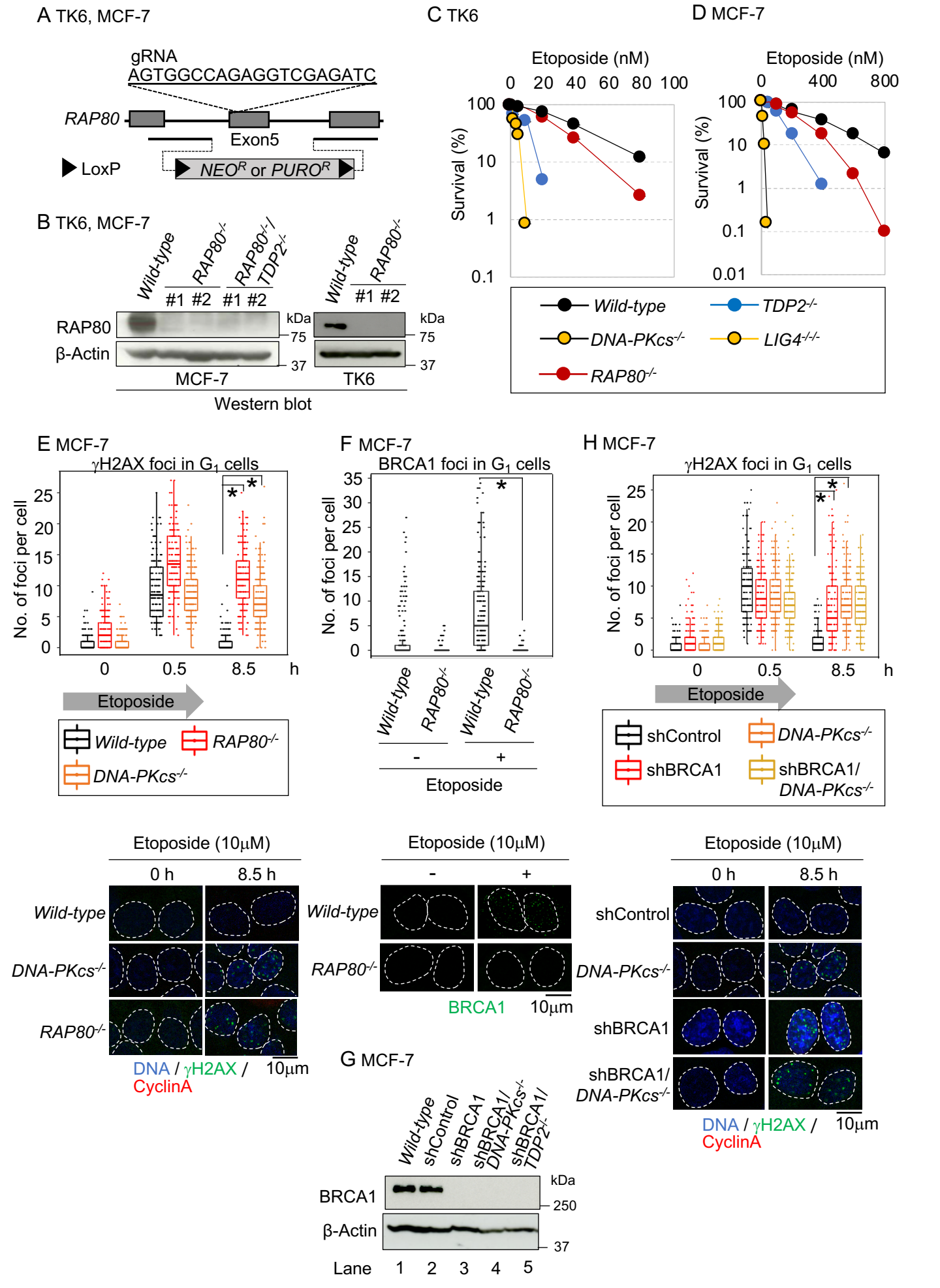
Figure S7 (related to Figure 5)

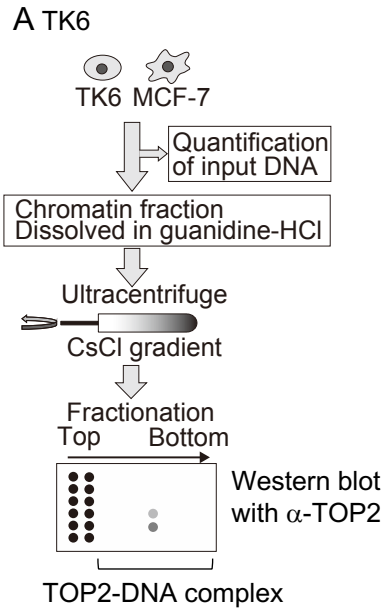
Disruption of the genes encoding *POLβ*, *POLθ*, *TDP1*, *PARP1*, *ARTEMIS*, *EXD2*, and *EXO1* in TK6 cells and radiosensitivity of the resultant mutants.

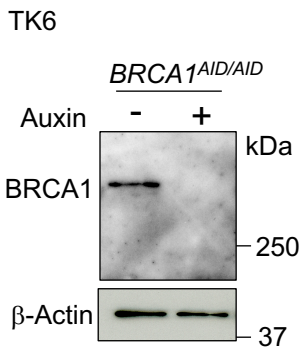
(A-D and F-H) Schematic diagram of the genomic loci, target location, gene-targeting constructs and guide RNA (gRNA) sequences of the indicated genes in TK6 (upper). The targeting events were confirmed by western blotting, Southern blotting, and/or reverse transcription PCR (RT-PCR) analyses. β-Actin is a loading control for western blotting analysis. β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are internal controls for RT-PCR analysis. Ponceau staining in (D) was used to show equal loading. Orange vertical and horizontal lines in (H) indicate *Hind*III restriction enzyme sites and probe for Southern blotting analysis, respectively.

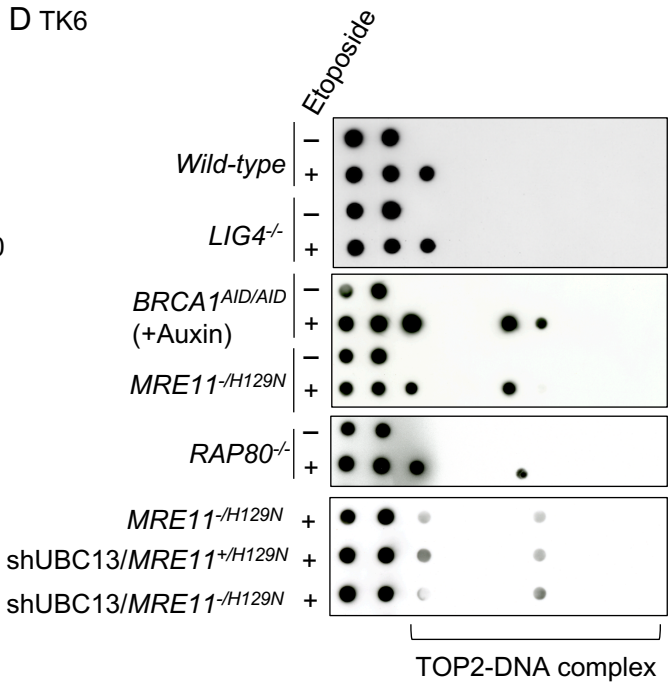
(E) The doses that reduce percent colony survival to 50% (LD_{50}) following the exposure of the indicated genotypes to IR. Error bars represent SD of the mean from three independent experiments. The single, double, and triple asterisks indicate $p = 8.4 \times 10^{-3}$ (*Wild-type* vs. *EXD2*$^{-/-}$), $p = 7.8 \times 10^{-3}$ (*Wild-type* vs. *ARTEMIS*$^{-/-}$) and $p = 4.4 \times 10^{-3}$ (*Wild-type* vs. *EXO*$^{-/-}$), respectively, which were calculated by a Student's *t*-test.

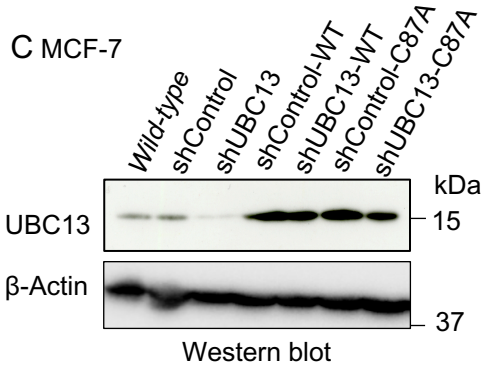

A MCF-7
Etoposide (10μM)
Wild-type (Asynchronous)
DNA / FK2 / 53BP1 10μm
shControl (Serum-starved)
shUBC13 (Serum-starved)
FK2 10μm
B MCF-7
BrdU uptake
DNA content (PI)
Serum + -
100%
75%
50%
25%
0%
G2/M
S
G1
C MCF-7
Wild-type
shControl
shUBC13
shUBC13/ TDP2-/-
shUBC13/ DNA-PKcs-/-
kDa
UBC13
β-Actin
15
37
Lane 1 2 3 4 5
Western blot
D MCF-7
FK2 foci
No. of foci per cell
Etoposide
shControl shUBC13
E MCF-7
γH2AX-Ub
γH2AX
kDa
15
Etoposide
Wild-type
shControl
shUBC13
F MCF-7
γH2AX foci in G1 cells
No. of foci per cell
0 0.5 8.5 h
Etoposide
shControl
shUBC13
DNA-PKcs-/-
shUBC13/ DNA-PKcs-/-
G MCF-7
gRNA#1 GACCCAGCGGCTGTTAGCGC
gRNA#2 CGTGGGCATCCGCCTGCTAG
Exon63 (206 bases)
DNA-PKcs
H MCF-7
Wild-type
DNA-PKcs-/-
#1 #2 #3
DNA-PKcs
β-Actin
kDa
37
Western blot
K MCF-7
RAP80-/-
Wild-type
RAP80-/-
+RAP80-WT
+RAP80-UIMΔ
RAP80
β-Actin
kDa
75
37
I MCF-7
BRCA1 foci in G1 cells
No. of foci per cell
Etoposide
shControl shUBC13
Etoposide (10μM)
shControl
shUBC13
DNA / BRCA1 / γH2AX 10μm
J MCF-7
RAP80 foci in G1 cells
No. of foci per cell
Etoposide
shControl shUBC13
Etoposide (10μM)
shControl
shUBC13
DNA / RAP80 / γH2AX 10μm
L MCF-7
RAP80 foci in G1 cells
No. of foci per cell
Etoposide
Wild-type
RAP80-/-
RAP80-WT
RAP80-UIMΔ
Etoposide (10μM)
Wild-type
RAP80-/-
RAP80-WT
RAP80-UIMΔ
DNA / RAP80 / γH2AX 10μm

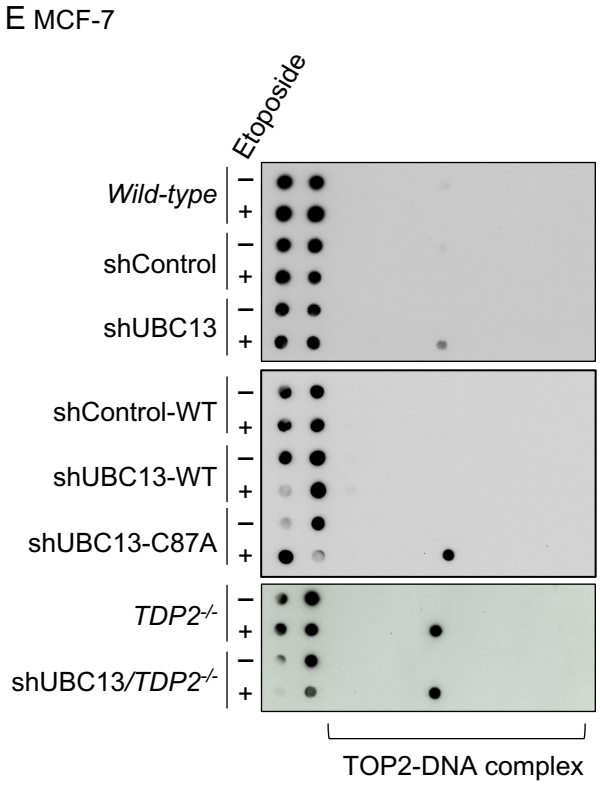

A TK6, MCF-7
gRNA
AGTGGCCAGAGGTCGAGATC
RAP80
Exon5
LoxP
NEOR or PUROR
B TK6, MCF-7
Wild-type
RAP80-/-
RAP80-/-/ TDP2-/-
#1 #2 #1 #2
Wild-type
RAP80-/-
#1 #2
RAP80
β-Actin
kDa
75
37
MCF-7
TK6
Western blot
C TK6
Etoposide (nM)
0 20 40 60 80 100
Survival (%)
100
10
1
0.1
D MCF-7
Etoposide (nM)
0 400 800
Survival (%)
100
10
1
0.1
0.01
Wild-type
TDP2-/-
DNA-PKcs-/-
LIG4-/-/-
RAP80-/-
E MCF-7
γH2AX foci in G1 cells
No. of foci per cell
0 5 10 15 20 25
0 0.5 8.5 h
Etoposide
Wild-type
RAP80-/-
DNA-PKcs-/-
F MCF-7
BRCA1 foci in G1 cells
No. of foci per cell
0 5 10 15 20 25 30 35
Wild-type
RAP80-/-
Wild-type
RAP80-/-
-
+
Etoposide
H MCF-7
γH2AX foci in G1 cells
No. of foci per cell
0 5 10 15 20 25
0 0.5 8.5 h
Etoposide
shControl
shBRCA1
DNA-PKcs-/-
shBRCA1/ DNA-PKcs-/-
Etoposide (10μM)
0 h
8.5 h
Wild-type
DNA-PKcs-/-
RAP80-/-
DNA / γH2AX / CyclinA
10μm
Etoposide (10μM)
-
+
Wild-type
RAP80-/-
BRCA1
10μm
Etoposide (10μM)
0 h
8.5 h
shControl
DNA-PKcs-/-
shBRCA1
shBRCA1/ DNA-PKcs-/-
DNA / γH2AX / CyclinA
10μm
G MCF-7
Wild-type
shControl
shBRCA1
shBRCA1/ DNA-PKcs-/-
shBRCA1/ TDP2-/-
BRCA1
β-Actin
kDa
250
37
Lane 1 2 3 4 5

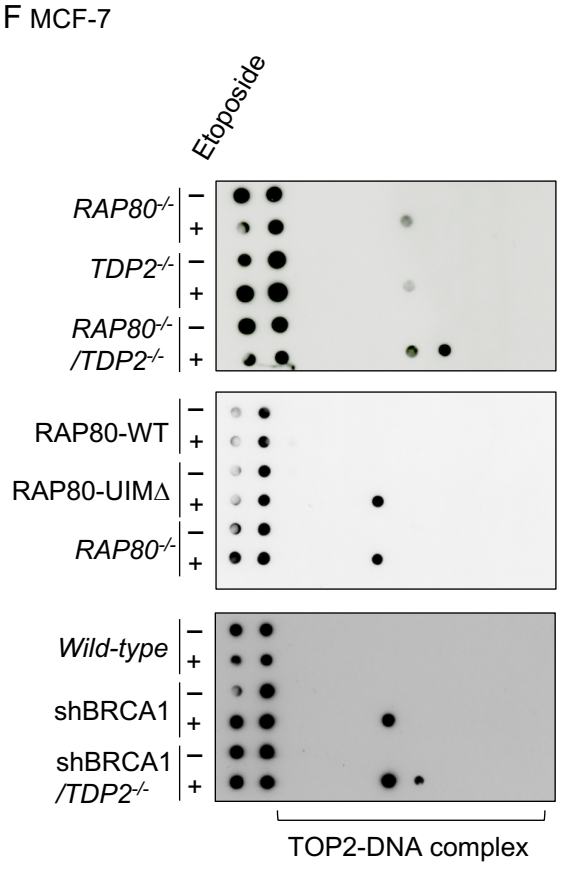

A TK6
TK6 MCF-7
Quantification of input DNA
Chromatin fraction Dissolved in guanidine-HCl
Ultracentrifuge
CsCl gradient
Fractionation
Top
Bottom
Western blot with α-TOP2
TOP2-DNA complex

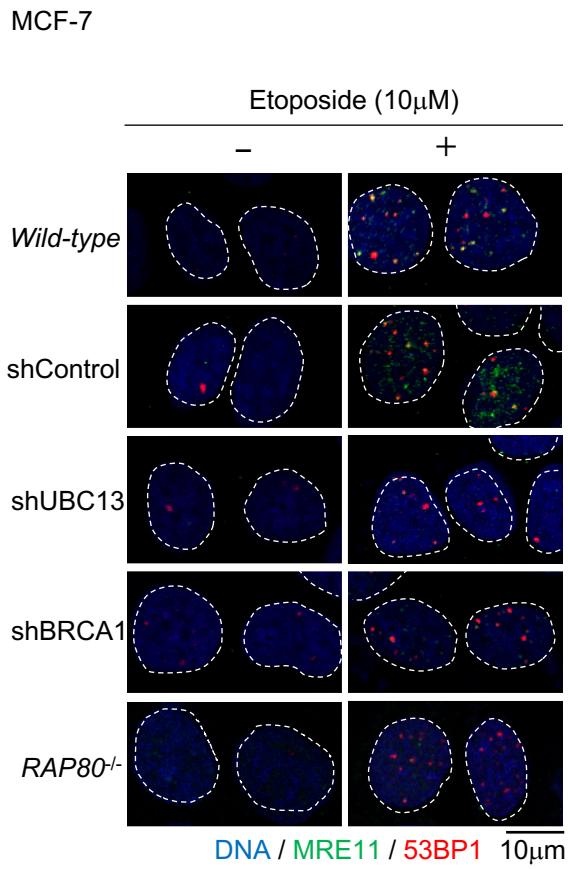

B TK6
BRCA1AID/AID
Auxin - +
kDa
BRCA1
250
β-Actin
37


D TK6
Etoposide
Wild-type
LIG4-/-
BRCA1AID/AID (+Auxin)
MRE11-/H129N
RAP80-/-
MRE11-/H129N
shUBC13/MRE11+/H129N
shUBC13/MRE11-/H129N
TOP2-DNA complex

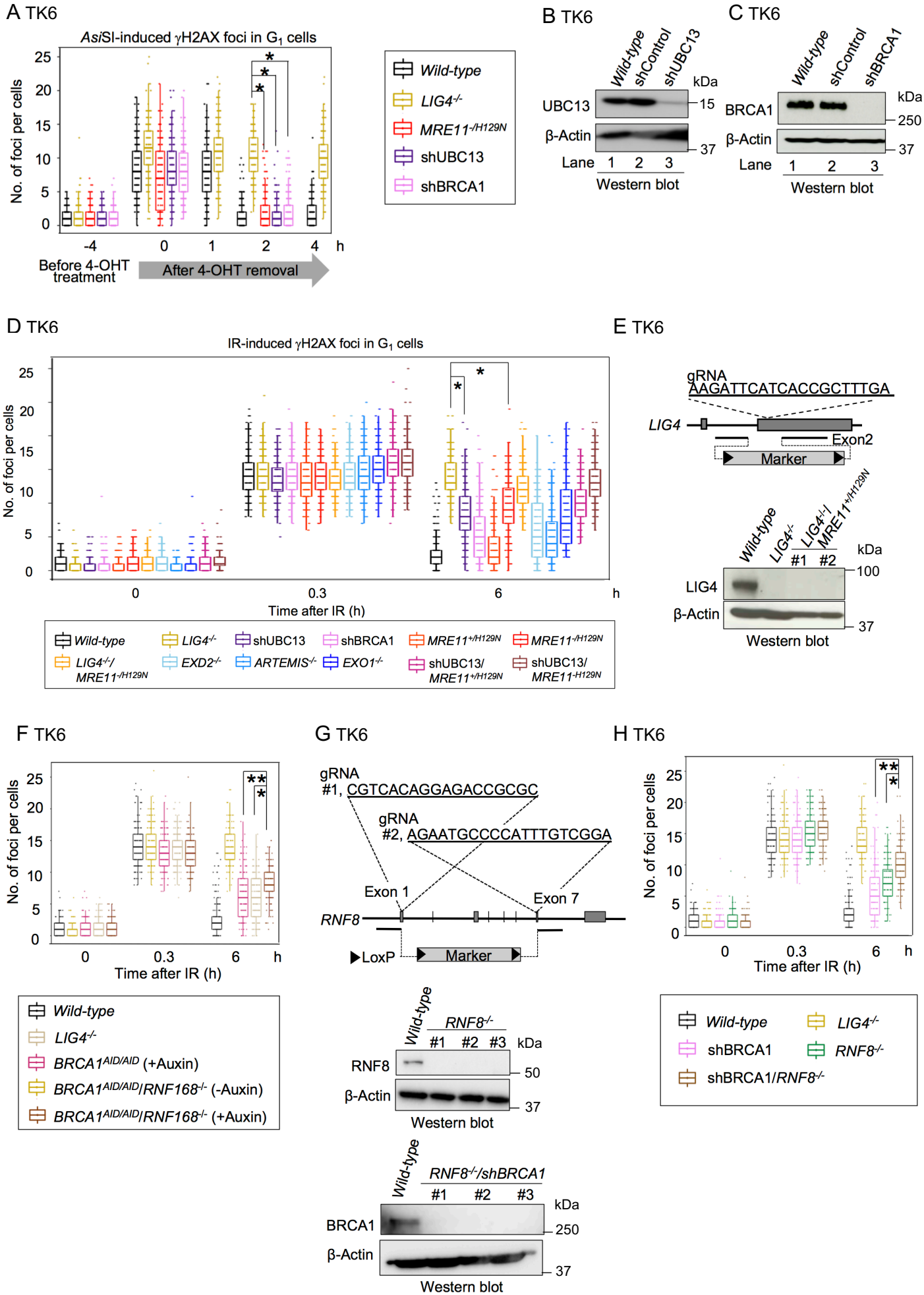

C MCF-7
Wild-type
shControl
shUBC13
shControl-WT
shUBC13-WT
shControl-C87A
shUBC13-C87A
kDa
UBC13
15
β-Actin
37
Western blot


E MCF-7
Etoposide
Wild-type
shControl
shUBC13
shControl-WT
shUBC13-WT
shUBC13-C87A
TDP2-/-
shUBC13/TDP2-/-
TOP2-DNA complex

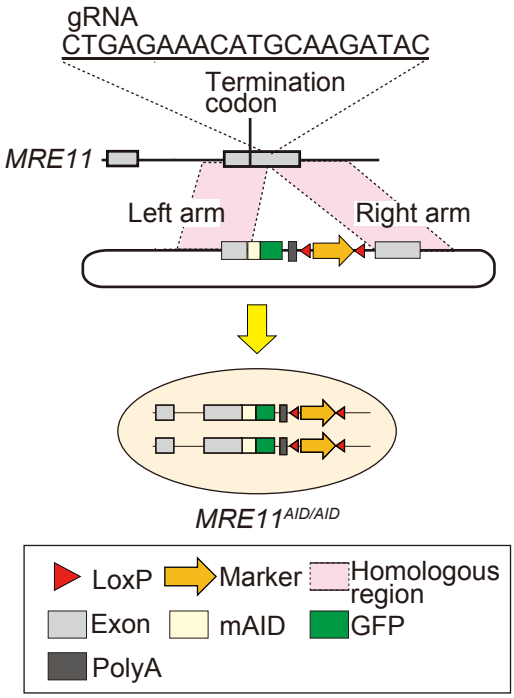

F MCF-7
Etoposide
RAP80-/-
TDP2-/-
RAP80-/- /TDP2-/-
RAP80-WT
RAP80-UIMΔ
RAP80-/-
Wild-type
shBRCA1
shBRCA1 /TDP2-/-
TOP2-DNA complex


MCF-7
Etoposide (10μM)
–
+
Wild-type
shControl
shUBC13
shBRCA1
RAP80-/-
DNA / MRE11 / 53BP1
10μm

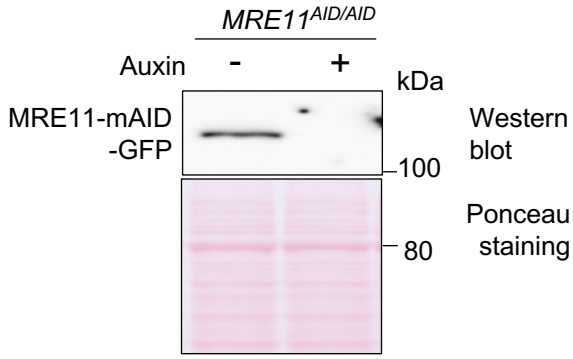
Akagawa, R., *et al*, Figure S5



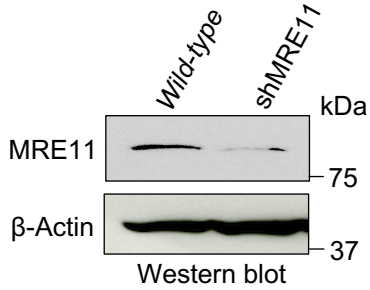
A HCT116



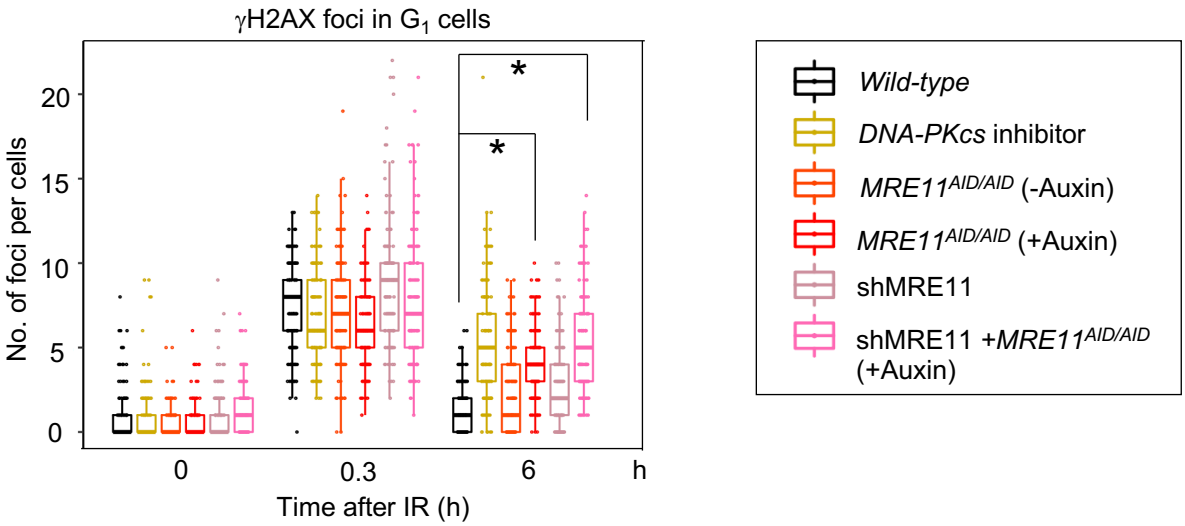
B HCT116

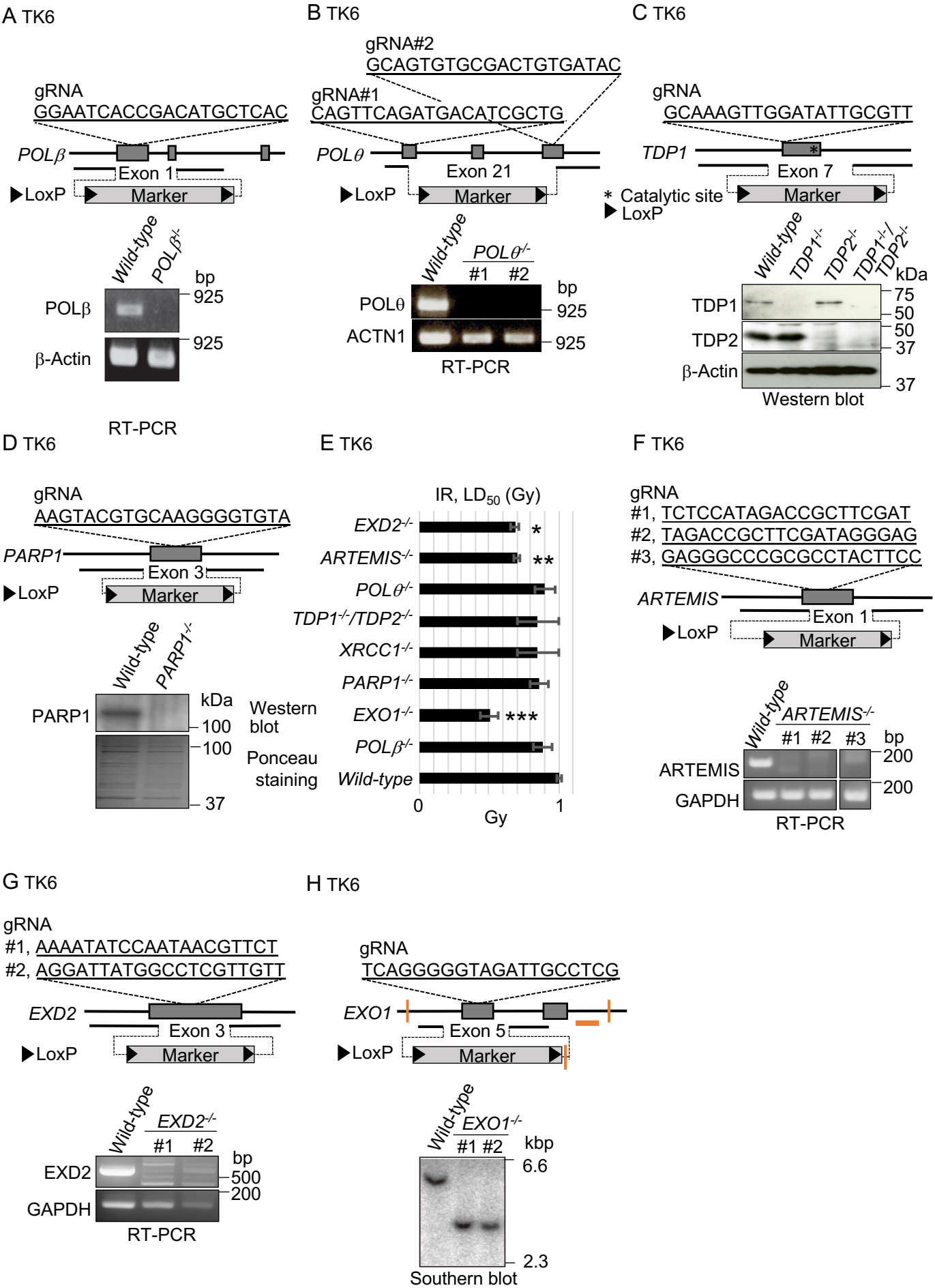


C HCT116



D HCT116




A TK6
gRNA
GGAATCACCGACATGCTCAC
POLβ
Exon 1
LoxP
Marker
Wild-type
POLβ-/-
bp
POLβ
925
β-Actin
925
RT-PCR
B TK6
gRNA#2
GCAGTGTGCGACTGTGATAC
gRNA#1
CAGTTCAGATGACATCGCTG
POLθ
Exon 21
LoxP
Marker
Wild-type
POLθ-/-
#1 #2
bp
POLθ
925
ACTN1
925
RT-PCR
C TK6
gRNA
GCAAAGTTGGATATTGCGTT
TDP1
Exon 7
* Catalytic site
LoxP
Marker
Wild-type
TDP1-/-
TDP2-/-
TDP1-/-/TDP2-/-
kDa
TDP1
75
50
TDP2
50
37
β-Actin
37
Western blot
D TK6
gRNA
AAGTACGTGCAAGGGGTGTA
PARP1
Exon 3
LoxP
Marker
Wild-type
PARP1-/-
kDa
PARP1
100
100
37
Western blot
Ponceau staining
E TK6
IR, LD50 (Gy)
EXD2-/-
*
ARTEMIS-/-
**
POLθ-/-
TDP1-/-/TDP2-/-
XRCC1-/-
PARP1-/-
EXO1-/-
***
POLβ-/-
Wild-type
0
1
Gy
F TK6
gRNA
#1, TCTCCATAGACCGCTTCGAT
#2, TAGACCGCTTCGATAGGGAG
#3, GAGGGCCCGCGCCTACTTCC
ARTEMIS
Exon 1
LoxP
Marker
Wild-type
ARTEMIS-/-
#1 #2 #3
bp
ARTEMIS
200
GAPDH
200
RT-PCR
G TK6
gRNA
#1, AAAATATCCAATAACGTTCT
#2, AGGATTATGGCCTCGTTGTT
EXD2
Exon 3
LoxP
Marker
Wild-type
EXD2-/-
#1 #2
bp
EXD2
500
GAPDH
200
RT-PCR
H TK6
gRNA
TCAGGGGGTAGATTGCCTCG
EXO1
Exon 5
LoxP
Marker
Wild-type
EXO1-/-
#1 #2
kbp
6.6
2.3
Southern blot