Supplementary Materials and Methods

Virus Infection

Lentivirus expressing Rad17 shRNA were generated with pTRIPZ inducible shRNA lentivirus vector using 293T cells. U2OS or Fadu cells were infected by lentivirus, followed by puromycin selection (1. 5 μg/ml) for two weeks to generate stable cells with doxycycline-inducible Rad17 shRNA. Retrovirus expressing shRNA-resistant Rad17 WT or Rad17 T622A mutants was generated with pQCXIN retrovirus vector using 293T cells. The above stable U2OS or Fadu cells expressing inducible Rad17 shRNA were further infected by retrovirus, followed by G418 (1 mg/ml) selection for two weeks to generate stable cells. Doxycyclin was used to induce down-regulation of endogenous Rad17 to generate U2OS or Fadu cells reconstituted with shRNA-resistant Rad17 WT or Rad17 T622A mutants.

Antibodies, Plasmids and siRNA

The antibodies used for immunoblotting, immunoprecipitation and immunofluorescence were purchased from Sigma (γ-Tubulin), Invitrogen (V5), Santa Cruz (Rad17, Rad51, BRCA1, RPA, DNA-PKcs and MRE11), Cell Signaling (ATR, ATM and ATM-pSer198, SMC1, SMC1 pS957), Bethyl Laboratories (RPA-pSer3/4), Millipore (γH2AX-pSer139, RPA, MDC1), Gene Tex (Rad51), Novus Biologicals (NBS1, NBS1-pSer343 and RAD50), BD Pharmingen (Orc2) and GE Healthcare (BrDU). Rad17-pT622 antibody was produced by Open BioSystems by immunizing rabbits with synthetic peptide (EESLGEP[pT]QATVPETWSL). DNA-PKcs siRNA (Cat. sc-35200) was purchased from Santa Cruz.

A tet-inducible Rad17 shRNA construct (pTRIPZ) was purchased from Open BioSystems; the target sequence of Rad17 shRNA is GGTTATTGTTTCCCAAAGAAA. A pCDNA3.1 vector containing Rad17 full-length cDNA was used as template to generate Rad17 phosphorylation mutant T622A using the Stratagene Quickchange II XL site directed mutagenesis kit. The Rad17 WT or Rad17 T622A mutant that is resistant to shRNA was produced using the same methodology to introduce four nucleotide mutations within the Rad17 shRNA target sequence. The full-length cDNA of shRNA-resistant Rad17 WT or Rad17T622A mutant was cut with BamHI and EcoRI from pCDNA3.1 vector and subcloned into the pQCXIN retroviral vector (Clontech). The pDR-GFP, I-SecI expressing vector (pCβASce), and control vectors (pCAGGS) were gifts from M. Jasin (Memorial Sloan-Kettering Cancer Center, USA). pBABE-HA-ER-I-PpoI retroviral vector, Flag-tagged ATM WT and KI constructs are gifts from Dr. Michael Kastan's laboratory (Duke University Cancer Institute)..

The siRNA sequences used are as follows:

Rad17 siRNA-1 AGGUUAUUGUUUCCCAAAG [dT][dT];

Rad17siRNA-2 CAGACUGGGUUGACCCAUC [dT][dT];

ATM siRNA GCCUCCAGGCAGAAAAAGA [dT][dT];

ATR siRNA CCUCCGUGAUGUUGCUUGA [dT][dT];

NBS1 siRNA GAAGAAACGUGAACUCAAG [dT][dT];

MDC1 siRNA UCCAGUGAAUCCUUGAGGU [dTdT];

H2AX siRNA CAACAAGAAGACGCGAAUC [dT][dT].

Primers used in sit-directed mutagenesis to incorporate the T622A mutation:

5’-CTCTGGGTGAACCCGCTCAAGCCACTGTG-3’

5-‘CACAGTGGCTTGAGCGGGTTCACCCAGAG-3’

Primers used in site-directed mutagenesis to introduce mutations within the Rad17 shRNA target sequence:

5’-GATAATAATCAAAGGTTGTTGTTCCCCAAGGAGATTCAGGAAGAGTG-3’

5’-CACTCTTCCTGAATCTCCTTGGGGAACAACAACCTTTGATTATTATC-3’

Immunoblotting and Immunoprecipitation

For immunoblotting, cells were lysed with NETN buffer (50 mM Tris, 150 mM NaCl , 1 mM EDTA, 1% NP40, 10% glycerol, 1 mM Na_3SO_4 and 10 mMNaF) supplemented with protease inhibitors (Sigma) and phosphatase inhibitors (Pierce). Protein concentrations were determined using the Bio-Rad protein assay, and 30 µg of total protein was loaded in each lane of 6%-12% SDS-PAGE gel. Proteins were transferred to PVDF membrane which were subsequently blocked with 4% BSA in tris-buffered saline with 0.1% Tween 20 (TBST) and then incubated with indicated antibodies (1:500-1:1000 dilution with TBST) at 4℃ with gentle shaking overnight. After w ashing with TBST three times, the membrane was incubated with secondary HRP-conjugated goat anti-rabbit (1:1000 dilution in TBST, Invitrogen, G21234) or goat anti-mouse (1:2000 dilution in TBST, Invitrogen, G21040) antibody for 1 hour at room temperature followed by four washes with TBST. Immunodetection was performed by enhanced chemiluminescence detection (Thermo Scientific). For γ-H2AX immunoblotting, cells were sonicated in

NETN buffer, and cellular debris was removed by centrifugation. The supernatant was analyzed by immunoblotting as described above.

For immunoprecipitation, cells were harvested and lysed in NETN buffer containing protease and phosphatase inhibitors. The clarified extract was precleared with protein G beads (GE Healthcare) for 1 hour at 4℃ followed by centrifugation (2,500×*g*, 3 min). 3 µg of appropriate primary antibody was added with protein G beads to the precleared supernatant and incubated for 3 hours or overnight at 4℃. Immunoprecipitates were washed four times in NETN buffer, boiled in SDS sample buffer for 5 min followed by centrifugation (2,500×g, 3 min). The resulting supernatants were then resolved on SDS-PAGE gel followed by Western blotting. The normal control rabbit or mouse IgG was purchased from Santa Cruz.

Immunofluorescent Staining

Cells were cultured on glass coverslips (22×22mm, No.1.5, VWR international) and treated with the indicated conditions before immunostaining. Cells were fixed with 3% paraformadehyde for 15 min and then permeabilized with 0.3% Triton X-100 for 2 min. After washing with PBS and incubation with blocking buffer (10% goat serum in PBS) for 30 min, the cells were incubated with primary antibodies (1:100-1:300 dilution in 1% goat serum in PBS) for 1 h at room temperature, followed by three PBS washes and incubation with the appropriate secondary antibodies (Alexa 594 or Alexa 488 conjugated secondary antibodies, 1:500-1:1000 dilution in 1% goat serum in PBS, Invitrogen) for 40 min at room temperature in the dark. After washing with PBS four times, coverslips were mounted onto slides using fluorescence mounting medium

containing DAPI (Vector Laboratories Inc.). Cells were visualized by fluorescence microscopy (Axio Imager A1, Zeiss, USA), and photographs were captured using MetaMorph (7.7.3.0) software. For detection of ssDNA foci under neutral condition, the method described in a published paper (Hu *et al*, 2011) was exactly followed. Briefly, cells were grown in culture medium containing 10 μM BrdU (Sigma) for 24 h followed by 5Gy IR. After 2h, cells were fixed and stained with anti-BrdU antibody (GE Healthcare). For laser irradiation, a partially customized 'laser-scissors' laser microdissection and capture system (Zeiss) in duke light microscopy core facility was used to introduce DNA damage in cultured cells. Following laser irradiation, cells were fixed at indicated time points and immunofluorescence staining was performed as described above.

***In vitro* Kinase Assay**

GST-Rad17 WT or phosphorylation mutants (residues 480-670 or 570-670) were bacterially expressed and purified as described above. 293T cells were transiently transfected with a Flag-ATR or Flag-ATM construct. The ATR or ATM kinase protein was then immunoprecipitated using an anti-Flag antibody. Immune complexes were washed twice with kinase buffer (50 mM Hepes, 150 mM NaCl, 4 mM $MnCl_2$, 6 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM dithiothreitol and 100 mM $NaVO_4$). Various 3 μg GST-Rad17 protein substrates, 10 μCi of ^{32}P adenosine triphosphate (ATP), and ATP to a final concentration of 20 μM were added and incubated at 30℃ for 15 min. After electrophoresis by SDS-PAGE, the gels were dried and exposed to autoradiography film at -80℃ for 4-12 hours.

Subcellular Fractionation

Isolation of nuclear soluble fractions and chromatin fractions was performed as previously described (Mendez *et al*, 2000). A total of ~ 3×10^6 cells were washed with PBS and resuspended in 200 µL of solution A (10 mM HEPES at pH7.9, 10 mM KCL, 1.5 mM $NaCL_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na_2VO_3, 1× protease inhibitor cocktail). Triton X-100 (0.1%) was added, and the cells were left on ice for 5 min. Nuclei were separated from cytoplasmic proteins by low speed centrifugation (1,300×*g* for 4 min). Isolated nuclei were washed once with solution A and lysed in solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT). After incubation on ice for 10 minutes, soluble nuclear proteins were separated from chromatin by centrifugation (1,700×*g* for 4 min). Isolated chromatin was washed once with solution B and collected by centrifugation (10,000×*g* for 1 min). Finally, chromatin was resuspended in SDS sample buffer and sheared by sonication (Fisher Scientific Sonic Dismembrator Model 150) on ice.

Cell Viability Assay

Cell viability was measured by the trypan blue exclusion method. Cells were trypsinized, harvested and stained with 0.4% trypan blue followed by counting the number of blue staining cells and the number of total cells. The percentage of viable cells was calculated as follows: % viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100.

Single-Cell Gel Electrophoresis (Comet Assay)

The repair kinetics of IR-induced DSB damage was evaluated by alkaline comet assay (Trevigen) according to the manufacturer's instructions. Briefly, cells were subjected to

ionizing radiation (10 Gy) and harvested at the indicated recovery time points for single cell electrophoresis, followed by staining of cell nuclei with SYBR green. The cell comets were visualized by fluorescence microscopy (Axio Imager A1, Zeiss, USA) and pictures were captured using MetaMorph (7.7.3.0) software. The data were evaluated by quantifying the tail moment of 75 cells per sample using the comet-analyzing program CometScore (Tritek)..

FACS Analysis

To determine cell cycle distribution, flow cytometry was performed by propidium iodide staining. Cells were digested with trypsin, washed twice with PBS and fixed with 70% ethanol overnight at 4℃. After washing twice with PBS, cells were incubated with 5 µg/ml propidium iodide and 50 µg/ml RNase A in PBS for 1h at room temperature, and analyzed by FACSCAN flow cytometer (Becton Dickinson). Data were analyzed using CELLQUEST software to reveal the percentage of cells in each cell cycle phase.

Clonogenic Survival Assay

Cells were plated at a density of 600 cells per well in six-well plates and treated with the indicated conditions. After treatment, cells were washed with PBS and allowed to recover in fresh medium. The cell cultures were then incubated for 10 to 12 days, with the medium being changed every 3 days. 0.5% crystal violet (Sigma)/20% ethanol was used to stain cell colonies, and only colonies containing 50 or more cells were counted.

Chromatin Immunoprecipitation

U2OS cells were transfected with control siRNA or Rad17 siRNA for two days. Cells were then infected by retrovirus containing HA-ER-I-PpoI followed by induction of 4-hydroxytamoxifen (4-OHT). The procedure of ChIP was performed as previously described (Berkovich et al, 2007). 1×10^7 cells were crosslinked by the addition of formaldehyde directly to the growth medium to a final concentration of 0.5%. Crosslinking was stopped after 10 min at room temperature by the addition of glycine to a final concentration of 0.125 M. Crosslinked cells were washed with PBS, scraped, washed with PBS with 1 mM PMSF, then resuspended in 2 ml buffer I (10 mM HEPES at pH 6.5, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100 supplemented with phosphatase and protease inhibitor cocktails (Thermo Scientific, Product Number 1861277). Cells were pelleted by centrifugation and then resuspended in 2 ml buffer II (10 mM HEPES at pH 6.5, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl and phosphatase/protease inhibitors). After centrifugation, nuclei were resuspended in 0.3-1 ml lysis buffer (50 mM Tris at pH 8.1, 10 mM EDTA, 1% SDS and phosphatase/protease inhibitors). The resulting chromatin was sonicated to an average size of 1000 bp, then microcentrifuged. 1% of the diluted supernatant was taken for protein and DNA concentration measurements. The same amount of protein-DNA from each sample was used for immunoprecipitations. After preclearing with BSA-IgG-t-RNA-blocked protein-A/G agarose beads, 2-3 µg anti-NBS1 (NB 100-143, Novus) antibody was added to each aliquot of chromatin and incubated on a rotating platform overnight at 4℃. Immunocomplexes were recovered with BSA t-R NA-blocked protein-A/G agarose beads. Following extensive washing, bound DNA fragments were eluted, purified and analysed by PCR or by SyberGreen (Applied Biosystems) real-time PCR.

SyberGreen real-time PCR dissociation curves showed that each primer set gave a single and specific product. Sequences of primers used for PCR and real-time PCR are the same as previously described (Berkovich et al, 2007). Experiments were repeated at least twice and, in each real-time PCR experiment, reactions were performed in duplicate.

Supplementary References

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