Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. Human U2OS and HEK 293T cell lines were purchased from ATCC and cultured in DMEM high-glucose medium with 10% (vol/vol) FBS and 1% penicillin/streptomycin at 37 °C in a CO_2 (5%; vol/vol) incubator. Plasmid or siRNA transfection was mediated by Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instruction.

Antibodies. Antibodies against BRUCE were obtained from Calbiochem (catalog no. AP1031) and Bethyl (catalog no. A300-367A); BRIT1 from Cell Signaling (catalog no. 4120) and Abcam (catalog nos. ab2612 and ab121277); USP8 from Bethyl (catalog no. A302); α-tubulin from Sigma (catalog no. T9026); FLAG (M2) from Sigma (catalog nos. A8592 and F3165); c-Myc (catalog nos. sc-40 and sc-789); GFP from Santa Cruz (catalog no. sc-9996); pATM from BD Biosciences (catalog no. DR1002); MDC1 from Novus (catalog no. NB100-395); NBS1 (catalog no. GTX30125) and Rad51 (catalog no. GTX100469) from GeneTex; γ-H2AX from Millipore (catalog no. 05-636); Orc2 from BD Biosciences (catalog no. 51-6875GR); ubiquitin from Cell Signaling (catalog no. 3933); BRM (catalog no. A301-016A), BRG1 (catalog no. A300-813A), and BAF170 (catalog no. A301-039A) from Bethyl.

Reagents and siRNAs. The comet assay kit was from Trevigen (catalog no. 4250-050-K); MNase was from Sigma (catalog no. N3755); the USP8 siRNA duplex pool was from Santa Cruz (catalog no. sc-76795, CCACAGAUUGAUCGUACUAdTdT, GGAUAGGGAACCUUCCAAAdTdT and CGACGUACAUAGUUUAACAdTdT) or was synthesized by Sigma (UGAAAUACGUGACUGUUUAUU). Three BRUCE siRNAs and one control siRNA were synthesized by Dharmacon. BRUCE siRNA sequences nos.1, 2, and 3 are GGUACAAUCACAUCUAGCAdTdT, GACCUUAAUGGAAUCUUGUdTdT, and GUUAUGAGCUGCUUGUAGAdTdT, respectively. The control siRNA sequence is UUCUCCGAACGUGUCACGUdTdT (1).

Preparation of Chromatin-Containing Whole-Cell Lysates. Cell pellets were lysed and sonicated to elute whole-cell lysate plus chromatin in NETN buffer [50 mM Tris-HCI (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40] with protease inhibitor tablets (Roche) and phosphatase inhibitors of 10 mM NaF and 50 mM β-glycerophosphate (2). The lysates were centrifuged at 15,000 × *g* for 20 min, and the supernatant was collected as cell-free extracts.

Preparation of Cytosol, Nuclear, and Chromatin-Enriched Fractions. Fractionation of U2OS cell extracts was conducted according to the protocol reported previously (3) with some modifications. A total of ~4×10^6 cells were washed with PBS and resuspended in 200 μL of solution A [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% (vol/vol) glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na_2VO_3, and protease inhibitor tablet]. Triton X-100 was added to a final concentration of 0.1%, and the cells were left on ice for 5 min. Cytoplasmic proteins (S1) were separated from nuclei (P1) by low-speed centrifugation (1,300 × *g* for 5 min). Isolated nuclei were washed once with solution A and lysed in 200 μL of solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT). After 10-min incubation on ice, soluble nuclear proteins (S2) were separated from chromatin (P2) by centrifugation (1,700 × *g* for 5 min). Isolated chromatin was washed once with solution B and spun down at high speed (10,000 × *g* for 1 min). Finally, chromatin was resuspended in 200 μL of NETN buffer and sheared by sonication (20% output, 15 s; Sonic Dismembrator, Model 100, Fisher Scientific). To digest chromatin with MNase, nuclei (P1) were resuspended in solution A containing 1 mM $CaCl_2$ and 50 U MNase (Sigma). After 2 min of incubation at 37 °C, nuclei were lysed and fractionated as described above. For the nuclear fraction, the nuclei (P1) were washed once with solution A, lysed in NETN buffer containing 0.1% SDS, and then sheared by sonication (20% output, 15 s; Sonic Dismembrator, Model 100, Fisher Scientific).

Immunoblotting and IP. Protein extracts (40–100 μg) were resolved by SDS/PAGE and transferred to a nitrocellulose filter. The filter was blocked with 5% (wt/vol) dry milk in PBS plus Tween 20 (PBST) for 1 h at room temperature, followed by incubation with primary antibody overnight at 4 °C or 2 h at room temperature. The filter was then washed in PBST five times for 5 min each, followed by incubation with HRP-conjugated secondary antibody for 1 h at room temperature. After washing with PBST, the filter was developed with ECL for 1 min and exposed to X-ray film. Coimmunoprecipitation was conducted in whole-cell lysate, nuclear fraction, or chromatin-enriched fraction. Cell extracts were incubated first with antibody for 3 h at 4 °C and then with protein A agarose beads for another 2 h. For FLAG IP, cell extracts were incubated directly with FLAG M2 agarose beads for 3 h at 4 °C. The agarose beads were washed four times with lysis buffer, once with high-salt lysis buffer (with 500 mM NaCl), and then with 1× Tris-buffered saline. The beads were eluted with 1× SDS sample loading buffer, and the elution was separated by SDS/PAGE.

Immunofluorescent Staining of Nuclear Foci Induced by DNA Damage. Cells cultured in eight-well chamber slides or on coverslips in six-well culture plates were irradiated with a Faxitron X-ray system (RX-650). For staining of pATM, MDC1, and γ-H2AX foci, cells were fixed in 4% (vol/vol) paraformaldehyde (PFA) on ice for 15 min and then were permeabilized in 0.5% Triton X-100 at room temperature for 12 min. For staining Rad51 foci, cells were pre-extracted for 6 min on ice in extraction buffer [10 mM Pipes (pH 6.8), 300 mM sucrose, 20 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100] and then were fixed with 4% (vol/vol) PFA on ice for 15 min. For staining of NBS1 and BRIT1 foci or coimmunostaining of BRIT1 with Myc-Ub, FLAG-USP8, or FLAG-BRUCEΔN, cells were fixed in methanol at −20 °C for 20 min. After blocking with 3% (wt/vol) BSA, samples were incubated with primary antibodies overnight on a shaker in a cold room or for 2 h at room temperature followed by incubation with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 for 1 h at room temperature. After washes, samples were examined under a Zeiss LSM 710 confocal microscope. For NaBu or TSA rescue experiments, cells were treated with 5 mM NaBu or 0.2 mg/L TSA for 12 h and then were subjected to nuclear foci staining.

U2OS Clone No. 16 with Stable Expression of DOX-Inducible shBRUCE. siRNA targeting BRUCE (GGCACAGCAGCTCTTATCA) was used to generate the shRNA (4): 5′-atcccGGCACAGCAGCTCTTATCAttcaagagaTGATAAGAGCTGCTGTGCCttttta-3′ and 5′-tcgaatttttCCGTGTCGTCGAGAATAGTaagttctctACTATTCTCGACGACACGGcc-3′. Eight tandem repeats of each shRNA, with an H1 promoter, were cloned into the pSUPERIOR.puro vector following the manufacturer's instructions (Oligoengine). The construct then was transfected with Lipofectamine 2000

into a U2OS cell line in which a Tet repressor (TetR) was stably expressed (gift of Xiaodong Wang, National Institute of Biological Sciences, Beijing, China). The transfected cells were selected for clones that integrated with both the shRNA construct and TetR by puromycin (1 μg/mL) and blasticidin (5 μg/mL), respectively. More than six positive stable clones were identified by immunoblotting in which DOX (1 μg/mL) treatment for 4–6 d ablated BRUCE expression. Clone no. 16 was used in this study, and results were confirmed in other clones.

Reconstitution of shRNA-Resistant Full-Length BRUCE in U2OS Clone No. 16. A pCI-Neo mammalian expression vector (Promega) containing cDNA encoding the full-length BRUCE protein consisting of 4,857 amino acids (pCI-Neo-FLAG-BRUCE) was made by piecing cDNA encoding a BRUCE isoform consisting of 4,829 amino acids together with a short N-terminal fragment encoding 28 amino acids with a FLAG tag engineered to the N terminus of the construct. Mutations in the cDNA were corrected and verified by DNA sequencing. Scramble/wobble mutations (shown in italics) *A*GCCCA*A*CA*A*CT*G*TT*G*TCC were introduced to this BRUCE construct to make it resistant to shRNA. This pCI-Neo-FLAG-BRUCE construct was transfected into U2OS clone no. 16 by Lipofectamine 2000. About 50 cell clones resistant to puromycin (1 μg /mL) and G418 (1 mg/mL) were obtained, and six were verified to by immunoblotting have stable expression of full-length BRUCE and an expression level the same as or similar to that of endogenous BRUCE; of these, stable cell line WT14, WT28 was used in this study.

Truncated Constructs and Mutagenesis. Full-length cDNAs expressing human BRUCE and BRIT1 were digested by restriction endonucleases at the sites indicated in Fig. 3 *A* and *D*. The resulting fragments were subcloned into a pCI-Neo expression vector with a FLAG, Myc, or GFP tag fused to the N terminus of the fragment. Lysine residues were mutated to arginine in the shaded regions of corresponding fragments as indicated in Fig. 4*H* [BRIT1(KR)-1, -2, or -3] by DNA synthesis (gBlocks gene fragments; Integrated DNA Technologies). The mutated fragments then were introduced into full-length BRIT1 cDNA to replace the original sequence. To create a human USP8 C786A point mutation, two complementary Ultramer oligonucleotides (Integrated DNA Technologies) that contain the C→A mutation were annealed, and the resulting short fragment was introduced into full-length USP8 cDNA to displace the corresponding wild-type fragment. Scramble/wobble mutations (shown in italics) TGAA*G*TA*T*GTCAC*G*GT*G*TA were introduced to full-length USP8 to make it resistant to USP8 siRNA.

Ubiquitination Assay. The BRTI1 ubiquitination assay was carried out as previously described with some modifications (5). Briefly, U2OS cells were cotransfected with FLAG-tagged BRIT1 and c-Myc–tagged Ub wild-type, K48R, and K63R constructs [gifts from Zhijian (James) Chen, University of Texas Southwestern Medical Center, Dallas, TX]. After 36 h, cells were harvested, lysed, and sonicated in extraction buffer [20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.2% SDS, 1 mM EDTA, and protease inhibitors]. The chromatin-containing cell lysate was used for IP with anti-Myc agarose beads (Sigma), and the IP products were analyzed by anti-FLAG immunoblotting. For the ubiquitination assay in the chromatin-enriched fraction, cells were lysed and fractioned following the protocol previously described with minor modifications (6). Briefly, U2OS cells were lysed in buffer I [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40, and protease and phosphatase inhibitors] for 5 min on ice. After centrifugation at 1,300 × *g* for 5 min at 4 °C, supernatant was removed, and the precipitate was washed once with buffer I and then extracted with buffer II [50 mM Tris·Cl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, protease, and phosphatase inhibitors] plus sonication. The extract was collected after centrifugation and subjected to c-Myc IP as described above.

RT-PCR Assay. mRNAs were prepared with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was carried out with SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Primers for amplification of endogenous BRUCE cDNA are TTACTATAGAGTGAGGGGTTG (forward) and TCCTCTC-TCCTCTACAGAGCCTC (reverse). Amplification of exogenous BRUCE was carried out using the forward primer used for amplification of endogenous BRUCE but a different reverse primer, TAA CCCTCACTAAAGGGAAGCGG.

Chromosome Studies and FISH. Metaphase chromosome spreads were prepared after hypotonic treatment of cells and were fixed in acetic acid-methanol. Telomeres in metaphase spreads were detected by FISH with a telomere-specific probe labeled with the fluorochrome Cy3 (red). Chromosomal aberrations were assessed by counting the chromosomal breaks, gaps, aneupolyploidy, and telomere association per metaphase (7).

Comet Assay. Cells were exposed to X-ray (Faxitron, RX-650) and analyzed by DSB-detecting neutral-pH comet assay following manufacture's instruction (Trevigen). Quantification of the results was performed according to the published method for USP8 samples (8) and MEFs (9).

MNase Digestion Assay. Cells cultured in 6-cm plates were treated with 10 Gy of IR; culture continued for 0.5–1 h; then medium was removed, and cells were treated with 1 mL of 0.01% egg lysolecithin in PS1 buffer [150 mM sucrose, 80 mM KCl, 35 mM Hepes (pH 7.4), 5 mM K_2PO_4, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$] for 90 s. After removal of PS1, cells were incubated with or without 2 U/mL MNase in 0.8 mL of PS2 buffer [20 mM sucrose, 50 mM Tris·Cl (pH 7.5), 50 mM NaCl, and 2 mM $CaCl_2$] at room temperature for the desired time. After digestion, PS2 was removed, and samples were treated with 1 mL of permeabilization stop solution [20 mM Tris·Cl (pH 8.0), 20 mM NaCl, 20 mM EDTA, and 1% SDS] containing 0.6 mg/mL proteinase K, followed by the addition of 0.5 mL of lysis dilution buffer (150 mM NaCl and 5 mM EDTA) and transfer of the solution to a 15-mL polypropylene tube for incubation at 37 °C overnight. Then Tris-EDTA buffer was added to the tube to reach a total volume of 3 mL. Genomic DNA was purified with phenol-chloroform extraction and was separated by electrophoresis in a 1% agarose gel.

HR Assay. The HR assay was performed in U2OS-DR-GFP cell lines (gift of Jeremy Stark, City of Hope, Duarte, CA) as described previously (10, 11). Cells cultured in six-well plates at 40% confluency were transfected twice with control or target siRNA. Twenty-four hours after the second round of siRNA transfection, cells were transfected with I-SceI–expressing pCBASce plasmid. After an additional 48 h of culture, cells were harvested, and GFP cells were analyzed by flow cytometry (BD LSRII analyzer).

NHEJ Assay. The NHEJ assay was carried out in U2OS-EJ5-GFP cell lines (gift of Jeremy Stark) as described previously (10, 11). Cells cultured in six-well plates at 70% confluency were transfected twice with control or BRUCE siRNA. Then the cells were transfected with I-SceI–expressing pCBASce plasmid. After an additional 48 h of culture, cells were harvested, and GFP^+ cells were analyzed by flow cytometry (BD LSR II analyzer).

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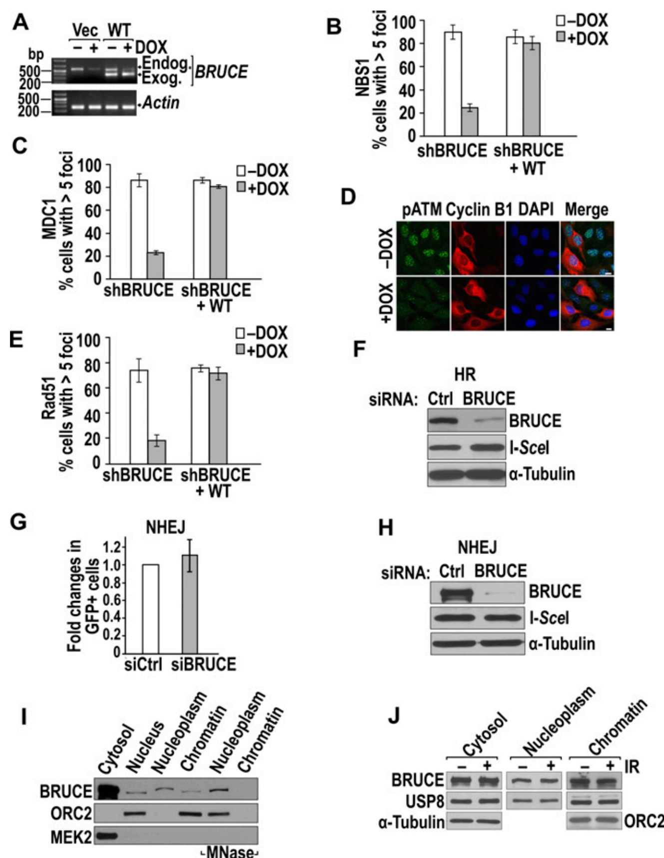


Fig. S1. (*A*) RT-PCR showing endogenous BRUCE mRNA was not transcribed after DOX treatment of the U2OS shBRUCE stable cell line, and exogenous BRUCE mRNA was expressed in both DOX-treated and nontreated cells in the shBRUCE+WT BRUCE cell line. (*B* and *C*) Quantification of NBS1 (*B*) and MDC1 (*C*) foci presented in Fig. 1 *E* and *F,* respectively. Error bars represent the SD of two independent experiments. (*D*) pATM foci (green) was reduced in both cyclin B1^{+} (red, G2/S) and cyclin B1^{-} (G1) cells after BRUCE depletion by DOX treatment. The cell nucleus was counterstained with DAPI (blue). (Scale bars, 10 μm.) (*E*) Quantification of RAD51 foci presented in Fig. 1*G*. Error bars represent SD of two independent experiments. (*F*) Immunoblot showing BRUCE knockdown by siRNA and the expression level of HA-tagged I-SceI in HR reporter U2OS-DR-GFP cells. (*G*) BRUCE depletion does not have a significant effect on NHEJ. Fold change of GFP^{+} cells is shown. NHEJ results after BRUCE depletion are relative to the percentage of GFP^{+} cells treated with control siRNA, which was set as 1. $P > 0.05$, Student's two-tailed *t* test. Error bars represent SD from three independent experiments. (*H*) Immunoblot showing BRUCE knockdown by siRNA and the expression level of HA-tagged I-SceI in NHEJ reporter U2OS-EJ5-GFP cells. (*I*) Immunoblot of subcellular localization of BRUCE with antibodies as indicated. MEK2, cytosol marker; ORC2, chromatin marker; MNase, micrococcal nuclease, which releases BRUCE from chromatin to nucleoplasm, indicating that part of BRUCE is chromatin-associated. (*J*) Immunoblot of BRUCE and USP8 across the subcellular fractionations as indicated. No obvious change in the levels of BRUCE and USP8 protein was observed before or after IR exposure across the fractions. These results also show that, in addition to their localization in cytosol, a fraction of BRUCE and USP8 also localizes in nuclear and chromatin fractions. Note that the blots were from different SDS/PAGE gels, and each blot had different X-ray film exposure, so that the amounts of proteins are not comparable across the three blots.

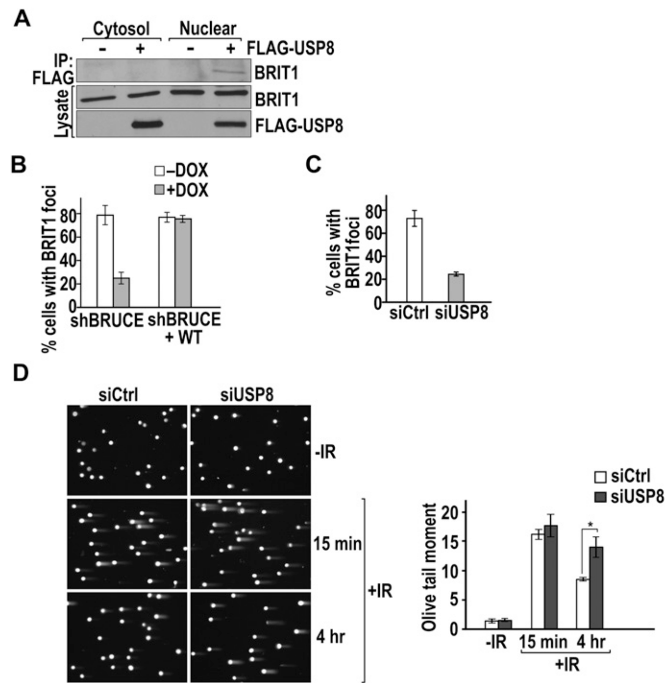


Fig. S2. (*A*) The binding of USP8 with BRIT1 is detected predominantly in the chromatin-containing nuclear fraction, although both also are present in the cytosol. Cytosolic and nuclear fractions were isolated from U2OS cells transfected with FLAG-USP8. USP8 was immunoprecipitated from both fractions, and the IP products were immunoblotted for BRIT1. (*B*) Quantification of the BRIT1 foci results presented in Fig. 2*E* showing that BRUCE depletion attenuates the formation of BRIT1 foci in U2OS cells. Error bars represent the SD of two independent experiments. (*C*) Quantification of the BRIT1 foci results presented in Fig. 2*F* showing that depletion of USP8 attenuates the formation of BRIT1 foci. Error bars represent the SD of two independent experiments. (*D*, *Left*) Comet assay results showing increased amount of DNA DSBs in USP8-knockdown cells compared with control 4 h after exposure to 5 Gy of IR. (*Right*) The combination of tail length and DNA content (Olive tail moment) as a measure of DNA damage according to the published method (8). *$P < 0.05$; Student's two-tailed *t* test.

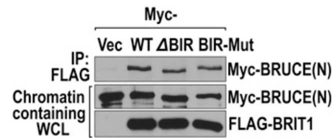


Fig. S3. The BIR domain is dispensable for BRUCE binding to BRIT1. U2OS cells were cotransfected with FLAG-BRIT1 and one of the three BRUCE constructs: BRUCE N (BRUCE fragment, amino acids 1–2025 ; see Fig. 3*A*), BRUCE N with BIR deleted (ΔBIR), or BRUCE N with BIR (BIR-Mut). BRIT1 was immunoprecipitated, and the IP products were immunoblotted for BRUCE (anti-Myc).

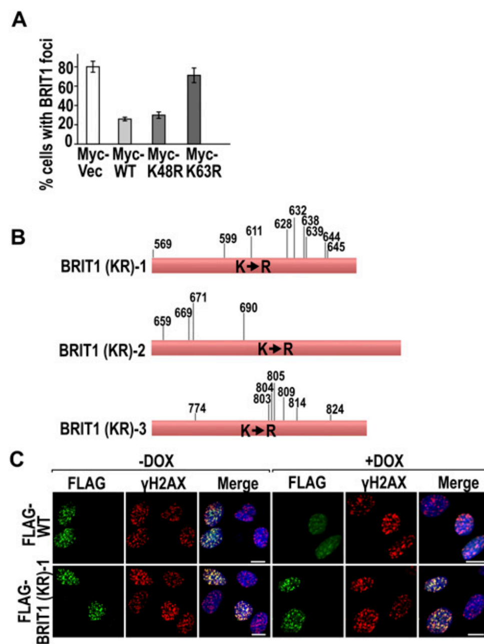


Fig. S4. (*A*) Quantification of BRIT1 foci results described in Fig. 4*G*; error bars represent the SD of two independent experiments. (*B*) Lysine-to-arginine mutations in BRIT1 (KR)-1, BRIT1 (KR)-2, and BRIT1 (KR)-3 variants. Numbers indicate the positions of mutated lysine. (*C*) Ubiquitination-resistant BRIT1 (KR)-1 formed IR-induced foci colocalizing with γ-H2AX. U2OS cells transfected with wild-type BRIT1 or BRIT1 (KR)-1 were irradiated and immunostained for BRIT1 (anti-FLAG, green) and γ-H2AX (red). (Scale bars, 10 μm.)

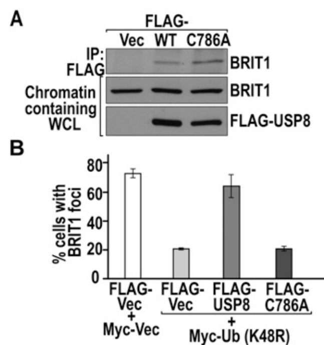


Fig. S5. (*A*) The USP8 catalytic mutant has slightly stronger binding than wild-type USP8 with BRIT1. U2OS cells were transfected with FLAG-USP8 (wild-type or C786A). After 48 h, IP of USP8 was performed in the chromatin-containing whole-cell lysates, and the products were immunoblotted for BRIT1. (*B*) Quantification of BRIT1 foci results presented in Fig. 5*F*. Error bars represent the SD of two independent experiments.

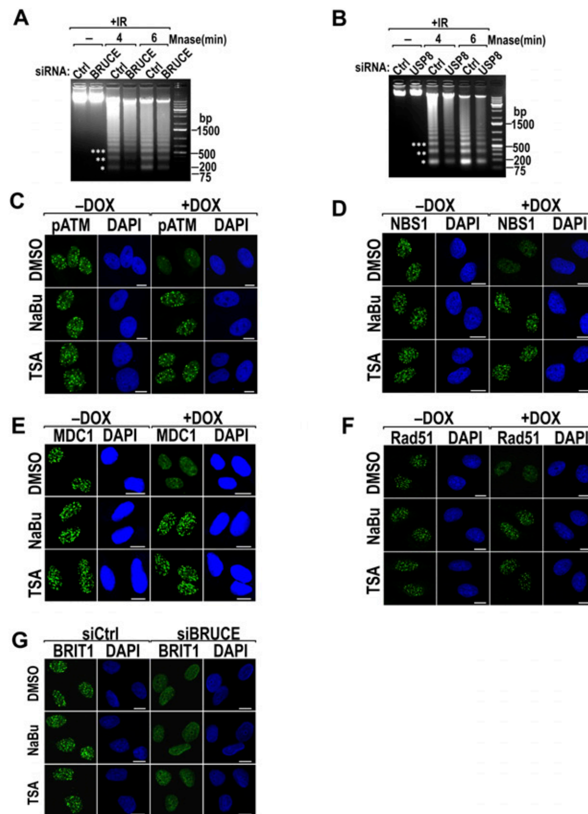
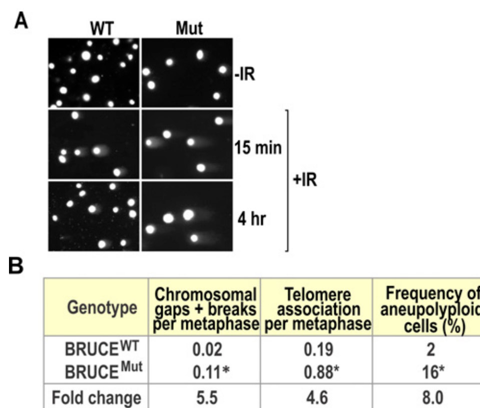


Fig. S6. (*A*) U2OS cells depleted of BRUCE by siRNA were irradiated (10 Gy), and after 30 min of continued culture the cells were subject to MNase digestion for 4 or 6 min or were not subjected to digestion (–). Chromatin relaxation was monitored by the release of nucleosomes. Mono(*), di-(**), and trinucleosomes (***) are indicated on the gel. (*B*) U2OS cells depleted of USP8 by siRNA were irradiated (10 Gy) and subject to MNase digestion as described in *A*. (*C–F*) Impaired DNA damage-induced foci formed by pATM (*C*), NBS1 (*D*), MDC1 (*E*), or Rad51 (*F*) are restored by incubation of the BRUCE-depleted cells (+DOX) with the chromatin-relaxing agents NaBu or TSA. (Scale bars, 10 μm.) (*G*) Impaired DNA damage foci formed by BRIT1 could not be restored by NaBu or TSA in BRUCE-depleted cells (Scale bars, 10 μm.)



Genotype	Chromosomal gaps + breaks per metaphase	Telomere association per metaphase	Frequency of aneupolyploid cells (%)
BRUCE^{WT}	0.02	0.19	2
BRUCE^{Mut}	0.11*	0.88*	16*
Fold change	5.5	4.6	8.0

Fig. S7. (*A*) Photographs of the MEF comet assay described in Fig. 7*A* showing sustained remaining DNA damage (longer and increased DNA content in the tail) in BRUCE mutant MEFs compared with wild-type MEFs 4 h after exposure to 5 Gy of IR. (*B*) Quantification and statistical analysis of the MEF telomere-FISH results shown in Fig. 7*B*, showing a 5.5-fold increase in chromosomal gaps and breaks, a 4.6-fold increase in telomere association, and an eightfold increase in aneupolyploidy per metaphase. *$P < 0.05$; χ^2 analysis.