Supporting Information

Feng et al. 10.1073/pnas.1010959107

SI Materials and Methods

Cells, Plasmids, and Transfections. MCF7, EUFA423, and HeLa cell lines were maintained in DMEM supplemented with 10% FBS, 20 mM Hepes, 100 μg/mL streptomycin, and 100 U/mL penicillin (Sigma). Capan-1 and HCC1937 cell lines were cultured in Iscove's modified Dulbecco's medium (Sigma) with 15% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine (1). The EUFA423/pcDNA3 and EUFA423/wtBRCA2 cell pair (Alan D. D'Andrea, Dana-Farber Cancer Institute, Boston, MA) (2) was cultured in complete DMEM containing 500 μg/mL G418 (Fisher). The HA-BRCA2 plasmid was a gift from Mien-Chie Hung (The M. D. Anderson Cancer Center, Houston, TX). The GFP-Rad52 plasmid was obtained from Zhimin Yuan (Harvard School of Public Health, Boston, MA) (3). All plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. To establish the Capan-1/Rad52 cell line, Capan-1 cells were transfected with the HA-tagged Rad52 plasmid (4) or the vector control plasmid (pcDNA3) using Lipofectamine 2000. Selection medium containing 750 μg/mL G418 was added to the cells 48 h posttransfection, and selection was continued for 7–10 d to obtain cell populations containing Capan-1/HA-Rad52 and Capan-1/pcDNA3. Cells were maintained in medium containing 500 μg/mL G418.

Rad52 and BRCA2 Knockdown. For the transient depletion of Rad52 in EUFA423 cells, a mixture of two siRNAs for Rad52 was used: siGENOME Duplex 3 or Duplex 4 (Dharmacon) mixed with Silencer siRNA (142431; Ambion). The control scramble siRNA (siGENOME Non-Targeting siRNA Pool #1) was obtained directly from Dharmacon Inc. The final working concentration for each siRNA used was 10 nM. All siRNA transfections were performed with Lipofectamine 2000 (Invitrogen). For stable knockdown, lentiviral particles expressing shRNA (Ambion), which target the same RNA sequence of human Rad52 as the Silencer siRNA, were used to infect EUFA423 cells, using a 1:1 mixture of virus stock and fresh culture medium to a final concentration of 8 μg/mL Polybrene (Millipore). The infected cells were maintained by growth in 300 μg/mL hygromycin. Control shRNA infections were used containing nontarget shRNA (SHC002V; Sigma Chemical).

Microscopy and Immunofluorescence. Cells were seeded into four-chamber tissue culture slides (Fisher) and incubated overnight. Adherent cells were treated with ionizing radiation using a Siemens Stabilipan-2 X-ray generator at 280 kVp, 12 mA, and a dose rate of 2.08 Gy/min. For BrdU treatment, cells were incubated with 25 μM of BrdU (Sigma) for 2 h and then were subjected to immunocytochemical staining. Cells were stained by fixing in 10% formaldehyde solution at room temperature for 30 min, followed by 10-min permeabilization in PBS containing 0.5% Triton X-100. All cells were washed in PBS, followed by PBS with 10% FBS to block nonspecific binding by antibody. Cells containing GFP-tagged Rad52 were visualized directly after fixation and permeabilization. Rad51 was detected by immunofluorescence using anti-Rad51 antibody Ab-2 (CalBiochem) at a 1:250 dilution. BrdU was detected using anti-BrdU monoclonal antibody (BD Biosciences) at a 1:10 dilution. Replication protein A2 (RPA2) was detected by anti-RPA2 monoclonal antibody (CalBiochem) at a 1:75 dilution. The secondary antibodies used were Alexa Fluor 594-labeled goat anti-mouse IgG (Molecular Probes) and Alexa Fluor 488-labeled chicken anti-rabbit IgG (Molecular Probes), each at a 1:500 dilution. Images were obtained using an Olympus BX51 microscope and processed using Adobe PhotoShop software.

Immunoblotting. Cells were irradiated with 10 Gy and incubated for various periods of time as indicated. Protein samples were loaded into polyacrylamide gels and subjected to SDS/PAGE electrophoresis. To detect the protein of interest, the membranes were incubated with an anti-Rad52 antibody [5H9 (GeneTex) diluted 1:500 in PBS, 3% milk], anti-BRC repeat region of :breast cancer 2, early onset (BRCA2) antibody [Ab-1 (CalBiochem) diluted 1:100 in PBS, 3% milk], anti-C terminus of BRCA2 antibody [Ab-2 (CalBiochem) diluted 1:100 in PBS, 3% milk], or anti-human β-actin diluted 1:2,000 in PBS, 3% milk (Sigma). Goat anti-mouse IgG [heavy and light chain (H&L)] and goat anti-rabbit IgG (H&L) labeled with HRP conjugate were used for secondary antibodies. Bands were detected using the ECL chemiluminescence detection method (Amersham Biosciences) and exposure on X-ray film.

Cell Number and Plating Efficiency. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the rate of cell proliferation in EUFA423 cells, treated either with control or Rad52 siRNA, and Capan-1 cells corrected with either HA-Rad52 plasmid or vector alone. Cells were seeded in a 24-well plate at a density of 1×10^4 cells/mL per well. At various time points, the MTT reagent (Sigma) was added, and the cells were incubated for a further 4 h before cell growth was measured by spectrophotometry (Biomate 3; Thermo Electron Corporation). Plating efficiency was measured in MCF7 cells by seeding $1 \times$ and 5×10^2, 10^3, and 10^4 cells into 6-cm dishes, with the total cell count made up to 5×10^4 with heavily irradiated feeder cells to maintain a constant number of plated cells. Colonies (>50 cells) were counted by fixing and staining with crystal violet.

1. Wang SC, et al. (2002) Inhibition of cancer cell growth by BRCA2. *Cancer Res* 62: 1311–1314.
2. Howlett NG, et al. (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* 297:606–609.
3. Kitao H, Yuan ZM (2002) Regulation of ionizing radiation-induced Rad52 nuclear foci formation by c-Abl-mediated phosphorylation. *J Biol Chem* 277:48944–48948.
4. Lau A, Kanaar R, Jackson SP, O'Connor MJ (2004) Suppression of retroviral infection by the RAD52 DNA repair protein. *EMBO J* 23:3421–3429.

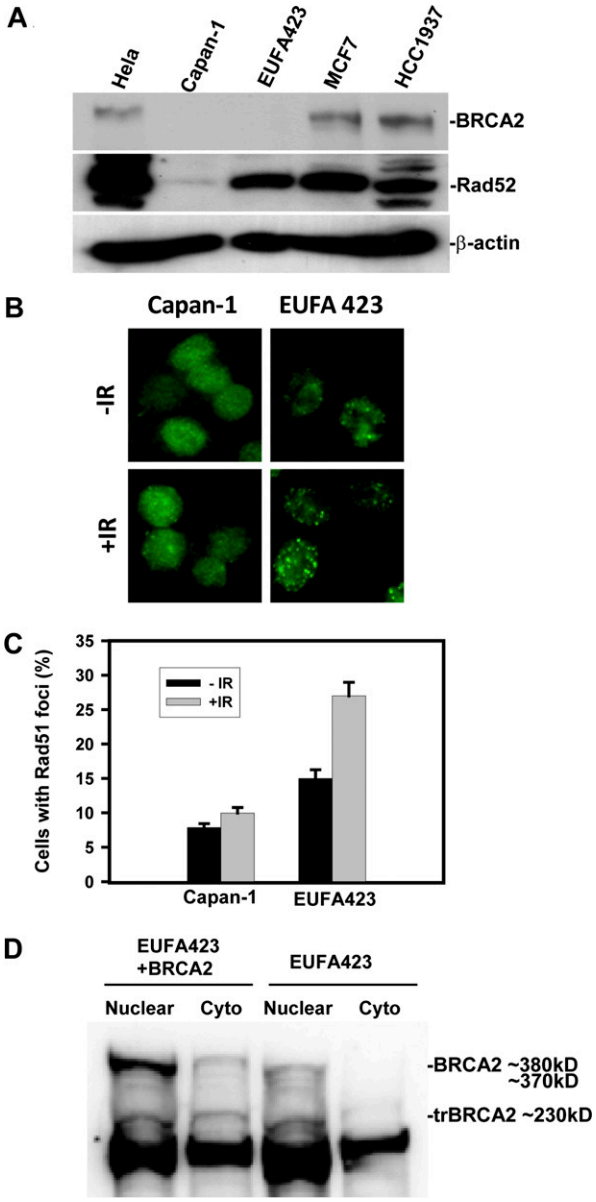

Fig. S1. Rad52 expression and Rad51 nuclear foci in BRCA2-defective cells. (*A*) Rad52 expression levels in whole-cell extracts isolated from BRCA2-positive cells (HeLa, MCF7, and HCC1937) and from BRCA2-deficient cells (EUFA423, Capan-1) subjected to SDS/PAGE and immunoblotted for BRCA2 and Rad52. A β-actin probe was used as a loading control. (*B*) The formation of spontaneous or ionizing radiation-induced Rad51 foci in two BRCA2-defective cell lines. EUFA423 and Capan-1 cells were treated with 10 Gy of ionizing radiation or were sham treated and were incubated for 8 h before imaging Rad51. (*C*) The percentages of EUFA423 and Capan-1 cells containing more than five Rad51 foci after treatment with ionizing radiation. More than 500 cells were counted per experiment. Bars represent means of three independent experiments; error bars indicate SE. (*D*) BRCA2 protein observed in EUFA423 cells by immunoblotting contains low levels of the 230-kDa and 370-kDa truncated proteins, which are seen in the cytoplasmic and nuclear fractions. Complementation with wild-type BRCA2 shows an increase in the nuclear level of full-length (380-kDa) protein.

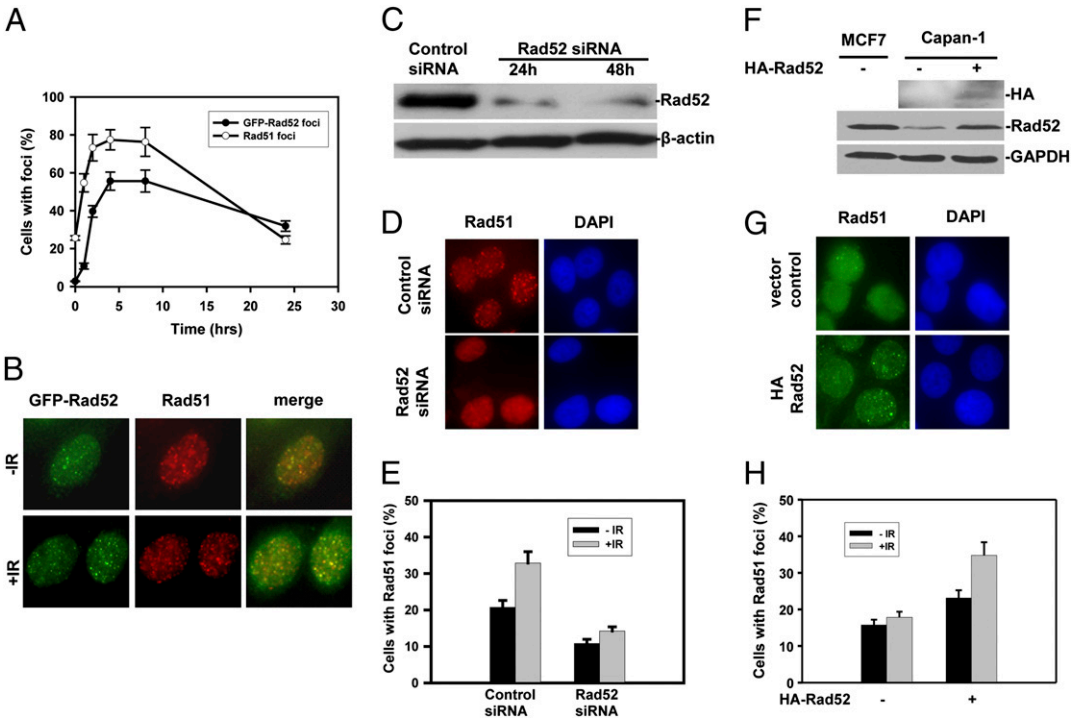

Fig. S2. Regulation of Rad51 nuclear foci by Rad52 in BRCA2-defective cells. (*A*) GFP-Rad52-MCF7 cells were exposed to 10-Gy ionizing radiation, and the percentage of cells containing five or more Rad51 and Rad52 foci were examined over a 24-h period. Open and closed circles represent the percentage of cells exhibiting Rad51 and GFP-Rad52 foci, respectively. Each data point represents the results (mean ± SE) from three independent experiments. (*B*) MCF7 cells expressing GFP-Rad52 were irradiated with 10 Gy, incubated for 8 h, and then subjected to immunofluorescence studies using anti-Rad51 antibody. (*C*) EUFA423 cells were treated with either control siRNA or Rad52-siRNA. Whole-cell extracts were analyzed by immunoblotting using anti-Rad52 and anti-β-actin antibodies. (*D*) Cells as in *C* showing spontaneous Rad51 foci. (*E*) Cells as in *C* exposed to 10-Gy ionizing radiation or sham treatment and incubated for 8 h. After incubation, Rad51 foci were scored by counting more than 500 cells per experiment. Bars represent means ± SE and are based on three independent experiments. Statistical comparisons between control and Rad52 siRNA, with or without ionizing radiation, are significant ($P < 0.0001$; Fisher's exact test). (*F*) Capan-1 cells transfected with HA-tagged Rad52 or vector alone and analyzed using an anti-HA antibody. MCF7 cells are shown as a control for normal Rad52 levels. (*G*) Cells as in *F* showing spontaneous Rad51 foci. (*H*) Cells as in *F* exposed to 10-Gy ionizing radiation or sham treated and incubated for 8 h; Rad51 foci were determined as previously. Statistical comparison between no exogenous Rad52 and Rad52 expression: $P = 0.0004$ (without ionizing radiation) and $P < 0.0001$ (with ionizing radiation) by Fisher's exact test.

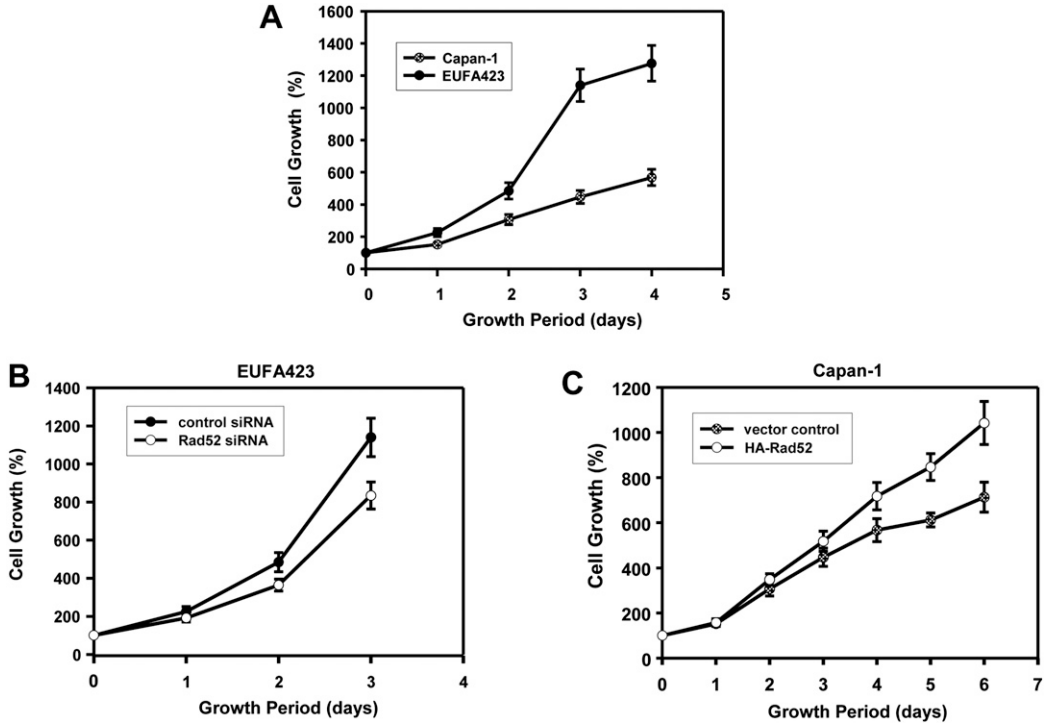

Fig. S3. Cellular proliferation is impaired in cells deficient in both BRCA2 and Rad52. (*A*) MTT cellular proliferation assay of Capan-1 and EUFA423 cell lines over a 4-d period. (*B*) EUFA423 cells were transiently transfected with control or Rad52 siRNA; 24 h later the cells were replated and subjected to the MTT assay as in *A*. (*C*) Capan-1 cells were stably transfected with HA-tagged Rad52 or empty vector and then counted by the MTT assay over a 6-d period. In all panels, the data are the average of three independent experiments (± SE).

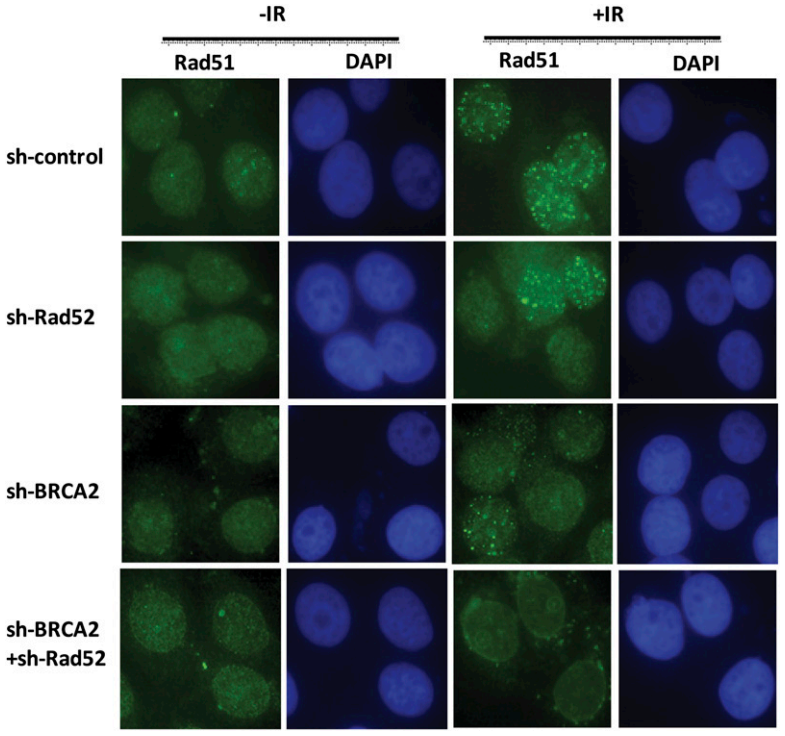

Fig. S4. Rad51 nuclear foci in MCF-7 cells with depletion of Rad52, BRCA2, or both. Representative examples of cells from four different genetic conditions achieved by shRNA, with or without exposure to ionizing radiation (10 Gy). Ionizing radiation-induced Rad51 foci (green) are observed in normal numbers in control shRNA and sh-Rad52 but are reduced in BRCA2-depleted cells and are almost undetectable in double-depleted cells. DAPI staining confirms the location of the cell nuclei.

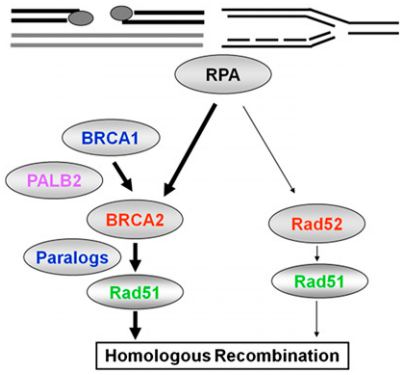

Fig. S5. Two independent pathways of Rad51-dependent homologous recombination (HR) control synthetic lethality. Double-stranded breaks in mammalian cells in S and G2 phases of the cell cycle are repaired preeminently by the BRCA2-Rad51 pathway of HR. However, in the absence of functional BRCA2, Rad52 can be used for their repair. The role of the Rad51 paralogs, some of which can be identified in yeast, is not known in relation to these two pathways. Some species have both pathways, some (*Drosophila*) have only the BRCA2-like pathway, and some (*Saccharomyces cerevisiae*) have only the Rad52 pathway. PALB2, partner and localizer of BRCA2.

Table S1. Summary of protein levels in each of the cell types used in this study

Cell type	BRCA2	Rad52	Rad51	RPA	BRCA1
Capan-1	No nuclear; total low	Low	Normal	Normal	Normal
Capan-1/BRCA2	Expressed and nuclear	Low	Normal	Normal	Normal
EUFA423	Two truncated proteins: 370 kDa and 230 kDa, both at low levels, limited nuclear BRCA2	Normal	Normal	Normal	Normal
EUFA423/BRCA2	Incremental expression of 380-kDa protein, which is detectable in nucleus	Normal	Normal	Normal	Normal
MCF7/control	Normal	Normal	Normal	Normal	Normal
MCF7/shRad52	Normal	<10%	Normal	Normal	Normal
MCF7/shBRCA2	<10%	Normal	Small reduction	Normal	Normal
MCF7/shRad52 + shBRCA2	<10%	<10%	Small reduction	Normal	Normal

Note that BRCA1 protein was not influenced by changes in BRCA2 or Rad52 protein. An acute reduction in BRCA2 does result in a small but detectable reduction in Rad51 protein. RPA is an abundant protein whose level is not altered by changing BRCA2 and Rad52 protein levels.