Supporting Information

Fan et al. 10.1073/pnas.1308206110

SI Methods

DNA Repair and Apoptosis Assays. *Comet assays.* After the indicated treatment, cells were incubated at 37 °C for different times and harvested for comet assays. Assays were performed as described (1), except that neutral lysis followed by neutral gel electrophoresis were used to detect DSBs. Agarose gel electrophoresis was carried out at 20 V and 300 mA for 20 min at 25 °C. The slides were stained with ethidium bromide (20 μg/mL), and the comet tail length was visualized by fluorescence microscopy. Fifty digitized images were analyzed per data point for duplicate slides in each experiment. Comets were scored by using a 40× objective on a Nikon Eclipse E800 fluorescence microscope. Experiments were repeated independently at least three times, and the results from representative experiments are shown.

DNA strand-rejoining assay. After the indicated cell treatment, nuclear lysates were prepared, and aliquots of lysate (75 μg of protein) were tested for their ability to rejoin a plasmid (pEGFP-1; Clontech) that had been linearized by using restriction enzyme SmaI (2). Reaction products were detected by Southern blotting, using a digoxigenin-labeled enhanced green fluorescent protein RNA probe. The percent of rejoined plasmids was determined by densitometry of bands for rejoined and unrejoined plasmid. The image shown is typical of three independent experiments.

Apoptosis assays. Apoptotic and necrotic cells were distinguished by analysis of phosphatidyl-serine on the outer leaflet of apoptotic cell membranes by using annexin V and propidium iodide staining. After the indicated treatment, cells (1×10^6/mL) were stained with annexin V-FITC (catalog no. 1013–1000) and propidium iodide (catalog no. 1056–1), according to the manufacturer's instructions (BioVision). To each tube, 500 μL of annexin binding buffer (catalog no. 1006–100; BioVision) was added and the stained cells were analyzed by using a flow cytometer (Becton Dickinson). The experiment shown is representative of three independent experiments.

Protein Phosphatase 2A Assay. After the indicated treatment, whole cell lysates were assayed for protein phosphatase 2A (PP2A) enzymatic activity by using a PP2A Immunoprecipitation Phosphatase Assay Kit (catalog no. 17–313; Millipore), according to the manufacturer's instructions. Briefly, lysates were immunoprecipitated by using antibody against the catalytic subunit of PP2A or nonimmune IgG as a negative control. The precipitates were tested for dephosphorylation of a phosphopeptide substrate (K-R-pT-I-R-R). Phosphate release was quantified by a colorimetric assay using malachite green. Values plotted are means ± SEMs of triplicate wells.

NF-κB Luciferase Assay. The 184A1 cells were cotransfected overnight with NF-κB–responsive reporter plasmid pNF-κB-Luc (BD Biosciences) and control plasmid pRSV-β-gal (to control for transfection efficiency) by using Lipofectamine reagent (Invitrogen), according to the manufacturer's instructions. Cells were then treated without or with DIM (0.3 mM) and without or with the selective cell permeant NF-κB activation inhibitor CAS 545380-34-5 (15 nM) for 4 h. The cells were then irradiated (6 Gy) or sham-treated, postincubated for another 4 h, and harvested for luciferase measurements. Luciferase activity was expressed relative to control conditions (0 DIM, 0 radiation, 0 inhibitor) and normalized by β-galactosidase activity to control for variations in transfection efficiency. Three independent experiments were carried out, each using triplicate wells for each assay condition. NF-κB inhibitor CAS 545380-34-5 was dissolved in DMSO and made to a stock solution of 10 mM before dilution into culture medium.

Statistical Methods. Animal survival was plotted by using Kaplan–Meier statistics, and survival curves were compared by using the log-rank test. Other comparisons were made by using two-tailed *t* tests, as appropriate. Dose-modifying factor values were calculated by Probit analysis.

Reagents. A formulation of 3,3′-diindolylmethane [BioResponse (BR)-DIM] was used in these studies. BR-DIM is a microencapsulated preparation with greater oral bioavailability than crystalline DIM (7). BR-DIM was provided by Michael Zeligs (BioResponse) or purchased from Nanjing R&M Pharm-Chem. The ATM inhibitor KU55933 was purchased from SelleckBio.com. Dimethyl sulfoxide (DMSO) and okadaic acid were purchased from Sigma-Aldrich.

Radiation Survival Curves. After the indicated treatment, subconfluent proliferating cells were irradiated with different doses of γ radiation by using a cesium-137 irradiator (Mark 1–68; J.L. Shepherd and Associates) (Georgetown University) at approximately 0.86 Gy/min. The cells were harvested and plated at different densities ± DIM for clonogenic survival assays (32). Values of surviving fraction are means ± SEMs of triplicate plates.

siRNA Treatment. ATM-siRNA (sc-29761) and control-siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. Cells were treated with ATM-siRNA or control-siRNA by using siRNA Transfection Reagent (Santa Cruz). The cells were exposed to the indicated siRNA (50 nM) for 48 h, and the knockdown was confirmed by Western blotting.

Western Blotting. Western blotting was performed as described (25, 43, 44). The primary antibodies were as follows: rabbit polyclonal anti-BRCA1 (sc-642; Santa Cruz); mouse monoclonal α-actin (sc-1616; Santa Cruz); anti–phospho-BRCA1 (S1387) (AB3257; Millipore); mouse monoclonal anti-ATM (sc-73615; Santa Cruz); anti–phospho-ATM (S1981) (Rockland Immunochemicals); mouse monoclonal anti-CHK2 (05-649; Millipore); anti–phospho-CHK2 (T68) (ab32055, rabbit monoclonal; Abcam); anti-p53 (ab2433, rabbit polyclonal; Abcam); anti–phospho-p53 (S15) (ab38497, rabbit polyclonal; Abcam); anti-NBS1 (ab23996; Abcam); anti-phospho-NBS1 (S343; Cell Signaling); anti-CHK1 (ab2845; Abcam); anti–phospho-CHK1 (S345) (ab47574; Abcam), anti–phospho-SMC1 (S957) (4801, rabbit polyclonal; Cell Signaling), and anti-SMC1 (4802, rabbit polyclonal; Cell Signaling). For mouse CHK1, the antibodies used were as follows: anti–phospho-CHK1 (2348, rabbit polyclonal; Cell Signaling) and anti-CHK1 (2360, mouse monoclonal; Cell Signaling). For tissue samples, protein extracts were prepared by using the TRI Reagent Protein Isolation Protocol (Life Technologies).

Animals and Treatments. Animals were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, which serves as the National Rodent Laboratory Animal Center, Shanghai Branch. All studies were performed at the Animal Laboratory Center, Soochow University Medical School, Suzhou, China. All animal protocols were approved by the Soochow University Animal Research Ethics Committee, and all experiments followed the Chinese Institutional Animal Care and Use Committee guidelines. Radioprotection/mitigation studies used female Sprague–Dawley rats (age 12–16 wk, weight 200 ± 30 g).

Rats were quarantined for at least 7 d before irradiation, housed 10 to a cage in a windowless laboratory room with automatic temperature control (22 ± 1 °C) and lighting control (12-h light/12-h dark), and fed standard laboratory chow and water ad libitum. Rats were randomly divided into experimental and control groups, with 20 animals per group. Animals were administered DIM or vehicle (DMSO in PBS, see below) by i.p. injection at the dose and schedule indicated, subjected to TBI by using Co-60 γ rays (0.96 Gy/min), and monitored for survival for 30 d. Animals that appeared moribund or died were considered not to have survived. DIM was dissolved in a small volume of DMSO and diluted to a final DMSO concentration of 0.2% in PBS. Five hundred microliters of DIM solution or vehicle was injected per rat.

s.c. Injection. In some experiments, DIM was delivered by s.c. injection on rat flanks or backs. As above, DIM was dissolved in DMSO and diluted to 0.2% DMSO in PBS. Control animals received the same injections without (see Fig. 1 for details).

Tumor Studies. For tumor studies, we used 8-wk-old female athymic (nu/nu) Swiss mice (weight approximately 20 g). To generate tumors, subconfluent proliferating MDA-MB-231 human breast carcinoma cells were harvested by using trypsin, counted, and prepared to a concentration of 2×10^7 cells/mL in a 1:1 mixture of serum-free DMEM and Matrigel (BD Biosciences). Tumor xenografts were initiated by injection of 0.2 mL of cell suspension (4×10^6 cells) into each flank (two injections per mouse). Two groups of mice were used, one of which received DIM and the other of which received vehicle only. Irradiation was performed by using a lead shield so that one flank was irradiated and the other flank and the rest of the mouse was shielded. The doses and schedules for administration of DIM and radiation are provided in the figure legend.

Radiation Source for Animal Studies. Animals received TBI or partial body irradiation by using a cobalt-60 γ radiation source (MDS Nordion) at the Applied Key Laboratory of Radiation Biology, Soochow University Medical School. The dose rate was 0.96 Gy/min. Unanesthetized animals were restrained in well-ventilated Perspex boxes and exposed to radiation at a distance of 85.5 cm from the source. For tumor irradiation, a lead shield was used (see above).

1. Martin FL, et al. (1997) DNA damage in breast epithelial cells: Detection by the single-cell gel (comet) assay and induction by human mammary lipid extracts. *Carcinogenesis* 18(12):2299–2305.

2. Müller-Tidow C, et al. (2004) The cyclin A1-CDK2 complex regulates DNA double-strand break repair. *Mol Cell Biol* 24(20):8917–8928.

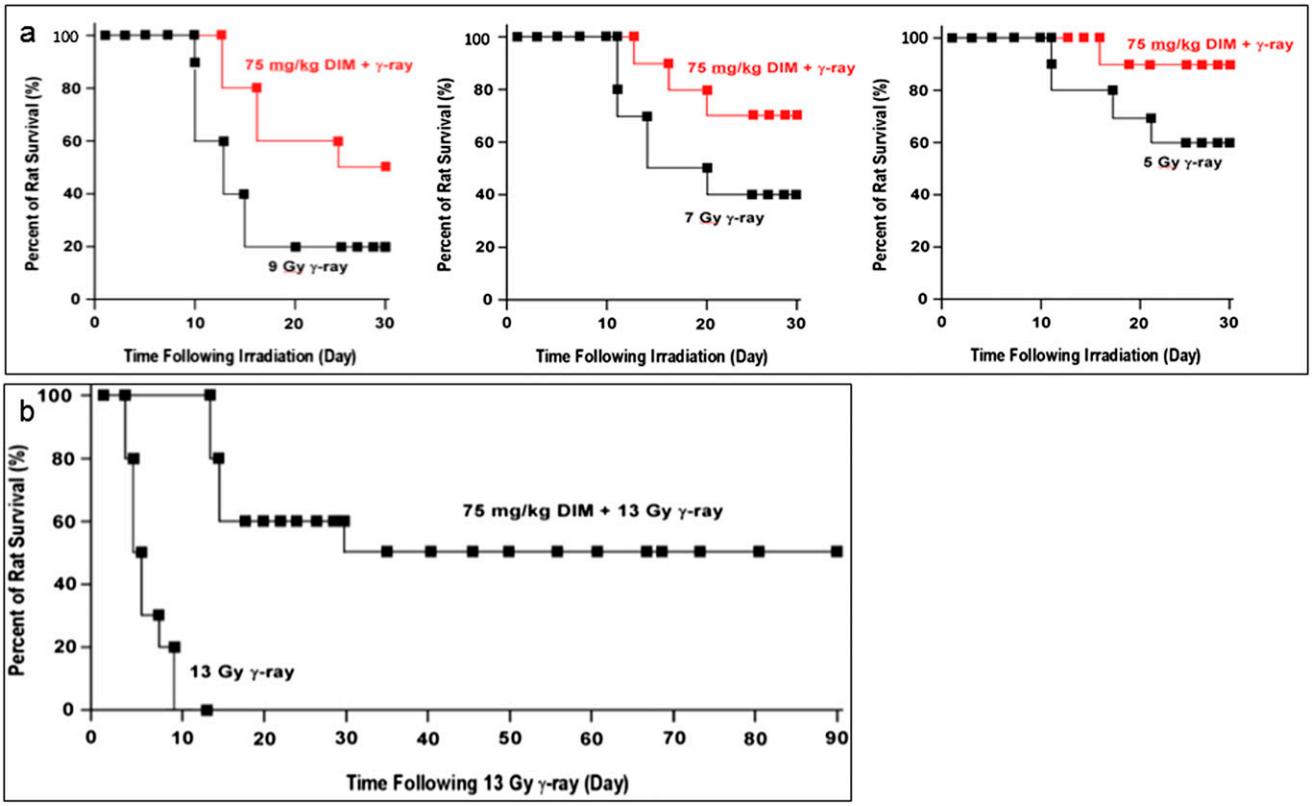

Fig. S1. Further studies on DIM mitigation of radiation injury. (*A*) DIM (3,3′-diindolylmethane) administered starting 24 h after total body irradiation (TBI). DIM (75 mg/kg) or vehicle were administered to SD rats by i.p. injection once per day for a total of 14 d starting 24 h after irradiation. The radiation-only groups were given the same injection schedule except with vehicle only. Twenty animals per group were used for each experimental and control group. (*B*) DIM administered starting 10 min after TBI. Experiments were carried out as described above, using a TBI dose of 13 Gy, except that the first DIM treatment was given 10 min after TBI and the rats were followed for up to 90 d.

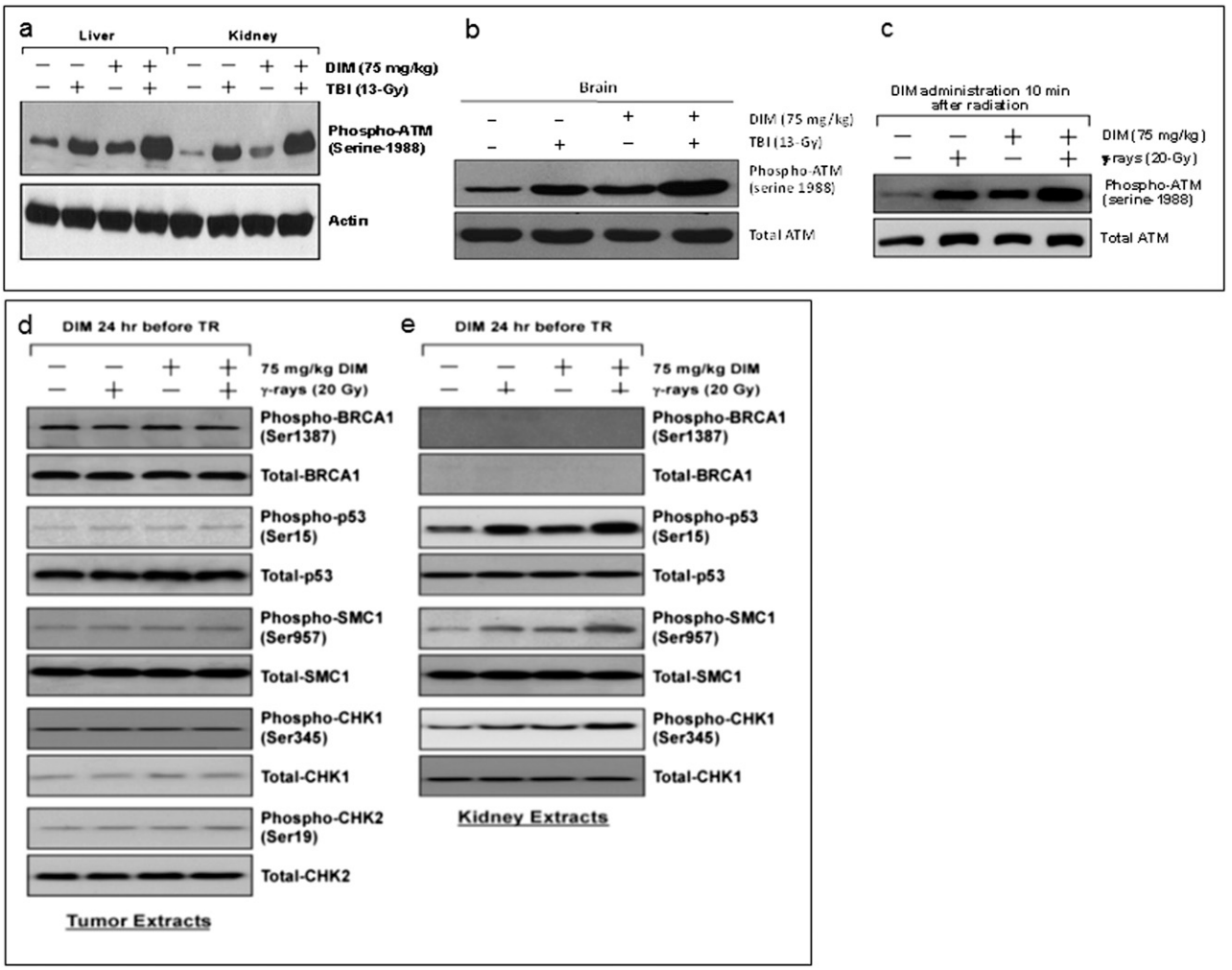

Fig. S2. DIM activates ATM in vivo. (*A*). Sprague–Dawley rats were treated without (–) or with (+) DIM (75 mg/kg) 24-h before sham treatment (–) or TBI (+). At 24 h after TBI, the animals were killed and the indicated tissues were extracted for Western blotting to detect phospho-ATM and actin. (*B*) Rats were treated without (–) or with (+) DIM (75 mg/kg) 1 h before sham treatment (–) or TBI (+). At 24 h after TBI, the animals were killed and the brain tissue was extracted for Western blotting for phospho-ATM or total ATM. (*C*) Rats were sham treated (–) or treated with TBI (20-Gy) (+) and administered vehicle (–) or DIM (+) at 10 min after radiation. The animals were killed 24 h after irradiation, and the kidneys were extracted for Western blotting as above. phospho-ATM (S1988) in rat corresponds to phospho-ATM (S1981) in humans. (*D*) Mice were treated as described in Fig. 2*C*, and tumor extracts were Western blotted for the indicated proteins. (*E*) Mice were treated as described in Fig. 2*D*, and tumor extracts were Western blotted for the indicated proteins.

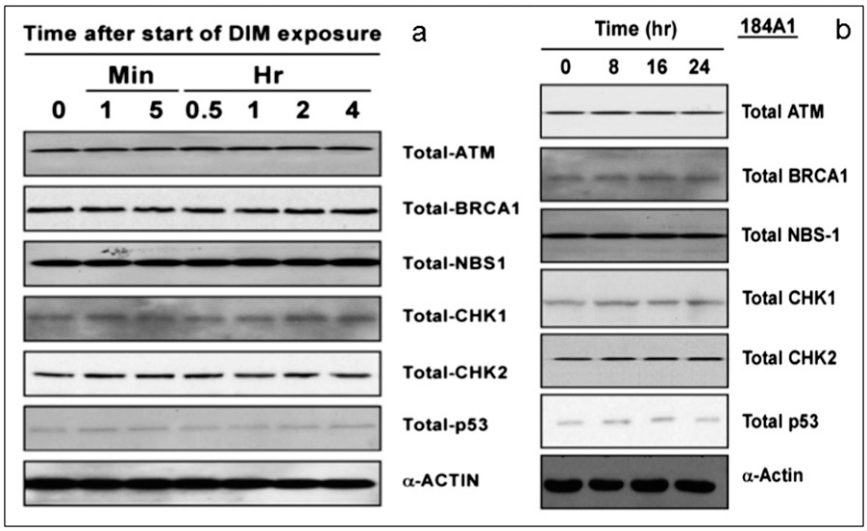

Fig. S3. Time course of DIM-induced signaling: control studies. Subconfluent proliferating 184A1 human mammary epithelial cells were exposed to DIM (0.3 μM) for different time intervals and harvested for Western blotting to detect the total levels of ATM and different DNA damage-response proteins, as indicated. *A* and *B* correspond to Fig. 4 *C* and *D*, respectively.

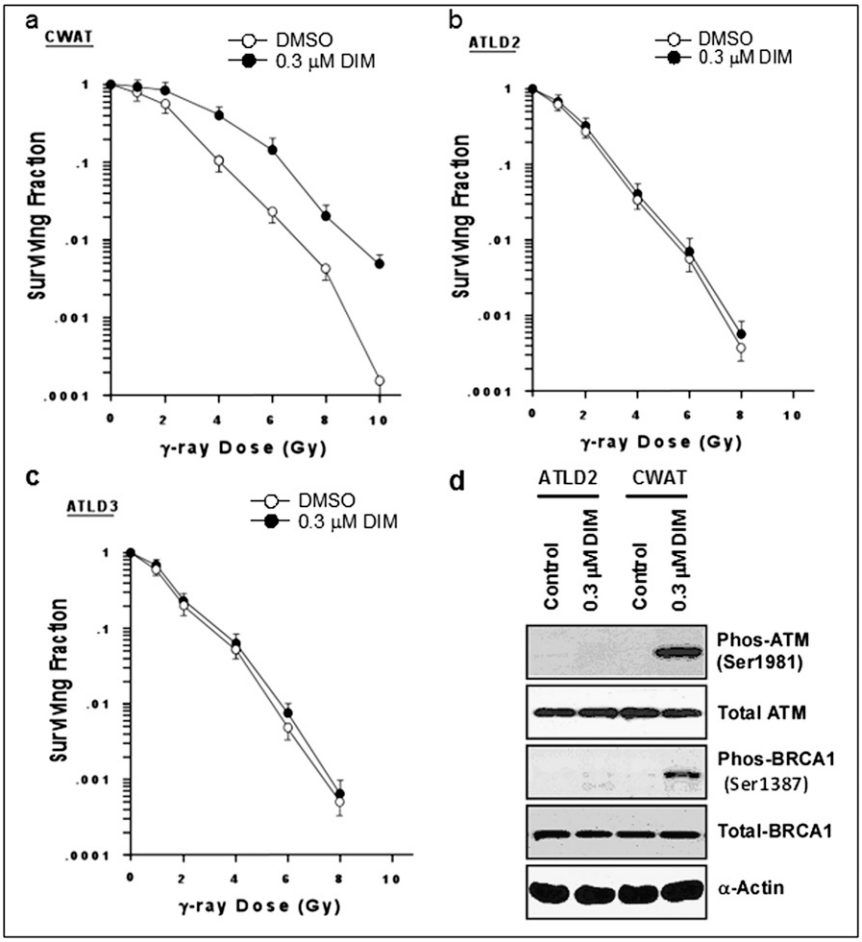

Fig. S4. DIM protects wild-type but not Mre11 mutant human dermal fibroblasts. (*A*–*C*) Subconfluent proliferating wild-type (CWAT) (*A*) or Mre11 mutant [ATLD2 (*B*) and ATLD3 (*C*)] human skin fibroblasts were treated with DIM (0.3 μM) or vehicle (DMSO) for 24 h, irradiated with different doses of ^{137}Cs γ rays, and harvested for clonogenic survival assays. Cell survival values are means ± SEMs of three replicate dishes. (*D*) Wild-type or Mre-11 mutant (ATLD2) fibroblasts were treated with DIM (0.3 μM) or vehicle for 30 min and then subjected to Western blotting to detect phospho-ATM (S1981), total ATM, phospho-BRCA1 (S1387), total BRCA1, or actin (loading control).

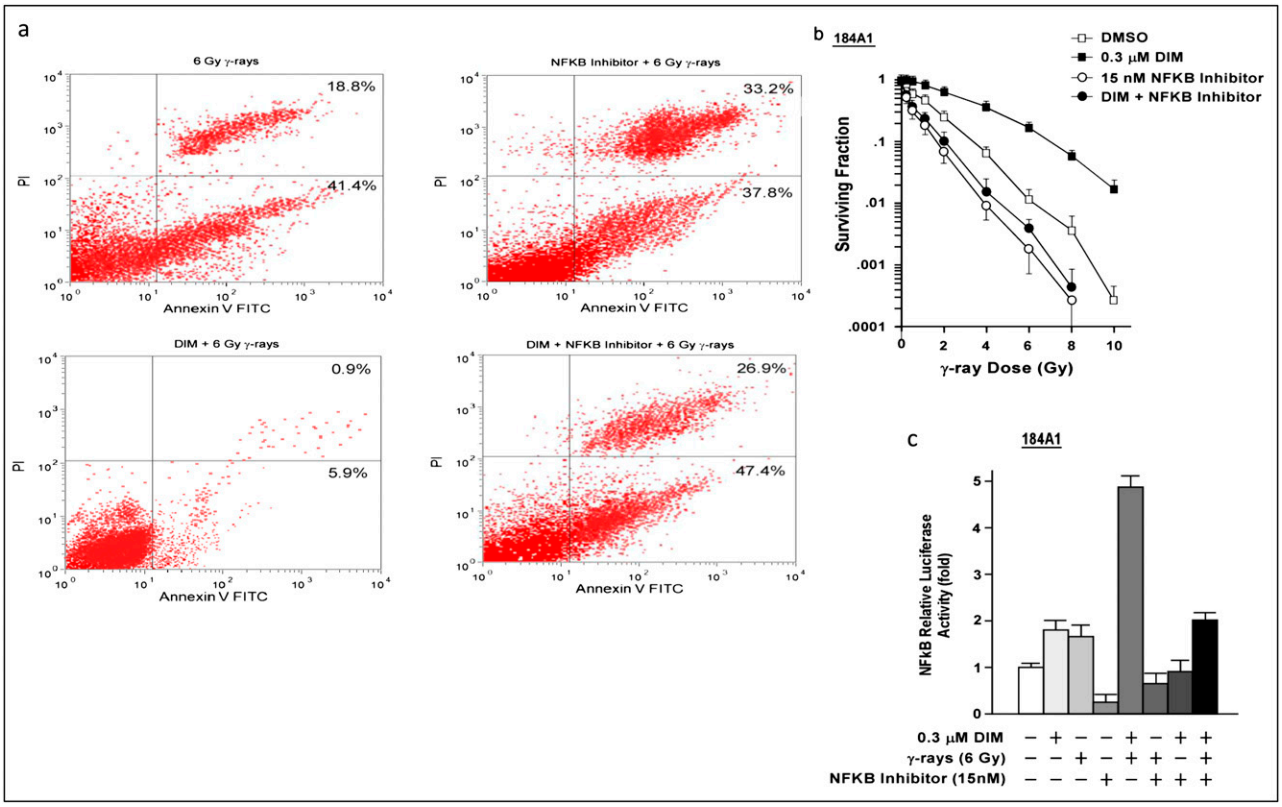

Fig. S5. Role of NF-κB in DIM-mediated radioprotection of 184A1 cells. (*A*) Apoptosis assays were performed as described in Fig. 6*D* except that the cells were incubated ±DIM (0.3 μM) in the absence (*Left*) or presence (*Right*) of the selective NF-κB inhibitor CAS 545380-34-5 (15 nM). (*B*) Radiation survival curves were determined as described in Fig. 3*A* except that pretreatment with ± DIM was carried out in the absence or presence of NF-κB inhibitor CAS 545380-34-5 (15 nM). (*C*) Cells were cotransfected with NF-κB–responsive luciferase reporter plasmid (pNFκ-B-Luc) and plasmid pRSV–β-gal (an internal control) overnight by using Lipofectamine. Cells were then treated ± DIM (0.3 mM) and ± NF-κB inhibitor (15 nM) for 4 h before irradiation at 6 Gy or sham treatment and then harvested for luciferase assays after a 4-h postirradiation incubation. Values are means ± SEMs of three independent experiments.

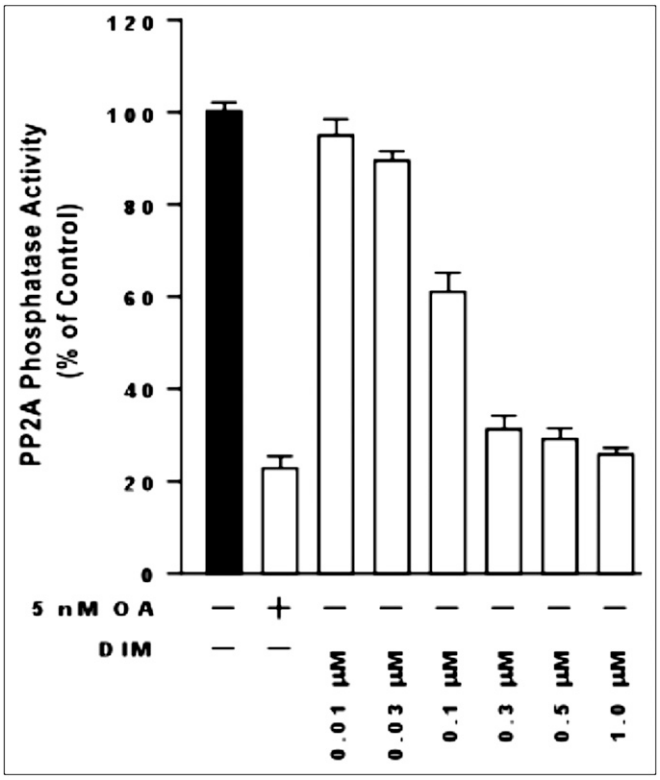

Fig. S6. DIM inhibits PP2A activity. Subconfluent proliferating 184A1 cells were treated with different concentrations of DIM for 30 min and then harvested for mmunoprecipitation (IP)-phosphatase assays, using a commercial assay kit (Millipore). IP was performed by using an antibody to the catalytic subunit of PP2A, and phosphatase activity was determined by using a synthetic phosphopeptide substrate (KR-pT-IRR), via a colorimetric assay. A selective pharmacologic inhibitor of PP2A (okadaic acid, OA) was used as a control.

Table S1. Variation in hematological parameters of mice treated with or without 75 mg/kg DIM and/or exposed to γ rays

Treatment	WBC, 10^9/L	RBC, 10^5/L	Hemoglobin, g/L	Platelets, 10^9/L	Hematocrit, %
Control	8.69 ± 0.65	9.92 ± 0.57	157 ± 8	989 ± 150	45.8 ± 0.7
75 mg/kg DIM	8.81 ± 0.63	9.83 ± 0.61	154 ± 9	987 ± 151	45.5 ± 0.8
2 Gy γ rays	6.36 ± 0.60*	7.65 ± 0.72*	128 ± 8*	903 ± 149	33.4 ± 0.7*
DIM + γ rays	7.86 ± 0.43†	9.74 ± 0.76†	145 ± 8†	922 ± 154	40.3 ± 0.9†
4 Gy γ rays	3.45 ± 0.65*	6.87 ± 0.87*	89 ± 8*	679 ± 153*	26.5 ± 0.7*
DIM + γ rays	6.97 ± 0.67†	8.93 ± 0.43†	130 ± 8†	881 ± 147†	41.9 ± 0.5†
6 Gy γ rays	3.01 ± 0.75*	4.23 ± 0.87*	72 ± 8*	466 ± 158*	21.6 ± 0.8*
DIM + γ rays	7.15 ± 0.64†	7.84 ± 0.66†	136 ± 8†	844 ± 147†	40.4 ± 0.7†

Mice were divided into different groups of 10 animals each and given 75 mg/kg DIM for 5 consecutive days. Twenty-four hours after the first injection the animals were exposed to different doses of TBI (γ rays). Blood was collected from the tail vein in a vial containing 0.5 M EDTA at 10 d after irradiation. Total WBC, RBC, hemoglobin, platelets, and hematocrit were determined by standard procedures. Values are means ± SDs.

*Control vs. γ rays, $P < 0.05$.

†γ rays vs. DIM + γ rays, $P < 0.05$.