Supporting Information

Otterbein et al. 10.1073/pnas.1102295108

SI Materials and Methods

Immunoblotting. Cells or tissues were lysed by a freeze-thaw cycle in ice-cold lysis buffer [1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM NaF] and sonication in the presence of a protease inhibitor mixture (Roche). Samples were centrifuged for 20 min at 14,000 × *g* at 4 °C, and the supernatants were collected. Protein concentration was measured using a BCA Protein Assay Kit (Pierce); 20–50 μg of each protein sample was electrophoresed on NuPAGE 4–12% Bis-Tris Gel (Invitrogen) in NuPAGE Mes SDS Running Buffer (Invitrogen). The proteins then were subjected to SDS/PAGE electrophoresis and transferred to PVDF membrane (Amersham). The membranes were blocked with 5% nonfat dry milk and probed with appropriate primary antibodies followed by HRP-conjugated secondary antibodies (Cell Signaling) at a dilution of 1:5,000. Bands were visualized using SuperSignal Chemiluminescent Substrate (Pierce) exposed on ECL Film (ISC BioExpress).

Nonhomologous End-Joining Assay. The colony-formation nonhomologous end-joining (NHEJ) assay was performed as previously described (1–3). Briefly, pPHW1 cells were transfected with I-SceI for 24 h. Cells were treated with CO (250 ppm) for the following 2 wk in the XHATM selection medium as previously described (2). The number of colonies was scored after 2 wk of selection.

Superoxide Measurement. HEK mirHO-1 and latent membrane protein (LMP) cells were cultured in 24-well plates with or without doxorubicin (10 μg/mL) or camptothecin (1 μg/mL) for 30 min together with 1 μM MitoSOX Red mitochondrial superoxide indicator (Invitrogen Molecular Probes). After 30 min, cells were washed three times with PBS and harvested in 100 μL of PBS. Fluorescence (excitation/emission, 510/580 nm) was measured by the ELISA plate reader.

Cell-Cycle Analysis. HEK mirHO-1 and LMP cells were grown to 50–70% confluency for 24 h; then the medium was replaced with serum-free medium for 24 h allowing cell synchronization in G0 phase. Cells were released from serum starvation for 12 h and stained with propidium iodide as previously described (4). The DNA profiles were evaluated using a Calibur Flow Cytometer (Becton Dickinson).

Immunofluorescence Staining. Fibroblasts were seeded on glass microscope slides 24 h before treatment. Cells were treated with 1 μg/mL camptothecin or 10 μg/mL doxorubicin for 4 h followed by three washes in 1× PBS. Bone marrow or mononuclear blood cells were isolated and cytospun on the glass slides. Cells were fixed in 2% paraformaldehyde and permeabilized with 0.05% Triton X-100. After washing, cells were blocked with 1% BSA in PBS, and primary antibodies against γ-H2AX, phosphorylated ataxia telangiectasia-mutated (P-ATM), or phosphorylated breast cancer 1, early onset (P-Brca1) were applied for 1 h at room temperature. Staining with secondary Alexa Fluor 594- or Alexa Fluor 488-conjugated antibody (Molecular Probes) was performed, and cells were costained with DAPI and covered with mounting medium (Gelvatol; Sigma-Aldrich). Slides were viewed in a Zeiss Apotome fluorescence microscope.

Immunohistochemistry. Kidneys, livers, spleens, and lungs of age-matched *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ BALB/c mice and C57BL/6 mice treated with irradiation or doxorubicin were harvested and fixed in 10% formalin and processed as previously described (5).

Plasmid and Transfection. HO-1-pcDNA3.1 plasmid was generated by subcloning heme oxygenase 1 (HO-1) from the human cDNA library into pcDNA3.1 (Invitrogen) vector. I-SceI plasmid was kindly provided by Ralph Scully (Beth Israel Deaconess Medical Center, Boston, MA). Homologous recombination/sister chromatid recombination (HR/SCR) U20S or pPHW1 cells were transfected transiently using Amaxa nucleofection techniques as previously described (6). Two micrograms of plasmid was used to transfect 1.5 × 10^6 cells. Cells were harvested 48 h later for analysis.

Reagents and Antibodies. Camptothecin and doxorubicin were purchased from Sigma and were used at 1 μg/mL and 10 μg/mL, respectively. CGK733, an inhibitor of ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) (Sigma), was used at 10 μM, and KU55933, an inhibitor of ATM, was used at 20 μM, 1 h before treatment with CO or for an additional 24 h posttransfection with HO-1/empty-control plasmids. Cells were harvested for analysis 24 h posttransfection.

The following antibodies were used: rabbit anti–phospho-ATR (Ser428); mouse anti–phospho-ATM (Ser1981); rabbit anti–phospho-BRCA1 (Ser1524); rabbit anti–phospho-Chk2 (Thr68); anti–phospho-histone H2AX (Ser139); rabbit anti-ATM; rabbit anti–phospho-p53 (Ser15); and total Akt (all from Cell Signaling); rabbit DNA-PK and p21 (from Santa Cruz Biotechnology); total p53 (Novocastra); anti-GAPDH (Calbiochem); rabbit anti–HO-1 (Fitzgerald Industries Intl or Novus Biologicals); and anti–β-Actin (Cell Signaling).

Marginal BM Transplantation. Mice were lethally irradiated and transplanted with 2.5 million cells i.v. immediately after irradiation. The amount of injected BM was sufficient for recovery of 40% of the mice.

Nu/nu mice from Taconic at 7 wk of age were used in a complementary experiment of an s.c. tumor model of PC3 cells injected into the right flank of mice. Doxorubicin (8 mg/kg) (Sigma) was given i.v. twice per week, and mice were exposed to CO (250 ppm) for 1 h daily. Tissues were harvested after 2 wk of treatment.

Cell Culture and Treatment. U203 HR/SCR cells were a kind gift from Ralph Scully (BIDMC, Boston, MA) and were maintained in DMEM (Gibco, Invitrogen) containing 10% FBS and antibiotics. pPHW1 cells (NHEJ model) were a kind gift of Henning Willers (Massachusetts General Hospital, Boston, MA) and were maintained in the DMEM with 10% (vol/vol) FBS and antibiotics. Mouse kidney fibroblasts were kindly given by Miguel Soares (Gulbenkian Institute, Lisbon, Portugal) and were obtained from adult *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ mice and were cultured between passages 4–7 in DMEM supplemented with 10% FBS and antibiotics. HEK293 cells that were transformed with SV40 large-T antigen (HEK293T, HEK) were purchased from American Type Culture Collection and were cultured following the manufacturer's protocol. For in vitro studies, cells were exposed to 250 ppm CO, 5% CO_2 in 95% N_2 for 2 min to 24 h as previously described (7). Cells were irradiated with 10 Gy and harvested at different time points after a single exposure.

MicroRNA-Adapted shRNA Retroviral-Mediated Transfections. MicroRNA-adapted shRNA (mirHO-1) for human HO-1 was purchased from Open Biosystems. mir–HO-1 was subcloned to MSCV-LTRmiR30-PIG (LMP) vector (Open Biosystems) with

XhoI and EcoRI restriction enzymes. The retrovirus for LMP-shRNAHO-1 and control vector were produced and used for transduction of HEK cells. Stable clones were generated by selection with 5 μg/mL puromycin (Sigma) for 2–4 wk.

1. Dahm-Daphi J, et al. (2005) Nonhomologous end-joining of site-specific but not of radiation-induced DNA double-strand breaks is reduced in the presence of wild-type p53. *Oncogene* 24:1663–1672.
2. Zhuang J, Jiang G, Willers H, Xia F (2009) Exonuclease function of human Mre11 promotes deletional nonhomologous end joining. *J Biol Chem* 284: 30565–30573.
3. Willers H, et al. (2006) Distinct mechanisms of nonhomologous end joining in the repair of site-directed chromosomal breaks with noncomplementary and complementary ends. *Radiat Res* 166:567–574.
4. Wegiel B, et al. (2010) Nitric oxide-dependent bone marrow progenitor mobilization by carbon monoxide enhances endothelial repair after vascular injury. *Circulation* 121:537–548.
5. Ollinger R, et al. (2005) Bilirubin: A natural inhibitor of vascular smooth muscle cell proliferation. *Circulation* 112:1030–1039.
6. Wegiel B, et al. (2005) A role for cyclin A1 in mediating the autocrine expression of vascular endothelial growth factor in prostate cancer. *Oncogene* 24:6385–6393.
7. Chin BY, et al. (2007) Hypoxia-inducible factor 1alpha stabilization by carbon monoxide results in cytoprotective preconditioning. *Proc Natl Acad Sci USA* 104: 5109–5114.

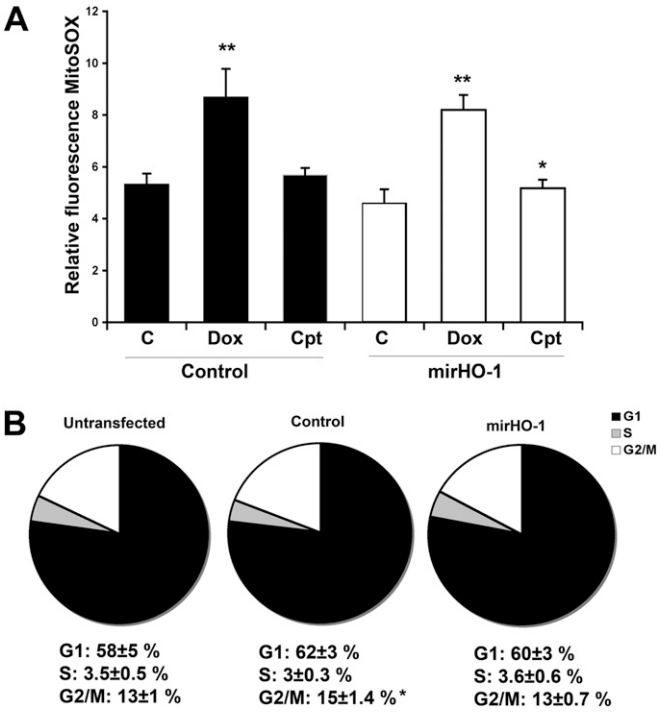

Fig. S1. (*A*) MitoSOX Red was used as an indicator of superoxide produced in the HEK293T (HEK) cells within 30 min of treatment with doxorubicin (10 μg/mL) or camptothecin (1 μg/mL). Data are representative of three independent experiments performed in triplicate. Averages ± SD are shown. $^{**}P < 0.0001$, doxorubicin (Dox) versus control (C); $^{*}P < 0.05$, camptothecin (Cpt) versus control; $^{*}P = 0.05$, Cpt + mirHO-1 versus Cpt + C; $P = 0.28$, Dox + mirHO-1 versus Dox + control. (*B*) Cell-cycle analyses of untransfected, control (LMP), and mirHO-1 HEK cells that were synchronized in G0 phase for 24 h by serum deprivation and released from starvation by the addition of medium containing 10% serum for 12 h. Quantitation of the number of cells in G1, S, and G2/M phases is shown. Data (averages ± SD) are representative for three experiments conducted in duplicate. $^{*}P < 0.05$, control versus mirHO-1 cells.

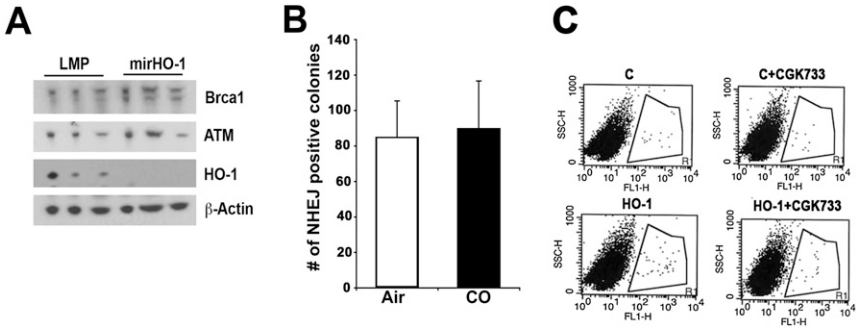

Fig. S2. (*A*) Immunoblot analysis in HEK control and mirHO-1 cells cultured for 24 h in medium with 10% serum. The levels of total Brca1, ATM, and HO-1 are shown. (*B*) pPHW1 cells were transfected with SceI plasmid for 24 h, and the $NHEJ^{+}$ colonies were treated with air and CO (250 ppm) and selected on the XHATM medium for 10 d. The colonies were counted; the number per well shown is representative of three independent experiments. (*C*) U20S-SCR reporter cells were cotransfected with SceI and HO-1 for 24 h, and CGK733 (10 μM) or DMSO (C, control) was applied for 24 h. Representative dot plots of the level of GFP^{+} cells as measured by flow cytometry are shown.

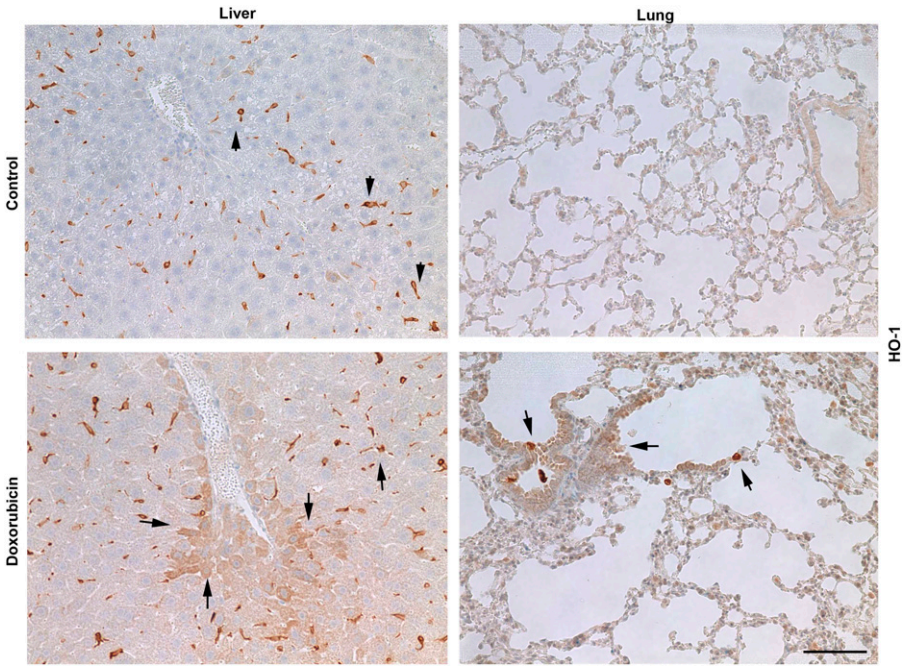

Fig. S3. Immunohistochemical analysis of HO-1 expression in the liver and lung of control mice and mice that were administrated doxorubicin as described in Fig. 6.

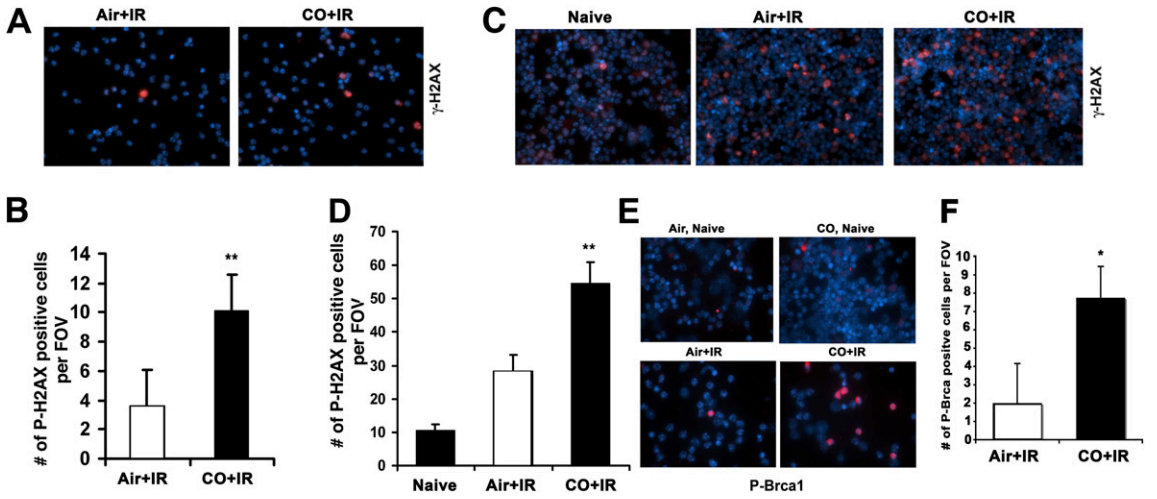

Fig. S4. (*A*–*D*) Immunostaining of mononuclear blood cells (*A* and *B*) and bone marrow cells (*C* and *D*) with antibodies against P-H2AX in mice that were treated with CO or air for 1 h before a lethal dose of irradiation (10 Gy). Blood and bone marrow cells were harvested 2 h after irradiation. Representative images are shown in *A* and *C*, respectively, and quantitation is shown in *B* and *D*, respectively. (*E* and *F*) Immunostaining of bone marrow cells with antibodies against P-Brca1 in nonirradiated (Naïve) mice and mice treated with irradiation (10 Gy). Bone marrow was harvested 2 h after irradiation. CO (250 ppm) was applied for 1 h before irradiation. Quantitation of the staining in *E* is shown in *F*. *$P < 0.001$, CO + irradiation versus air + irradiation.