Supporting information

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SI Materials and Methods

Cell Culture Growth Conditions. A549, U2OS, and MCF7 DR-GFP cells were grown in high-glucose DMEM supplemented with 10% FBS (Invitrogen). RKO-neo and RKO-E7 cell lines were grown in high-glucose DMEM supplemented with 10% FBS and 0.4 mg/mL geneticin (Invitrogen). H460 and MCF7 cells were grown in RPMI Medium 1640 supplemented with 10% FBS (Invitrogen). Wild-type polymerase β and E295K dominant negative cells were grown in high-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 0.25 mg/mL hygromycin B (Invitrogen).

Clonogenic Survival Assays. Cells were exposed to either normoxic or hypoxic conditions for 48 h and then seeded at 100–500 cells per well, depending upon the cell type into 6-well dishes and treated with various concentrations of PARP inhibitors in triplicate for 48–72 h, after which the media containing inhibitor was removed and replaced with fresh media. Colonies were fixed with 0.9% saline solution and stained with crystal violet 10–12 days later. Colonies consisting of >50 cells were counted. For the radiation survival experiments, cells were seeded into six-well dishes and treated or not with 200 μM PHEN for 48 h then irradiated using an X-RAD 320 irradiation system (Precision X-Ray) to a range of doses. The colonies were fixed and stained as described above. For the siRNA normoxia/hypoxia experiments, cells were treated with siRNA for 24 h and then placed into normoxia/hypoxia for 48 h and then reseeded and treated with a range of doses of PHEN for 72 h under normoxic conditions. For the radiation survival experiments, cells were treated with siRNA for 48–72 h then reseeded into six-well dishes and treated or not with 200 μM PHEN for 48 h then irradiated using an X-RAD 320 irradiation system to a range of doses. For the overexpression experiments, cells were transfected with the protein expression vector or empty vector control and then 72 h later reseeded into six-well dishes and treated with a range of doses of PHEN for 72 h.

Immunoblotting. The primary antibodies used for Western blotting were as follows: mouse monoclonal anti-BRCA1 (D-9; Santa Cruz Biotechnology), mouse monoclonal anti-α-tubulin (B-5-1-2; Sigma-Aldrich), rabbit polyclonal anti-Parp (Cell Signaling Technology), mouse monoclonal anti-GAPDH (6C5; Abcam), mouse monoclonal anti-RAD51 (3C10; Upstate), mouse monoclonal anti-β-actin (C4; Santa Cruz Biotechnology), mouse monoclonal anti-Rb2 (p130) (10; BD Transduction Laboratories), mouse monoclonal anti-E2F1 (KH95; Santa Cruz Biotechnology), and mouse monoclonal anti-E2F4 (GG22-2A6; Millipore). The antibodies used for immunoprecipitations were mouse monoclonal anti-E2F1, anti-p130, rabbit polyclonal anti-Parp (listed above), and mouse monoclonal anti-E2F4 (C-20; Santa Cruz Biotechnology).

Quantitative Real-Time PCR Analysis. cDNA was synthesized from total RNA samples using the SuperScript III First-Strand Synthesis System (Invitrogen) and were used in PCR reactions containing TaqMan Universal PCR Master Mix (Applied Biosystems) and premixed Taqman probes and primers for BRCA1, RAD51, and 18S (Applied Biosystems). The Mx3000p real-time PCR system (Stratagene) was used to monitor fluorescence intensity in real-time and calculate cycle thresholds.

PARP Activity Assay. Cells were treated with 200 μM PHEN or 200 μM ANI for 72 h, and the HT Universal Colorimetric PARP Assay Kit (Trevigen) was used according to the manufacturer's instructions.

Luciferase Assay. The *BRCA1* promoter–luciferase assay and construction of the vectors has been previously described (1). A549 cells were seeded at 80,000 cells per well in 12-well dishes and transfected (in triplicate) with 1 μg each reporter construct using Fugene 6 transfection reagent (Roche Applied Science). The cells were then treated 24 h later with PHEN and lysed 24 h later with Passive Lysis Buffer (Promega), and firefly luciferase activity was measured using a Luciferase Reporter Assay System kit (Promega). Luciferase activity was normalized to total protein and then to a control vector containing the luciferase coding region driven by the SV40 early promoter.

1. Bindra RS, et al. (2005) Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. *Cancer Res* 65:11597–11604.

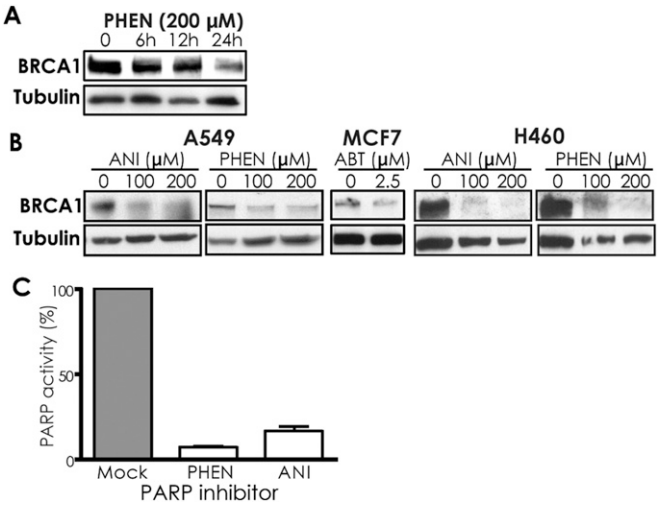


Fig. S1. PARP inhibition causes decreased BRCA1 protein levels at early time points. (*A*) Analysis of BRCA1 protein levels in A549 cells by immunoblot at various time points, as indicated, after exposure to PHEN. (*B*) BRCA1 protein levels are decreased after 48 h PARP inhibitor treatment in A549, MCF7, and H460 cells. (*C*) A549 cells treated with the indicated PARP inhibitors were assayed for PARP activity in cell lysates by ELISA.

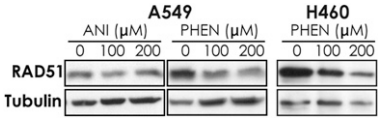


Fig. S2. PARP inhibition causes decreased RAD51 protein levels after 48 h in A549 and H460 cells.

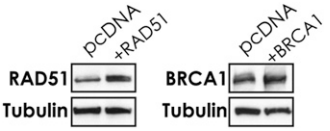
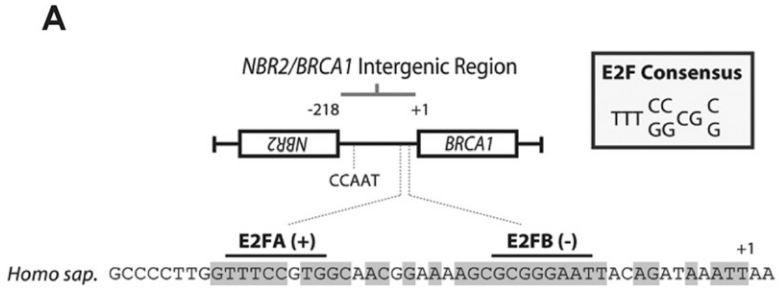


Fig. S3. Forced expression of BRCA1 and RAD51 in A549 cells. The respective cDNAs were cloned into the expression vector pcDNA3.1 and used to transfect MCF7 cells. Expression was assayed by immunoblot using the respective antibodies.



B

Relative luciferase expression

	No inhibitor	PHEN	Decrease in expression (%)
WT	0.38	0.13	66
E2FA	0.5	0.23	54
E2FB	0.64	0.32	50
E2FA/B	2.2	1.22	45

Fig. S4. Suppression of *BRCA1* promoter expression by PARP inhibition is mediated through two E2F consensus binding sites. (*A*) A schematic of the *BRCA1* proximal promoter region is shown with the locations of the two consensus E2F sites, E2FA and E2FB, as indicated. Site-directed mutations were introduced within the E2FA and E2FB sites in the luciferase expression constructs, as indicated. The promoter–luciferase reporter constructs consisted of the wild-type *BRCA1* promoter or the *BRCA1* promoter with site-directed mutations in either the E2FA consensus site, the E2FB site, or in both the E2FA and E2FB sites. (*B*) Effects of E2FA and/or E2FB site mutations within the *BRCA1* promoter–luciferase reporter vector on luciferase expression after transfection into A549 cells treated or not with PHEN (A549 cells were transfected with the promoter–luciferase constructs, 24 h later treated or not with PHEN, and harvested for measurement of luciferase expression in cell lysates after another 24 h). For each construct, relative expression normalized to that of the parental construct with the SV40 early promoter driving luciferase is shown. Constructs with disruption of one or both E2F sites had elevated baseline expression in untreated cells compared with the wild-type construct. Importantly, however, these mutant constructs exhibited less suppression than the wild-type in cells treated with PHEN. The double-site mutant had the least suppression. These results provide further evidence linking E2F-related factors to regulation of *BRCA1* by PARP.

Fig. S5. Lack of interaction between PARP-1 and either E2F4 or p130. No association between PARP-1 and either E2F4 or p130 is detected by coimmunoprecipitation using the indicated antibodies in total cell extracts from A549 cells treated or not with PHEN.

Fig. S6. siRNA mediated knockdown of BRCA1 or PARP-1 does not significantly add to the radiosensitization of PARP inhibitor treatment. (*A*) Survival by colony formation to ionizing radiation (9 Gy) of A549 cells treated with PHEN or not and siRNAs against either GAPDH, PARP-1, or BRCA1, as indicated. (*B*) siRNA-mediated knockdown of the respective factors was confirmed by immunoblot.