Deficiency in the Repair of DNA Damage by Homologous Recombination and Sensitivity to Poly(ADP-Ribose) Polymerase Inhibition

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Abstract

Deficiency in either of the breast cancer susceptibility proteins BRCA1 or BRCA2 induces profound cellular sensitivity to the inhibition of poly(ADP-ribose) polymerase (PARP) activity. We hypothesized that the critical role of BRCA1 and BRCA2 in the repair of double-strand breaks by homologous recombination (HR) was the underlying reason for this sensitivity. Here, we examine the effects of deficiency of several proteins involved in HR on sensitivity to PARP inhibition. We show that deficiency of RAD51, RAD54, DSS1, RPA1, NBS1, ATR, ATM, CHK1, CHK2, FANCD2, FANCA, or FANCC induces such sensitivity. This suggests that BRCA-deficient cells are, at least in part, sensitive to PARP inhibition because of HR deficiency. These results indicate that PARP inhibition might be a useful therapeutic strategy not only for the treatment of BRCA mutation-associated tumors but also for the treatment of a wider range of tumors bearing a variety of deficiencies in the HR pathway or displaying properties of 'BRCAness.' (Cancer Res 2006; 66(16): 8109-15)

Introduction

Multiple cellular roles have been attributed to the breast cancer susceptibility proteins BRCA1 and BRCA2, including a role in DNA repair (1, 2). Cells defective in BRCA1 or BRCA2 have a defect in the repair of double-strand breaks (DSB) by the error-free mechanism of homologous recombination (HR) by gene conversion (3–6). This defect results in the repair of such lesions by error-prone mutagenic pathways, such as single-strand annealing (SSA) and nonhomologous end joining, culminating in genomic instability. We have exploited the DNA repair defect in BRCA1- and BRCA2-deficient cells in the design of new therapies to treat BRCA-associated cancers (7, 8). We showed a profound sensitivity of BRCA-deficient cells to inhibition of poly(ADP-ribose) polymerase (PARP), which resulted in cell cycle arrest, chromosome instability, and cell death (8). The sensitivity was dependent on the use of potent inhibitors of PARP (9, 10). PARP is an important mediator of the base excision repair pathway, important for the repair of DNA single-strand breaks and gaps. Loss of PARP-1 has been shown to cause an increase in RAD51 foci and sister chromatid exchanges (SCE; ref. 11). Because PARP does not directly play a role in HR (11), loss of PARP activity results in an increase in the number of lesions normally repaired by HR. This might be lethal in a BRCA-defective background. We therefore hypothesized that the sensitivity of BRCA-deficient cells to PARP inhibition was due to a defect in HR by gene conversion rather than a deficiency in BRCA1 or BRCA2 per se. To understand the basis of PARP inhibition sensitivity and how this relates to BRCA1 and BRCA2 function, we examined the effect of PARP inhibition in cells with loss or depletion of several other HR-related genes.

Materials and Methods

Small-molecule inhibitors. The PARP inhibitors KU0058684 (IC_{50}, 3.2 nmol/L), KU0058948 (IC_{50}, 3.4 nmol/L), and the control drug KU0051529 (IC_{50}, 730 nmol/L) are described in ref. 8. KU0058684 and KU0058948 are potent and specific inhibitors of PARP-1 and PARP-2 but not of vault PARP or tankyrase PARP (8). The ataxia-telangiectasia mutated (ATM) inhibitor KU0055933 (IC_{50}, 13 nmol/L) has been previously validated (12). Caffeine was purchased from Sigma (Poole, United Kingdom), dissolved in DMSO, and stored at −20°C.

Cell lines. Mouse fibroblasts from wild-type (WT) *Fancd2*$^{-/-}$, *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, and *Fanca*$^{-/-}$*c*$^{-/-}$ animals were obtained from the Fanconi Anemia Cell Repository, Oregon Health and Science University (Portland, OR). These cells were maintained in DMEM supplemented with FCS (10%, v/v), glutamine, and antibiotics. *RAD54*- and *RAD52*-deficient embryonic stem cells were kind gifts of R. Kanaar and J.H. Hoeijmakers (Erasmus Medical Center, Rotterdam, the Netherlands), respectively. Embryonic stem cells were grown in DMEM supplemented with FCS (15%, v/v), glutamine, antibiotics, nonessential amino acids, 2-mercaptoethanol, and leukemia inhibitory factor. HeLa (CCL-2) and U20S cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with FCS (10%, v/v), glutamine, and antibiotics. SV40-transformed NBS1 human fibroblast cell lines were described previously (13–15). They were maintained in Ham's F10 medium (Life Technologies, Paisley, United Kingdom) supplemented with FCS (10%, v/v), glutamine, and antibiotics. NBS1-LBI cells containing human chromosome 8 were cultured under selective pressure of 300 μg/mL geneticin (G418 sulfate, Life Technologies).

Clonogenic survival assays to measure PARP inhibition sensitivity. For measurement of sensitivity to PARP inhibitors, exponentially growing embryonic stem cells were seeded at various densities in six-well plates on a confluent monolayer of mitomycin C (MMC)–inactivated mouse embryonic fibroblasts. Likewise, exponentially growing mouse fibroblasts were seeded at various densities in six-well plates onto a subconfluent monolayer of MMC-inactivated mouse embryonic fibroblasts. Where appropriate, cells were continuously treated with inhibitors 18 hours after plating.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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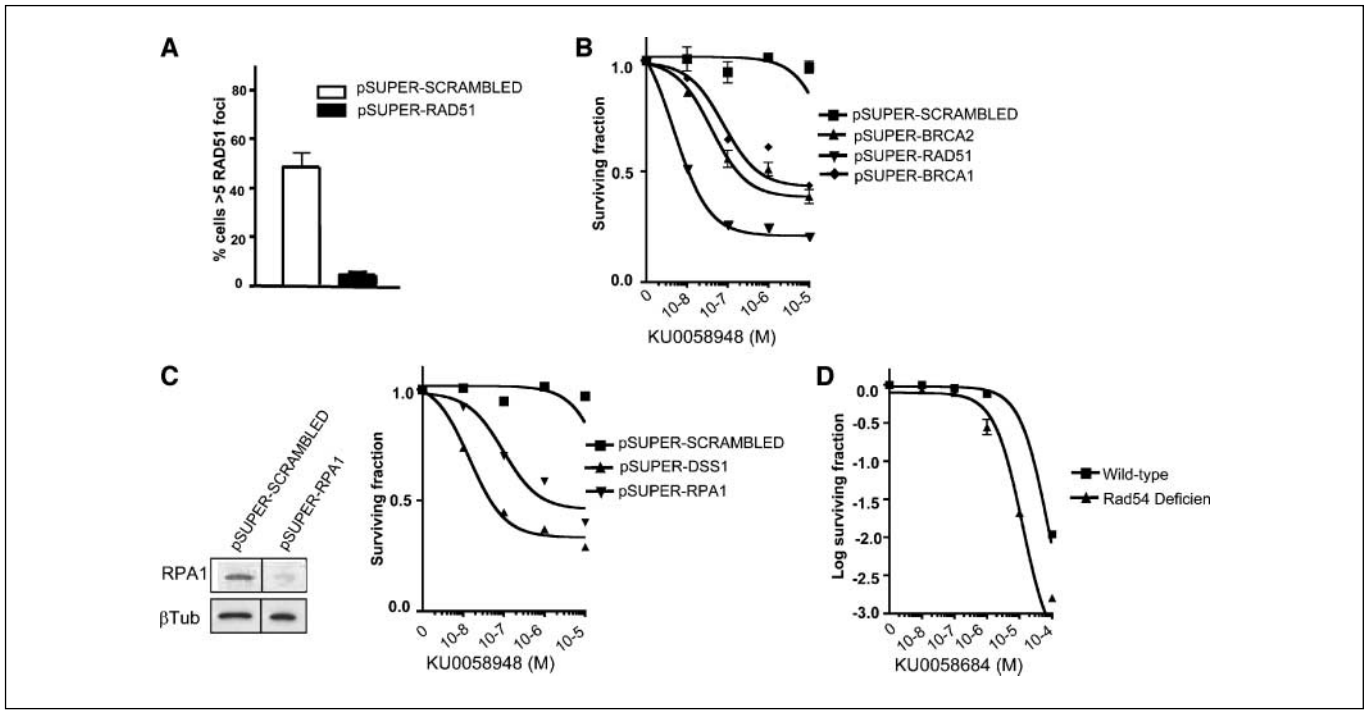

Figure 1. Deficiency of proteins integral to HR induces sensitivity to PARP inhibition. *A,* quantification of radiation-induced RAD51 foci formation in HeLa cells transfected with either pSUPER-SCRAMBLED or pSUPER-RAD51. Cells containing more than five RAD51 foci per cell were counted as positive and quantified. *Columns,* mean; *bars,* SE. *B,* cell viability curves of pSUPER-SCRAMBLED, pSUPER-BRCA1, pSUPER-BRCA2, or pSUPER-RAD51 transfected HeLa cells under constant exposure to a range of concentrations of PARP inhibitor KU0058948 for 5 days. *Points,* mean; *bars,* SE. The SF_{50} for pSUPER-SCRAMBLED-transfected cells is >10 μmol/L, 500 nmol/L for pSUPER-BRCA1, 100 nmol/L for pSUPER-BRCA2, and 10 nmol/L for pSUPER-RAD51. *C,* Western blots of lysates from HeLa cells 48 hours after transfection with either pSUPER-SCRAMBLED or pSUPER-RPA1. Cell viability curves of pSUPER-SCRAMBLED, pSUPER-RPA1, or pSUPER-DSS1 transfected HeLa cells under constant exposure to a range of concentrations of PARP inhibitor KU0058948 for 5 days. *Points,* mean; *bars,* SE. SF_{50} for pSUPER-SCRAMBLED-transfected cells is >10 μmol/L, 1 μmol/L for pSUPER-RPA1-transfected cells, and 47 nmol/L for pSUPER-DSS1-transfected cells. *D,* clonogenic survival curves of *Rad54* WT and *Rad54*-deficient embryonic stem cells under continuous exposure to a range of concentrations of PARP inhibitor KU0058684 for 12 to 14 days. *Points,* mean; *bars,* SE. SF_{50} for WT cells is 7.5 μmol/L and 830 nmol/L for isogenic *Rad54*-deficient cells.

Exponentially growing human fibroblasts were seeded at various densities in six-well plates or 10-cm plates and continuously treated with PARP inhibitors. Cell medium was replaced every 4 days, and inhibitors were replenished. After 10 to 14 days, cells were fixed in methanol, stained with crystal violet, and counted. The plating efficiencies were calculated as the number of colonies divided by the number of cells plated for each drug concentration. The surviving fraction (SF) for a given dose was calculated as the plating efficiencies for that dose divided by the plating efficiencies of vehicle-treated cells. Survival curves were generated as described previously (8).

Use of RNA interference to assess PARP inhibition sensitivity. RNA interference (RNAi) constructs were generated by cloning gene-specific RNAi target sequences into pSUPER-CFP as described previously (8). The RNAi target sequences used were as follows: scrambled, CATGCCTGATCCGCTAGTC; BRCA1, GTAGCTGATGTATTGGACG; BRCA2, ACAACAATTACGAACCAAA; RAD51, TGTAGCATATGCTCGAGCG; replication protein A1 (RPA1), GGCGACAAGCCGGACTACT; deleted in split-hand/split-foot syndrome 1 (DSS1), GATGAAGATGCACATGTCT; ATM, ACTGTAAAGCTGCAATGAA; ATM and Rad3-related (ATR), ATGTCAATCGTAGAGAGAT; and checkpoint kinase 2 (CHK2), AGAAGCTACCTGCAAGCTC.

For 96-well plate-based cell viability assays, HeLa cells were transfected with pSUPER-RNAi vectors and a vector expressing blasticidin resistance (pEF-Bsd, Invitrogen, Paisley, United Kingdom) at a ratio of 10:1 (pSUPER/pEF-Bsd) using Fugene 6 (Roche, Basel, Switzerland). Twenty-four hours after transfection, cells were plated into replica plates, and this was followed by treatment with PARP inhibitors and blasticidin 48 hours after transfection. Medium containing PARP inhibitors and blasticidin was replenished 5 days after transfection. Cell viability was measured 7 days after transfection using the 96-well plate CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Southampton, United Kingdom) according to the manufacturer's instructions. Survival fractions were calculated by dividing the cell viability for a given drug dose by the cell viability of the vehicle-treated cells. To silence checkpoint kinase 1 (CHK1) expression, a synthetic 19-bp RNA duplex was transfected into HeLa cells using DharmaFECT 3 (Dharmacon, Lafayette, CA) according to the manufacturer's instructions.

Immunofluorescence and fluorescence-activated cell sorting analysis. Formation and quantification of RAD51 foci, γH2AX foci, and DNA content analysis after PARP inhibitor treatment were carried out as described in ref. 8.

Validation of gene silencing by RNAi. Transfected cell pellets were lysed in 20 mmol/L Tris (pH 8), 200 mmol/L NaCl, 1 mmol/L EDTA, 0.5% (v/v) NP40, 10% (v/v) glycerol, and protease inhibitors. Lysates were electrophoresed on Novex precast gels (Invitrogen) and immunoblotted with the following antibodies: anti-RAD51, 3C10 (NeoMarkers, Fremont, CA); anti-ATM, ab2631 (Abcam, Cambridge, United Kingdom); anti-ATR, ab2905 (Abcam); anti-CHK2, SC9094 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-RPA1, ab12320 (Abcam); and anti-β-tubulin, T4026 (Sigma). This was followed by incubation with anti-IgG-horseradish peroxidase and chemiluminescent detection (enhanced chemiluminescence, Amersham, Buckinghamshire, United Kingdom). Immunoblotting for β-tubulin was used as a loading control. The plasmid encoding RNAi of DSS1 was previously validated (16). The synthetic RNA duplex used to silence CHK1 has been previously validated (17).

Western blot analysis. Lysates and Western blotting analysis after PARP inhibitor treatment or ionizing radiation (IR) for NBS1 phosphorylation analysis was carried out as above. The NBS1 antibody was from Novus Biologicals (Littleton, CO; NB100-143), and phosphorylated Ser^{343} NBS1 antibody was from Cell Signaling Technology (Danvers, MA).

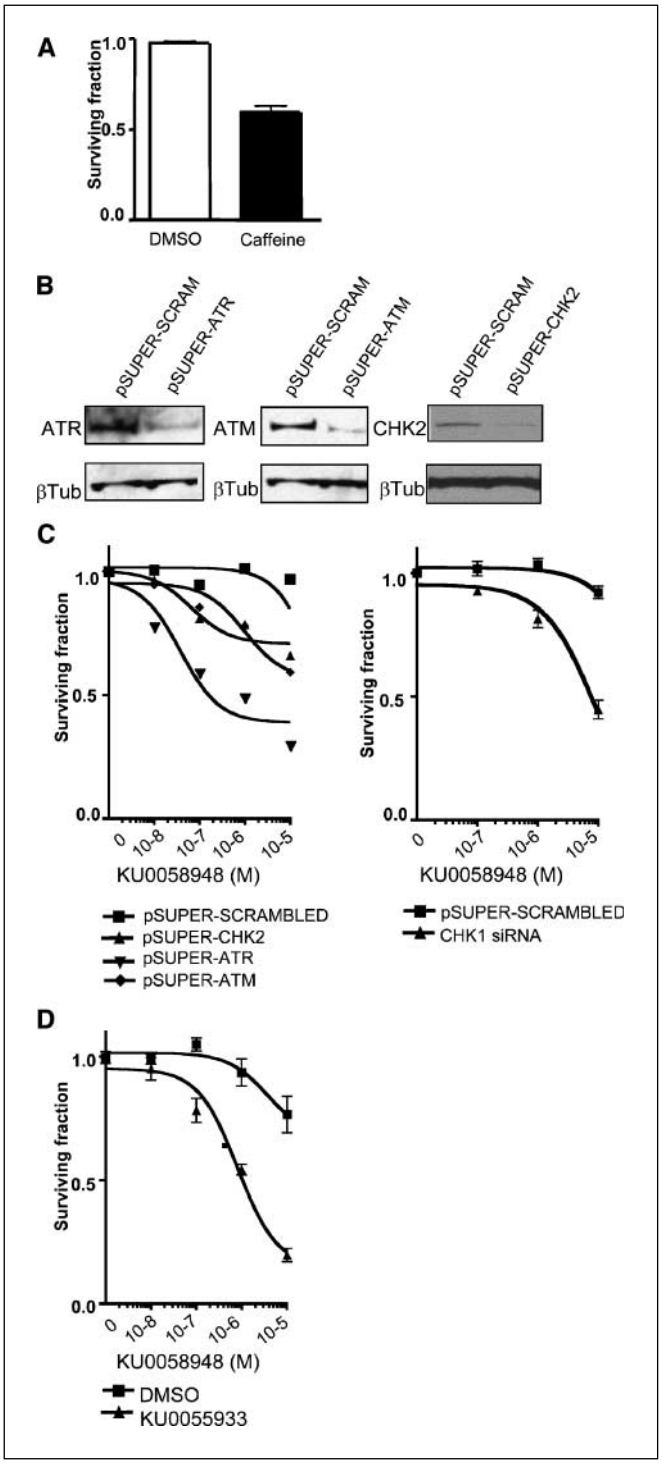

Figure 2. Deficiency in DNA damage signaling proteins induces sensitivity to PARP inhibition. *A,* cell viability graph of HeLa cells exposed to KU0058948 and caffeine. Cells were exposed to 1 μmol/L KU0058948 for 5 days in the presence of 1 mmol/L caffeine in DMSO or DMSO alone. *Columns,* mean; *bars,* SE. *B,* Western blots of lysates from HeLa cells 48 hours after transfection with either pSUPER-SCRAMBLED or gene-specific pSUPER constructs. *C,* cell viability curves of pSUPER-SCRAMBLED, pSUPER-ATM, pSUPER-ATR, pSUPER-CHK2, or CHK1 siRNA transfected HeLa cells under constant exposure to a range of concentrations of PARP inhibitor KU0058948 for 5 days. *Points,* mean; *bars,* SE. *D,* cell viability curves of HeLa cells exposed to 10 μmol/L ATM inhibitor KU0055933 or DMSO and treated with PARP inhibitor KU0058948 at varying doses and assessed after 5 days. *Points,* mean; *bars,* SE.

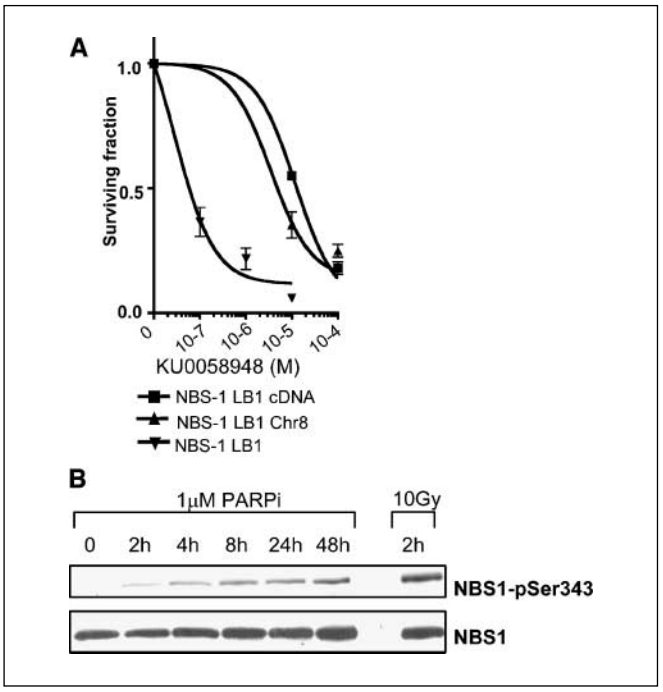

Figure 3. Deficiency in the DNA damage signaling protein NBS1 induces sensitivity to PARP inhibition. *A,* clonogenic survival curves of human SV40-transformed immortal fibroblasts deficient for NBS1 (*NBS1-LBI*) and isogenic cells complemented with either chromosome 8 (*NBS1-LBI Chr8*) or a *NBS1* cDNA (*NBS1-LBI cDNA*) under continuous exposure to a range of concentrations of PARP inhibitor KU0058948 for 12 to 14 days. *Points,* mean; *bars,* SE. *B,* Western blot analysis showing ATM-dependent phosphorylation of NBS1 on Ser^{343} (*NBS1-pSer343*) after PARP inhibition. U20S osteosarcoma cells were treated with PARP inhibitor for 2, 4, 8, 24, and 48 hours, and lysates were collected. Lysates were also collected following 10 Gy IR exposure as a control. Western blotting of total NBS1 was used as a loading control.

Results

Deficiency in proteins integral to HR induces sensitivity to PARP inhibition. To examine whether PARP inhibition was selectively lethal to cells deficient in the expression of key HR genes, we tested the sensitivity of cells deficient or depleted in RAD51, RAD54, DSS1, and RPA1. RAD51 stimulates DNA strand exchange reactions between homologous DNA sequences, a critical step in HR by gene conversion. To deplete RAD51 protein expression, we used a pool of three plasmids, each expressing a small interfering RNA (siRNA) targeting human *RAD51* mRNA in HeLa cells. Following transient transfection, these constructs caused a 5-fold reduction in RAD51 focus formation after radiation compared with a control plasmid expressing scrambled siRNA (Fig. 1*A*; Supplementary Fig. S1A and B; Supplementary Table S1). Cells were transfected with either the *RAD51* siRNA plasmids or a control plasmid encoding a scrambled siRNA and continuously treated with a range of concentrations of the PARP inhibitor KU0058948. This resulted in >1,000-fold reduction in the cell viability of *RAD51* siRNA-transfected cells compared with scrambled control-transfected cells (Fig. 1*B*). Similar experiments using siRNA-expressing plasmids targeting human *BRCA1* and *BRCA2* in HeLa cells showed profound sensitivity of these cells to PARP inhibition compared with a plasmid expressing a scrambled control RNAi, showing the validity of the assay (Fig. 1*B*).

DSS1 encodes a small acidic protein, which interacts with BRCA2 (18). Depletion of DSS1 protein by RNAi results in defective

RAD51 focus formation and chromosomal instability consistent with a defect in gene conversion (16). RPA1, in conjunction with BRCA2, facilitates the DNA strand exchange activities of RAD51, and mutation of RPA1 results in a failure in DSB repair consistent with a deficiency in HR (19). Human *DSS1* and *RPA1* mRNAs were targeted using RNAi-expressing plasmids that effectively reduce expression of the relevant proteins (Fig. 1*C*; ref. 16). HeLa cells were transfected with these plasmids and cell viability assays were done in the presence of the PARP inhibitor KU0058948. This showed enhanced sensitivity of cells transfected with DSS1 and RPA1 RNAi plasmids to PARP inhibition compared with cells transfected with a control plasmid expressing scrambled RNAi (Fig. 1*C*).

RAD54, a RAD51 paralogue and a member of the SWI/SNF protein family, exhibits chromatin remodeling activity and facilitates the opening up of DNA important for the DNA strand exchange activity of RAD51 (20). *Rad54*-deficient embryonic stem cells have been shown to exhibit defects in HR (21). Clonogenic survival assays of *Rad54*-deficient embryonic stem cells showed greater sensitivity (9-fold) to PARP inhibition compared with isogenic WT cells (Fig. 1*D*).

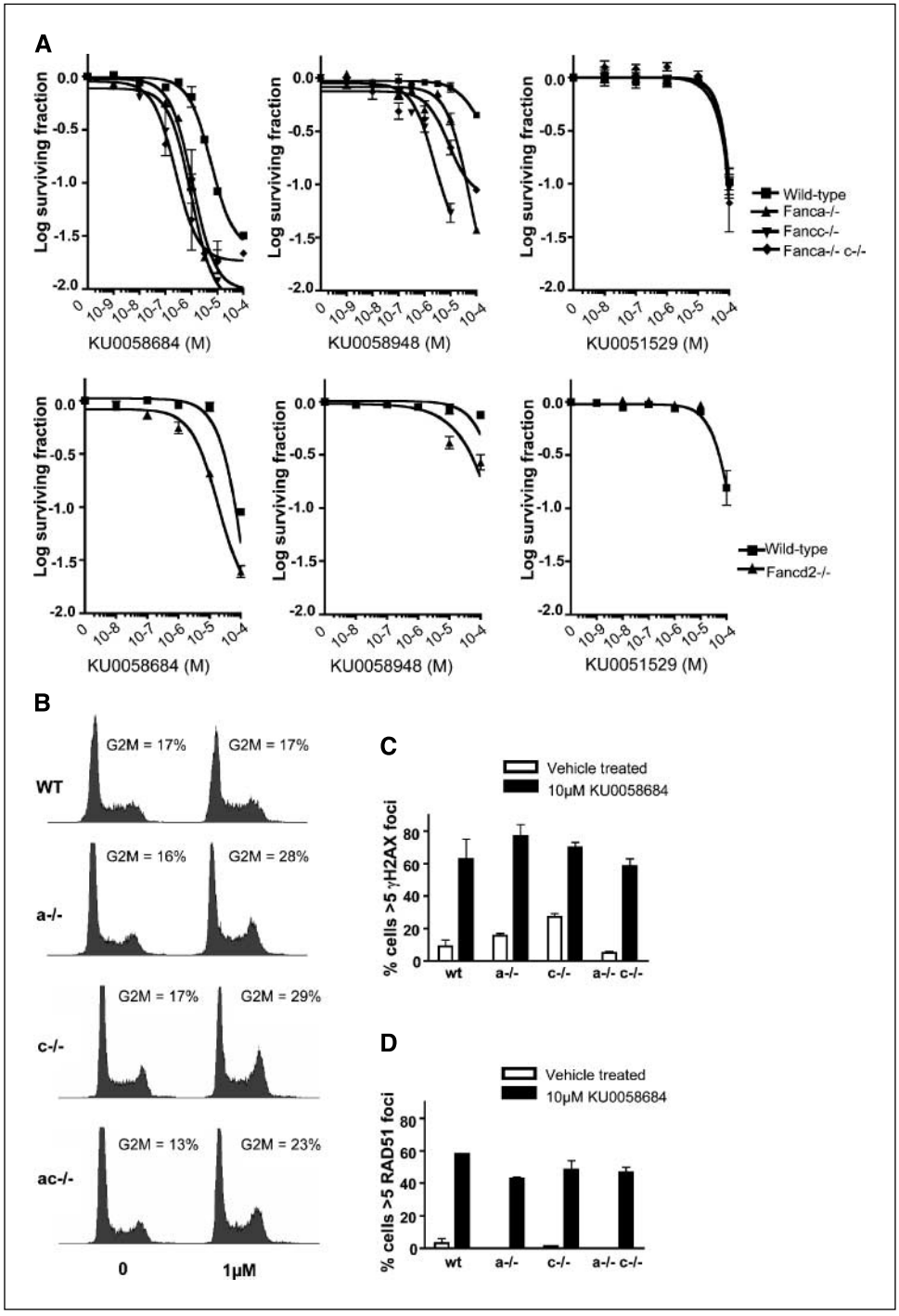

Figure 4. Deficiency in Fanconi anemia proteins induces sensitivity to PARP inhibition. *A,* clonogenic survival curves of WT mouse fibroblasts and isogenic *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, *Fanca*$^{-/-}$*c*$^{-/-}$, and *Fancd2*$^{-/-}$ fibroblasts under continuous exposure to a range of concentrations of PARP inhibitors KU0058684, KU0058948, and KU0051529 for 12 to 14 days. *Points,* mean; *bars,* SE. *B,* exposure to PARP inhibitor for 48 hours results in G_2-M arrest in *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, and *Fanca*$^{-/-}$*c*$^{-/-}$ fibroblasts. Fibroblasts were mock treated or treated with KU0058684 for 48 hours at 1 μmol/L, and DNA content was analyzed by FACS. The increase in proportion of FANC-deficient cells in the G_2-M phase of the cell cycle after PARP inhibitor treatment was statistically significant compared with that of WT cells ($P = 0.0328$, *Fanca*$^{-/-}$ compared with WT using Student's *t* test; $P = 0.0182$, *Fancc*$^{-/-}$ compared with WT using Student's *t* test; $P = 0.0299$, *Fanca*$^{-/-}$*c*$^{-/-}$ compared with WT using Student's *t* test). *C,* exposure to PARP inhibitor for 48 hours results in an increase in γH2AX foci formation in WT and isogenic *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, and *Fanca*$^{-/-}$*c*$^{-/-}$ fibroblasts. Quantification of γH2AX foci formed following exposure to PARP inhibitor. Cells were exposed to 10 μmol/L KU0058684 for 48 hours. The percentage of cells with more than five foci per cell was quantified. *Columns,* mean; *bars,* SE. *D,* exposure to PARP inhibitor for 48 hours results in RAD51 foci formation in WT and isogenic *Fanca*$^{-/-}$, *Fancc*$^{-/-}$, and *Fanca*$^{-/-}$*c*$^{-/-}$ fibroblasts. Quantification of RAD51 foci formed following exposure to PARP inhibitor. Cells were exposed to 10 μmol/L KU0058684 for 48 hours. The percentage of cells with more than five foci per cell was quantified. *Columns,* mean; *bars,* SE.

Deficiency in DNA damage signaling proteins induces sensitivity to PARP inhibition. ATM and ATR are members of the phosphoinositol 3-kinase–related kinase family and are involved in mediating the cellular response to DSBs and replication stress. Deficiency in ATM and reduction of ATR kinase activity result in defects in HR (22, 23). We used caffeine, an inhibitor of the kinase activity of ATM and ATR (24), to determine if loss of these DNA damage signaling pathways might induce sensitivity to PARP inhibition. Treatment of HeLa cells with 1 mmol/L caffeine resulted in increased sensitivity to the PARP inhibitor KU0058948 (Fig. 2*A*). This suggested that defects in the ATM and ATR signaling pathways might result in sensitivity to PARP inhibition. To further investigate this, we generated siRNA plasmids expressing RNAi targeting human ATM and ATR to effectively deplete protein expression (Fig. 2*B*). Furthermore, it has previously been shown that RNAi silencing of CHK1 leads to a reduction in HR (17) and elimination of the kinase activity of CHK2 also results in reduction in HR (25). Therefore, we also generated a similar plasmid to deplete CHK2 protein expression (Fig. 2*B*) and used previously validated siRNA oligos to silence CHK1 protein expression (17). Cell viability assays using HeLa cells with silenced human ATM, ATR, CHK1, and CHK2 expression showed increased sensitivity to the PARP inhibitor KU0058948 compared with scrambled controls (Fig. 2*C*). Furthermore, similar results were obtained when HeLa cells were treated with the ATM inhibitor KU0055933 in combination with the PARP inhibitor KU0058948 (Fig. 2*D*). These findings highlight the importance of these DNA signaling kinases in the response to a variety of DNA damage, including damage caused by the use of PARP inhibitors, and suggest that lesions generated by PARP inhibition require an intact DNA damage signaling cascade for repair.

NBS1 is a component of the MRE11/RAD50/NBS1 complex, which has an important role in DNA processing at sites of DNA breaks and in facilitating ATM- and ATR-dependent DNA damage signaling (26). NBS1-deficient cells display reductions in both gene conversion and SCEs, suggesting that they might be sensitive to PARP inhibition (27). Exposure of human fibroblasts deficient in NBS1 to PARP inhibition showed profound sensitivity when compared with the same cells complemented with either chromosome 8 (*NBS1* maps to chromosome 8q21-24) or a *NBS1* cDNA clone, respectively (Fig. 3*A*). Furthermore, treatment of the human U20S osteosarcoma cells with 1 μmol/L PARP inhibitor resulted in a time-dependent increase in the phosphorylation of NBS1 on Ser^{343}, a known ATM phosphorylation site (Fig. 3*B*), suggesting that, in cells competent for HR, the inhibition of PARP activity may result in an ATM-dependent S-phase checkpoint response. These data further emphasize the importance and interplay of these signaling cascades in response to the damage generated after PARP inhibitor treatment.

Deficiency in Fanconi anemia proteins induces sensitivity to PARP inhibition. Fanconi anemia is a genetically heterogenous disease associated with a greatly increased risk of cancer. Seven of the 11 Fanconi anemia proteins identified (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCM) form a nuclear core complex, which interacts with FANCL and results in monoubiquitination of FANCD2 following DNA damage. This modification is required for the repair of DNA cross-links and the accumulation of FANCD2 at sites of DNA damage where it associates with BRCA1 and BRCA2 (28–33). Recently, deficiencies in HR have been ascribed to cells with defects in FANCA, FANCC, or FANCD2 (34–37); hence, we examined whether Fanconi anemia cells were sensitive to PARP inhibition.

We did clonogenic survival assays with mouse fibroblast cells to investigate whether Fanconi anemia cells are sensitive to the inhibition of PARP. These experiments showed that deficiencies in FANCD2, FANCA, or FANCC and deficiency in both FANCA and FANCC result in sensitivity to the potent PARP inhibitors KU0058684 and KU0058948 compared with control isogenic WT cells (Fig. 4*A*). In contrast, there was no difference in sensitivity between the WT and FANC-deficient cells following treatment with the control compound KU0051529, suggesting that this is a specific effect of PARP inhibition. In our previous work, we showed that the loss of clonogenic survival following exposure to PARP inhibitors was initially due to cell cycle arrest (8). To assess whether the same mechanism of sensitivity was responsible for the reduced clonogenic survival of the FANC-deficient cells, we did fluorescence-activated cell sorting (FACS) analysis of PARP inhibitor-treated cells. This showed a greater percentage of $Fanca^{-/-}$, $Fancc^{-/-}$, and $Fanca^{-/-}c^{-/-}$ cells in the G_2-M phase of the cell cycle after PARP inhibition compared with WT cells (Fig. 4*B*). We examined γH2AX as a marker of DNA damage and were able to show a similar phenotype to that seen in the BRCA-deficient PARP inhibitor-treated cells (Fig. 4*C*; ref. 8). However, we also examined the ability of the FANC-deficient cells to form RAD51 foci in response to PARP inhibition, a hallmark of the activity of HR by gene conversion. This analysis showed that FANC-deficient cells are proficient in the ability to form RAD51 foci (Fig. 4*D*) unlike many HR-deficient cells (16, 38, 39). The proficiency of RAD51 focus formation in FANC-deficient cells has been reported elsewhere (35, 40).

Deficiency in Rad52 does not induce sensitivity to PARP inhibition. RAD52 is a critical component of the SSA pathway of HR in mammalian cells but is apparently dispensable for DSB repair by gene conversion (5). To investigate whether a defect in RAD52 resulted in sensitivity to PARP inhibition, we did clonogenic survival assays in *Rad52*-deficient embryonic stem cells. These experiments showed that *Rad52*-deficient cells were

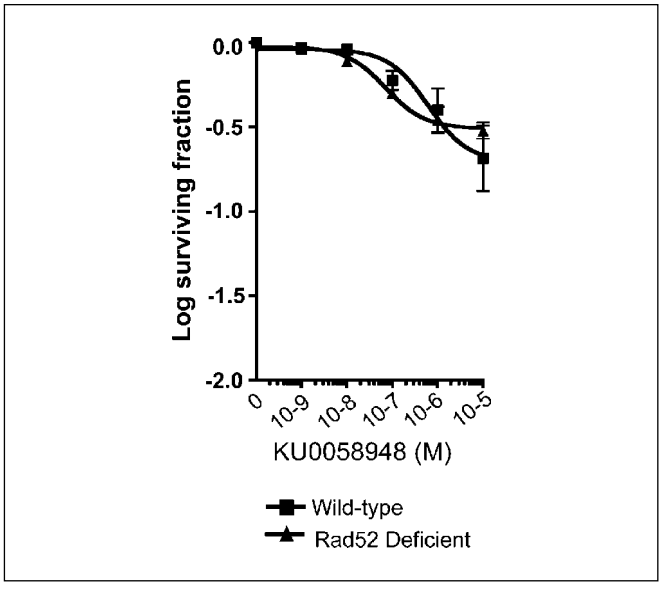

Figure 5. Deficiency in Rad52 does not induce sensitivity to PARP inhibition. Clonogenic survival curves of *Rad52* WT and *Rad52*-deficient embryonic stem cells under continuous exposure to a range of concentrations of PARP inhibitor KU0058948 for 12 to 14 days. *Points,* mean; *bars,* SE.

no more sensitive to the PARP inhibitor KU0058948 than isogenic WT cells (Fig. 5). This suggested that the sensitivity to PARP inhibition is not conferred by a defect in the RAD52/SSA pathway of DSB repair.

Discussion

In a previous study, we hypothesized that the sensitivity of BRCA-deficient cells to PARP inhibitors was based on the defective repair of DNA DSBs by the error-free mechanism of gene conversion. We have confirmed this hypothesis by showing sensitivity of cells with several defects in the HR pathway. Interestingly, the lack of sensitivity in RAD52-deficient cells suggests that SSA is not a major pathway used for the repair of DNA damage that persists as a result of PARP inhibition, implying that gene conversion is the predominant HR mechanism involved. In addition, by examination of NBS1, ATM, ATR, CHK1, and CHK2 deficiency, we have shown that sensitivity to PARP inhibition can also be caused by the inability to sense DNA damage. The demonstration that FANC-deficient cells are sensitive to PARP inhibition while being proficient in RAD51 focus formation suggests that the Fanconi anemia genes are either downstream of RAD51 focus formation or they act in another parallel pathway (37).

The sensitivity of cells deficient in proteins involved in HR to PARP inhibition suggests that treatment with PARP inhibitors may be a useful therapeutic strategy for tumors displaying properties of 'BRCAness' or with defects in the HR pathway (41). Sporadic breast (11-14%) and ovarian (5-31%) cancers have been shown to inactivate the *BRCA1* gene by methylation (41). Although there is little evidence for methylation of the *BRCA2* gene, other modes of *BRCA2* inactivation have been proposed in sporadic breast and ovarian cancers (41).

The observation that ATM and CHK2 depletion resulted in sensitivity to PARP inhibition further suggests that PARP inhibition would be beneficial for a wide variety of cancers with dysfunction of genes involved in the DNA damage response. Mutations in the *ATM* gene have been found in patients with leukemia (42, 43) and breast cancer (44). Heterozygous CHK2 germ-line mutations have been observed in a variety of tumors (45, 46). In addition, a significant proportion of sporadic cancers has been shown to inactivate the Fanconi anemia pathway through methylation of the *FANCF* promoter (47, 48).

In summary, our data support the hypothesis that a deficiency in HR is a determinant of sensitivity to PARP inhibition and not BRCA1 or BRCA2 deficiency per se. This suggests that a wider range of cancers may benefit from the use of PARP inhibition other than solely BRCA1 or BRCA2 germ-line-associated breast cancers.

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