

# RAD51 Up-regulation Bypasses *BRCA1* Function and Is a Common Feature of *BRCA1*-Deficient Breast Tumors

Richard W. Martin,<sup>1,2</sup> Brian J. Orelli,<sup>1,2</sup> Mitsuyoshi Yamazoe,<sup>4</sup> Andy J. Minn,<sup>1,3</sup> Shunichi Takeda,<sup>4</sup> and Douglas K. Bishop<sup>1,2</sup>

Departments of <sup>1</sup>Radiation and Cellular Oncology and <sup>2</sup>Molecular Genetics and Cell Biology and <sup>3</sup>Ludwig Center for Metastasis Research, University of Chicago, Chicago, Illinois, and <sup>4</sup>Radiation Genetics, Faculty of Medicine, Kyoto University, CREST Research Project, Japan Science and Technology, Kyoto, Japan

## Abstract

The breast cancer susceptibility gene *BRCA1* encodes a large protein thought to contribute to a variety of cellular processes, although the critical determinants of *BRCA1*-deficient tumorigenesis remain unclear. Given that *BRCA1* is required for cell proliferation, suppressor mutations are believed to modify *BRCA1* phenotypes and contribute to the etiology of *BRCA1*-deficient tumors. Here, we show that overexpression of the homologous recombinase RAD51 in a DT40 *BRCA1*<sup>Δ/Δ</sup> mutant rescues defects in proliferation, DNA damage survival, and homologous recombination (HR). In addition, epistasis analysis with *BRCA1* and the DNA end-joining factor KU70 indicates that these factors operate independently of one another to repair double-strand breaks. Consistent with this genetic finding, cell synchronization studies show that the ability of *BRCA1* to promote radioresistance is restricted to the late S and G<sub>2</sub> phases of the cell cycle, as predicted for genes whose function is specific to homology-mediated repair rather than nonhomologous end-joining. Notably, retrospective analyses of microarray expression data reveal elevated expression of RAD51 and two of its late-acting cofactors, RAD54 and RAD51AP1, in *BRCA1*-deficient versus sporadic breast tumors. Taken together, our results indicate that up-regulation of HR provides a permissive genetic context for cells lacking *BRCA1* function by circumventing its requirement in RAD51 subnuclear assembly. Furthermore, the data support a model in which enhanced HR activity contributes to the etiology of *BRCA1*-deficient tumors. [Cancer Res 2007; 67(20):9658–65]

## Introduction

Mutations in the breast cancer susceptibility gene *BRCA1* confer a high risk of breast and ovarian cancer (1). Extensive genetic and molecular analysis of *BRCA1* function indicates that it participates in a variety of cellular processes. Importantly, *BRCA1* is known to participate in the cellular response to DNA damage, a role in which it serves to maintain genomic stability (2). However, neither the critical contribution that *BRCA1* makes to tumor suppression nor its precise molecular role in maintaining genome stability is clear.

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

R.W. Martin and B.J. Orelli contributed equally to this work.

**Requests for reprints:** Douglas K. Bishop, Cummings Life Science Center, Box 13, Room 821, 920 East 58th Street, Chicago, IL 60637. Phone: 773-702-9211; Fax: 773-834-9064; E-mail: dbishop@uchicago.edu.

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One important function, with particular relevance to the results presented here, is the role of *BRCA1* in repair of DNA damage. The link between *BRCA1* and DNA repair was established by the seminal demonstration that *BRCA1* colocalizes with the homologous recombinase RAD51 in subnuclear immunostaining foci (3). Subsequent genetic analyses indicated that *BRCA1* is critical for both the repair of DNA double-strand breaks (DSB) by homologous recombination (HR; refs. 4, 5) as well as the subnuclear assembly of RAD51 after DNA damage (6–8). *BRCA1* is likely to contribute to the HR reaction in conjunction with the breast cancer susceptibility gene *BRCA2* (8, 9). However, although significant mechanistic insight into the role of *BRCA2* has been achieved (ref. 9 and references therein), the details of *BRCA1* involvement in the HR reaction remain unknown.

The HR pathway is critical for cell viability in an otherwise “normal” cellular context (10, 11). It is therefore likely that the embryonic and cellular lethality associated with *BRCA1* disruption is due to deficiency in HR (12–15). Indeed, the proliferative defects and apoptosis observed in *BRCA1*-deficient mouse models mirror the phenotypes of RAD51-deficient systems, in which a toxic load of endogenous damage accumulates, leading to cell cycle arrest and apoptosis (10, 11). These observations highlight the fact that the development of *BRCA1*-deficient tumors is likely to depend on additional modifications of the “normal” genome that compensate for *BRCA1* growth defects. This possibility is consistent with genetic studies in mice showing that elimination of cell cycle checkpoints or apoptotic controls allow for enhanced proliferation and rescue of *BRCA1* mutant viability (15–17). Given this, identifying modifiers of *BRCA1* relevant to tumor progression is an important goal toward understanding the etiology of *BRCA1*-mediated disease.

Here, we use a *BRCA1*-null derivative of the vertebrate DT40 cell line to provide evidence that up-regulation of the HR pathway is capable of rescuing the growth and DNA repair defects associated with loss of *BRCA1* function. In addition, epistasis and cell cycle analyses support the assignment of *BRCA1* as a bona fide HR factor. These observations support the conclusion that the HR reaction is the principal, evolutionarily conserved effector pathway controlled by *BRCA1* after DNA damage. Moreover, they raise the possibility that elevated HR provides a permissive genetic context for the development of *BRCA1*-deficient tumors. Consistent with this view, breast tumor microarray data bear evidence of significant up-regulation of RAD51 and two of its accessory factors in *BRCA1*-deficient tumors. Together, our results provide insight into the mechanism by which *BRCA1* suppresses tumorigenesis as well as the manner in which its disruption may give rise to genomic instability and cancer.

## Materials and Methods

**Cell culture and transfections.** The chicken lymphoma B-cell line DT40 was cultured at 39°C, 5% CO<sub>2</sub> in RPMI medium (Life Technologies)

supplemented with 10% fetal bovine serum (Atlas), 1% chicken serum (Sigma), and 50  $\mu\text{mol/L}$   $\beta$ -mercaptoethanol (Sigma). Targeting vectors were created to disrupt the *BRCA1* locus by PCR amplification of genomic sequence 5' of the translational start site and at the 3' end of exon 11 and insertion flanking selection cassettes for histidinol or puromycin resistance. Twenty micrograms of linearized targeting plasmid were electroporated (550 V, 25  $\mu\text{F}$ ) into  $10^7$  cells, and stable transformants were isolated by selection in 1.5 mg/mL histidinol and 0.5  $\mu\text{g/mL}$  puromycin, respectively. To generate *BRCA1* <sup>$\Delta/\Delta$</sup>  *KU70* <sup>$\Delta/\Delta$</sup>  cells, the same targeting strategy was repeated beginning with a *KU70* <sup>$\Delta/\Delta$</sup>  derivative of DT40 (18). Clones were screened for correct integration by Southern analysis. To generate the RAD51-over-expressing cell lines, a human *RAD51* cDNA expression construct (19) was transfected and clones stably expressing high levels of ectopic RAD51 were isolated. For homologous targeting assays, a construct designed to disrupt the immunoglobulin light chain was transfected as described above, and transfectants were selected in 20  $\mu\text{g/mL}$  blasticidin.

**Growth and clonogenic survival assays.** Doubling times were determined by trypan blue exclusion using a Levy Brightline hemacytometer. Cell synchronization (20) and methylcellulose clonogenic assays (19) were done as previously described. Cells were irradiated using a Maxitron generator calibrated for this purpose (250 kVp photons, 15 mA, 0.5 mm Cu filtration, dose rate 1.55 Gy/min). *Cis*-platinum was added directly to the methylcellulose medium.

**Immunoblotting.** SDS-PAGE and Western blotting were conducted by standard methods. Human RAD51 antibody was generously provided by Akira Shinohara. Mouse monoclonal  $\beta$ -tubulin antibody (Sigma) was used at 1:1,000.

**Immunofluorescence.** For Rad51 immunofluorescence assays, cells were spun onto glass slides and stained as described (21). Images were collected on a Zeiss Apotome-equipped Axioplan 2 microscope ( $\times 60$  objective, 0.25  $\mu\text{m}$  optical sectioning) with an ORCA2-ER camera (Hamamatsu) and processed with Axiovision 4.2 imaging software (Zeiss). Images shown are projected Z-series reconstructions.

**Flow cytometry.** Bromodeoxyuridine (BrdUrd) labeling and fluorescence-activated cell sorting (FACS) analysis were conducted as previously described (18). Annexin V assays were conducted using an Annexin V-FITC conjugate (BD PharMingen) according to the protocol supplied by the manufacturer. Surface IgM expression was assayed as previously described (22) using a chicken anti-IgM antibody conjugated to FITC (Bethyl Labs). FACS data were collected on a FACScanII (Becton Dickinson) and analyzed with FloJo version 6.2 software (Tree Star) with at least 20,000 live cells used for each analysis.

**CHEF gel analysis.** Cells ( $5 \times 10^5$ ) were embedded in low-melt agarose, digested with proteinase K and RNase A, and run through a 0.5% agarose gel in  $0.5 \times$  Tris-borate EDTA on a CHEF DR II apparatus (Bio-Rad). Gels were stained with Cybergreen (Molecular Probes), visualized on a STORM phosphorimaging system (Molecular Dynamics), and quantitated using ImageQuant software (Molecular Dynamics).

**Microarray data analysis.** The data set containing microarray expression profiles of 117 primary breast tumors was obtained from the Rosetta Inpharmatics web site (23). Analyses were done using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam and available on the National Cancer Institute web site.<sup>5</sup> Class comparisons were computed by random variance *t* tests between the 18 BRCA1 tumors and the 99 non-BRCA1 tumors (including two BRCA2-defective tumors) based on eight genes comprising the core HR reaction that do not have known roles beyond HR. These genes were also used in hierarchical clustering using average linkage and a Pearson correlation as the similarity metric.

Out of 172 S-phase genes previously described (24), 144 were mapped to the Rosetta microarray platform. The evaluation of whether this S-phase gene list was differentially expressed between BRCA1 and non-BRCA1 samples was done using a functional class scoring analysis described by Pavlidis et al. (25). For each gene, the *P* value for comparing BRCA1 versus non-BRCA1 was computed and the set of *P* values for a class was

summarized by two summary statistics: (a) The LS summary is the average log *P* values for the genes in that class and (b) the KS summary is the Kolmogorov-Smirnov statistic computed on the *P* values for the genes in that class. The statistical significance of the S-phase class containing 144 genes represented on the array was evaluated by computing the empirical distribution of these summary statistics in random samples of 144 genes. The functional class scoring analysis was done using BRB-ArrayTools.

## Results

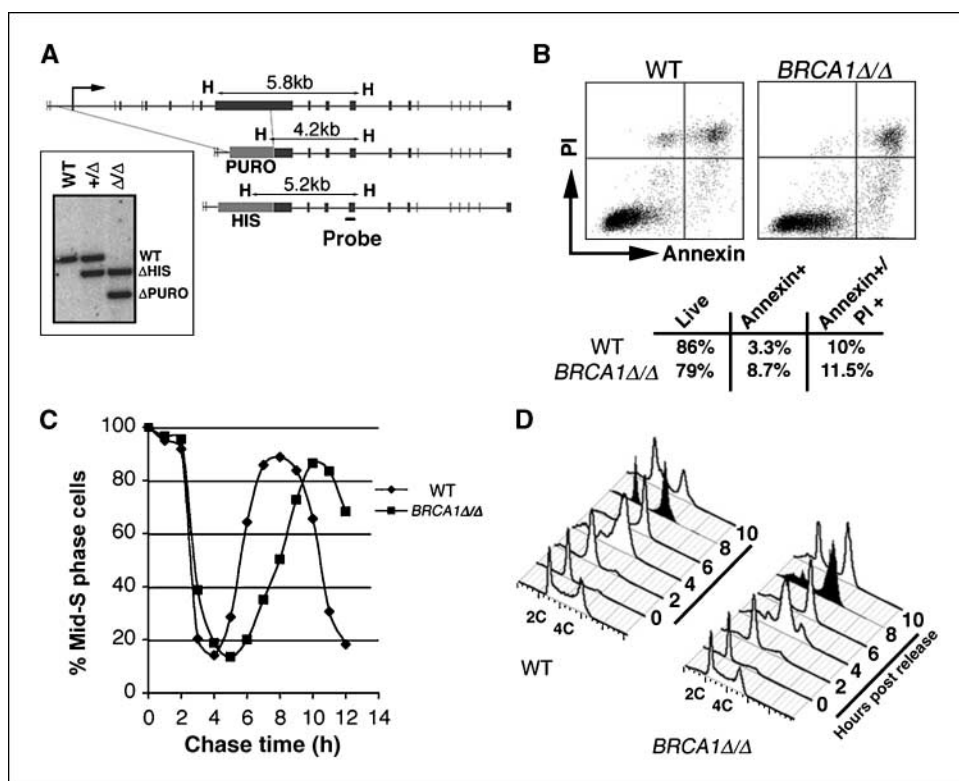
**Generation and cell cycle characterization of a *BRCA1* <sup>$\Delta/\Delta$</sup>  DT40 derivative.** To study BRCA1 function in a genetically tractable, isogenic model, we generated a *BRCA1*-null mutant of the vertebrate cell line DT40. The schematic and Southern confirmation of the targeted knockout are presented in Fig. 1A. Western blot analysis using an antibody raised against chicken BRCA1 protein confirmed the absence of expression in the homozygous knockout line.<sup>6</sup>

BRCA1 is not essential for survival in DT40 cells (ref. 26 and the present study). This result is likely to depend on DT40 p53 deficiency (27) as loss of p53 is known to rescue *BRCA1* <sup>$-/-$</sup>  viability (15, 16). However, *BRCA1* <sup>$\Delta/\Delta$</sup>  DT40 cells exhibit a proliferative defect resulting in a 1.35-fold increase in doubling time relative to isogenic controls (wild-type 9.1 h versus *BRCA1* <sup>$\Delta/\Delta$</sup>  12.3 h; Fig. 2B). Thus, our DT40 model recapitulates the proliferative defects associated with BRCA1 ablation in murine models (13, 15). FACS methods were used to show that the observed slow growth of the mutant results from a combination of an increase in the frequency of apoptosis (Fig. 1B) and an  $\sim 2$ -h increase in the length of the cell cycle (Fig. 1C). Synchronous release from a G<sub>1</sub>-S arrest followed by time course FACS analysis indicates that the delay occurs between late S and the completion of mitosis in the *BRCA1* <sup>$\Delta/\Delta$</sup>  mutant (Fig. 1D; Supplementary Fig. S1). *BRCA1* <sup>$-/-$</sup>  cells are known to accumulate chromosome aberrations as a consequence of failing to repair spontaneous and induced damage (data not shown and ref. 16). Therefore, we speculate that the longer duration of G<sub>2</sub>-M in *BRCA1* <sup>$\Delta/\Delta$</sup>  DT40 cultures reflects a checkpoint response to spontaneous damage incurred during the previous S phase.

BRCA1 has been implicated in the ionizing radiation-induced G<sub>2</sub>-M checkpoint in mammalian cells (28–31). However, there is also evidence for BRCA1-independent G<sub>2</sub>-M arrest in the presence of damage (32, 33). Consistent with the latter data set, we find that *BRCA1* <sup>$\Delta/\Delta$</sup>  DT40 cells display a robust, ionizing radiation-induced G<sub>2</sub>-M arrest (Supplementary Fig. S2A–C) as well as efficient activation of the checkpoint effector Chk1 (Supplementary Fig. S2D), indicating that BRCA1 is not needed for the G<sub>2</sub>-M checkpoint in these cells under the conditions examined. The bases for the discrepant reports concerning the role of BRCA1 in this checkpoint are unclear, but could involve differences in degree of redundancy of checkpoint functions in different cell types and/or differences in the methodology used to detect the checkpoint. In the latter regard, our data indicate that high levels of phosphohistone H3 in *BRCA1* <sup>$\Delta/\Delta$</sup>  DT40 cells 1 h after irradiation are a consequence of delayed mitotic exit in these cells rather than failure of G<sub>2</sub>-M arrest (Supplementary Fig. S2A). Consistent with this interpretation, incubation of cells in nocodazole to block both *BRCA1* <sup>$\Delta/\Delta$</sup>  and wild-type cells from exiting mitosis confirms an intact ionizing radiation-induced block to mitotic entry in *BRCA1* <sup>$\Delta/\Delta$</sup>  DT40 cells (Supplementary Fig. S2B).

<sup>5</sup> <http://linus.nci.nih.gov/BRB-ArrayTools.html>

<sup>6</sup> Ö. Yildez and D.K. Bishop, unpublished observations.



**Figure 1.** Generation and cell cycle characterization of a *BRCA1* $\Delta/\Delta$  derivative of DT40. **A**, targeting strategy and Southern confirmation for generation of the *BRCA1* $\Delta/\Delta$  DT40 line. The targeted deletion is from intron 2 (which lies upstream of the translational start site) to exon 11. Correctly targeted clones were identified by Southern hybridization analysis using a probe for sequences 3' of those used for targeting. Genomic DNA was digested with *Hind*III. **B**, cultured cells were incubated with Annexin V and propidium iodide (PI), and  $2 \times 10^5$  were analyzed by two-color FACS. **C**, cultures of DT40 cells were pulse labeled with BrdUrd and followed for 12 h by two-color FACS with anti-BrdUrd-FITC and propidium iodide. Data are expressed as the percentage of BrdUrd-labeled cells occupying mid-S phase at the indicated times. **D**, cells were released from a nocodazole/mimosine  $G_1$  arrest and assayed at various times for DNA content by propidium iodide staining and FACS analysis.

**Rescue of *BRCA1* $\Delta/\Delta$  phenotypes by overexpression of the homologous recombinase RAD51.** *BRCA1* $^{-/-}$  cells exhibit reduced HR-mediated DSB repair (4, 5) and defective RAD51 subnuclear assembly (6), phenotypes similar to those of the RAD51 mediators. Overexpression of RAD51 suppresses the phenotypes of mediator mutants in both yeast and vertebrates (19, 34, 35). To determine if RAD51 overexpression is also capable of suppressing *BRCA1* $\Delta/\Delta$  phenotypes, the *BRCA1* $\Delta/\Delta$  DT40 cell line was stably transfected with a construct that directs expression of human RAD51 protein. Clones expressing high levels of RAD51 (~15-fold overexpression relative to endogenous protein) were selected (Fig. 2A). Overexpression of RAD51 partially rescued the proliferative defect of *BRCA1* $\Delta/\Delta$  cells and also restored normal  $G_2$ -M progression ( $T_d = 11$  h; Fig. 2B; Supplementary Fig. S3). Furthermore, RAD51 overexpression rescued the sensitivity of *BRCA1* $\Delta/\Delta$  cells to both X-rays and the cross-linking agent *cis*-platinum (Fig. 2C and D), although at higher doses rescue of the mutant phenotype was not complete (data not shown). These suppression results were confirmed for an independent *BRCA1* $\Delta/\Delta$  transfectant expressing a similar level of RAD51 (data not shown). Importantly, when RAD51 was overexpressed in wild-type DT40 cells, there was no effect on growth rate (Fig. 2B) and no increase in damage resistance; resistance to X-rays was the same as that in wild-type cells (Fig. 2C) and resistance to cisplatin was somewhat lower (Fig. 2D). These results indicate that rescue of *BRCA1* $\Delta/\Delta$  damage sensitivity by RAD51 overexpression is specific to the mutant background.

To determine if BRCA1 is required for RAD51 assembly in DT40 and if RAD51 overexpression suppresses such a requirement, we exposed cells to 4 Gy X-rays followed by fixation at 4 h and staining with RAD51 antibodies. Exposure of wild-type DT40 cells to this dose resulted in focus induction with kinetics similar to what has been previously reported for this cell line (36). Consistent with

results from mammalian cells, *BRCA1* $\Delta/\Delta$  DT40 cells show a 4-fold reduction in damage-induced RAD51 foci relative to isogenic wild-type controls (Fig. 3A and B). RAD51 overexpression in the *BRCA1* $\Delta/\Delta$  background significantly increased the number of radiation-induced nuclear foci (Fig. 3A and B; 2.5-fold, Wilcoxon rank-sum test  $P = 0.004$ ). High levels of cytoplasmic staining in the overexpressing line may have reduced detection of foci at the nuclear periphery. Thus, the suppression of the focus defect may be more efficient than suggested by these data.

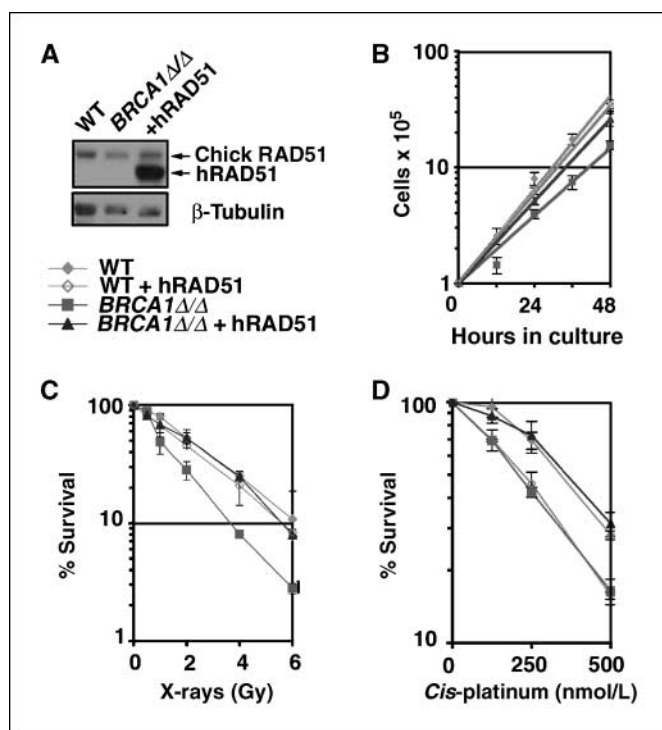
To assess the effect of BRCA1 deficiency and RAD51 overexpression on HR in the DT40 system, we used a knockout construct targeting the immunoglobulin light chain locus and assayed loss of IgM surface expression by FACS to identify targeted transfectants (22). BRCA1 ablation reduced HR-targeting frequency at the DT40 immunoglobulin light chain locus from 12% to 0.35% (32 of 272 transfectants compared with 1 of 283 transfectants). RAD51 overexpression significantly improved the targeting efficiency of *BRCA1* $\Delta/\Delta$  cells 10-fold to 3.7% (12 of 318 transfectants;  $P = 0.02$  Fisher's exact test). Suppression of the *BRCA1* $\Delta/\Delta$  DNA damage sensitivity, RAD51 assembly, and HR defects by RAD51 overexpression provides critical support for our previous proposal that defective recombinase assembly can account for these phenotypes of *BRCA1* $^{-/-}$  cells (6). Partial suppression of *BRCA1* $\Delta/\Delta$  phenotypes may reflect incomplete or aberrant restoration of the HR pathway and/or roles of *BRCA1* in other damage responses.

**Nonpistaxis with KU70 and late S- $G_2$ -specific radiosensitivity of *BRCA1* $\Delta/\Delta$  cells.** The results presented above provide genetic evidence that BRCA1 promotes damage survival largely through its effects on RAD51 assembly rather than through effects on other aspects of the DNA damage response. Given the multitude of functions assigned to the BRCA1 protein, however, we sought to use our DT40 genetic model to investigate this further. To this end,

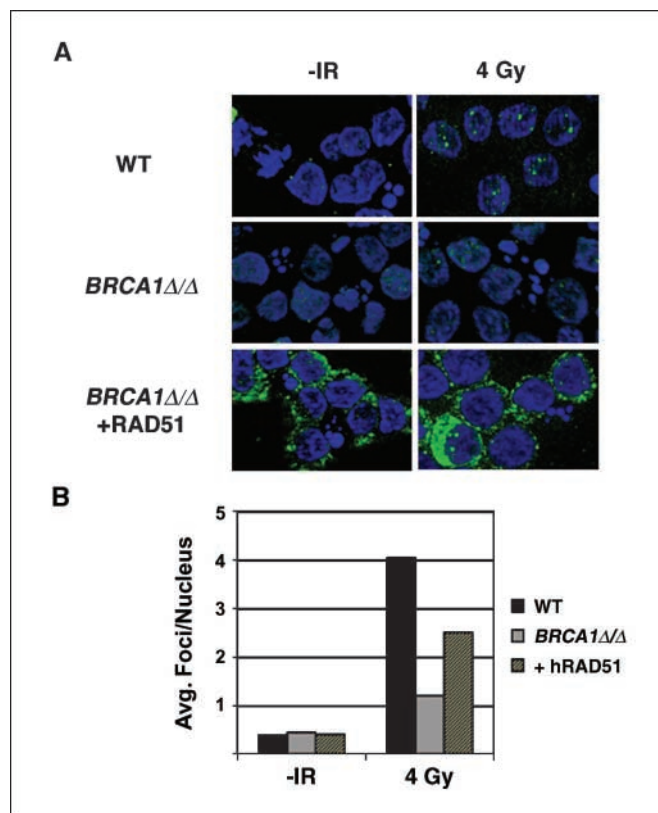
we conducted epistasis analysis between *BRCA1* and the nonhomologous end-joining (NHEJ) gene *KU70*. *BRCA1* has been implicated in regulation of NHEJ (37, 38), although conflicting results have been reported (4, 15). Homozygous deletion of *KU70* in *BRCA1*<sup>Δ/Δ</sup> DT40 cells did not exacerbate the proliferative defect of *BRCA1*<sup>Δ/Δ</sup> cells consistent with a limited role for NHEJ in repair of spontaneous lesions that arise during replication (Fig. 4A). Exposure of either single mutant to X-rays resulted in loss of clonogenic survival relative to wild-type cells, although at high dose *KU70*<sup>Δ/Δ</sup> cells exhibit radioresistance (Fig. 4B). This phenotype was previously interpreted to signify channeling of DSBs by upstream components of the NHEJ pathway into toxic repair intermediates and away from high-fidelity homology-directed repair (39). Strikingly, exposure of the double-mutant cell line to X-rays revealed dramatic synergy in X-ray sensitivity relative to either single mutant (Fig. 4B).

To determine if the synergistic survival defect of the double mutant is explained by the ability of *BRCA1* and *KU70* to act independently in DSB repair, we analyzed the fate of ionizing radiation-damaged chromosome fragments by CHEF gel electrophoresis. This method revealed a severe repair defect in the *KU70*<sup>Δ/Δ</sup> *BRCA1*<sup>Δ/Δ</sup> double mutant relative to wild-type or either single mutant (Fig. 4C). Lack of epistasis between these genes indicates that the *KU70*-dependent NHEJ pathway is largely or completely intact in *BRCA1*<sup>Δ/Δ</sup> DT40 cells, whereas *BRCA1*-dependent repair is largely or completely intact in the absence of NHEJ.

Previous studies indicate that NHEJ predominates in the G<sub>1</sub> phase of the cell cycle, whereas HR is the preferred mode of DSB repair in late S and G<sub>2</sub> cells (18). Furthermore, *BRCA1* is trans-



**Figure 2.** RAD51 overexpression rescues *BRCA1*<sup>Δ/Δ</sup> phenotypes. *A*,  $\alpha$ -Rad51 immunoblot of whole-cell lysates from wild-type (WT), *BRCA1*<sup>Δ/Δ</sup>, and *BRCA1*<sup>Δ/Δ</sup> + hRAD51 cells. *B*, growth curves for the indicated lines assessed by trypan blue staining and hemacytometry. Clonogenic survival in response to X-rays (*C*) or chronic *cis*-platinum exposure (*D*). Points, mean ( $n = 3$ ) expressed as the percentage of surviving cells relative to values from untreated controls; bars, SD.

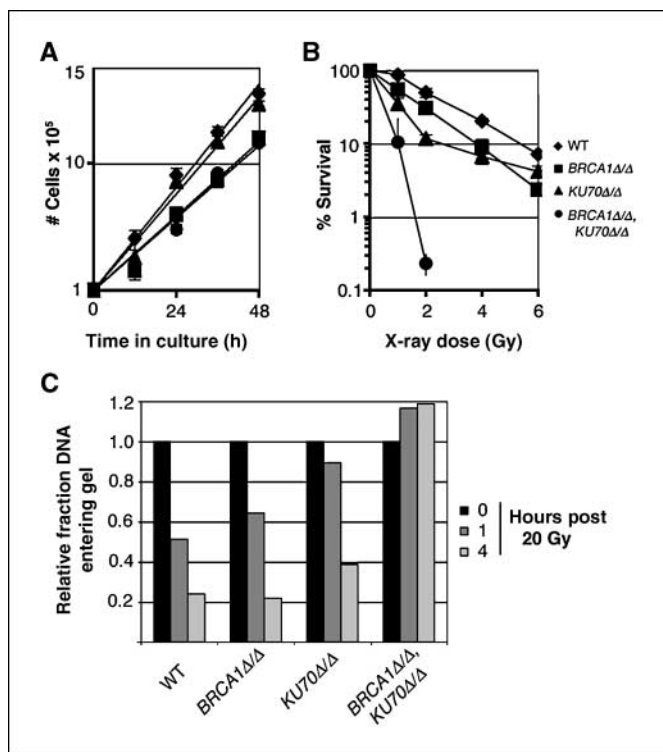


**Figure 3.** RAD51 overexpression restores damage-induced RAD51 focus formation. *A*, cells were exposed to 4 Gy X-rays or left untreated, fixed at 4 h post-ionizing radiation, and stained with anti-RAD51 antibodies followed by FITC-conjugated secondary antibody. Images are of projected Z-series (0.25  $\mu$ m sectioning distance) acquired with an Apotome-equipped fluorescence microscope. *B*, average focus counts of >50 cells for each sample.

criptionally (40) and posttranslationally (41, 42) regulated in a manner consistent with S and G<sub>2</sub> functions. To determine if the ability of *BRCA1* to promote radioresistance varies during the cell cycle, cells were synchronized at the G<sub>1</sub>-S boundary by successive nocodazole and mimosine exposure and released into S phase (Fig. 5A). The two synchronized cell populations were exposed to X-rays at 1 h (G<sub>1</sub>-early S) and 6 h (late S-G<sub>2</sub>) and plated to assay viability (Fig. 5B). As reported previously, wild-type DT40 cells were more sensitive to X-rays in G<sub>1</sub> than in G<sub>2</sub> (18). Strikingly, the ionizing radiation hypersensitivity of *BRCA1*<sup>Δ/Δ</sup> cells was entirely accounted for by the sensitivity of the late S and G<sub>2</sub> phases; *BRCA1*<sup>Δ/Δ</sup> cells showed sensitivity identical to wild-type in G<sub>1</sub>. We also confirmed that deletion of *KU70* specifically sensitizes G<sub>1</sub> cells (18) and showed that the *KU70*<sup>Δ/Δ</sup> *BRCA1*<sup>Δ/Δ</sup> double mutant is sensitized in both cell cycle stages (data not shown). Therefore, *BRCA1* contributes to the radiation resistance of late S and G<sub>2</sub> cells at a time when HR is the predominant DSB repair pathway.

**Evidence for up-regulation of HR genes in *BRCA1*-deficient tumors.** The identification of RAD51 as an extragenic, dosage-dependent suppressor of *BRCA1*<sup>Δ/Δ</sup> phenotypes raises the possibility that elevated HR enhances or is required for proliferation of *BRCA1*-deficient cells during tumorigenesis. To address this possibility, we sought evidence for elevated expression of *RAD51* transcripts in *BRCA1*-deficient tumors (hereafter "BRCA1 tumors"). This was done by analysis of microarray data on RNAs from 117 primary breast tumors previously published by van't Veer et al. (23).





**Figure 4.** *BRCA1*<sup>Δ/Δ</sup> *KU70*<sup>Δ/Δ</sup> epistasis analysis. **A**, cell growth assays via trypan blue exclusion and a hemacytometer. Points, mean ( $n = 3$ ); bars, SD. **B**, clonogenic survival after X-ray treatment. Colonies were counted 10 d after plating on methylcellulose medium. Points, mean for each condition ( $n = 3$ ); bars, SD. **C**, CHEF gel assay for resolution of ionizing radiation-induced chromosome fragments. Numbers plotted are the fraction of DNA that enters the gel/total DNA as determined by phosphorimaging. Fractions are normalized to those obtained immediately after irradiation ( $t = 0$ ). Fragments were induced by a dose of 20 Gy X-rays.

We analyzed the expression differences between BRCA1 and non-BRCA1 tumors for *RAD51* itself, as well as two sets of genes encoding *RAD51* accessory factors, *RAD51* assembly factors (also known as mediators), and postassembly accessory factors. First, and most important, analysis of *RAD51* indicated a significant correlation between *RAD51* expression levels and BRCA1 status (one-tailed  $t$  test,  $P = 0.0007$ ); BRCA1 tumor RNA contained 2.5-fold higher levels of *RAD51* transcript on average compared with RNA from sporadic tumors. There are two known *RAD51* accessory factors that function after assembly: *RAD54* and *RAD51API* (also called *PIR51*; ref. 43).<sup>7</sup> Like *RAD51*, both of these factors showed significant association with BRCA1 status; *RAD54* was expressed an average of 4-fold higher ( $P = 1.1 \times 10^{-6}$ ) and *RAD51API/PIR51* an average of 2-fold higher ( $P = 0.007$ ). In contrast, no significant difference in expression levels was found for the five transcripts encoding *RAD51* assembly factors, including *RAD51B*, *RAD51C*, *RAD51D*, *XRCC3*, and *RAD52*. Hierarchical clustering of tumors based on the expression profiles of these eight HR genes using a Pearson correlation metric emphasizes that the majority of BRCA1 tumors fall into a cluster with elevated levels of *RAD51*, *RAD54*, and/or *RAD51API/PIR51* (Fig. 6). We note that these results are in agreement with a cytologic analysis of protein levels in tissue microarrays from a separate set of tumors, which also indicates

<sup>7</sup> C. Weis, E. Dray, P. Sung, and D. Schild, personal communication.

higher levels of nuclear *RAD51* in BRCA1 tumors compared with sporadic specimens (44).

BRCA1 tumors tend to be of higher grade than sporadic tumors. Given this difference, it was important to eliminate the possibility that, rather than resulting from up-regulation at the transcript level, high recombinase levels in BRCA1 tumor samples simply reflect higher proliferative index and/or a larger fraction of cells in S-phase. This concern is particularly important because *RAD51*, *RAD54*, and *RAD51API/PIR51* are specifically expressed in S-phase. To directly address a possible enrichment in S-phase cells among the BRCA1 population, we conducted gene-set enrichment analysis of the tumor data set using a list of S-phase expressed genes (defined by their expression pattern during a synchronous HeLa cell cycle; ref. 24). Based on analysis of 144 S-phase genes, there was no significant enrichment among BRCA1 tumors (LS permutation  $P = 0.172$ , KS permutation  $P = 0.116$ ; see Materials and Methods for details). To ensure that the method used was capable of detecting relevant differences, the same analysis was also applied to the breast cancer samples classified as either poor prognosis or good prognosis by the 70-gene signature described by van't Veer et al. (23). Proliferation-related genes are a common denominator of the 70-gene signature (45). Consistent with this, our analysis shows that the 144 S-phase gene list is significantly enriched in the poor prognosis patients relative to the good prognosis patients (LS permutation  $P = 1 \times 10^{-7}$ , KS permutation  $P = 0.00016$ ). We therefore conclude that *RAD51*, and its two late-functioning accessory factors, are expressed at higher levels in BRCA1 tumors than in sporadic tumors independent of cell cycle variables.

## Discussion

The results presented above have two principal implications. First, the ability of *RAD51*, an HR-specific protein, to bypass BRCA1 function indicates that the principal conserved effector pathway controlled by BRCA1 in response to DNA damage is HR. Our epistasis and cell cycle analysis further corroborate this interpretation and add to evidence that BRCA1 is not required for the DNA end-joining pathway. A second implication is that up-regulation of the HR pathway may contribute to the etiology of BRCA1-deficient breast cancers.

Previous work showed that overexpression of *RAD51* can enhance resistance to DNA damage and increase the frequency of recombination in certain cell lines that are presumed or known to be *BRCA1*<sup>+/+</sup> (46). Furthermore, a variety of tumor types have been found to display high levels of *RAD51* relative to normal tissue (46). These findings suggest that the role of *RAD51* overexpression in tumorigenesis is not limited to compensating for BRCA1 deficiency. Here, we used a genetic model to show high copy suppression of a BRCA1 mutant by *RAD51*. Importantly, we found that overexpression of *RAD51* had no effect the rate of proliferation or the DNA damage sensitivity of wild-type (*BRCA1*<sup>+/+</sup>) DT40 cells. This feature of our model system made it possible to show specific suppression of *BRCA1*<sup>Δ/Δ</sup> defects by *RAD51*. We also show here that elevation of HR can enhance cellular growth rate in *BRCA1*<sup>Δ/Δ</sup> cells. This novel finding leads us to the hypothesis that elevation of HR efficiency promotes tumorigenesis via suppression of the growth defect associated with BRCA1 deficiency.

Rescue of damage sensitivity, *RAD51* focus formation, and HR by *RAD51* overexpression indicates that BRCA1 contributes to DNA

repair by regulating recombinase assembly in a manner functionally analogous to that of the RAD51 assembly factors, including the so-called RAD51 mediators. Previous studies showing that RAD51 fails to assemble at damaged sites in the absence of BRCA1 indicated its requirement at this early step (6–8). The suppression of defects in damage survival and gene targeting by a method that restores RAD51 assembly suggests that the contribution of BRCA1 to HR is likely to be limited to the RAD51 assembly step, with postassembly stages of recombination proceeding with relatively little dependence on BRCA1.

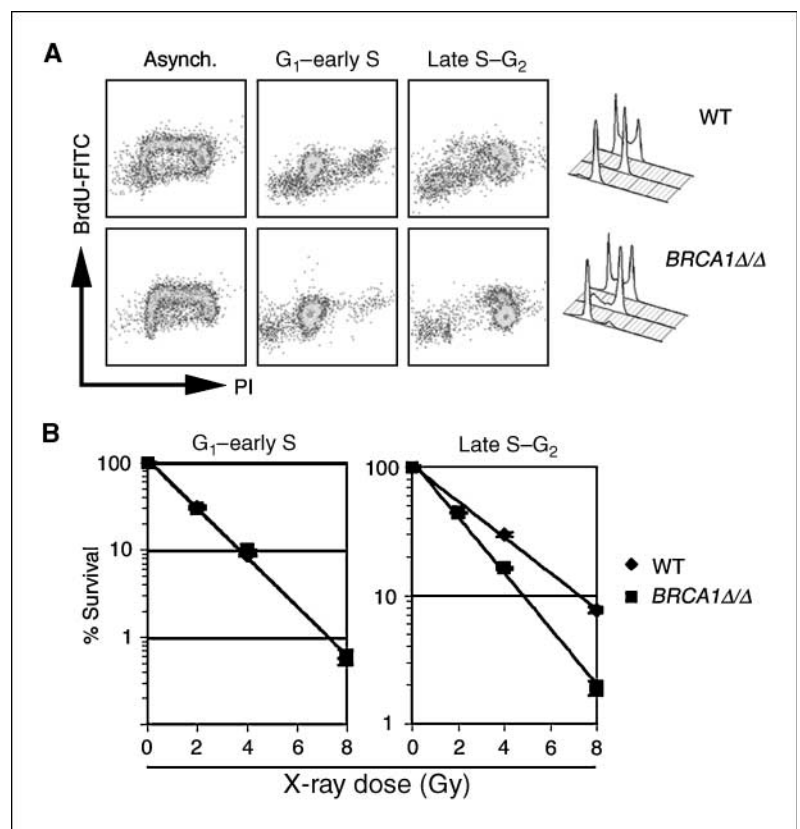
Current evidence suggests that BRCA1 may exert control over RAD51 assembly by either a direct mechanism involving protein-protein interactions, or indirectly via a signaling function. RAD51 interacts directly with several of its assembly factors including the RAD51 paralogs and BRCA2 (3, 9, 47). Interestingly, a recent report shows that BRCA1 is required for correct localization of BRCA2, suggesting that BRCA2 may physically link BRCA1 and RAD51 in the HR reaction (8). A nonexclusive alternative to direct physical regulation is that BRCA1 may promote assembly of BRCA2 and RAD51 indirectly by mediating damage-induced signals to the repair machinery via its well-established interactions with this signaling apparatus (48, 49). This view is consistent with the recent identification of a CHK2-dependent phosphorylation site on BRCA1 required for RAD51 assembly (7).

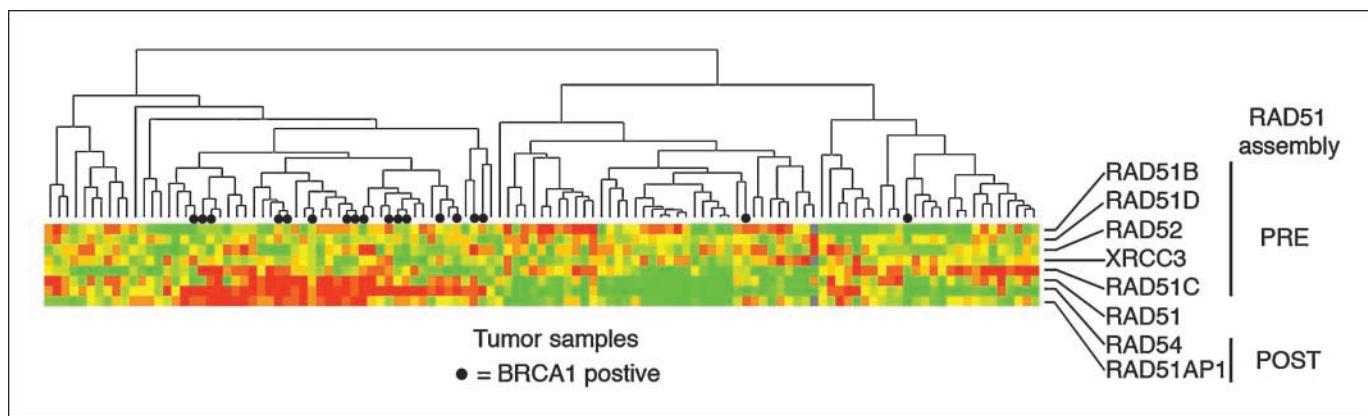
Given that increased recombinase expression can bypass the function of assembly factors, what function did assembly factors evolve to serve? There is increasing experimental support for the idea that unrestrained HR is as deleterious to genome stability as HR deficiency (50, 51). Tracks of ssDNA are normal intermediates during processes such as DNA replication and transcription. As such, barriers to recombinase assembly may have evolved to block

recombinase access to such substrates when no damage is present or when a less risky repair pathway, such as excision repair during replication or NHEJ in G<sub>1</sub> cells, might be used to resolve a lesion. Thus, BRCA1 and related factors regulating recombinase assembly may provide a means to induce assembly specifically at lesions that can only be accurately repaired by HR (e.g., DSBs). Interestingly, frequent chromosomal abnormalities present in BRCA1-deficient cells, including balanced translocations, quadriradials, and triradials, are likely to result from aberrant HR rather than HR deficiency. These observations led Scully et al. (52) to propose that loss of BRCA1 function results in deregulated sister chromatid recombination. The data presented here lead us to further propose that, rather than resulting from BRCA1 deficiency alone, chromosome instability in BRCA1 tumors results from a combination of BRCA1 deficiency and compensatory elevation of recombinase activity.

The growth and viability defects of BRCA1-deficient cells are difficult to reconcile with the deregulated growth of BRCA1-deficient tumors. The findings we present here suggest that one mechanism by which this apparent paradox is resolved is via genetic suppression of BRCA1 phenotypes by up-regulation of HR. The fact that the majority of BRCA1-deficient tumors examined exhibit significantly up-regulated expression of HR genes suggests either that there is a selection for such up-regulation during tumor development or that a high level of expression is a precondition for tumorigenesis of BRCA1-deficient cells. Previously defined modifiers of *BRCA1*<sup>-/-</sup> phenotypes in mice and human tumors include deficiency of checkpoint proteins p53 (15, 16, 53) and CHK2 (17). Overexpression of the antiapoptotic regulator BCL2 also ameliorates *BRCA1*<sup>-/-</sup> phenotypes (15). We now add elevated RAD51 to this list of modifiers of BRCA1 deficiency. We postulate that

**Figure 5.** *BRCA1*<sup>Δ/Δ</sup> DT40 cells are specifically sensitized to DSBs during late S–G<sub>2</sub> phases of the cell cycle. Cells were synchronized at the G<sub>1</sub>–S boundary by nocodazole/mimosine, released from the block, and fractions were collected at 1 h (G<sub>1</sub>–early S) and 6 h (late S–G<sub>2</sub>). **A**, FACS analysis of cell aliquots after BrdUrd pulse labeling (10 min) and staining with both BrdUrd antibody and propidium iodide; the 1 h fraction contains predominantly prereplicative cells with 2C DNA content, whereas the 6 h fraction contains mostly postreplicative 4C cells. **B**, cells from the two fractions assayed in **A** were exposed to X-rays and plated in methylcellulose medium. Colonies were counted 10 d after plating. Points, mean (n = 3); bars, SD.





**Figure 6.** Hierarchical clustering of 117 primary breast tumors based on the gene expression profiles of HR genes. Clustering was done as described in Materials and Methods. Each column represents a tumor sample. ●, BRCA1-positive tumors. Each row corresponds to the gene listed on the right. Relative levels of expression are indicated by color: high (red) and low (green). Functional assignments for accessory factors at pre- and post-RAD51 assembly steps of the recombination pathway are indicated at right.

up-regulated HR facilitates repair of both spontaneous and induced lesions, thereby avoiding the cell cycle arrest and apoptotic responses normally elicited by damage in the *BRCA1*<sup>-/-</sup> background.

Previous microarray analysis identified a subtype of breast cancer exhibiting a “basal-like” signature of gene expression, and BRCA1 tumors belong to this subtype (54). Interestingly, the expression levels of RAD51, RAD54, and RAD51AP1 in the van’t Veer tumor data set predict basal status, suggesting that up-regulated recombinase is common among basal-like tumors (76% accuracy by *k*-nearest neighbors).<sup>8</sup> The association of high gene expression of the three late-acting RAD genes with basal-like tumors suggests two possibilities; either recombinase expression is elevated in a common progenitor population for both BRCA1 and non-BRCA1 basal-like tumors, or acquisition of this feature is selected for during basal-like tumor progression. We view the first of these possibilities as less likely because no differences in expression levels of the RAD genes were seen by SAGE analysis of normal breast cell types, including the myoepithelial cells thought to be the progenitors of basal-like tumors (55).<sup>9</sup> The alternative possibility, that enhanced RAD expression is a selected compensatory trait, is consistent with the recent proposal that all basal-like tumors are

deficient in BRCA1 function (56). These alternatives pose intriguing questions for further study.

Given that the functional studies described above involved a chicken cell line, it will be important to test for genetic suppression of BRCA1 deficiency by RAD51 in human cells. It should be noted, however, that if loss of BRCA1 function results in a strong selection for elevated HR activity, established BRCA1-deficient tumor cell lines, such as the highly characterized HCC1937 line, may already have undergone selection for optimal RAD51 levels. If so, further elevation of RAD51 may show no effect in established tumor lines. If this turns out to be the case, it will be necessary to develop alternative approaches to showing that the ability of RAD51 to suppress BRCA1 deficiency has been conserved among vertebrates. The human tumor data discussed here provide a strong rationale for these studies.

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<sup>8</sup> A.J. Min, R.W. Martin, and D.K. Bishop, unpublished observation.

<sup>9</sup> <http://cgap.nci.nih.gov/SAGE>

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