Association of BRCA1 with the hRad50-hMre11-p95 Complex and the DNA Damage Response

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BRCA1 encodes a tumor suppressor that is mutated in familial breast and ovarian cancers. Here, it is shown that BRCA1 interacts in vitro and in vivo with hRad50, which forms a complex with hMre11 and p95/nibrin. Upon irradiation, BRCA1 was detected in discrete foci in the nucleus, which colocalize with hRad50. Formation of irradiation-induced foci positive for BRCA1, hRad50, hMre11, or p95 was dramatically reduced in HCC/1937 breast cancer cells carrying a homozygous mutation in *BRCA1* but was restored by transfection of wild-type *BRCA1*. Ectopic expression of wild-type, but not mutated, *BRCA1* in these cells rendered them less sensitive to the DNA damage agent, methyl methanesulfonate. These data suggest that BRCA1 is important for the cellular responses to DNA damage that are mediated by the hRad50-hMre11-p95 complex.

BRCA1 is a tumor-suppressor gene linked to familial breast and ovarian cancers (*1*). The hallmarks of BRCA1 protein include an NH_2-terminal RING finger domain and BRCA1 COOH-terminal (BRCT) repeats that mediate binding to CtIP (*2*). Several lines of evidence have indicated that BRCA1 is involved in DNA repair; BRCA1-deficient embryonic stem cells are hypersensitive to ionizing radiation and are defective in transcription-coupled repair of oxidative DNA damage (*3*). Upon DNA damage, BRCA1 becomes hyperphosphorylated and shows alterations in subnuclear localization (*4*) and CtIP binding (*2*). BRCA1 exon 11 deletion cells display a defective G_2/M checkpoint after ionizing radiation and methyl methanesulfonate (MMS) treatments (*5*).

To determine potential binding partners of BRCA1 that might elucidate its role in DNA repair, we immunoprecipitated ^{35}S-methionine–labeled T24 human bladder carcinoma cells with BRCA1 antibodies, and three coprecipitated cellular proteins (150, 95, and 84 kD) were revealed (*6*). The largest (150-kD) protein was confirmed to be hRad50 by reprecipitation with specific α-hRad50 (*7*), co-migrating with the immunoprecipitated and in vitro translated hRad50 (Fig. 1A). Following the cell cycle, BRCA1 was coimmunoprecipitated with hRad50, and this interaction appeared to peak at 33 hours after release from density arrest (Fig. 1B). This corresponds to late S and G_2, a time when BRCA1 phosphorylation is maximal (*6*), suggesting that BRCA1 may be involved in DNA recombination during the normal cell cycle.

To delineate the specific binding sites of hRad50 and BRCA1, we performed a glutathione S-transferase (GST) pull-down assay with in vitro translated hRad50 and various bacterially expressed GST-BRCA1 fusion proteins (Fig. 1, C through E). A fragment containing amino acids from 341 to 748 (BRCA-Bgl in Fig. 1, C and D) was found to bind to hRad50 specifically (Fig. 1E). A yeast two-hybrid binding assay yielded similar results (Fig. 1F). The NH_2-terminal half of hRad50 was required for BRCA1 binding (Fig. 1G).

Rad50, Mre11, and p95/nibrin form a complex that functions in homologous recombination, nonhomologous end joining (NHEJ), meiotic recombination, the DNA damage response, and telomere maintenance (*8*). Rad50 is a coiled-coiled structural maintenance of chromosomes–like protein with adenosine 5′-triphosphate–dependent DNA binding activity (*9*). Mre11 has been proposed to have both structural (DNA end holding) and catalytic activities, including DNA exo- and endonuclease activities (*10*). Mutation of the *NBS1* gene encoding p95 is responsible for Nijmegen Breakage Syndrome (NBS), a disease characterized by an increased cancer incidence, cell cycle checkpoint defects, and sensitivity to ionizing radiation (*11, 12*). A deficiency of p95 in NBS cells abrogates the formation of ionizing radiation–induced hMre11-hRad50 foci (*12*). In normal human diploid fibroblasts, hMre11 localizes to DNA breaks within 30 min of irradiation (*13*). These observations have prompted speculation that the Mre11-Rad50-p95 complex functions as a sensor of DNA damage.

To examine BRCA1 and hRad50 interactions upon DNA damage, we treated T24 cells with gamma irradiation or MMS and coimmunoprecipitated cell lysates with α-BRCA1 6B4 or α-hRad50 13B3 monoclonal antibodies (mAb's). As observed previously (*2, 4*), these treatments resulted in the slower migration of BRCA1, consistent with its phosphorylation (Fig. 2A). The treatments did not appear to change the amount of the BRCA1-hRad50 complex (Fig. 2A), suggesting that it exists even in the presence of DNA damage.

Considering that the level of their interactions does not change after DNA damage, as assessed by protein amounts in co-immunoprecipitates in Fig. 2A, relocalization of the component proteins to sites of damaged DNA may be a crucial aspect of BRCA1 function during the repair process. Both BRCA1 and hRad50 display discrete nuclear foci after treatment of cells with genotoxic agents (*14, 15*). The BRCA1 dot pattern appears in untreated T24 cells. Upon irradiation, the BRCA1 dots were disrupted within 1 hour (*4*) then gradually reassembled into bright foci. The BRCA1 irradiation-induced foci (IRIF) appear in 70 to 90% of nuclei at 6 to 8 hours after irradiation and remain until 12 hours (*16, 17*). The hRad50 IRIF pattern is consistent with the reported pattern, reaching its peak at 6 to 8 hours and declining at 12 hours after irradiation (*15*) (Table 1).

We tested whether radiation-induced foci containing hRad50 colocalize with those containing BRCA1. T24 cells irradiated with 12-gray (Gy) gamma radiation demonstrated the punctate pattern of immunostaining for BRCA1 with mAb's Ab-1 (*4*) or 17F8 (*16, 18*); this pattern overlaps hRad50-containing foci identified with rabbit α-hRad50 antiserum (*15*) (Fig. 2B). Among cells displaying both hRad50 and BRCA1 foci, >90% showed substantial colocalization. Irradiation-induced colocalization of hRad51 and BRCA1 foci were also observed (Fig. 2B), similar to the observation of the colocalization of these two proteins upon hydroxyurea or ultraviolet treatments (*4*).

Cells appear to have one of two types of BRCA1 foci: Most colocalize with hRad51, and a portion of the cells colocalize with hRad50. The percentage of BRCA1 foci-containing cells associating with either hRad50 or hRad51 foci varies after irradiation (Table 1), and these two types of foci appear to be mutually exclusive because cells with both hRad50- and hRad51-asso-

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ciated foci have seldom been observed (*15, 16*). With the specific antibodies or antisera (Fig. 3A), radiation-induced hMre11 and p95 foci were also examined in T24 cells, which display a pattern similar to that displayed by hRad50 foci (Fig. 3B) and colocalize with BRCA1 and hRad50 foci (*16*).

To explore the relation of BRCA1 to these foci, we assayed, for IRIF (*17*), HCC1937 cells that express a COOH-terminally truncated BRCA1 protein (*19*). BRCA1 foci were diminished in these cells, and the nuclear staining of BRCA1 was homogenous, albeit much dimmer, in HCC1937 cells regardless of treatment (Fig. 3B). Interestingly, hRad50, hMre11, and p95 IRIF were dramatically reduced in HCC1937 cells. Most of the irradiated cells displayed a diffuse nuclear pattern of hRad50, hMre11, or p95 immunostaining similar to that seen in untreated HCC1937 cells. In contrast, IRIF that were positive for hRad51 antibodies were readily and efficiently detected in both T24 and HCC1937 cells (Fig. 3B).

In addition to BRCA1 mutation, HCC1937 also harbors many other genetic changes (*19*). To determine whether the BRCA1 deficiency was responsible for the defect in IRIF formation, we transiently transfected hemagglutinin (HA)–tagged wild-type *BRCA1* into HCC1937 cells and irradiated cells 40 hours later. Of the transfected cells, 18 to 28% reconstitute HA-BRCA1 foci, and among these cells, ~29 to 38% had immunoreactive hRad50, hMre11, or p95 foci, colocalizing with BRCA1 foci (Fig. 3C). Cells mock transfected or transfected with a vector showed no or very few foci after radiation (Fig. 3C) (*16*). These results indicate that BRCA1 is responsible for defective hRad50, hMre11, and p95 IRIF response in HCC1937 cells.

To test whether defective BRCA1-hRad50-hMre11-p95 foci formation may be due to mutated *BRCA1* gene product in HCC1937 cells, we examined the integrity of the BRCA1-hRad50-hMre11-p95 complex in HCC1937, and nuclear extracts prepared from the cells that were untreated or treated with MMS or gamma radiation (Fig. 3C) were co-immunoprecipitated with α-hRad50. Both hMre11 and p95 were in the complex, similar to T24 cells, but the truncated BRCA1 (Fig. 3D), which is expressed at detectable levels, was not. The disruption of BRCA1-hRad50 interaction in HCC1937 cells may be due to conformation change, lower expression, and possibly inefficient nuclear transportation (*16*).

To explore the biological consequence of BRCA1 deficiency in HCC1937 cells, we assayed cell survival after treatment with MMS. Relative to T24 and another breast cancer cell line, MCF7, both of which express full-length *BRCA1*, HCC1937 cells were hypersensitive to MMS treatment (Fig. 4A). Transfection of wild-type *BRCA1*, but not *BRCA1* mutants (Fig. 4B) with alterations at the NH_2-terminal RING finger domain ($Cys^{61} \rightarrow Gly^{61}$) (*20*) or the COOH-terminal BRCT domain ($Ala^{1708} \rightarrow Glu^{1708}$) (*21*), substantially increased the survival of MMS-treated HCC1937 cells (Fig. 4C). In contrast, transfection with wild-type *BRCA2* did not affect cell survival under similar conditions (Fig. 4C). The expression of these constructs was confirmed by protein immunoblot analysis with α-BRCA1 COOH-terminus antibody, C20 (Fig. 4D). These results are consistent with a defective G_2/M checkpoint upon MMS treatment in BRCA1 exon 11–deficient cells (*5*), and they also suggest that the BRCA1 RING finger domain and the BRCT repeats may be critical for a similar DNA damage response.

In summary, our results suggest that formation of the BRCA1-hRad50 complex does not change in response to DNA damage; rather, it is the nuclear partitioning of the complex that changes. BRCA1 is

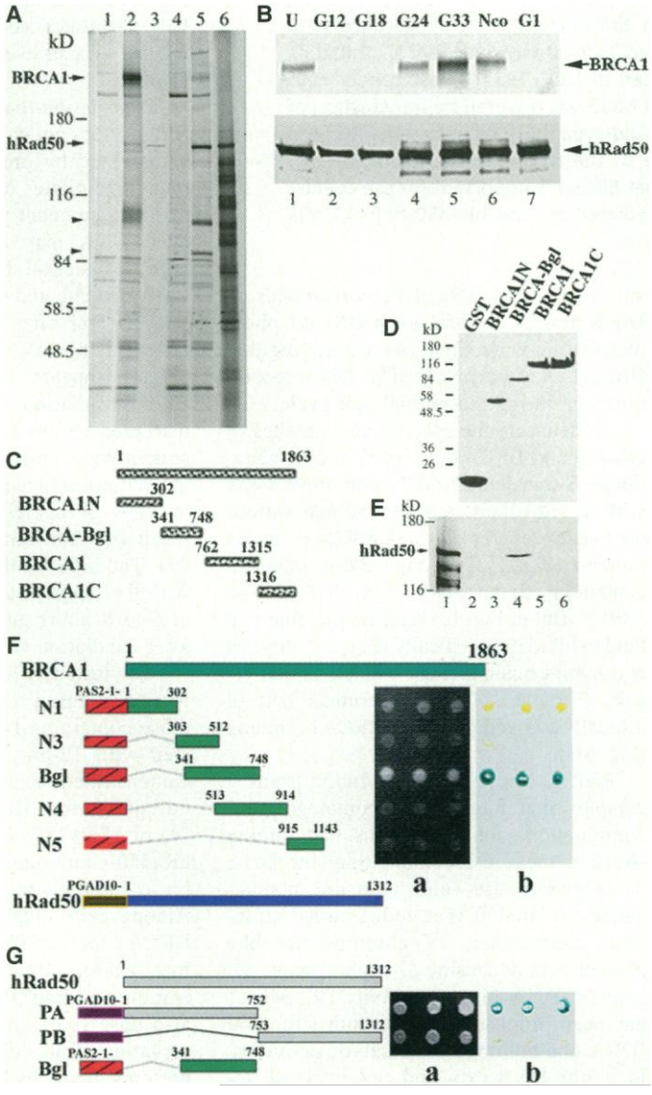

Fig. 1. BRCA1 interacts with hRad50 in vivo and in vitro. (**A**) Lysates, labeled with ^{35}S-methionine, from T24 cells were immunoprecipitated with preimmune serum (lanes 1 and 4), α-BRCA1 or α-hRad50 (lanes 2 and 5, respectively), or α-BRCA1 followed by dissociation and reprecipitation with α-Rad50 (lane 3). In vitro translated hRad50 served as a control (lane 6). Arrows mark bands that may contain p95 or hMre11. (**B**) BRCA1 associates with hRad50 in a cell cycle–dependent manner. Density-arrested T24 cells were released and collected at the times indicated (*26*) (U, unsynchronized; G12, G_1; G18, G_1/S; G24, S; G33, G_2; Nco, M; and G1, G_0/G_1), and cell extracts were immunoprecipitated with α-hRad50 mAb 13B3. hRad50 and BRCA1 proteins were detected by protein immunoblot analysis. Co-immunoprecipitated BRCA1 peaks in the late S and G_2 phases. (**C**) Schematic diagrams of the BRCA1-GST fusions and (**D**) the expressed and purified fusions from *Escherichia coli*. (**E**) In vitro translated hRad50 and the binding results with the BRCA1-GST fusions indicated in (D). Only the BRCA-Bgl (amino acids 341 through 748) binds to hRad50 (lane 4). Lane 1 shows the total input of translated hRad50. (**F**) BRCA1 interacts with hRad50 in a yeast two-hybrid assay. The indicated regions of BRCA1 were fused to the DNA binding domain of GAL4 in pAS2-1. hRad50 was fused to the activation domain of GAL4 in pGAD10. These plasmids were cotransformed from yeast strain Mav203 and (a) scored for colony growth on Ura^-/His^-/Trp^-/Leu^- plates and (b) color assayed for β-galactosidase activity. BRCA-Bgl binds to hRad50. (**G**) NH_2-terminus of hRad50 binds BRCA1 in a yeast two-hybrid assay. NH_2- or COOH-terminal fragments of hRad50 were fused to the transactivation domain of GAL4 in pGAD10, and these plasmids were cotransformed with the BRCA-Bgl fragment fused to the DNA binding domain of GAL4 in pAS2-1 as in (F).

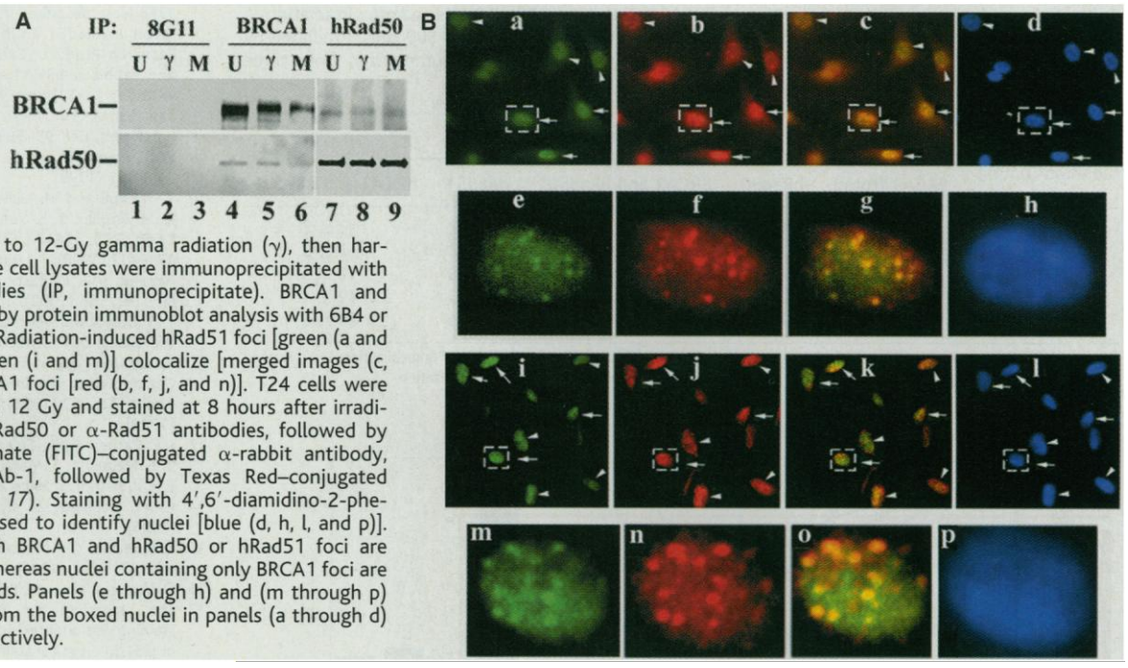

Fig. 2. Co-immunoprecipitation and co-localization of BRCA1 with hRad50. (**A**) BRCA1 co-immunoprecipitated with hRad50. T24 cells were mock treated (U) or treated with 0.05% MMS (M) for 1 hour or were exposed to 12-Gy gamma radiation (γ), then harvested 1 hour later. The cell lysates were immunoprecipitated with the indicated antibodies (IP, immunoprecipitate). BRCA1 and hRad50 were detected by protein immunoblot analysis with 6B4 or 13B3, respectively. (**B**) Radiation-induced hRad51 foci [green (a and e)] or hRad50 foci [green (i and m)] colocalize [merged images (c, g, k, and o)] with BRCA1 foci [red (b, f, j, and n)]. T24 cells were gamma irradiated with 12 Gy and stained at 8 hours after irradiation with rabbit α-hRad50 or α-Rad51 antibodies, followed by fluorescein isothiocyanate (FITC)–conjugated α-rabbit antibody, and α-BRCA1 mAb Ab-1, followed by Texas Red–conjugated α-mouse antibody (*7*, *17*). Staining with 4′,6′-diamidino-2-phenylindole (DAPI) was used to identify nuclei [blue (d, h, l, and p)]. Nuclei containing both BRCA1 and hRad50 or hRad51 foci are marked with arrows, whereas nuclei containing only BRCA1 foci are marked with arrowheads. Panels (e through h) and (m through p) are enlarged images from the boxed nuclei in panels (a through d) and (i through l), respectively.

present in both hRad50 and hRad51 foci upon irradiation; however, cells containing hRad50-hMre11-p95 foci have no detectable hRad51 foci, and vice versa (*15*, *16*). BRCA1 is crucial for hRad50-hMre11-p95 foci assembly but not for hRad51 foci in HCC1937 cells. All these data suggest that BRCA1 has distinct roles in each complex in response to DNA damage.

The Rad50-Mre11-p95 complex participates in NHEJ or homologous recombination in DNA double-strand breaks. In homologous recombination, it is postulated that the Rad50-Mre11-p95 complex is responsible for end processing, and Rad51 is involved in strand exchange during a subsequent phase. BRCA1 may facilitate the coupling of these two steps. This hypothesis is supported by evidence that BRCA2 is associated with hRad51 (*22*) and that BRCA1 interacts with BRCA2 (*23*). Also, BRCA1 could be involved in NHEJ through interactions with the hRad50-hMre11-p95 complex.

There is no evidence for a BRCA1-like protein in the well-studied DNA repair systems in yeast. It follows that BRCA1 may function as an accessory DNA repair protein, perhaps in mammalian cells facilitating, coordinating, or sensing DNA dam-

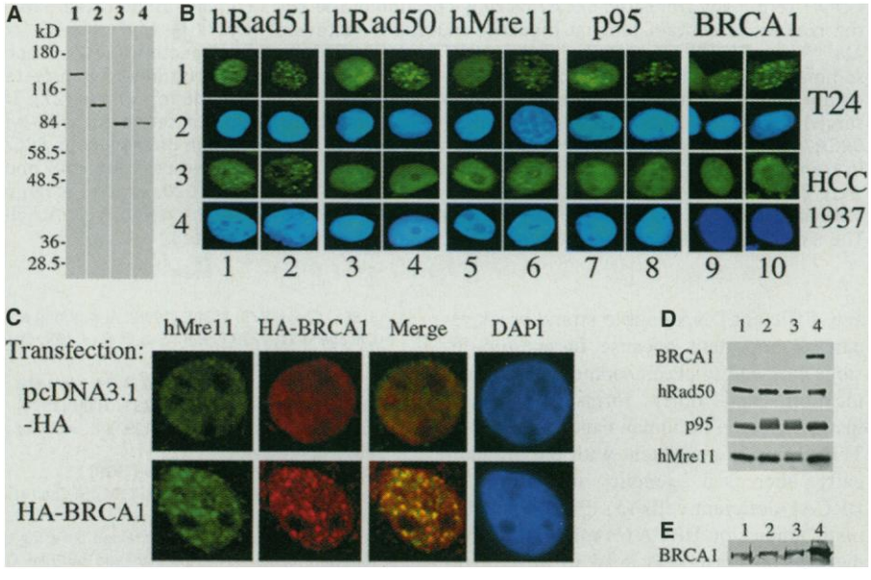

Fig. 3. BRCA1 is crucial for the formation of hRad50-hMre11-p95 IRIF. (**A**) Antibodies specific for hRad50 [α-hRad50 mAb 13B3 (lane 1)], p95 [α-p95 polyclonal antiserum (lane 2)], and hMre11 [α-hMre11 polyclonal antiserum (lane 3) and α-hMre11 mAb 12D7 (lane 4)] (*7*) were tested by straight protein immunoblot with T24 lysates. (**B**) hRad51, hRad50, hMre11, p95, and BRCA1 subnuclear partitioning in HCC1937 and T24 cells in response to gamma irradiation. Cells were mock treated (columns 1, 3, 5, 7, and 9) or treated with 12-Gy gamma rays (columns 2, 4, 6, 8, and 10) and stained with indicated antibodies at 8 hours after irradiation (*17*); rows 1 and 3 were stained with FITC, and rows 2 and 4 were stained with DAPI. HCC1937 cells contain hRad51 foci but do not contain hRad50, hMre11, p95, and BRCA1 foci. (**C**) Ectopic expression of *BRCA1* restores formation of hRad50-hMre11-p95 IRIF in HCC1937 cells. Expression plasmid containing *HA-BRCA1* (10 μg) or vector (PcDNA3.1-HA) alone was transfected into HCC1937 cells by lipofection (*27*). The cells were treated with 12-Gy gamma rays and stained with α-hMre11 mAb, 12D7, and rabbit α-HA (Y-11) (*16*, *17*) as indicated. HA-BRCA1 foci colocalize with hMre11 foci in cells transfected with the *HA-BRCA1* cDNA. (**D**) Formation of hRad50-hMre11-p95 and BRCA1 complexes. Lysates from HCC1937 cells that were mock treated (lane 1), treated with 0.05% MMS for 1 hour (lane 2), or treated with 12-Gy gamma rays (lane 3) or lysates from untreated T24 cells (lane 4) were immunoprecipitated with α-hRad50 mAb 13B3. The immunoprecipitates were analyzed by protein immunoblot probed with α-BRCA1 mAb 6B4, α-hRad50 mAb 13B3, α -p95, and α -hMre11, as indicated. BRCA1 is present in the hRad50-hMre11-p95 complex of T24 but not in HCC1937 cells. (**E**) Full-length or truncated BRCA1 was detected by protein immunoblot with α-BRCA1 mAb, 6B4, in lysates used in (D).

Table 1. Focus formation and colocalization of BRCA1, hRad51, and hRad50 after gamma irradiation. A cell nucleus displaying >10 foci was counted as a foci-containing cell. At least 500 cells, irradiated by 12-Gy gamma rays, were analyzed for each experiment, and results were summarized from three independent experiments.

Time after radiation (hours)	Foci contained in cells (%)				
	BRCA1 (total)	hRad50	BRCA1 and hRad50*	hRad51	BRCA1 and hRad51†
0	37 ± 8	1 ± 1	0 ± 0	7 ± 2	3 ± 2
1	13 ± 6	2 ± 2	1 ± 1	10 ± 2	7 ± 3
3	43 ± 7	17 ± 5	15 ± 3	20 ± 6	17 ± 5
6	81 ± 13	30 ± 7	28 ± 6	53 ± 6	49 ± 4
12	79 ± 11	13 ± 6	11 ± 5	74 ± 4	64 ± 6

*Among cell nuclei containing both BRCA1 and hRad50 foci, 85 to 95% of cells show >50% of colocalization. †Among cell nuclei containing both BRCA1 and hRad51 foci, 75 to 83% of cells show >50% of colocalization.

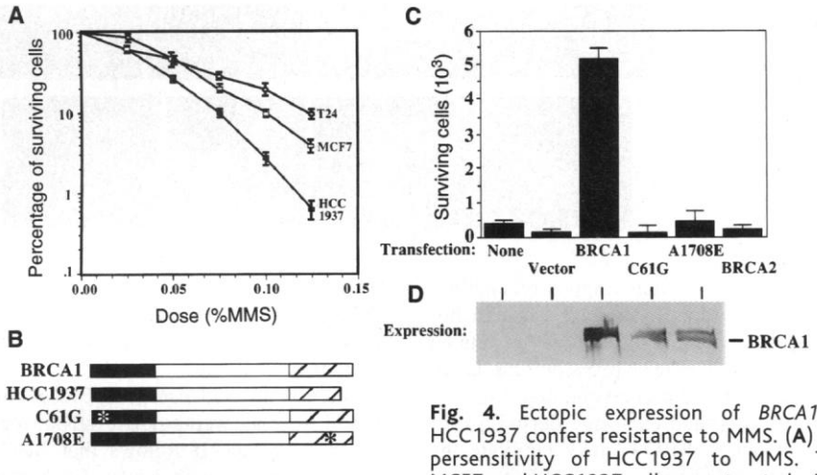

Fig. 4. Ectopic expression of *BRCA1* in HCC1937 confers resistance to MMS. (**A**) Hypersensitivity of HCC1937 to MMS. T24, MCF7, and HCC1937 cells were treated with a dose of MMS (indicated by the *x* axis) for 50 min, and the number of surviving cells was counted by trypan blue exclusion assay with hematocytometry 48 hours after treatment. These experiments were repeated three times. Error bars indicate SD. (**B**) Schematic diagrams of the *BRCA1* cDNA used to rescue resistance of HCC1937 to MMS. These cells express a COOH-terminally truncated *BRCA1* lacking a portion of the BRCT domain, as indicated. Two of the constructs contain familial missense mutations (asterisks) [$Cys^{61} \rightarrow Gly^{61}$ (C61G) and $Ala^{1708} \rightarrow Glu^{1708}$ (A1708E)] in the RING and BRCT domains, respectively. (**C**) Graphic summary of cell survival in response to 0.1% MMS treatment. Parallel cultures of transfected, empty vector, or untransfected cells (none) were treated with 0.1% MMS for 50 min (*27*). The surviving cells were counted and plotted (*y* axis). Only transfection of cells with wild-type *BRCA1* restored resistance to MMS. Error bars indicate SD. (**D**) Expression of exogenous *BRCA1* in transfected cells. Lysates from parallel cultures of *BRCA1*-transfected cells after 48 hours were prepared and immunoprecipitated by rabbit α-BRCA1 antibody, C20, which recognizes full-length but not COOH-terminally truncated BRCA1, and detected by α-BRCA1 mAb 6B4. The expected 220-kD BRCA1 full-length or mutant proteins are indicated.

age. Efficient DNA double-strand break repair is important because unrepaired lesions can lead to chromosome break, translocation, and other forms of genetic instability seen in human cancer cells (*24*). This notion is consistent with the dramatically increased genetic instability of BRCA1-deficient cells (*5*). Further mechanistic studies on BRCA1's role in the DNA damage response may lead to new therapeutic strategies for breast and ovarian cancer patients.

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7. Polyclonal antibodies were obtained by using the following bacterially expressed and purified GST fusion proteins as antigens: GST-hRad50-15A5, containing amino acids 211 through 575 of hRad50; GST-MM, containing amino acids 82 through 582 of hMre11; GST-NBS1, containing amino acids 12 through 754 of p95; GST-hRad51, containing full-length hRad51; and GST alone (for anti-GST mAb 8G11), to generate polyclonal or monoclonal antibodies (*25*). BRCA1 mAb's 6B4 and 17F8 were described in (*18*). BRCA1 mAb Ab-1 and rabbit α-hRad51 antibodies were from Oncogene Research Product (Cambridge, MA). Affinity-purified rabbit α-BRCA1 antibody (C-20) and α-HA antibody (Y-11) were from Santa Cruz Biotechnology (Santa Cruz, CA).
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17. The procedure for indirect immunofluorescence staining was adopted and slightly modified from that in (*25*). In a typical staining experiment, 500 to 1000 cells were counted. For the foci-containing cells, only nuclei containing >10 foci were counted as a foci-containing cell. Each experiment was repeated at least three times, and the results were reproducible with the antibodies indicated here. The results did not vary according to the conditions of fixation. The foci formation studies were performed following different time courses after irradiation (at 1, 3, 6, 8, and 12 hours). Because all of the studied foci show relatively high quality and quantity at 8 hours after irradiation, the results at this time point are mostly used as representatives here.
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26. Synchronization of T24 human bladder carcinoma cells was performed by density arrest, then released at time zero by replating at a density of 2×10^6 cells per 10-cm plate. At various time points thereafter (12 hours for G_1, 18 hours for G_1/S, 24 hours for S, 33 hours for G_2/M, and 1 hour for arrest in G_0/G_1), cells were harvested. To obtain cells in M phase, we added nocodazole (0.4 μg/ml) to the culture medium for 10 hours before harvest.
27. Constructs based on plasmid pcDNA3.1 (Invitrogen, San Diego, CA) were used for lipofectin-mediated transfection of HCC1937 cells with BRCA1 cDNA. Cells were harvested after 48 hours for immunoprecipitation and protein immunoblot analysis. Parallel cultures were treated with 0.1% MMS for 50 min, and surviving cells were counted after 8 days. The experiments were repeated at least three times, and the results were consistent.

28. We thank J. Petrini for rabbit antisera to hRad50, P. Garza and D. Jones for antibody production, and N. Ting and T. Boyer for critical reading. Supported by grants from NIH (CA 58183 and CA 30195), the McDermott endowment (W.H.L.), and the Susan G. Komen Foundation for Breast Cancer Research (P.L.C.).

30 March 1999; accepted 25 June 1999