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Advances in Brief

Both DNA Topoisomerase II-binding Protein 1 and BRCA1 Regulate the G_2-M Cell Cycle Checkpoint[1]

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Abstract

Cell cycle checkpoints play a central role in genomic stability. The human DNA topoisomerase II-binding protein 1 (TopBP1) protein contains eight BRCA1 COOH terminus motifs and shares similarities with Cut5, a yeast checkpoint Rad protein. TopBP1 also shares many features with BRCA1. We report that, when expression of TopBP1 protein is inhibited in BRCA1 mutant cells, mimicking a TopBP1, BRCA1 double-negative condition, the G_2-M checkpoint is strongly abrogated and apoptosis is increased after ionizing radiation. However, a BRCA1-negative or a TopBP1-negative background resulted in only partial abrogation of the G_2-M checkpoint. The BRCA1 mutant and TopBP1-reduced condition specifically destroys regulation of the Chk1 kinase but not the Chk2 kinase, suggesting involvement in the ataxia telangiectasia-related pathway. These results indicate that both TopBP1 and BRCA1 specifically regulate the G_2-M checkpoint, partially compensating each function.

Introduction

Cell cycle checkpoints induced by DNA damage are essential for maintaining genetic integrity (reviewed in Refs. 1 and 2). Some signals of DNA damage lead to cell cycle arrest to prevent transfer of damaged genetic information to the daughter cells. Checkpoint responses are considered to be a major mechanism to reduce both initiation and progression of cancer, which can be caused by incomplete DNA repair resulting in genetic alterations of tumor suppressors and proto-oncogenes. For example, ATM[3] is a responsible protein in the genetic disease ataxia telangiectasia and plays a central role in checkpoint controls (3). Cultured fibroblasts and lymphoid cells from patients with ataxia telangiectasia demonstrate genomic instability, and affected patients show a cancer-predisposition phenotype, indicating a tight relationship between malfunction of checkpoint control and oncogenesis.

The human TopBP1 protein contains eight BRCT motifs and associates with topoisomerase II *in vitro* (4). After DNA damage, TopBP1 protein localizes at IR-induced nuclear foci and is phosphorylated by ATM kinase (5). TopBP1 protein shares sequence and structural similarities with the *Schizosaccharomyces pombe* Cut5 (Rad4) protein, which has four BRCT motifs and regulates the G_2-M and S-phase checkpoints (6, 7). The Cut phenotype is made up of a typical G_2-M checkpoint defect and/or a cell division defect that is thought to represent an uncoupling of cytokinesis and chromosomal segregation. Cut5 is the one of the checkpoint Rad proteins (including Rad3, Rad17, Rad9, Rad1, Hus1, Cut5, Crb2, and Rad26) that are essential for cell cycle checkpoint responses (1).

BRCA1 is a tumor suppressor that can be mutated in familial breast and ovarian cancer (8). The BRCA1 protein has two BRCT motifs that are also found in several proteins involved in cell cycle checkpoints and DNA repair (9, 10). A few reports suggest that the BRCA1 protein is transiently and partially involved in the G_2-M checkpoint (see "Discussion"). This checkpoint involvement may directly link genomic instability and familial breast and ovarian cancer to a malfunction of mutated BRCA1.

There are several common features that link BRCA1 and TopBP1 proteins (5). First, both are BRCT-containing proteins, and TopBP1 also shares other sequence homology with BRCA1 at the COOH-terminal wide region (4, 11). Second, BRCA1 and TopBP1 proteins are strongly induced during S phase with similar induction patterns. Third, both are phosphorylated by ATM in response to DNA damage and DNA replication stress (12). Fourth, both colocalize at IR-induced nuclear foci after DNA damage, and both also colocalize with proliferating cell nuclear antigen (PCNA) at stalled replication forks after a replication block (11, 13). On the basis of these observations, we hypothesize that TopBP1 and BRCA1 may have partially overlapping functions, obscuring clear results when only one of the two proteins is absent.

Because we reported previously that TopBP1-negative cells have limited viability and cannot be passaged (5), we mimicked BRCA1 and TopBP1 double-negative conditions in this study using a BRCA1-mutant human breast cancer cell line and antisense morpholino oligomers against TopBP1 to suppress the protein level. We report that the G_2-M checkpoint control is strongly abrogated only under conditions mimicking a TopBP1 and BRCA1 double negative. This double-negative-mimicking background also resulted in a marked reduction in colony formation, induction of apoptosis after irradiation, and failure of a critical phosphorylation of Chk1 after irradiation. A wild-type *BRCA1* transfection reversed these results, indicating that BRCA1 and TopBP1 have overlapping functions.

Materials and Methods

Cell Culture and IR. HCC1937 cells were established from a primary breast carcinoma and are homozygous for a BRCA1 mutation that includes a base insertion at codon 1755 (14), corresponding to a site between the two BRCT repeats. HCC1937 cells were transfected with a Myc-epitope-tagged wild-type *BRCA1* gene on a pcDNA3 (Invitrogen)-based vector. To avoid isolation of special cells in these genetically unstable cells, the total neomycin-resistant clones were isolated and termed HCC1937+wtBRCA1. All of the cells were grown in RPMI 1640 supplemented with 10% FCS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were irradiated in a JL Shepherd ^{137}Cs radiation source at a rate of 4 Gy/min. The cells were then returned under the culture conditions and maintained for the indicated times.

Transfection. The morpholino oligomers with artificial nonhydrolyzable backbone structures (Gene Tools; Philomath, OR) were used to decrease the level of TopBP1 protein. The two antisense oligomers used were As1, 5′-

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[3] The abbreviations used are: ATM, ataxia telangiectasia-mutated; TopBP1, DNA topoisomerase II binding protein 1; BRCT, BRCA1 COOH terminus; MSH2, MutS homologue-2; TUNEL, terminal UTP nick end labeling; ATR, ataxia telangiectasia-related; IR, ionizing radiation; Cut, cell untimely torn.

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TTGGGACACATCGCTGGTGGTGCAT (at the translational initiation site), and As2, 5′-AAACGGTTCTTTGTCATTTCTGGAC (at 5′ untranslated region). The two antisense controls were scrambled As2 (termed Sc2), 5′-CAACAGTTCGTTGCCATCGCTGCAC, and the general control from Gene Tools was 5′-CCTCTTACCTCAGTTACAATTTATA. Unless otherwise stated, the control was the general control. Cell suspensions (60 ml) in medium without antibiotics were mixed with these morpholino oligomers (500 mM; 4.7 ml) and were subjected to electroporation at 200 V, 10 ms, in a cuvette with a 0.4-cm gap and with a single pulse (BTX, Holliston, MA). After 3–5 days, the cells were again transfected with the identical oligomers using a second electroporation (200 V, 10 ms, double pulse with 100-ms interval). We call this a tandem transfection. All of the transfectants were cultured again for 3–7 days, and used for all of the experiments. The efficiency of the tandem transfection is estimated to be >85%, based on a colony formation assay.

Western Blotting. Total cell extracts were prepared by trichloroacetic acid precipitation to detect TopBP1, BRCA1, and MSH2. After extraction of trichloroacetic acid with ether, DNA was sheared by sonication before loading onto gels. Antibodies were obtained from Transduction Laboratory (Franklin Lakes, NJ; TopBP1) and Oncogene Research Products (San Diego, CA; Ab-2, MSH2). The MS110 monoclonal antibody was used for BRCA1 (15). The two antibodies against the phosphorylated Chk1 and Chk2 were obtained from Cell Signaling Technology (Beverly, MA).

Flow Cytometry. Cells were fixed with 90% ethanol at –20°C for 60 min to 2–3 days, were incubated with RNase, were stained with propidium iodide, and were subjected to flow cytometry (Epics XL-MCL; Coulter, Miami, FL). For TUNEL staining, the Apo-Direct kit was used (eBioscience, San Diego, CA).

G_2-M Checkpoint Assay. Two identical plates were prepared for each transfectant. Nocodazole (0.3 mg/ml; Sigma, St. Louis, MO) was added to the medium of both plates immediately after 0 or 10 Gy of IR at 4°C. After incubation under the culture conditions for the indicated times in figure legend 3, total cell extracts were prepared by sonication of cells in PBS and subjected to Western blotting. An antibody against the phosphorylated serine 10 of histone H3 was obtained from Upstate (Waltham, MA). Normal anti-histone H3 antibody (C-16) was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). To determine the extent (%) of abrogation of the G_2-M checkpoint, the following calculation was performed:

$$\text{Checkpoint abrogation (\%)} = \frac{A}{B} \times 100$$

where A = the compensated band intensity after irradiation (derived from phosphorylated histone H3) and B = the compensated band intensity without irradiation (derived from phosphorylated histone H3). Compensation was performed by a series of quantitatively diluted positive signals using Western blotting.

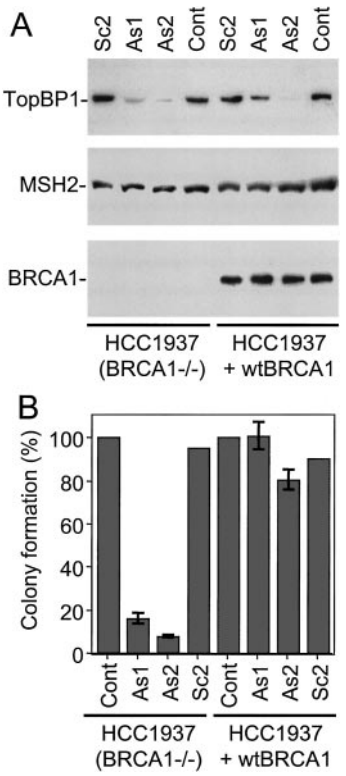

Fig. 1. The inhibition of colony formation by the antisense oligomers against TopBP1 is strongly reversed by wild-type BRCA1 transfection. *A*, the morpholino oligomers used were two different antisense oligomers, As1 and As2, against the TopBP1 mRNA 5′ region and two antisense controls (*Cont*, a general control, and *Sc2*, a scrambled As2). These oligomers were transfected into HCC1937 and HCC1937+wtBRCA1 by electroporation. The whole-cell lysates were analyzed by Western blotting using antibodies against TopBP1, BRCA1, and MSH2 (a control protein), respectively. A small amount of mutant BRCA1 in HCC1937 cell lysate was visible only when it was overexposed (data not shown) *B*, colony formation assays were performed using the above transfectants. After ~20 days, colonies were stained and counted. The averages of three independent experiments [*Cont* (the general control), *As1* and *As2*] are shown with the SDs. The colony number of transfectants with the general control was normalized to 100%. The results of one experiment using the scrambled control oligomer (*Sc2*) are also shown.

Results

The Inhibition of Colony Formation by the Antisense Oligomers against TopBP1 Is Strongly Reversed by Wild-Type BRCA1 Transfection. We reported previously (5) that the inhibition of *TopBP1* gene expression results in enhanced cytotoxicity, suggesting that TopBP1 is required for basic cell viability, similar to ATR or hChk1 (16–19). Because TopBP1-negative cell lines are not currently available, we mimicked BRCA1 and TopBP1 double-negative conditions using a BRCA1-mutant human breast cancer cell line, HCC1937 (14), and two antisense morpholino oligomers against TopBP1. We also examined the possible relationship of BRCA1 and TopBP1 using HCC1937 cells stably transfected with wild-type BRCA1 cDNA (HCC1937+wtBRCA1; Fig. 1*A*). The two different antisense oligomers, As1 and As2, inhibited the expression of the *TopBP1* gene in both cell lines (Fig. 1*A*).

The two different antisense oligomers (As1 and As2) against TopBP1 also strongly inhibited colony formation in the BRCA1-mutant cells (Fig. 1*B*). In contrast, no significant effects on colony formation were found in the BRCA1(+) transfectants using the two TopBP1 antisense oligomers (Fig. 1*B*). Although the data reflected in Fig. 1*B* are the result of a tandem transfection, a single transfection of the antisense oligomers gave similar cytotoxicity results (data not shown). It should be noted that the constitutive expression of BRCA1 under a cytomegalovirus promoter in the HCC1937 cells seems to confer greater resistance to the anti-TopBP1 oligomer, explaining the difference in clonogenic survival in HCC1937+wtBRCA1 cells compared with our previously published data using the same oligomers in HeLa cells (5).

Apoptosis Is Induced by Inhibition of TopBP1 Expression on a BRCA1-negative Background, in Response to IR. When cellular debris in the medium was removed after transfection, the cell cycle profiles were similar in all of the samples (Fig. 2*A*, 0 h). After irradiation (10 Gy), sub-G_1 populations were increased in antisense-treated cells, suggesting that apoptosis was induced by irradiation (48 h, HCC1937, As1 and As2). The remaining G_0-G_1 population at 48 h in HCC1937 was not caused by a reduction of TopBP1, because it was also observed in the control. In the BRCA1-transfected HCC1937 cells, the sub-G_1 populations were markedly reduced, and almost the entire population was at G_2-M at 48 h after irradiation. These data also clearly suggest that TopBP1 and BRCA1 do not regulate a G_1-S phase checkpoint response in this cell line. Interestingly, DNA damage-induced apoptosis was observed only by a tandem antisense transfection and not by single transfection (data not shown), suggesting that a small amount (20–30% of the control in HCC1937) of TopBP1

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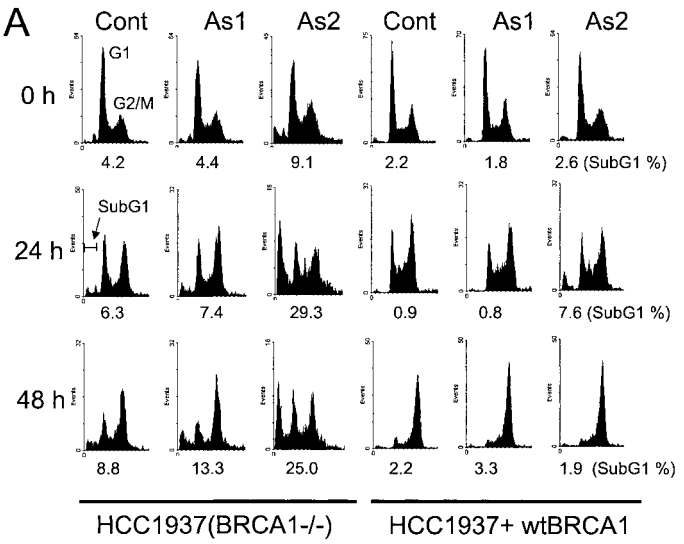

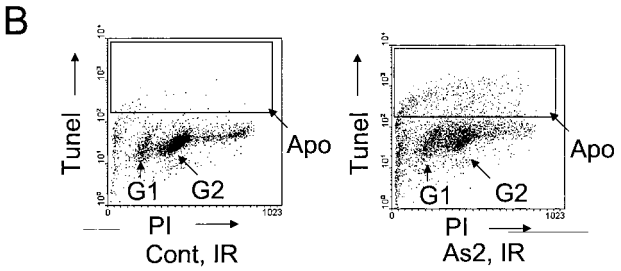

Fig. 2. Apoptosis is induced by inhibition of TopBP1 expression in BRCA1-mutant cells, in response to IR. The antisense (*As1* and *As2*) and general control (*Cont*) morpholino oligomers were transfected into HCC1937 or HCC1937 + wild type BRCA1 cells (*HCC1937+ wtBRCA1*). Cells were then irradiated to 10 Gy, and returned to the incubator. *A,* nonirradiated control cells (0 h), and cells at 24 h and 48 h after IR, were fixed, stained with propidium iodide, and subjected to flow cytometry. The percentage of sub-G_1 phase in the total cell population is shown under each panel. Sub- G_1, G_1, and G_2-M peaks are indicated *on the left panels*. All of the cell cycle profiles are similar to those at 0 h, at least for up to 5 days without irradiation. *B,* transfected HCC1937 cells with either the control oligomer or As2 were irradiated to 10 Gy, and incubated for 48 h. Cells were fixed with formaldehyde and ethanol, and then were double stained by the TUNEL method using FITC-labeled UTP and propidium iodide. The apoptotic cells are labeled as APO.

protein as found after a single transfection was enough to reduce DNA damage-induced apoptosis. The flow cytometry results in the scrambled antisense control (Sc2) were similar to those of the general antisense control (data not shown).

To better define the apoptotic profile in As2-transfected HCC1937 cells at 48 h after irradiation, we performed flow cytometry using two-dimensional staining with propidium iodide and TUNEL using FITC-labeled UTP. Before staining, cells were partially cross-linked by formaldehyde to delineate the cell cycle distribution of apoptotic populations. As shown in Fig. 2*B*, the apoptotic cells appear to be distributed throughout the cell cycle but mostly in the sub-G_1 fraction in As2-transfected HCC1937, BRCA1-mutant cells. The apoptotic cell population was much lower in the control cells.

Both TopBP1 and BRCA1 Control the G_2-M Checkpoint. To examine whether TopBP1 and BRCA1 can affect the G_2-M checkpoint, we used an antibody against the phosphorylated serine 10 of histone H3, as a mitotic marker (19, 20). After 0 or 10 Gy of IR, nocodazole was added to arrest cells in M phase, as a general technique. In irradiated cells, the band of phosphorylated histone H3 almost completely disappeared under the TopBP1 and BRCA1 double-positive condition (Fig. 3*A, top panel, Lane 8*), indicating that a G_2-M checkpoint after IR damage was activated to inhibit cells from entering into M phase. However, under conditions mimicking a TopBP1 and BRCA1 double negative, the phosphorylated band was clearly detected after irradiation (Fig. 3*A, Lanes 3–6*), indicating that the G_2-M checkpoint was only partially abrogated (Fig. 3*B, As1*: 33%, *As2*: 93%). Partial abrogation (Fig. 3*A, Lane 2*) was also observed with the control oligomer in HCC1937. The results of the scrambled control (Sc2) were similar to those of the general control (data not shown). BRCA1 transfection strongly reversed the observed G_2-M checkpoint abrogation. These results indicate that both TopBP1 and BRCA1 control the G_2-M checkpoint induced by IR. In addition to G_2-M control, the stronger reduction of TopBP1 by As2 in HCC1937 might inhibit the entry into mitosis, because the band was already reduced before irradiation (Fig. 3*A, top panel, Lane 5*). We also investigated the time course and an IR-dose response on G_2-M checkpoint abrogation after TopBP1 inhibition by As2 in HCC1937 cells (Fig. 3, *C* and *D*). In the TopBP1-reduced, BRCA1-mutant cells (double negative), a near complete abrogation of the G_2-M checkpoint remains constant to 48 h after 5 and 10 Gy of IR (Fig. 3, *C* and *D*).

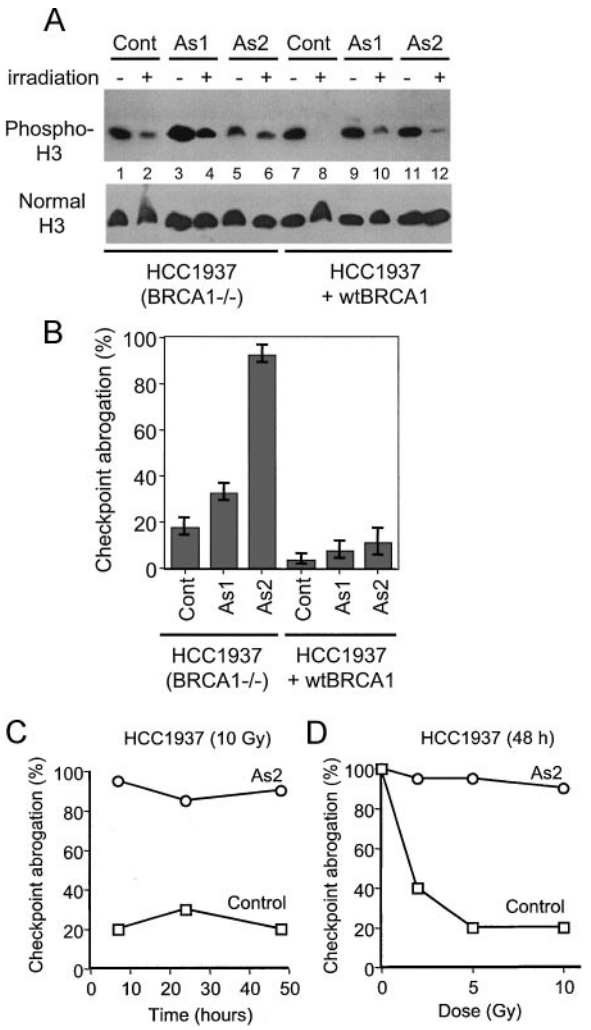

Fig. 3. Both TopBP1 and BRCA1 control G_2-M checkpoint. The antisense (*As1* and *As2*) and general control (*Cont*) morpholino oligomers were transfected into HCC1937 or HCC1937+wild type BRCA1 cells. *A,* nocodazole was added to duplicate plates of each transfectant after 0 or 10 Gy of IR. After incubation for 48 h, cell extracts from each transfectant were subjected to Western blotting using an antibody against the phosphorylated serine 10 of histone H3. *B,* the percentage of G_2-M checkpoint abrogation was quantified from data in *A* as described in "Materials and Methods." The results were from two independent experiments. *C,* after irradiation (10 Gy), nocodazole was added. The transfectants were harvested at indicated times. The data were quantified as described above. *D,* after irradiation at indicated doses, nocodazole was added. The transfectants were harvested after 48 h. The data were quantified as described above.

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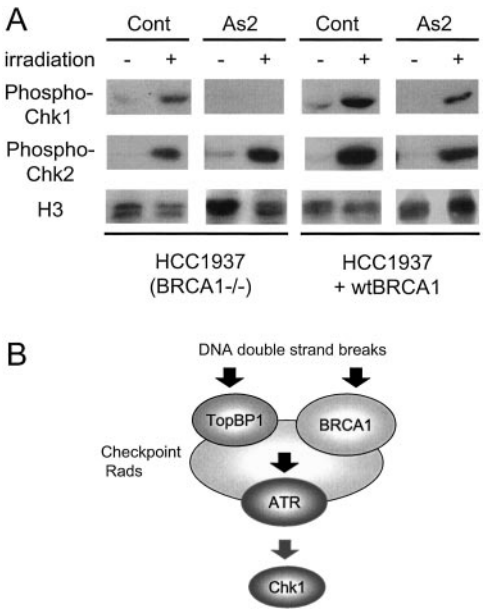

Fig. 4. Regulation of Chk1 but not Chk2 is destroyed in BRCA1-mutant and TopBP1-reduced cells. The antisense (*As2*) and general control (*Cont*) morpholino oligomers were transfected into HCC1937 or HCC1937+wild type BRCA1 cells. After irradiation (0 or 10 Gy), each transfectant was incubated for 2 h under the culture conditions. The cell lysates were subjected to Western blotting using an antibody against the phosphorylated serine 317 of Chk1. The identical lysates were also subjected to Western blotting using an antibody against the phosphorylated threonine 68 of Chk2. The control was Western blotting using an antibody against normal histone H3. *B*, *arrows*, signal flows for G_2-M checkpoint activation generated by DNA double-strand breaks.

Regulation of Chk1 but not Chk2 Is Reduced in BRCA1-mutant and TopBP1-reduced Cells. We used an antibody against the phosphorylated serine 317 of Chk1 to examine the involvement of the checkpoint kinase Chk1 in our system. The phosphorylation of serine 317 is essential for the full activation of Chk1 (21). After irradiation (10 Gy) and a 2-h incubation, the phosphorylation of Chk1 was strongly reduced in the absence of TopBP1 and BRCA1 (Fig. 4*A*, *As2, HCC1937*), indicating that the TopBP1-reduced and BRCA1-mutant condition specifically destroyed regulation of Chk1 but not of Chk2. The phosphorylation was also reduced in the absence of BRCA1 [Fig. 4*A*, Control (*Cont, HCC1937*)]. The phosphorylation of threonine 68 of Chk2 (22, 23) was observed under all conditions. These results suggest that the TopBP1 protein with eight BRCTs is functionally related to the checkpoint Rad protein Cut5 with four BRCTs, whose mutation also strongly reduces the phosphorylation of Chk1 in yeast (24). TopBP1 and BRCA1 proteins may control the ATR pathway, because it has been reported that ATR activates Chk1 (18, 25).

Discussion

In this study, we addressed the hypothesis that TopBP1 and BRCA1 proteins share common functions in determining cell cycle regulation. To examine this hypothesis, we used two different antisense morpholino oligomers against TopBP1, a BRCA1-mutant human breast cancer cell line (HCC1937) and a wild-type BRCA1 cDNA transfectant of HCC1937. One of our questions was whether TopBP1 and BRCA1 regulate the G_2-M checkpoint after DNA damage by IR. We found that a BRCA1-mutant or a TopBP1-reduced background resulted in only partial abrogation of the G_2-M checkpoint, whereas the combined TopBP1-reduced and BRCA1-mutant background resulted in a nearly complete abrogation (Fig. 3). It is important to examine whether BRCA1 can regulate G_2-M or other checkpoints, because checkpoint regulations by BRCA1 may explain the genomic instability of BRCA1-negative cells and the marked predisposition for human breast and ovarian cancer development. Our results indicate that both TopBP1 and BRCA1 regulate G_2-M checkpoint compensating each function in part.

Our data on regulation of the G_2-M cell cycle checkpoint with and without BRCA1 protein expression may clarify two previous reports. BRCA1 was first reported to be involved in a transient G_2-M arrest (mainly 1–3 h) after IR damage using transgenic mouse cells deleted in exon 11 of BRCA1 (26). More recently, partial involvement of BRCA1 in G_2-M checkpoint was also reported using the HCC1937 cells (20). In the HCC1937 (BRCA1 mutant) cells, we found a partial (18%) abrogation of G_2-M after 10 Gy compared with only a 4% abrogation in the HCC1937+wtBRCA1 cells (Fig. 3B). Our data indicate that the partial abrogation of the G_2-M checkpoint in HCC1937 cells persists from 7 to 48 h after 10-Gy irradiation (Fig. 3*C*). However, a BRCA1-mutant and TopBP1-reduced background showed the near complete abrogation from 7 to 48 h, explaining why a BRCA1-mutant background alone showed only a reduced G_2-M checkpoint response. Although another previous study showed no induction of the major G_2-M peak after IR damage in HCC1937 cells (27), our flow cytometry data (Fig. 2) and another recent report (28) clearly demonstrate induction of the G_2-M peak in HCC1937 cells. Whereas HCC1937 is an extensively characterized cell line (14, 15, 20, 29), there are no reports indicating such a G_2-M checkpoint defect using only propidium iodide staining and flow cytometry.

Our data also suggest that TopBP1 and BRCA1 are checkpoint Rad proteins, regulating Chk1 kinase (Fig. 4*A*). TopBP1 and BRCA1 proteins may affect the ATR pathway, because it has been reported that ATR activates Chk1 (18, 25). Because ATR defects result in G_2-M checkpoint abrogation after IR (18, 30, 31), our data are consistent with these observations. We propose that TopBP1 and/or BRCA1, and other checkpoint Rad proteins may cooperatively activate ATR to transduce a DNA damage signal to activate Chk1 kinase (Fig. 4*B*). On the other hand, Chk2 phosphorylation was detected even under conditions of the mutant BRCA1 and a TopBP1-reduced cell (Fig. 4*A*). This may be related to the observation that a Chk2 knockout and antisense inhibition of Chk2 displayed a very weak defect in the G_2-M checkpoint control (32–34). We have demonstrated previously (5) that TopBP1 can colocalize with Nijmegen breakage syndrome 1 and BRCA1 (5), forming IR-induced nuclear foci, which are considered to be a DNA damage recognition site *in vivo* (35, 36). Because we have also reported that BRCTs of TopBP1 and BRCA1 bind to DNA breaks directly *in vitro* (37, 38), it is possible that TopBP1 and BRCA1 proteins may participate in the initial recognition of DNA damage.

In the HCC1937 cells, the tandem transfection protocol mimicked a BRCA1 and TopBP1 double negative and resulted in a marked reduction in colony formation, which was not seen in the HCC1937+wtBRCA1 cells (Fig. 1*B*). These results suggest that TopBP1 and BRCA1 proteins are important in determining overall cell viability. Our clonogenic survival results using this human breast cancer cell line are also consistent with published reports in yeast, in which *S. pombe* cells deficient in Cut5 (6) and *Saccharomyces cerevisiae* cells deficient in Dpb11 (39), both putative homologues of TopBP1, are lethal, whereas yeast deficient in most other checkpoint Rad proteins remain viable. It should be noted that the constitutive expression of BRCA1 under a cytomegalovirus promoter in HCC1937 cells seems to confer greater resistance to the anti-TopBP1 oligomer than we previously reported in HeLa cells (5). We also found that apoptosis was induced by IR DNA damage only under the conditions mimicking a BRCA1 and TopBP1 double negative (Fig. 2). DNA damage-induced apoptosis was not observed by a single transfection (data not shown), suggesting that a small amount (20–30% of con-

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trols) of TopBP1 protein is sufficient to prevent this response. In the BRCA1-transfectant cells, DNA damage-induced apoptosis was strongly suppressed, and almost the entire population of cells was detected in the G_2-M fraction at 48 h after irradiation. The G_2-M peak was still detected even under the TopBP1-reduced and BRCA1-defective conditions after irradiation (Fig. 2).

In summary, this is the first report that demonstrates that TopBP1 and BRCA1 proteins have overlapping functions in regulating the G_2-M cell cycle checkpoint response after DNA damage by IR. Our data also suggest functional symmetry between Cut5-Chk1 pathway (24) in yeast and TopBP1/BRCA1-Chk1 pathway in humans.

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Both DNA Topoisomerase II-binding Protein 1 and BRCA1 Regulate the G $_2$-M Cell Cycle Checkpoint

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