

**BRCA1 Contributes to Cell Cycle Arrest and Chemoresistance in Response to
Anticancer Agent, Irofulven**

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Abbreviations: IR, ionizing radiation; HR, homologous recombination; DSBs, DNA double-strand breaks; sh-RNA, short-hairpin RNA; RDS, radioresistant DNA synthesis assay; PFGE, pulse-field gel electrophoresis; FISH, fluorescent *in situ* hybridization.

ABSTRACT

Tumor suppressor gene *BRCA1* is frequently mutated in familial breast and ovarian cancer. BRCA1 plays pivotal roles in maintaining genomic stability by interacting with numerous proteins in cell cycle control and DNA repair. Irofulven (6-hydroxymethylacylfulvene, HMAF, MGI 114, NSC#: 683863) is one of a new class of anticancer agents that are analogs of mushroom-derived illudin toxins. Preclinical studies and clinical trials have demonstrated that irofulven is effective against several tumor cell types. The exact nature of irofulven-induced DNA damage is not completely understood. Previously, we have demonstrated that irofulven activates ATM and its targets, NBS1, SMC1, CHK2 and p53. In this study, we hypothesize that irofulven induces DNA double-strand breaks and that BRCA1 may affect chemosensitivity by controlling cell cycle checkpoints, DNA repair and genomic stability in response to irofulven treatment. We observed that irofulven induces the formation of chromosome breaks and radials as well as the activation and foci formation of γ -H2AX, BRCA1 and RAD51. We also provided evidence that irofulven induces the generation of DNA double-strand breaks. By using BRCA1-deficient or proficient cells, we demonstrated that in response to irofulven, BRCA1 contributes to the control of S and G2/M cell cycle arrest, is critical for repairing DNA double-strand breaks and for RAD51-dependent homologous recombination. Furthermore, we found that BRCA1 deficiency results in increased chromosome damage and chemosensitivity after irofulven treatment.

INTRODUCTION

Tumor suppressor *BRCA1* is frequently mutated in familial breast and ovarian cancer (Narod and Foulkes, 2004; Venkitaraman, 2004). More than ten percent of women with breast or ovarian cancer carry *BRCA1* mutations (Narod and Foulkes, 2004; Venkitaraman, 2002). *BRCA1* is involved in multiple cellular processes including cell cycle checkpoint control, chromosome remodeling, transcriptional regulation, DNA repair and apoptosis (Narod and Foulkes, 2004; Venkitaraman, 2004; Zhou and Elledge, 2000). It is required for both S and G2/M checkpoints in response to ionizing radiation (IR). Moreover, it plays important roles in multiple DNA repair pathways including homologous recombination (HR) and transcription-coupled nucleotide excision repair (TC-NER) (D'Andrea and Grompe, 2003; Narod and Foulkes, 2004; Venkitaraman, 2004). In response to DNA double-strand breaks (DSBs), proteins such as H2AX, RAD51, MRE11, RAD50, NBS1 and *BRCA1* are rapidly phosphorylated by ATM and/or ATR kinases and form foci at the damaged sites. *BRCA1* interacts with many of these DNA damage signaling and DNA repair proteins including γ -H2AX and RAD51 (Narod and Foulkes, 2004; Scully et al., 1997; Venkitaraman, 2004; Zhou and Elledge, 2000). The γ -H2AX foci formation functions to recruit DNA repair factors to the damaged sites, enforcing HR repair of DNA DSBs and linking chromatin remodeling to DNA repair (Morrison et al., 2004; Riballo et al., 2004; van Attikum et al., 2004; Xie et al., 2004). RAD51 is a DNA recombinase and an essential protein in initiating the HR process by mediating DNA strand exchange during recombination. *BRCA1* is required for RAD51

foci assembly in response to IR-induced DNA DSBs (Narod and Foulkes, 2004; Scully et al., 1997; Venkitaraman, 2004).

Irofulven (6-hydroxymethylacylfulvene, HMAF, MGI 114, NSC#: 683863) is one of a new class of anticancer agents that are analogs of mushroom-derived illudin toxins. Preclinical studies and clinical trials have demonstrated that irofulven is effective against several tumor cell types (Britten et al., 1999; Friedman et al., 2001; Hidalgo et al., 1999; Kelner et al., 2002; MacDonald et al., 1997; Murgo et al., 1999; Sato et al., 2001; Senzer et al., 2005; Woo et al., 2005). Earlier studies have suggested that the DNA damage caused by the illudin family of compounds might be repaired by the nucleotide excision repair (NER) pathway (Kelner et al., 1994; Kelner et al., 1995). Recent studies suggested that TC-NER was the exclusive repair pathway in repairing illudin S and irofulven-elicited DNA lesions and that irofulven cytotoxicity was influenced by the expression of excision endonuclease XPG (Jaspers et al., 2002; Koepfel et al., 2004). However, the HR pathway for DSB repair was not evaluated in these studies (Jaspers et al., 2002; Kelner et al., 1994; Kelner et al., 1995; Koepfel et al., 2004), even though it was suggested as a potential mechanism likely affecting sensitivity to irofulven (Jaspers et al., 2002). Nonetheless, the structure and nature of DNA damage caused by irofulven have not been characterized. Recent reports indicated that ATM and CHK2 were specifically activated by IR or drug (calicheamicin)-induced DSBs (Bakkenist and Kastan, 2003; Buscemi et al., 2004; Ismail et al., 2005; Lee and Paull, 2004; Lee and Paull, 2005). Previously, we have demonstrated that irofulven activates ATM and its targets, NBS1, SMC1, CHK2 and p53 (Wang et al., 2004). Based on these findings, we hypothesize that irofulven induces DNA DSBs and, as a result, BRCA1 may confer chemoresistance to irofulven by

controlling cell cycle checkpoints, DNA repair and genomic stability. Therefore, BRCA1 deficiency might be a useful target and predictive marker for chemotherapy by irifolven.

To further understand the mechanisms of action involved with irifolven, we investigated the role that BRCA1 might play in irifolven-induced DNA damage response. We have observed that irifolven induces the formation of chromosomal breaks and radials as well as the activation and foci formation of γ -H2AX, BRCA1 and RAD51. We have provided evidence that irifolven induces the generation of DSBs. Furthermore, we have demonstrated that in response to irifolven, BRCA1 controls S and G2/M checkpoints and is critical for repairing DNA double-strand breaks through RAD51-dependent homologous recombination, and BRCA1 deficiency results in increased chromosome damage and chemosensitivity.

MATERIALS AND METHODS

Cell culture. All cell lines were maintained in various media supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO₂ atmosphere. Ovarian cancer cell lines A2780, CAOV3 and OVCAR3 were cultured in RPMI 1640; SKOV3 was cultured in McCoy's 5A medium. The vector and BRCA1-transfected breast cancer cell line HCC1937 cells (generously provided by Professor Ralph Scully of Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) were cultured in ACL4 medium as described (Scully et al., 1999). The vector and short-hairpin BRCA1 (sh-BRCA1) stably transfected SKOV3 cells were cultured in McCoy's 5A medium containing 200 µg/ml of G418 (Invitrogen, Carlsbad, CA).

Clonogenic survival assay. To determine chemosensitivity and 1xIC₅₀ concentration, clonogenic survival assay was performed as described previously (Wang et al., 2004) on 60-mm cell culture dishes. Cells were treated with different concentrations of irifolven for one hour followed by drug-free incubations for about 10 days. Colonies were stained with crystal violet and colonies with 50 or more cells were counted.

Metaphase spread. Cells were treated with irifolven. Colcemid (400 ng/ml) (Biosciences, La Jolla, CA) was added to medium four hours before harvesting. After trypsinization, cells were washed once with PBS. Cell pellets were re-suspended in 75 mM KCl and placed in a 37°C incubator for eight minutes. After centrifugation, cells were fixed for two hours at 4°C using 3:1 absolute methanol to glacial acetic acid and

then washed twice with fixative. Cells were re-suspended in fixative and dropped onto slides. Slides were air-dried at room temperature and stained with 5% Gurr's Giemsa stain (Biomedical Specialties, Santa Monica, CA) for seven minutes. Slides were rinsed twice with distilled water and air-dried. The images were recorded by an Olympus Provis AX70 light/fluorescence microscope (Olympus, Melville, NY) and Spot digital camera and software (Diagnostic Instruments, Sterling Height, MI).

Western blotting. Western blot was performed as described previously (Wang et al., 2004). Antibodies used were: monoclonal anti-actin (Sigma, St Louis, MO) and monoclonal anti-BRCA1 (Oncogene Research Products, San Diego, CA).

Immunofluorescent staining and confocal microscopy. Cells were plated on coverslips and treated with 1xIC50 concentration of irofulven for one hour followed by 12 hours of drug-free incubation. Cells were then fixed and stained with polyclonal antibody against BRCA1 or RAD51 (Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal antibody against γ -H2AX (Upstate, Charlottesville, VA). After staining with Alexafluor 488-conjugated goat anti-mouse or goat anti-rabbit; or Alexafluor 546-conjugated goat anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR), slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 5 ng/ml of DAPI. Single color staining images were captured by an Olympus Provis AX70 fluorescence microscope (Olympus, Melville, NY) and Spot digital camera and software (Diagnostic Instruments, Sterling Height, MI). Confocal staining images were captured by a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, NY).

Radioresistant DNA synthesis (RDS) assay. The RDS assay was performed as described (Xu et al., 2002). Briefly, cells in the logarithmic phase of growth were pre-labeled by culturing in medium containing 10 nCi of [^{14}C]-thymidine (PerkinElmer, Boston, MA) for 24 hours. The medium was then replaced with normal medium, and cells were incubated for another 24 hours. Cells were treated with iriffulven for one hour and incubated in drug-free medium for 12 hours. Cells were then pulse-labeled with 2.5 μCi of [^3H]-thymidine (PerkinElmer, Boston, MA) for 15 minutes. Cells were harvested, washed twice with PBS, and fixed in 70% methanol for at least 30 minutes. Cells were then transferred to Whatman filters (Whatman, Clifton, NJ) and washed sequentially with 70% and then 95% methanol. The filters were air-dried and the amount of radioactivity was quantified in Wallac 1410 liquid scintillation counter (PerkinElmer, Downers Grove, IL). The resulting ratio of ^3H counts per minute to ^{14}C counts per minute, corrected for those counts per minute that were the results of channel crossover, was a measure of DNA synthesis.

Phosphorylated histone H3 staining and flow cytometry. The phospho-histone H3 staining was performed as described (Xu et al., 2002). Briefly, the vector and BRCA1-transfected HCC1937 cells were treated with 1 μM of iriffulven for one hour followed by one hour of drug-free incubation. Cells were harvested and fixed in 70% ethanol. The fixed cells were washed twice with PBS and made permeable with 0.25% Triton X-100 in PBS on ice for 10 minutes. Cells were rinsed in 1% BSA/PBS and then stained with rabbit anti-phospho-S10 histone H3 antibody (Upstate, Charlottesville, VA) for two hours

at room temperature. Cells were rinsed in 1% BSA/PBS and stained with Alexafluor 488-conjugated anti-rabbit secondary antibody for 30 minutes at room temperature. Cells were washed twice with PBS and suspended in PBS containing propidium iodide (0.25 $\mu\text{g/ml}$) and RNase A (20 $\mu\text{g/ml}$). Flow cytometry was performed on FACSCalibur with CellquestPro software (Becton Dickinson, San Jose, CA). Thirty thousands events were recorded for each sample. The percentage of mitotic cells was determined as those cells that were Alexafluor-positive and contained 4N DNA content.

Mitotic index. Cells were plated on cover-slips and treated with 1xIC50 concentration of irrofulven for one hour followed by 24 hours of drug-free incubation. Cells were then fixed and stained with DAPI (5 ng/ml). Staining images were captured by an Olympus Provis AX70 fluorescence microscope (Olympus, Melville, NY) and Spot digital camera and software (Diagnostic Instruments, Sterling Height, MI). In each group, about 4,000 cells were counted. Mitotic index was calculated as the percentage of cells in mitosis.

Pulse-field gel electrophoresis (PFGE). The PFGE was conducted as follows. Cells were scraped from the dish and washed with ice-cold embedding buffer (15 mM Tris-HCl, pH 7.4, 1mM EGTA, 2mM EDTA, 60mM KCl, 15mM NaCl, 0.5mM spermidine and 0.15 mM spermine). Cells were then re-suspended in embedding buffer, mixed well, and incubated for 30 seconds in a 30°C water bath before adding 1.6% low melting point agarose pre-warmed to 55°C. After thorough mixing, the cell suspension was aspirated into 2.3 mm inner diameter tubing using a syringe. The tube was immediately placed in ice-cold water for 5 minutes to allow the agarose to harden. The agarose core was

incubated in extraction buffer (10mM Tris-HCl, pH 9.5, 10mM NaCl, 25 mM EDTA, 1.5% SDS and 0.1% mercaptoethanol) overnight at room temperature with gentle agitation. Extraction was performed three more times for two hours each followed by three washes of two hours in TE buffer. The agarose core was then cut into 6 mm-long plugs. A 1% agarose gel (PFGE-certified, Bio-Rad, Hercules, CA) in 0.5X TBE (Cambrex, Rockland, ME) was cast and plugs were inserted into gel wells. The concatenated chromosomes of lambda phage (48.5 kb) (Bio-Rad, Hercules, CA) were used as the standard for DNA size. The DNA was resolved by direct current of 100 V for 20 minutes followed by 17 hours of pulse current using a programmable power inverter PPI-200 (MJ Research, Watertown, MA) and program number 6. DNA was visualized by staining with 0.5 µg/ml of ethidium bromide (Invitrogen, Carlsbad, CA) and pictures were captured using Eagle Eye II system and software (Stratagene, La Jolla, CA).

Comet Assay. The comet assay (Trevigen Inc., Gaithersburg, MD) was performed according to manufacturer's protocol by using neutral conditions to mainly detect double-strand breaks. Briefly, cells were harvested, washed with cold PBS, and combined with molten LMAgarose, and 75 µl (500-1000 cells) was immediately added to Comet Slide. After hardening, slides were incubated for 30 minutes in lysis solution at 4°C, then rinsed with 1X TBE before electrophoresis for 60 minutes at 30V. Slides were rinsed with dH₂O and placed in 70% ethanol for 10 minutes, then air-dried. To visualize DNA, 50 µl of a 1:1000 dilution of SYBR Green (Molecular Probes, Eugene, Oregon) in PBS was added to each slide. Slides were visually scored using Olympus Provis AX70 microscope (Olympus, Melville, NY) from 0-4 based on tail length and intensity, and a total score of

75 cells was used to determine relative amount of double-strand breaks for each time point. Images were captured using Spot digital camera and software (Diagnostic Instruments, Sterling Height, MI).

Fluorescent *in situ* hybridization (FISH). Slides were rinsed at room temperature in 2xSCC solution for 30 minutes and then rinsed in PBS for 15 minutes. Slides were then fixed in 3.7% formaldehyde/PBS solution for 15 minutes, followed by a 5% pepsin/0.01 M HCl solution at 37°C for 15 minutes. Slides were washed in PBS for 5 minutes at room temperature. Slides were put into 95% ethanol for 5 minutes and then air-dried. For FISH hybridization, the Whole Chromosome 1 Probe (Oncor, Gaithersburg, Maryland) was pre-warmed to 37°C for 5 minutes. Aliquots of 3 µl of probe were applied to the slides, covered with 12 mm diameter round cover-slips and sealed. The slides were then co-denatured at 74°C for 6 minutes and placed in a 37°C water bath overnight. Slides were washed according to manufacturer's protocol and detected using the Rhodamine-labeled anti-Digoxigenin detection reagent (Insitus, Albuquerque, New Mexico). Slides were then counterstained with DAPI and evaluated using an AxioPlan II epi-fluorescence microscope (Zeiss, Thornwood, NY) and CytoVision software (Applied Imaging, San Jose, California).

RNA interference. Three pairs of 65-nt short-hairpin BRCA1 (sh-BRCA1) oligos containing target sequences of AACCTGTCTCCACAAAGTGTG, AAAGTACGAGATTTAGTCAAC and AAGCAGCGGATACAACCTCAA were designed and synthesized. After annealing, these three 65-bp double-strand sh-BRCA1

fragments were inserted into pSilencer 2.1-U6-neo vector (Ambion, Austin, TX) and transfected into SKOV3 cells. The pSilencer 2.1-U6-neo vector containing the scrambled sequence was transfected as the non-specific control. Stable cell lines were established by selecting in medium containing G418.

RESULTS

Irofulven induces chromosome aberrations and activates BRCA1.

To characterize the DNA damage caused by irofulven and to examine whether DNA DSBs were generated after treatment, mitotic spread experiments were performed in breast cancer cell line HCC1937 and ovarian cancer cell line SKOV3. HCC1937 cell line is known to express a truncated BRCA1 protein (Scully et al., 1999; Tomlinson et al., 1998). SKOV3 cell line is known to harbor a functional BRCA1 (Husain et al., 1998). Cell lines were treated with irofulven for one hour followed by 24 hours of drug-free incubation. The mitotic spread results clearly demonstrated the induction of chromosome breaks and radials in these cells (Fig. 1A). Similar chromosome breaks and radials were also observed in ovarian cancer cell lines A2780, CAOV3 and OVCAR3 after treatment (data not shown). These results demonstrate that irofulven indeed induces the generation of DNA DSBs.

Upon the induction of DSBs, histone variant H2AX is rapidly phosphorylated (γ -H2AX) and forms discrete nuclear foci co-localizing with many other DNA repair proteins such as RAD50, RAD51 and BRCA1 (Paull et al., 2000; Rothkamm and Lobrich, 2003). The γ -H2AX foci formation also allows a sensitive detection of DSBs (Franco et al., 2006; Greenberg et al., 2006; Paull et al., 2000; Rogakou et al., 1998; Rothkamm and Lobrich, 2003; Sedelnikova et al., 2002; Stucki et al., 2005). To further confirm that irofulven induces the generation of DSBs, SKOV3 cells were treated with irofulven and immunofluorescently stained utilizing antibodies that recognize γ -H2AX and BRCA1. Confocal microscopic images indicated that γ -H2AX and BRCA1 form co-

localizing foci after treatment (Fig. 1B). Taken together, these findings demonstrate that irofulven induces the generation of DNA DSBs, which results in the activation and foci formation of γ -H2AX and BRCA1.

BRCA1 contributes to the control of S and G2/M checkpoints in response to irofulven-induced DNA damage.

To explore the possible role that BRCA1 activation might play in regulating cell cycle progression after irofulven treatment, we first characterized the cell cycle arrest at S phase by the radioresistant DNA synthesis (RDS) assay. The vector and BRCA1-transfected HCC1937 cells were treated with increasing concentrations of irofulven for one hour followed by 12 hours of drug-free incubation. The results of the RDS assay demonstrated that DNA synthesis was significantly inhibited in the BRCA1-transfected HCC1937 cells compared with the vector-transfected cells (Fig. 2A). This indicates that BRCA1 does contribute to the control of S phase cell cycle arrest in response to irofulven.

It has been reported that there are two distinct G2/M checkpoints in response to IR-induced DSBs, which control the transient G2/M transition and prolonged G2/M accumulation, respectively. BRCA1 is involved in controlling both G2/M checkpoints (Xu et al., 2002). To study the role that BRCA1 plays in modulating the G2/M checkpoints, immunofluorescent staining for phospho-histone H3, a marker for mitosis, and FACS analysis were performed to assess the transient G2/M checkpoint. The vector and BRCA1-transfected HCC1937 cells were treated with irofulven for one hour followed by one hour of drug-free incubation. The FACS analysis results indicated that

the phospho-histone H3-positive population was increased from 1.14% to 1.63% in vector-transfected cells; while in BRCA1-transfected cells it was dramatically decreased from 1.25% to 0.55% (Fig. 2B). These results indicate that BRCA1 controls the G2/M checkpoint in response to irifolven treatment.

The cumulative effect of BRCA1 on S and G2/M checkpoints was also reflected by assessing the mitotic index. The vector and BRCA1-transfected HCC1937 cells were treated with irifolven for one hour followed by 24 hours of drug-free incubation. The mitotic index decreased only 19% (from 2.7% to 2.18%) in vector-transfected HCC1937 cells; while it dropped 90% (from 1.56% to 0.15%) in BRCA1-transfected HCC1937 cells after treatment (Fig. 2C). Taken together, these results demonstrate that BRCA1 contributes to both S and G2/M checkpoints in response to irifolven-induced DNA damage.

BRCA1 is critical for repairing irifolven-induced DSBs and for RAD51-mediated homologous recombination.

From the data described above, it was demonstrated that irifolven induces chromosome aberrations (breaks and radials) and the foci formation of γ -H2AX and BRCA1. These findings indicate that irifolven induces the generation of DSBs and activation of BRCA1. Therefore, we hypothesize that BRCA1 might play a critical role in regulating the repair of irifolven-induced DSBs. To examine this hypothesis, the BRCA1-proficient and deficient HCC1937 cell lines were again used to compare the DNA repair dynamics and to assess the foci formation of DNA repair factors.

We first compare the differences in the occurrence and repair of irifolven-induced DSBs by pulse-field gel electrophoresis (PFGE). As shown in Fig. 3A, after one-hour of treatment, the large genomic DNA fragments from 50 to >400 kb gradually increased in vector-transfected HCC1937 cells over the period of 6-48 hours. In BRCA1-transfected HCC1937 cells, these DSBs were significantly repaired under the same treatment conditions (Fig. 3A).

To confirm these results, we performed the Comet assay under neutral electrophoresis conditions that will predominantly detect DSBs. The results again indicated that irifolven-induced DSBs were significantly repaired in BRCA1-transfected HCC1937 cells 48 or 72 hours after treatment ($p < 0.05$) or $p < 0.01$, respectively) (Fig. 3, B and C).

To further examine the differences in repair dynamics of irifolven-induced DSBs, we stained cells for γ -H2AX foci formation over the time period of 72 hours after treatment. The immunofluorescent staining results indicated that the percentage of cells containing γ -H2AX foci started decreasing dramatically in BRCA1-transfected HCC1937 cells 24 hours after treatment (Fig. 3D). Taken together, these results demonstrate that BRCA1 plays an important role in repairing irifolven-induced DSBs.

RAD51 is a DNA recombinase and an essential protein for initiating the strand invasion process in the HR repair of DNA DSBs (Narod and Foulkes, 2004; Venkitaraman, 2004). RAD51 forms foci in response to IR-induced DNA DSBs and BRCA1 is required for RAD51 foci formation (Scully et al., 1997; West, 2003). We therefore examined the RAD51 foci formation in the vector and BRCA1-transfected HCC1937 cells. Immunofluorescent staining results demonstrated that, upon irifolven

treatment, more RAD51 foci were assembled in BRCA1-transfected HCC1937 cells (from 4.7% to 40.3%) than in vector-transfected HCC1937 cells (from 2.8% to 13.2%) (Fig. 3, E and F). When the γ -H2AX foci formation was evaluated under the same conditions, it was observed that γ -H2AX assembled foci to the similar extent in both cells (Fig. 3, E and F). These results demonstrate that a similar amount of DSBs were induced by iroutilven and RAD51 foci formation is dependent on BRCA1. Taken together, these results demonstrate that BRCA1 plays an important role in repairing iroutilven-induced DSBs, RAD51-dependent HR repair is involved and BRCA1 is critical for this process.

BRCA1 contributes to maintaining chromosome integrity upon exposure to iroutilven.

Since BRCA1 controls S and G2/M cell cycle arrest and is important in repairing iroutilven-induced DSBs, we hypothesize that BRCA1 contributes to maintaining chromosome integrity upon exposure to iroutilven. Mitotic spread experiments were again performed in the vector and BRCA1-transfected HCC1937 cells. A dramatic increase in chromosome breaks and radials were observed in vector-transfected cells compared with BRCA1-transfected cells (Fig. 4A).

To further illustrate the chromosome aberrations induced by iroutilven, FISH analysis was carried out in vector-transfected HCC1937 metaphase cells with the whole chromosome 1 FISH paint probe. Images again revealed that iroutilven induces a significant amount of chromatid/chromosome breaks and radials involving chromosome 1 (Fig. 4B). These results suggest that the repair of iroutilven-induced DSBs is largely

impaired in BRCA1-deficient cells, and BRCA1 plays a pivotal role in maintaining chromosome integrity in response to irifolven-induced DNA damage.

To verify the role that BRCA1 plays in maintaining chromosome integrity, and especially in chemosensitivity in response to irifolven-elicited DNA damage, we employed the RNA interference approach to stably knock-down BRCA1 in SKOV3 cells. The effectiveness of three sh-BRCA1 (short-hairpin BRCA1) constructs (sh-B1, sh-B2 and sh-B3) in knocking-down the endogenous BRCA1 levels was determined by Western blot. As shown in Fig. 5A, the sh-B2 most effectively reduced BRCA1 protein level, therefore, was chosen for subsequent studies.

Chromosome aberrations were again evaluated in BRCA1-depleted mitotic SKOV3 cells. As shown in Fig. 5B, chromosome breaks were increased in untreated sh-BRCA1 (sh-B2)-transfected SKOV3 cells, which was further exacerbated after irifolven treatment (Fig. 5B). Strikingly, the majority of sh-B2-transfected SKOV3 cells displayed more severe chromosome damage after treatment. In these metaphase cells, chromosomes were damaged to the point where all of the chromosomes appeared to be broken or fragmented (Fig. 5C).

FISH analysis was also performed in sh-B2-transfected SKOV3 metaphase cells with the whole chromosome 1 FISH paint probe. Similar to what was observed above, images displayed extensive fragmentation of chromosome 1 after irifolven treatment (Fig. 5D). Taken together, these results demonstrate that BRCA1 plays an important role in maintaining chromosome integrity in response to irifolven-induced DNA damage.

BRCA1 confers chemoresistance to irifolven.

To determine whether BRCA1 might affect chemosensitivity to irifolven, the clonogenic survival assay was carried out. The vector and BRCA1-transfected HCC1937 cells were treated with different concentrations of irifolven for one hour followed by drug-free incubations. When IC₅₀ concentrations were compared, the results demonstrated that the vector-transfected cells were two-fold more sensitive than the BRCA1-transfected cells (Fig. 6A). We also conducted clonogenic assay with longer exposure time to verify that BRCA1-deficient cells are more sensitive. The results indicated that at 0.25 μ M, a concentration that caused no difference in chemosensitivity between the vector and BRCA1-transfected HCC1937 cells in Fig. 6A, the BRCA1-transfected HCC1937 cells are four-fold more resistant when treated for six hours, and 19-fold more resistant when treated for 24 hours, than vector-transfected HCC1937 cells (Fig. 6B). These results demonstrate that BRCA1 deficiency renders cancer cells more sensitive to irifolven.

To corroborate these results, clonogenic survival assay was also performed in the vector and sh-BRCA1 transfected SKOV3 cells. Cells were treated with different concentrations of irifolven for one hour followed by drug-free incubations. The results demonstrated that knocking-down the endogenous BRCA1 levels resulted in more than two-fold increase in chemosensitivity to irifolven when IC₅₀ concentrations were compared (Fig. 6C). Therefore, it can be concluded that BRCA1 confers chemoresistance to irifolven.

DISCUSSION

In this study, we have observed that iriffulven induces the formation of chromosome breaks and radials as well as the formation of γ -H2AX, RAD51 and BRCA1 foci. We have also provided evidence that iriffulven induces the generation of DSBs. Furthermore, we have demonstrated that BRCA1 contributes to the control of S and G2/M cell cycle arrests, and is critical for RAD51-dependent HR repair of DSBs, chromosome integrity and chemosensitivity in response to iriffulven.

BRCA1 is frequently mutated in familial breast and ovarian cancer. Cancers that arise in mutation carriers have often lost the second allele through somatic alterations that must occur during tumor progression (Narod and Foulkes, 2004; Venkitaraman, 2002; Venkitaraman, 2004; Zhou and Elledge, 2000). It has previously been shown in several tumor cell lines that continuous exposure to iriffulven resulted in a few to several hundred-fold difference in cytotoxicity based solely on increased exposure times (Kelner et al., 1997; Kelner et al., 1999; Kelner et al., 1990). We found that BRCA1-deficient cells are more sensitive to iriffulven treatment. We also found that when being treated for a longer period of time at lower concentration, greater sensitivity can be reached in BRCA1-deficient cells. This suggests that by maintaining a low level of drug through consecutive exposures to iriffulven, BRCA1 deficiency might be exploited clinically to achieve preferential therapeutic outcomes.

BRCA1 plays an important role in regulating cell cycle checkpoints after IR (Narod and Foulkes, 2004; Venkitaraman, 2004). However, a recent study demonstrated that BRCA1-deficient MEFs arrested at S and G2/M phases in response to mitomycin C

treatment (Bhattacharyya et al., 2000). In this study we have found that BRCA1 controls both S and G2/M checkpoints in response to irifolven-induced DNA damage.

To date, the structure and nature of irifolven-induced DNA damage have not been fully characterized. Earlier studies have suggested that the DNA damage caused by the illudin family of compounds might be repaired by the NER pathway (Kelner et al., 1994; Kelner et al., 1995). Recent studies suggested that TC-NER was the exclusive repair pathway in repairing illudin S and irifolven-elicited DNA lesions and that irifolven cytotoxicity was influenced by the expression of excision endonuclease XPG (Jaspers et al., 2002; Koepfel et al., 2004). However, the HR pathway for DSB repair was not evaluated in these studies. In this study, we have provided evidence that irifolven induces the generation of DSBs, and BRCA1 plays an important role in RAD51-dependent HR repair, chromosome integrity and chemosensitivity in response to irifolven-induced DSBs. These findings are consistent with our previous observations that irifolven induces the activation of ATM and its target genes NBS1, SMC1 and CHK2 (Wang et al., 2004). A distinct possibility exists that irifolven is able to produce multiple types of DNA lesions. Since BRCA1 plays important roles in multiple DNA repair pathways including HR and TC-NER (D'Andrea and Grompe, 2003; Narod and Foulkes, 2004; Venkitaraman, 2004), it remains to be determined whether BRCA1 might also be involved in TC-NER of irifolven-induced DNA lesions.

Notably, we observed that there was some degree of RAD51 foci formation in vector-transfected-HCC1937 cells after irifolven treatment. HCC1937 cells lack the wild-type allele but retain the mutant allele (5382insC) of *BRCA1*. As a result, this cell line expresses a BRCA1 protein truncated at amino acid 1755 of the C-terminus, resulting

in loss of the second BRCT domain (Greenberg et al., 2006; Scully et al., 1999; Tomlinson et al., 1998; Yu et al., 2003). Due to its known BRCA1 mutation status, this cell line is widely used for the study of BRCA1 functions (Chen et al., 1998a; Greenberg et al., 2006; Scully et al., 1997; Scully et al., 1999; Tomlinson et al., 1998; Yu et al., 2003). Earlier investigations have demonstrated that in response to DNA damage, BRCA1 forms a large protein complex with a group of proteins including MSH2, MSH6, MLH1, ATM, BLM, and the MRE11-RAD50-NBS1 (M/R/N) protein complex, indicating that BRCA1 may function as a coordinator of multiple activities required for the maintenance of genomic integrity and DNA repair (Wang et al., 2000; Wu et al., 2000; Zhong et al., 1999). Recent studies indicate that in response to DSBs, BRCA1 complexes with multiple protein partners, BRCA1/BARD1/BACH1/TopBP1, BRCA1/BARD1/CtIP/M/R/N or BRCA1/BARD1/BRCA2/RAD51, integrating the activities of these partners in cell cycle checkpoints and HR repair of DSBs (Greenberg et al., 2006; Scully et al., 1997). Recent studies also demonstrate that the tandem BRCT domains of BRCA1 function as phospho-serine- or phospho-threonine-specific binding modules that recognize substrates phosphorylated by ATM. The two BRCT domains of BRCA1, but not the individual BRCT domains alone, displayed phospho-specific binding (Manke et al., 2003; Yu et al., 2003). Therefore, the impaired RAD51 foci formation in HCC1937 cells in response to irrofulven- or IR/laser-induced DSBs observed in this and other studies (Greenberg et al., 2006), could be due to the decrease in binding of partner proteins, such as BRCA2, which is critical for RAD51 foci formation (Chen et al., 1998a; Chen et al., 1999; Chen et al., 1998b; Greenberg et al., 2006; Scully et al., 1997; Scully and Livingston, 2000; Sharan et al., 1997; Wong et al., 1997). It could also be due to the

impaired interaction with the M/R/N complex at the DSB sites (Wang et al., 2000; Wu et al., 2000; Zhong et al., 1999) or to the loss of interaction with the ATM-phosphorylated substrates, such as BACH1 (Greenberg et al., 2006; Yu et al., 2003). In support of this, HCC1937 cells displayed barely detectable association of BRCA1-associated proteins, BARD1, RAD51, BRCA2, and BACH1; and decreased association of truncated BRCA1 with TopBP1 (albeit not absent), compared with BRCA1-transfected HCC1937 cells in response to DSBs (Greenberg et al., 2006). In addition, some degree of RAD51 foci formation was also observed in mouse *BRCA1*^{-/-} (deleted for exon 11) ES cells compared with *BRCA1*^{+/+} ES cells in response to IR-induced DSBs (Bhattacharyya et al., 2000).

The chromosome breaks, tri-radials and quadri-radials formed after iroutilven treatment are reminiscent of Fanconi anemia and BRCA2-deficient cells treated with IR or mitomycin C (D'Andrea and Grompe, 2003; Venkitaraman, 2002; Venkitaraman, 2004). Based on the roles that BRCA1 plays in RAD51-dependent HR, chromosome integrity and chemosensitivity in response to iroutilven, it can be postulated that cells deficient in other important proteins involved in the HR pathway of DSB repair, such as FANCD2, BRCA2 or RAD51, might also show increased sensitivity. *FANCD2* and *BRCA2* are found frequently mutated or repressed in many types of cancers (D'Andrea and Grompe, 2003; Turner et al., 2004; Venkitaraman, 2002; Venkitaraman, 2004).

In summary, we have observed that iroutilven induces the formation of chromosome breaks and radials as well as the formation of γ -H2AX, RAD51 and BRCA1 foci. We have also provided evidence that iroutilven induces the generation of DSBs. We have demonstrated that BRCA1 is critical for S and G2/M phase cell cycle checkpoints, RAD51-dependent HR, chromosome integrity and chemosensitivity in response to

irofulven. These findings will enhance our understanding of the mechanisms of action involved with irofulven, and more specifically, the proteins and mechanisms that might affect irofulven-induced chemosensitivity. They will also provide insight for future studies of targeted therapy by irofulven in BRCA1-deficient familial breast and ovarian cancers.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Irofulven induces chromosome breaks, triradials and quadriradials and activates BRCA1. **(A)** HCC1937 and SKOV3 were treated with 1xIC50 concentrations of irofulven (1.0 and 2.3 μM , respectively) for one hour followed by 24 hours of drug-free incubation. Pictures showed the mitotic spread staining of cells treated with irofulven. Arrows indicate chromosome breaks, triradials and quadriradials. **(B)** Confocal microscopic images of immunofluorescent staining for BRCA1 and $\gamma\text{-H2AX}$. SKOV3 cells were treated with 1xIC50 concentration of irofulven for one hour followed by 12 hours of drug-free incubation.

Fig. 2. BRCA1 controls S and G2/M checkpoints in response to irofulven-induced DNA damage. **(A)** DNA synthesis was determined by RDS assay. The vector and BRCA1-transfected HCC1937 cells were treated with 1, 2 or 3 μM of irofulven for one hour followed by 12 hours of drug-free incubation. DNA synthesis rates were presented as the average and standard error of triplicate experiments. **(B)** The mitotic population of cells was determined by staining for phosphorylated histone H3 and flow cytometry. The vector and BRCA1-transfected HCC1937 cells were treated with 1 μM of irofulven for one hour followed by one hour of drug-free incubation. The percentage of phospho-histone H3-positive population was presented as the mean and standard deviation of triplicate experiments. **(C)** The mitotic index of vector and BRCA1-transfected HCC1937 cells. Cells were treated with 1 μM of irofulven for one hour followed by 24 hours of drug-free incubation. In each group, about 4,000 cells were counted.

Fig. 3. BRCA1 is critical for repairing irofulven-induced DSBs and for RAD51-dependent HR. The vector and BRCA1-transfected HCC1937 cells were treated with 1 μ M of irofulven for one hour followed by different times of drug-free incubation. **(A)** Genomic DNA samples were extracted and separated by PFGE. **(B and C)** Comet assay was performed using neutral conditions to specifically detect double-strand breaks **(B)**. The comet tail movement was quantified by visual scoring. The statistical significance was analyzed by Student's *t*-test and marked as * ($p < 0.05$) and ** ($p < 0.01$) **(C)**. **(D)** The DNA repair dynamics was characterized by counting the γ -H2AX foci formation. **(E and F)** Cells were immunofluorescently stained for RAD51, γ -H2AX and DAPI **(E)**. Cells with five or more foci were counted as positive for staining. The percentage of cells with RAD51 or γ -H2AX foci was exhibited as the mean and standard deviation of triplicate counts of 1,000 cells **(F)**.

Fig. 4. Chromosome aberrations induced by irofulven are related to BRCA1 status. The vector and BRCA1-transfected HCC1937 cells were treated with 1 μ M of irofulven for one hour followed by 24 hours of drug-free incubation. **(A)** Mitotic spread staining was performed. The percentage of mitotic cells with four or more chromosome breaks or with radials was presented. In each group, 100 mitotic cells were counted. **(B)** FISH was performed to specifically characterize aberrations involving chromosome 1 in the vector-transfected HCC1937 cells treated with irofulven. The images show the largest portion of chromosome 1 within the HCC1937 cells **(a and b)**. The asterisk (*) indicates an inherent

translocation on chromosome 1. Arrows indicate the chromatid/chromosome breaks (**c** and **d**) and a quadri-radial (**e** and **f**) involving chromosome 1 after irifolven treatment.

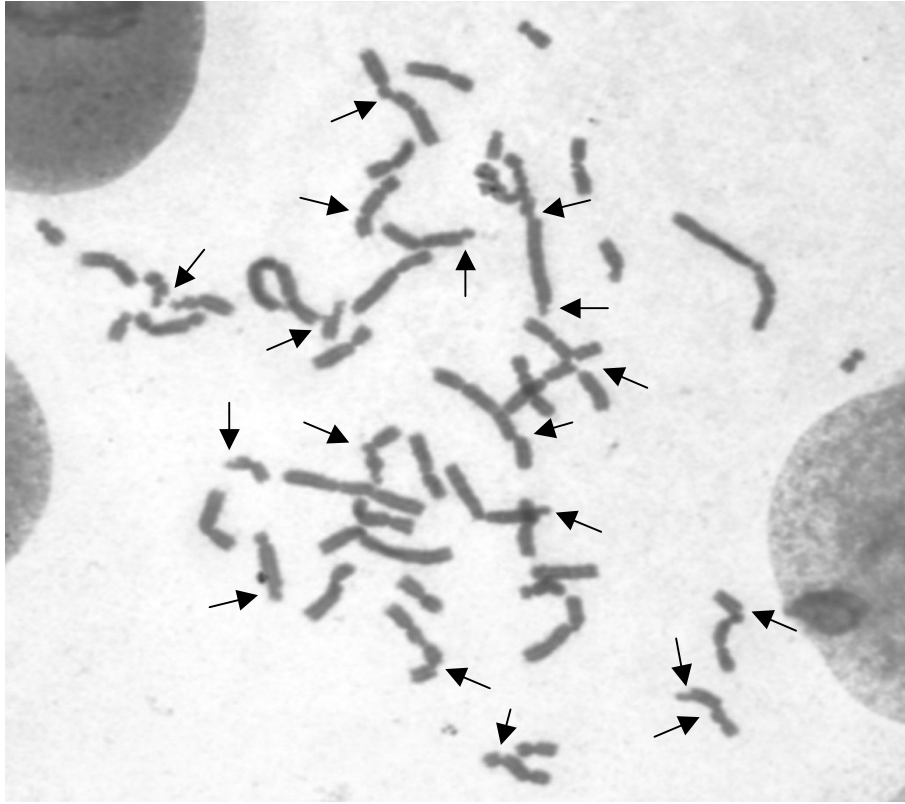
Fig. 5. Knocking-down BRCA1 protein levels by RNA interference results in increased chromosome aberrations. **(A)** SKOV3 cells were stably transfected with the vector (sh-V) or sh-BRCA1 (short-hairpin BRCA1) constructs (sh-B1, B2, B3), respectively. The efficacy of sh-BRCA1 constructs in knocking-down BRCA1 protein levels was determined by Western blot analysis with the anti-BRCA1 antibody. The blot for actin served as loading control. **(B through D)** Mitotic spread staining was performed. The sh-V and sh-B2-transfected SKOV3 cells were treated with 1xIC50 concentration of irifolven for one hour followed by 24 hours of drug-free incubation. The percentage of mitotic cells with four or more chromosome breaks was presented. In each group, 100 mitotic cells were counted **(B)**. Representative picture of metaphase sh-B2-transfected SKOV3 showed widespread chromosome fragmentation after irifolven treatment **(C)**. FISH was performed to specifically characterize the chromosome 1 damage in sh-B2-transfected SKOV3 cells treated with irifolven. The images show chromosome 1 within the sh-B2-transfected SKOV3 cells (**a** and **b**). Arrows indicate the highly altered chromosome 1 after irifolven treatment (**c** through **f**) **(D)**.

Fig. 6. BRCA1 confers chemoresistance to irifolven. Irifolven-induced chemosensitivity was determined by clonogenic survival assay in the vector and BRCA1-transfected HCC1937 cells or in the vector and sh-BRCA1-transfected SKOV3 cells. **(A through C)**

Cells were treated with irofulven for one hour (**A** and **C**), or one, six or 24 hours (**B**). The mean and standard deviation of triplicate experiments were demonstrated.

Figure 1A

HCC1937



SKOV3

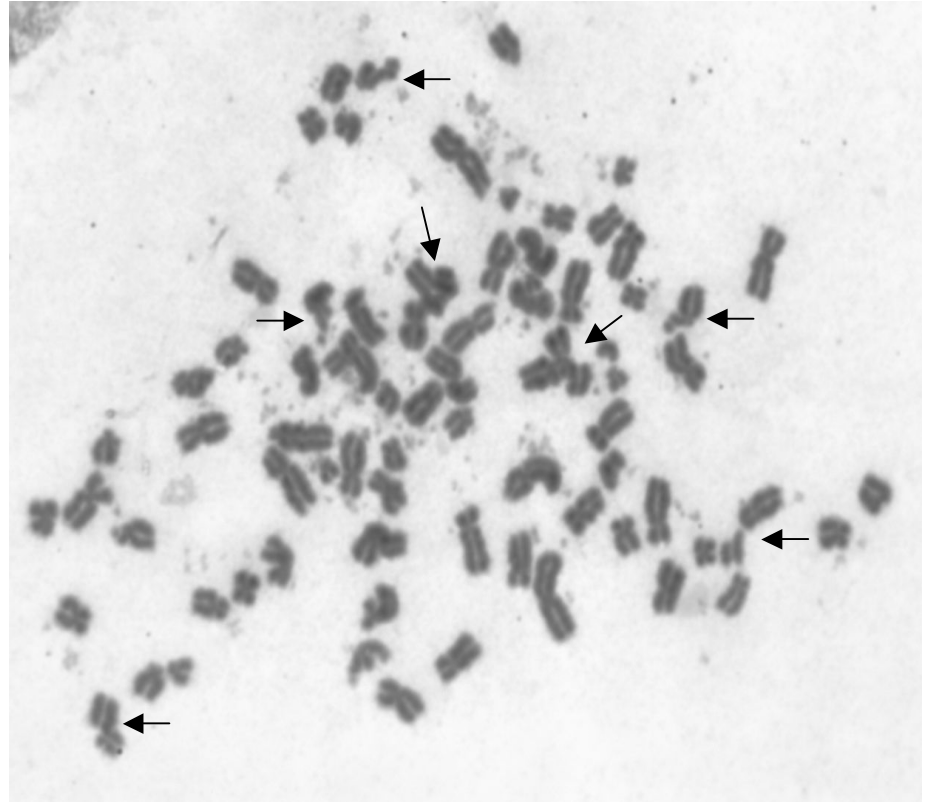


Figure 1B

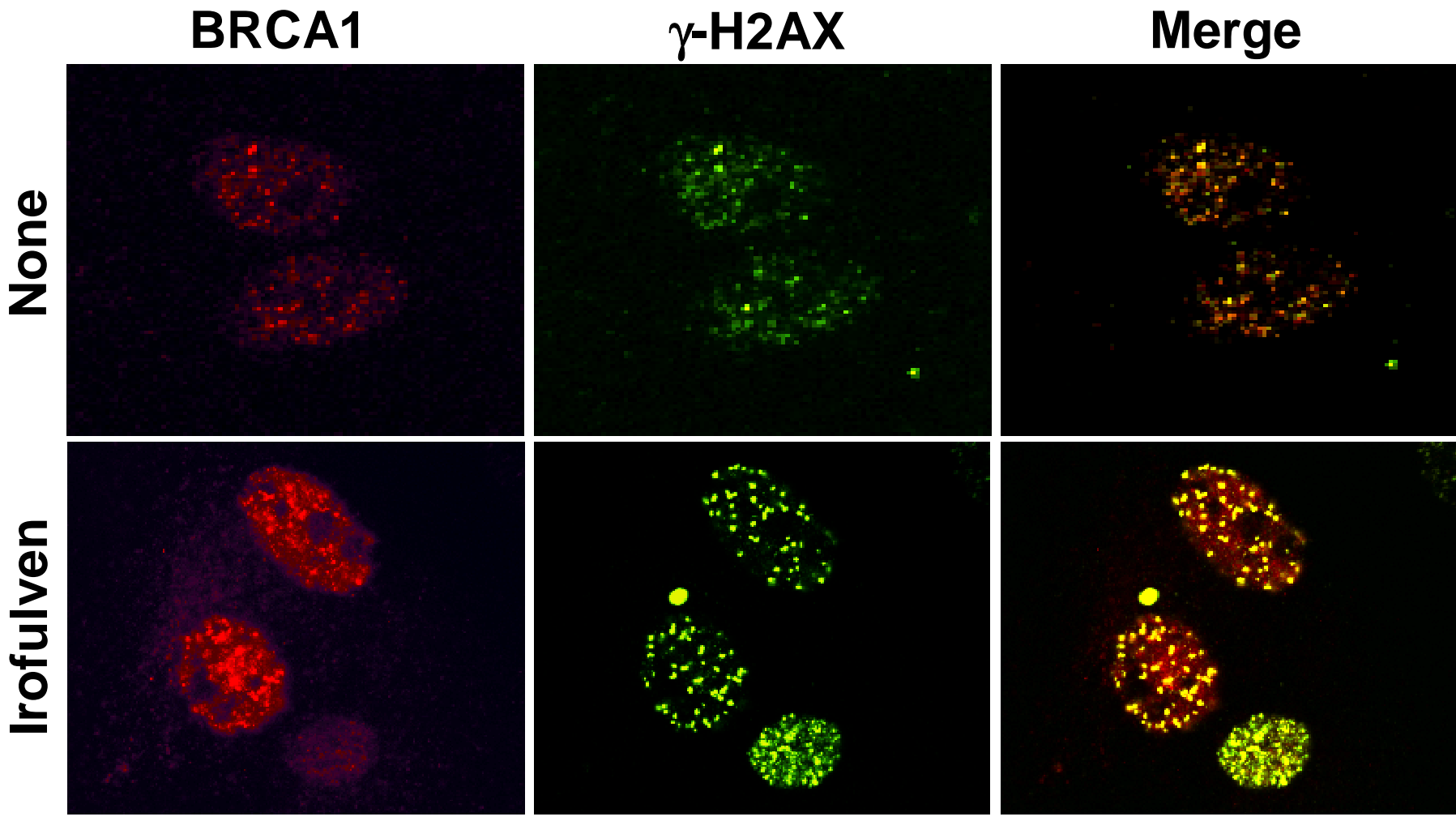


Figure 2A

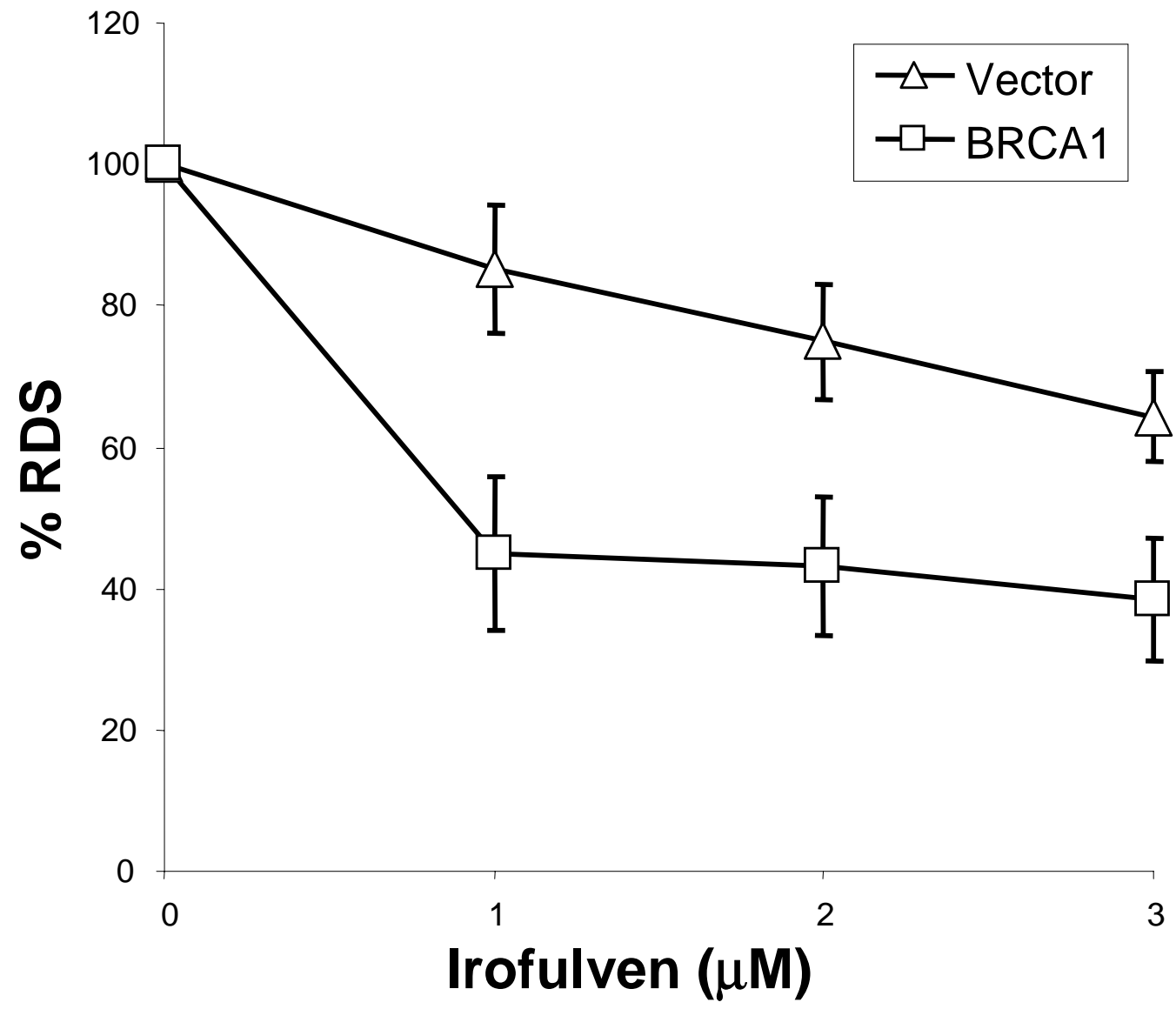


Figure 2B

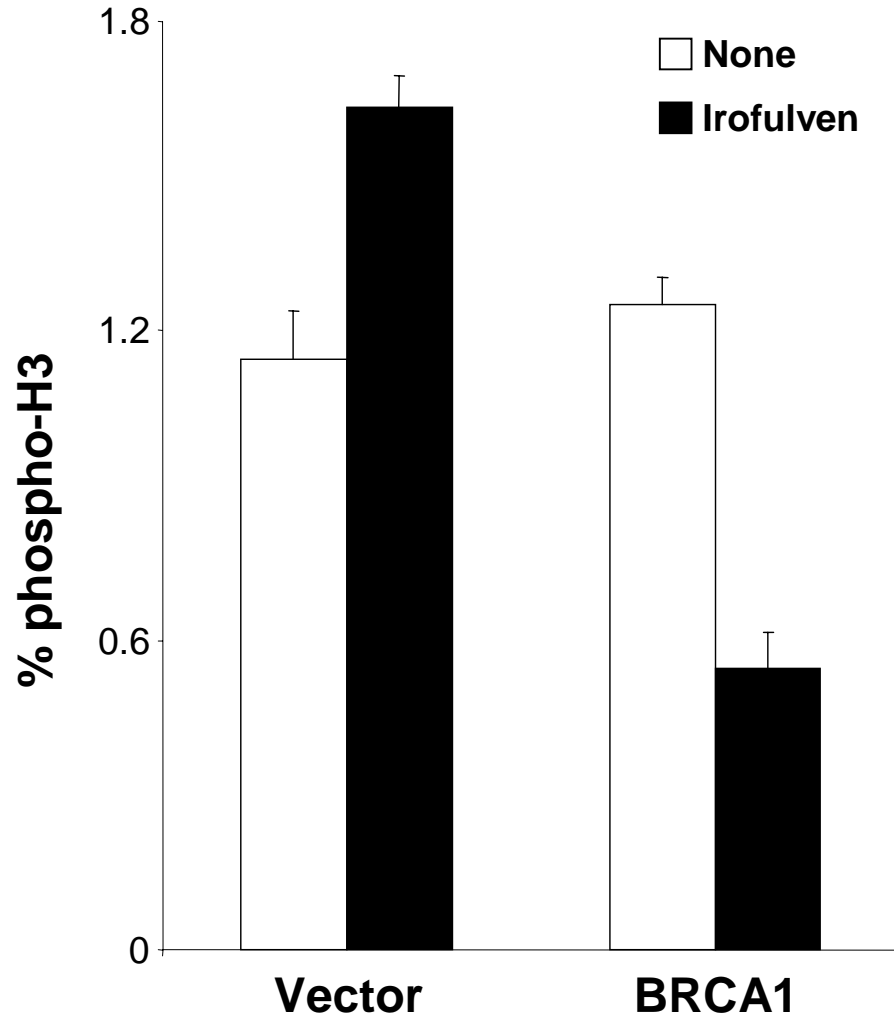


Figure 2C

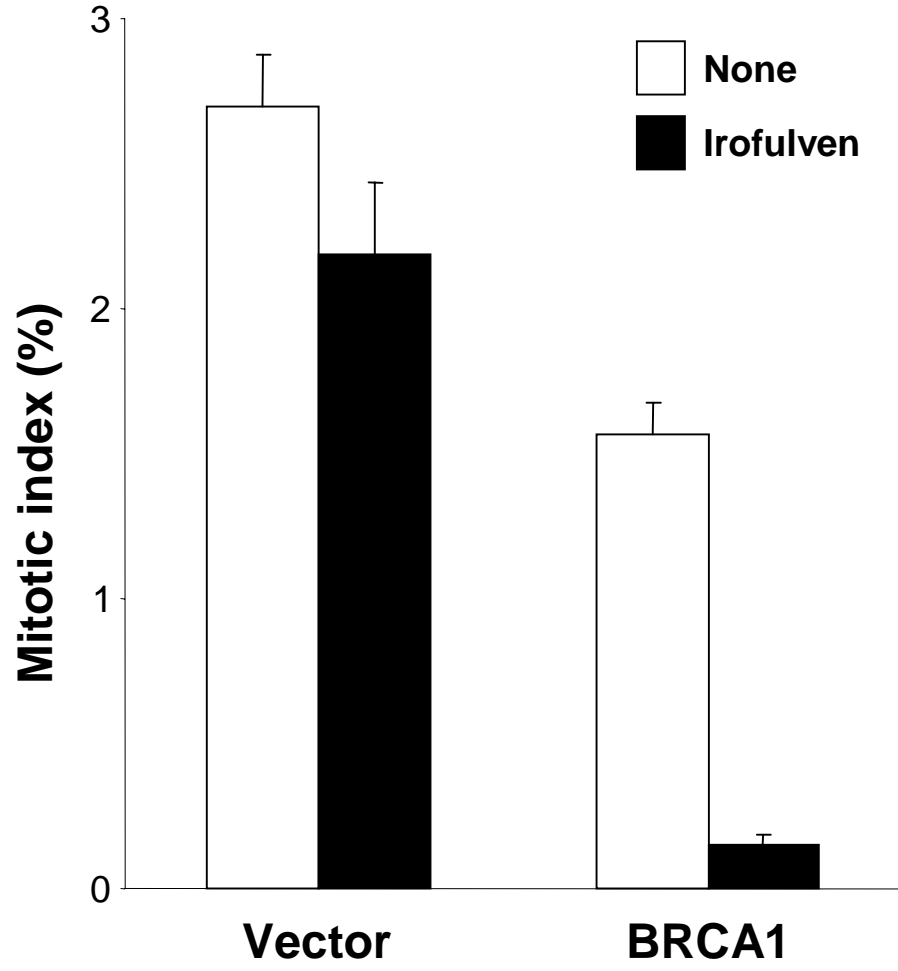


Figure 3A

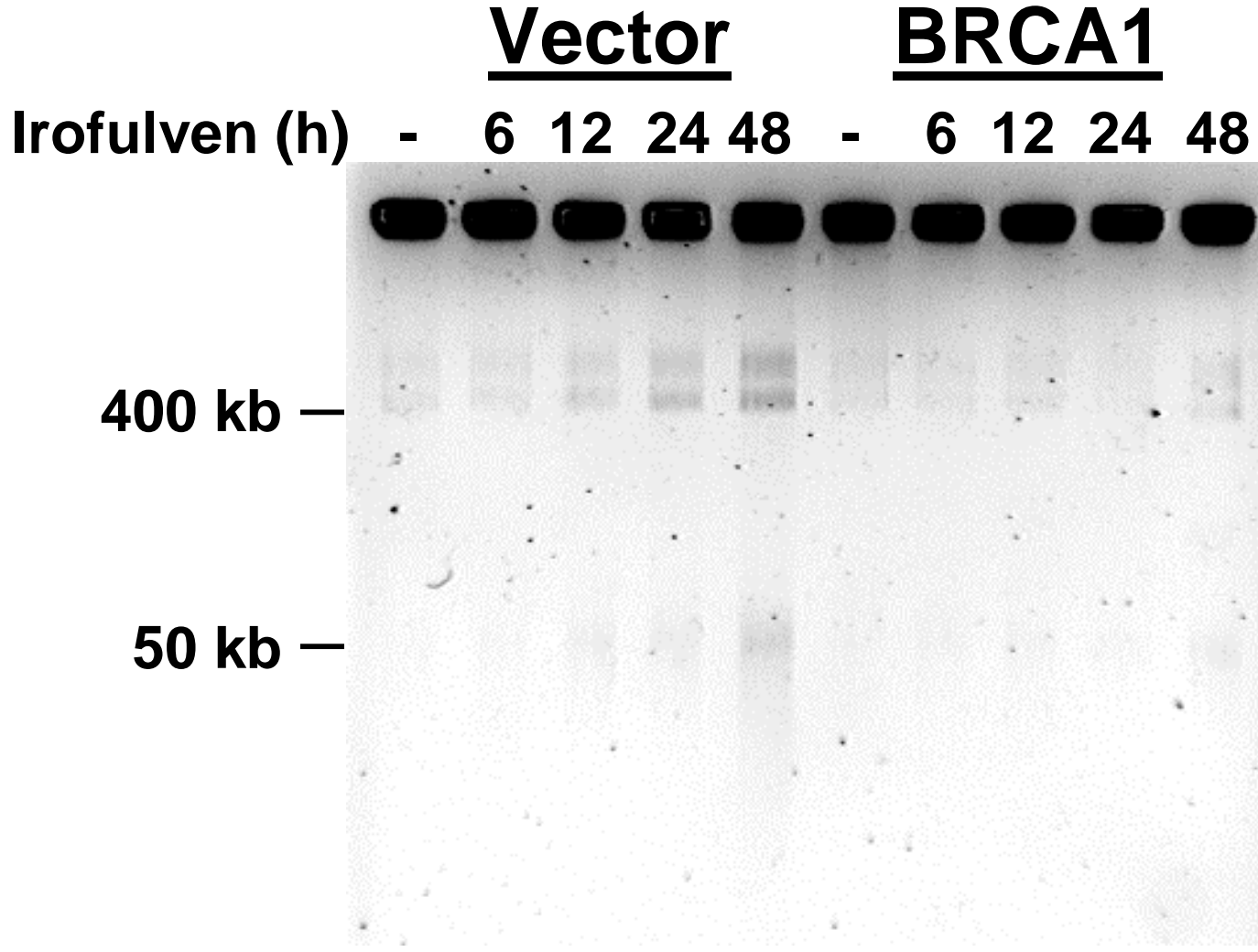


Figure 3B

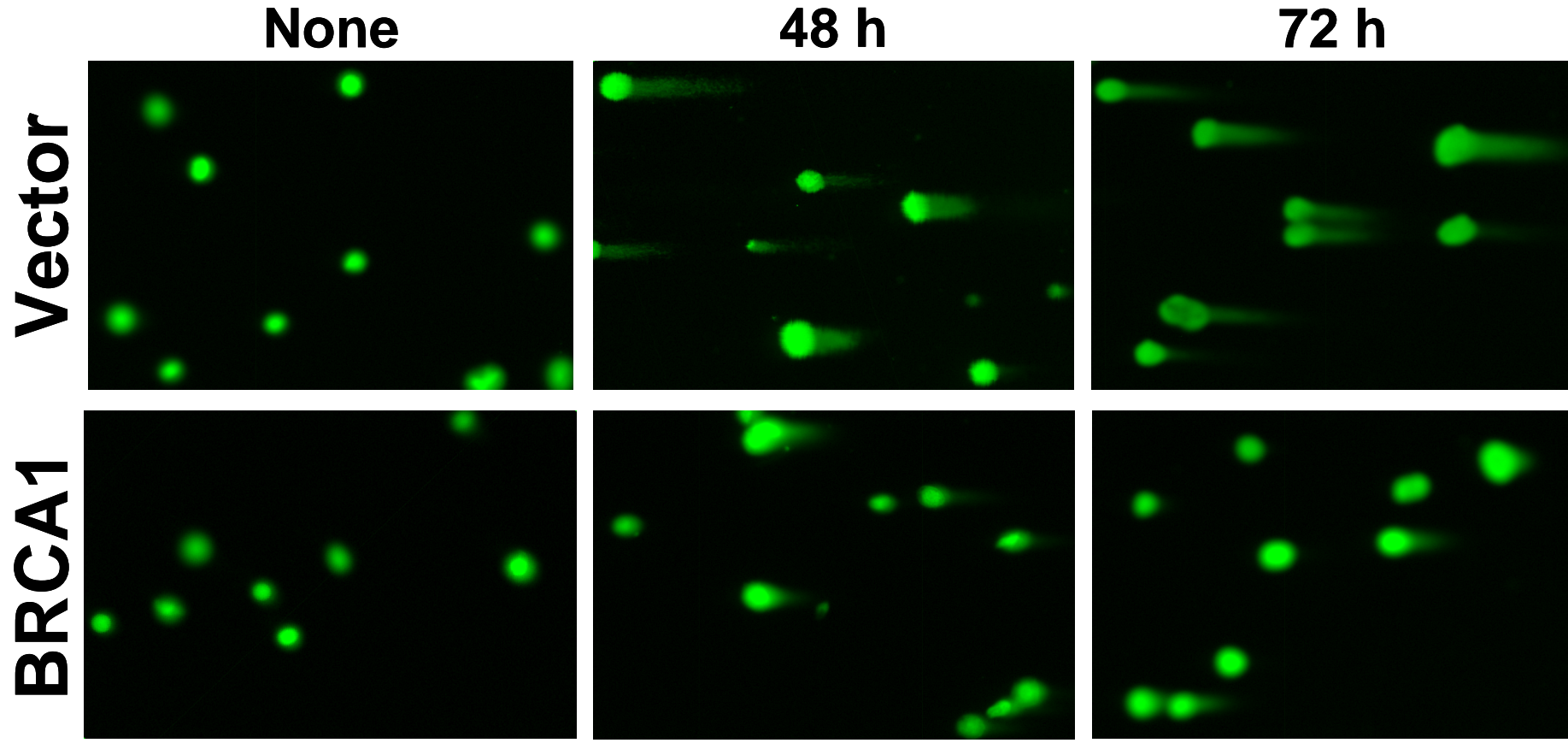


Figure 3C

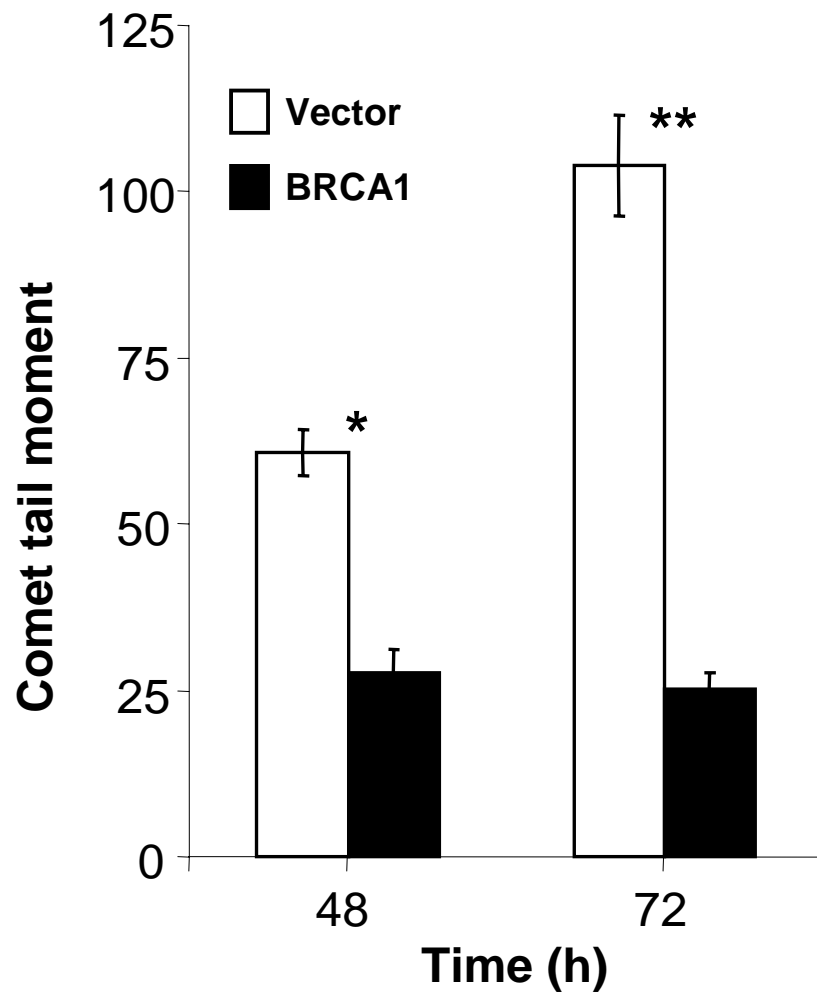


Figure 3D

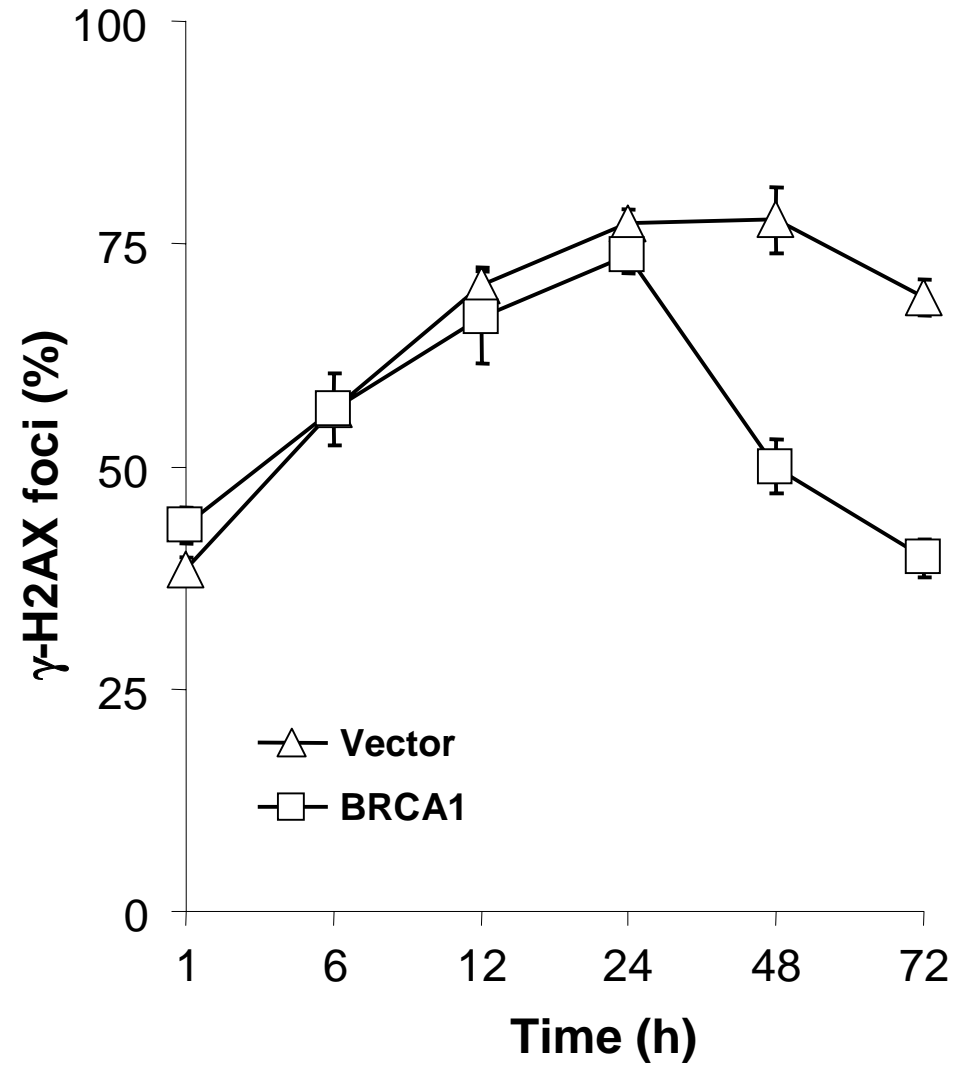
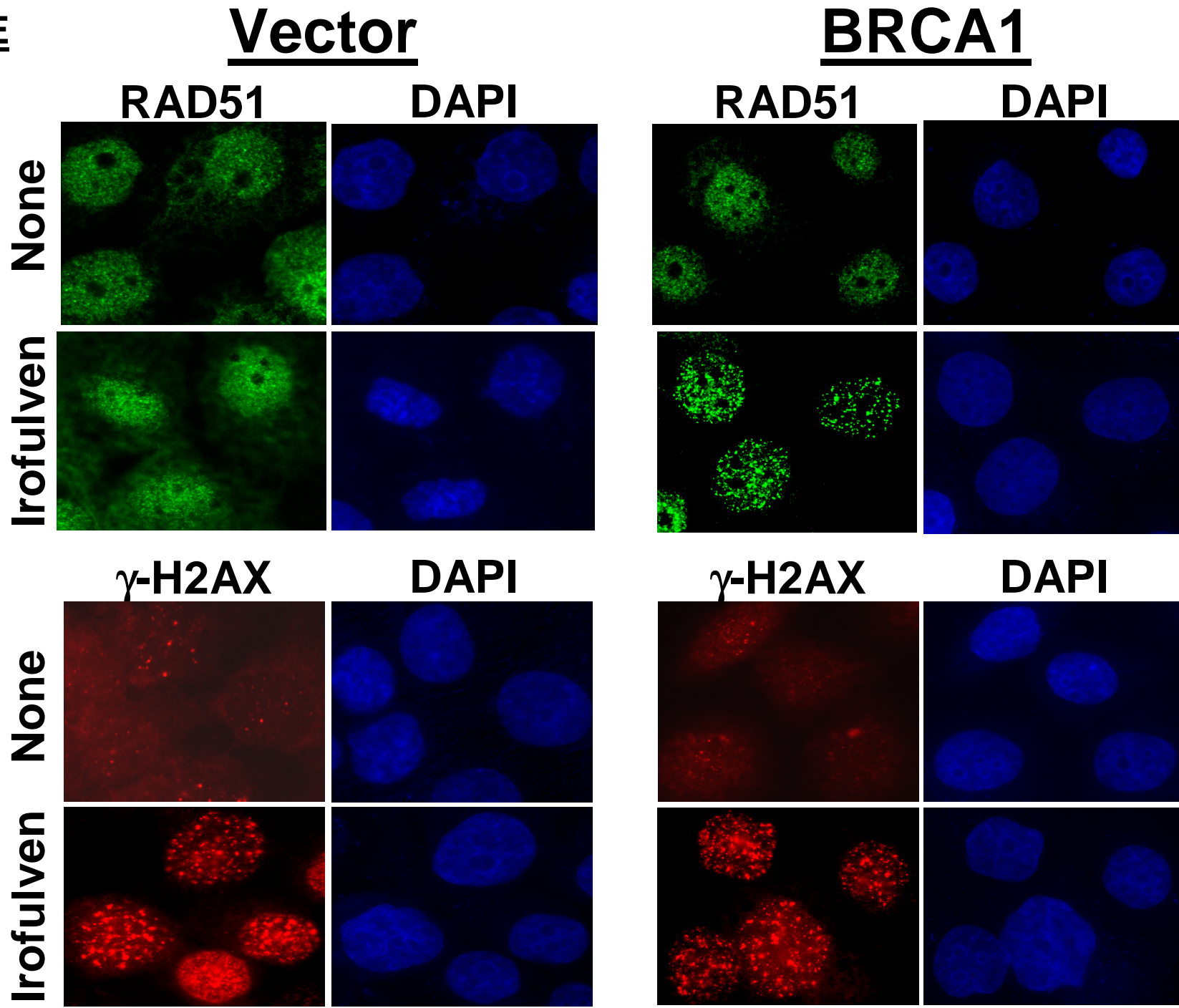


Figure 3E



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This article has not been certified and formatted. The final version may differ from this version.

Figure 3F

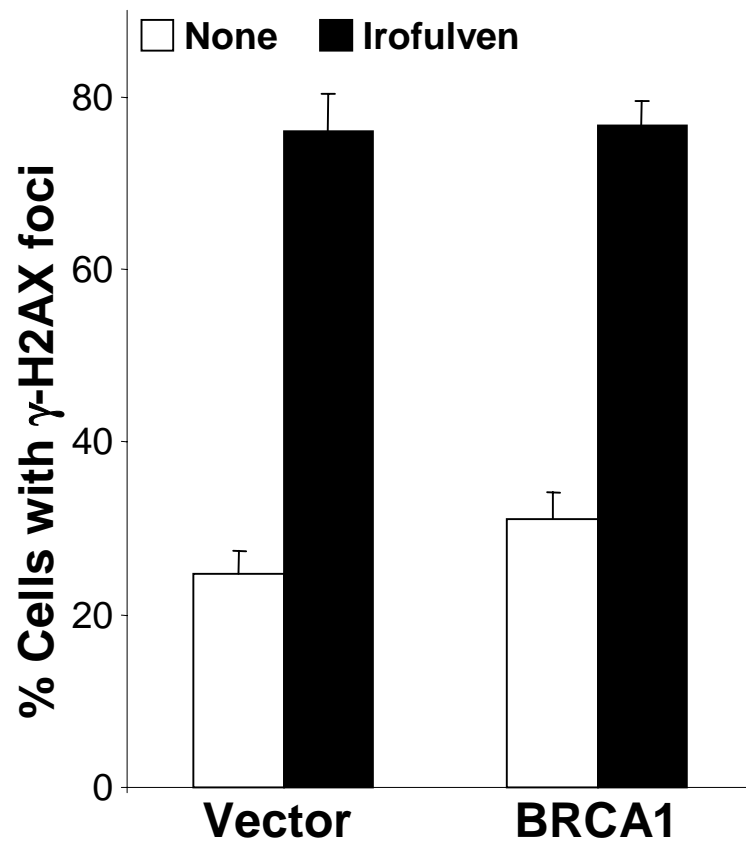
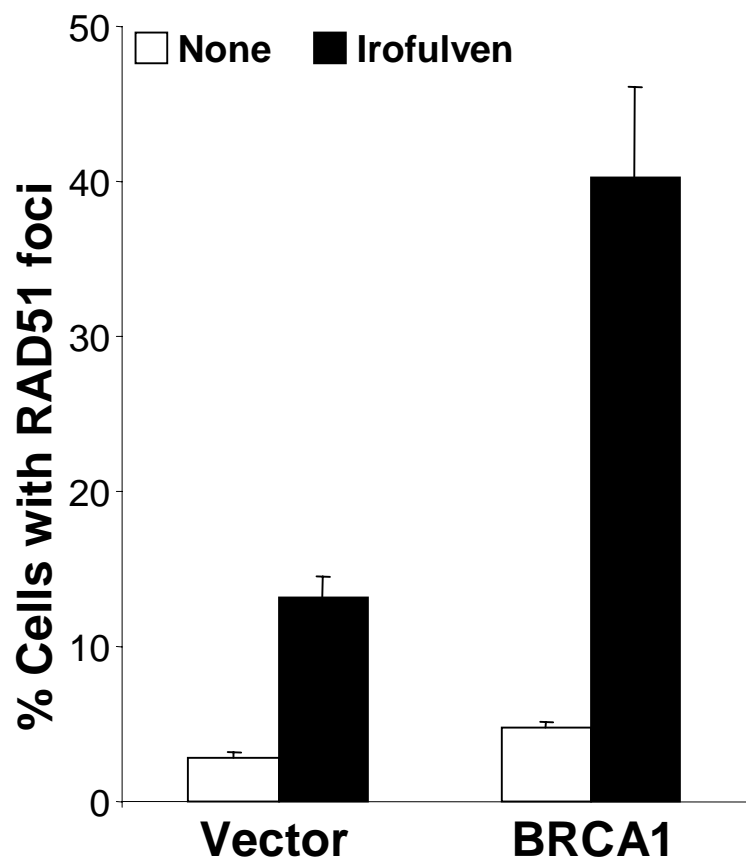


Figure 4A

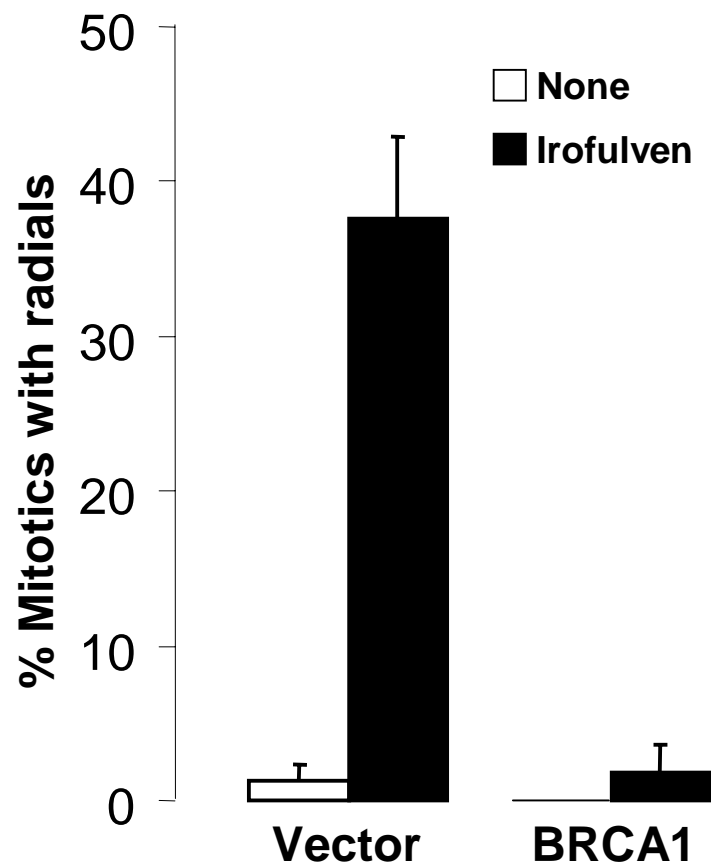
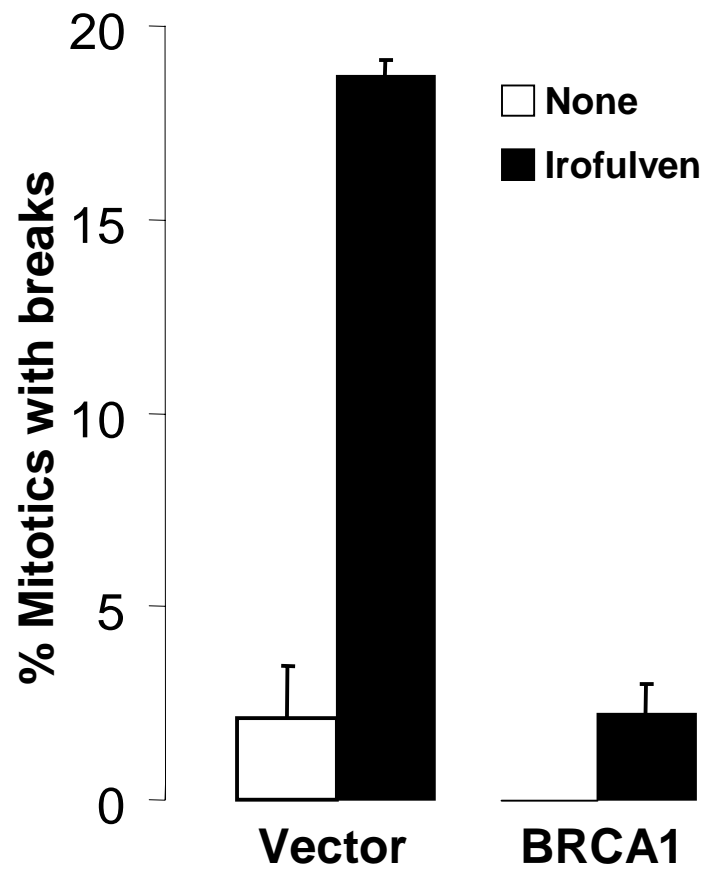


Figure 4B

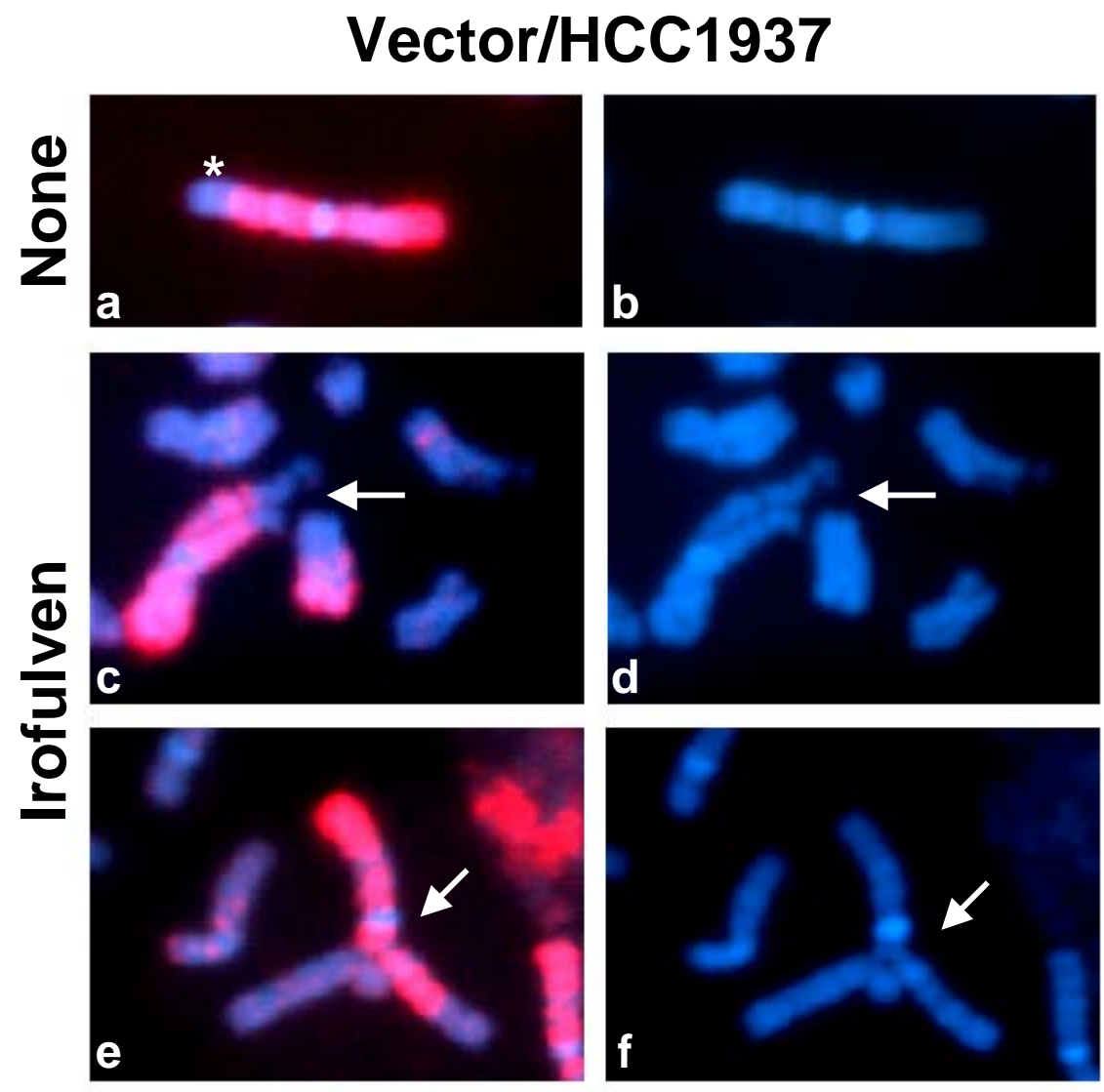


Figure 5A

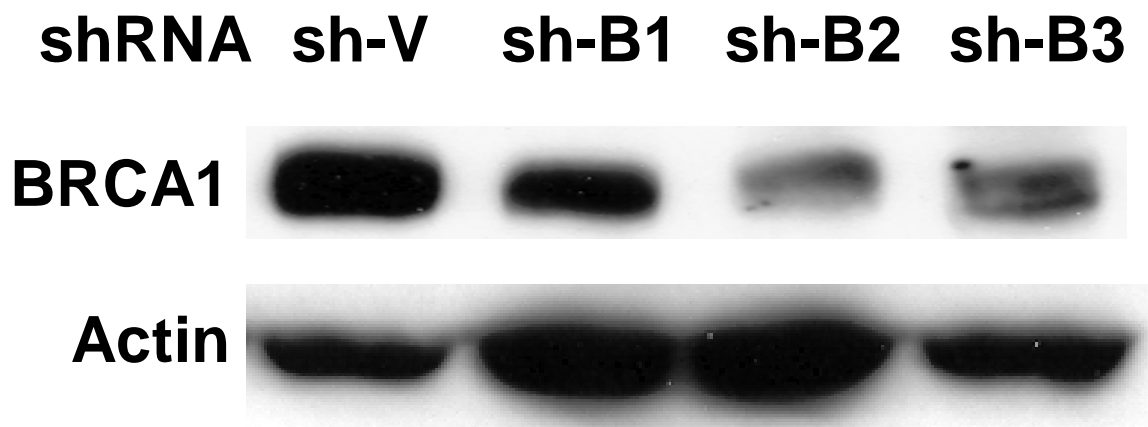
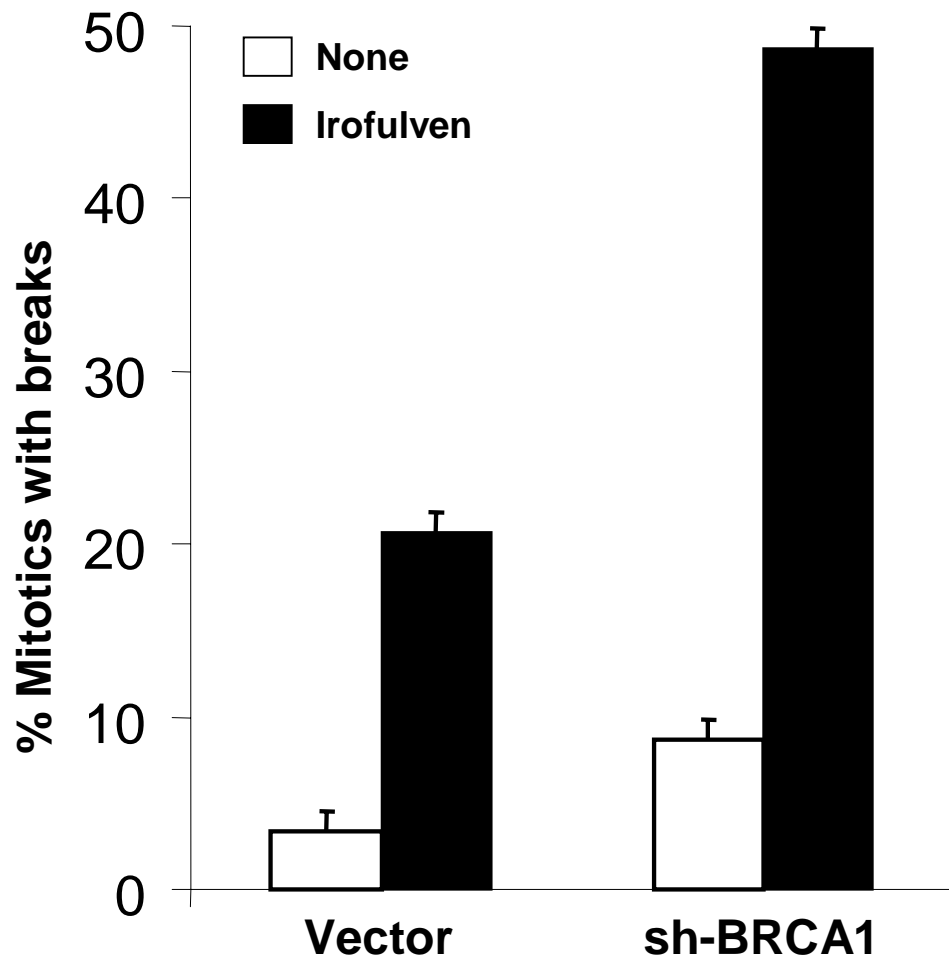
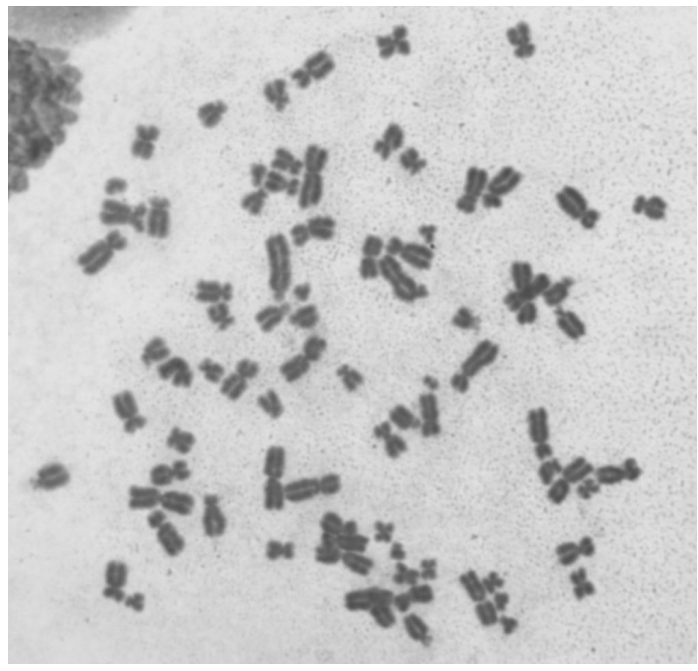


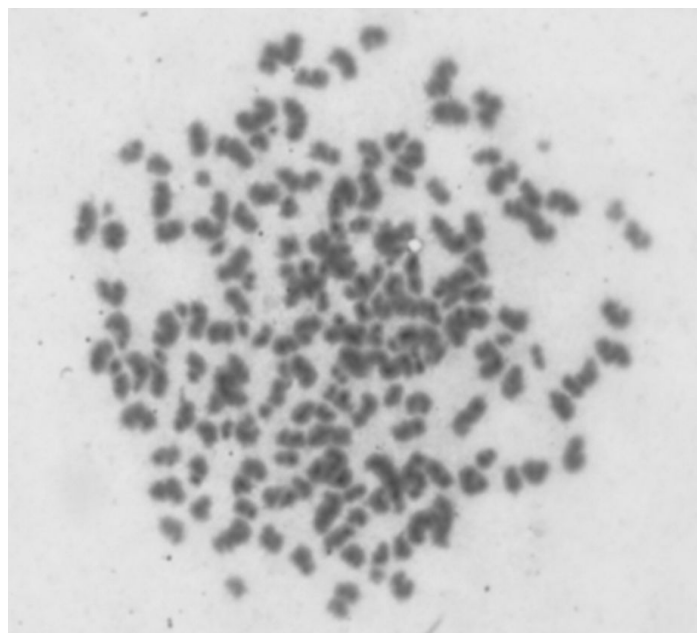
Figure 5B



sh-BRCA1



None



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Figure 5C

Figure 5D

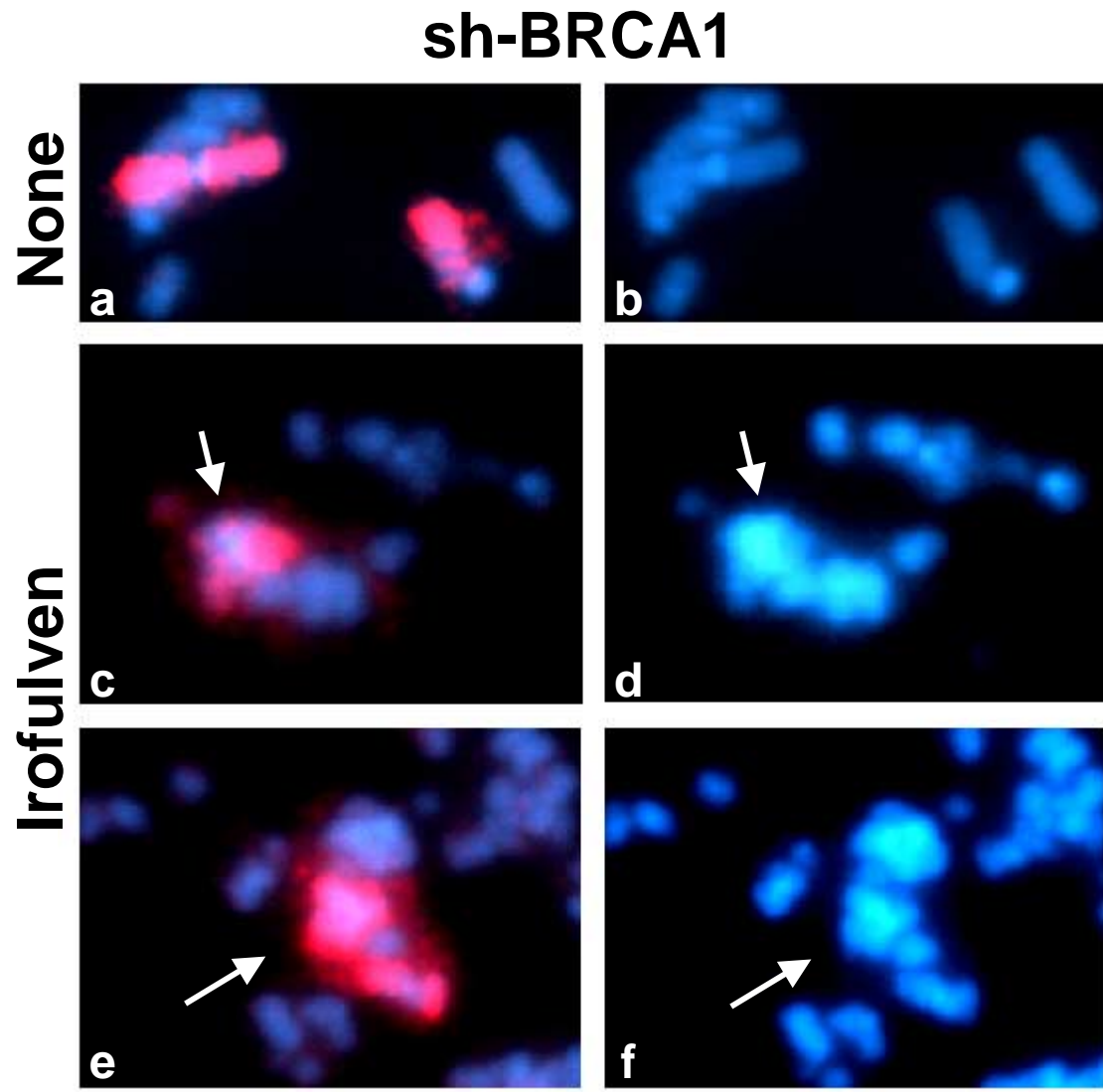


Figure 6A

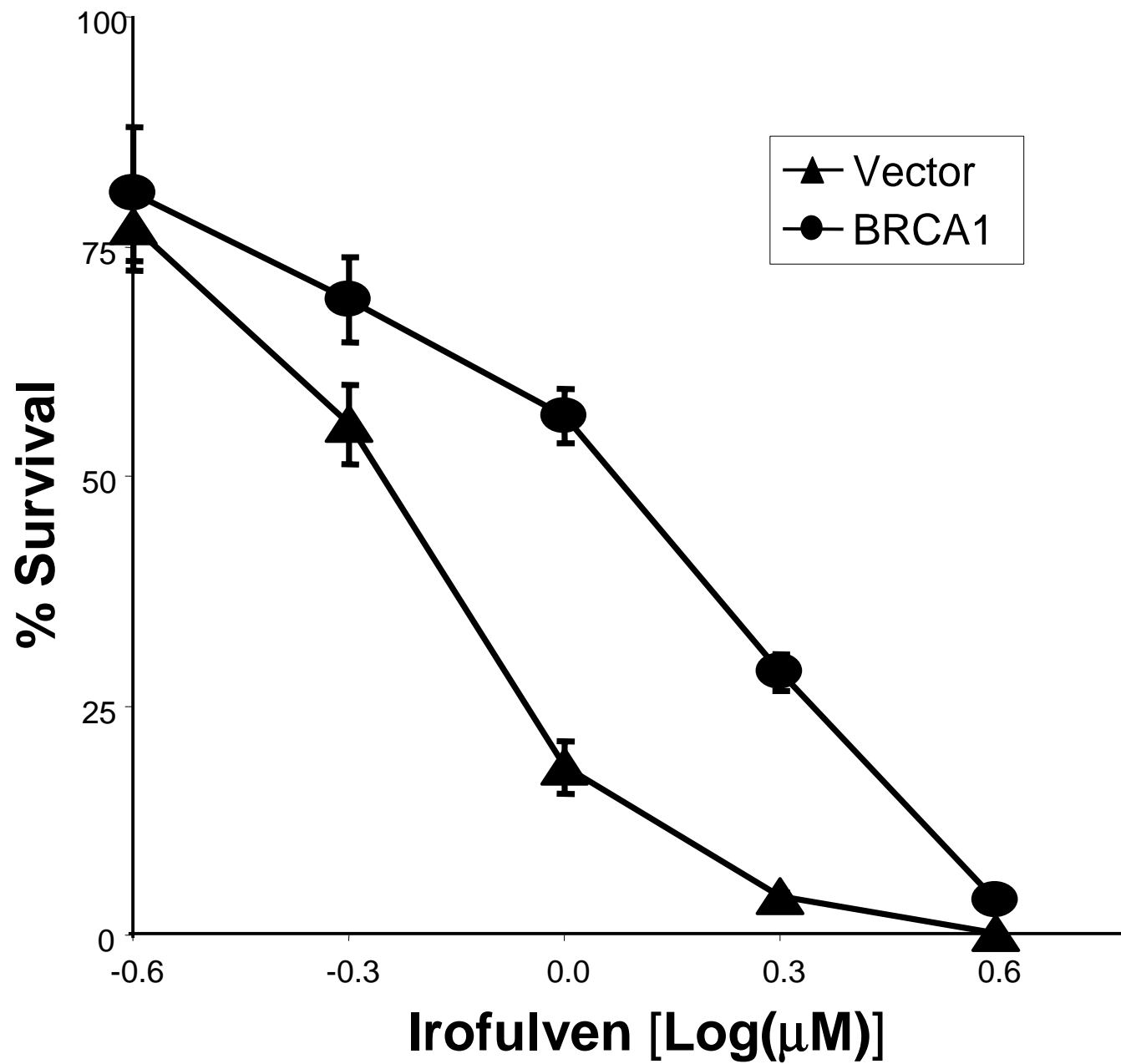


Figure 6B

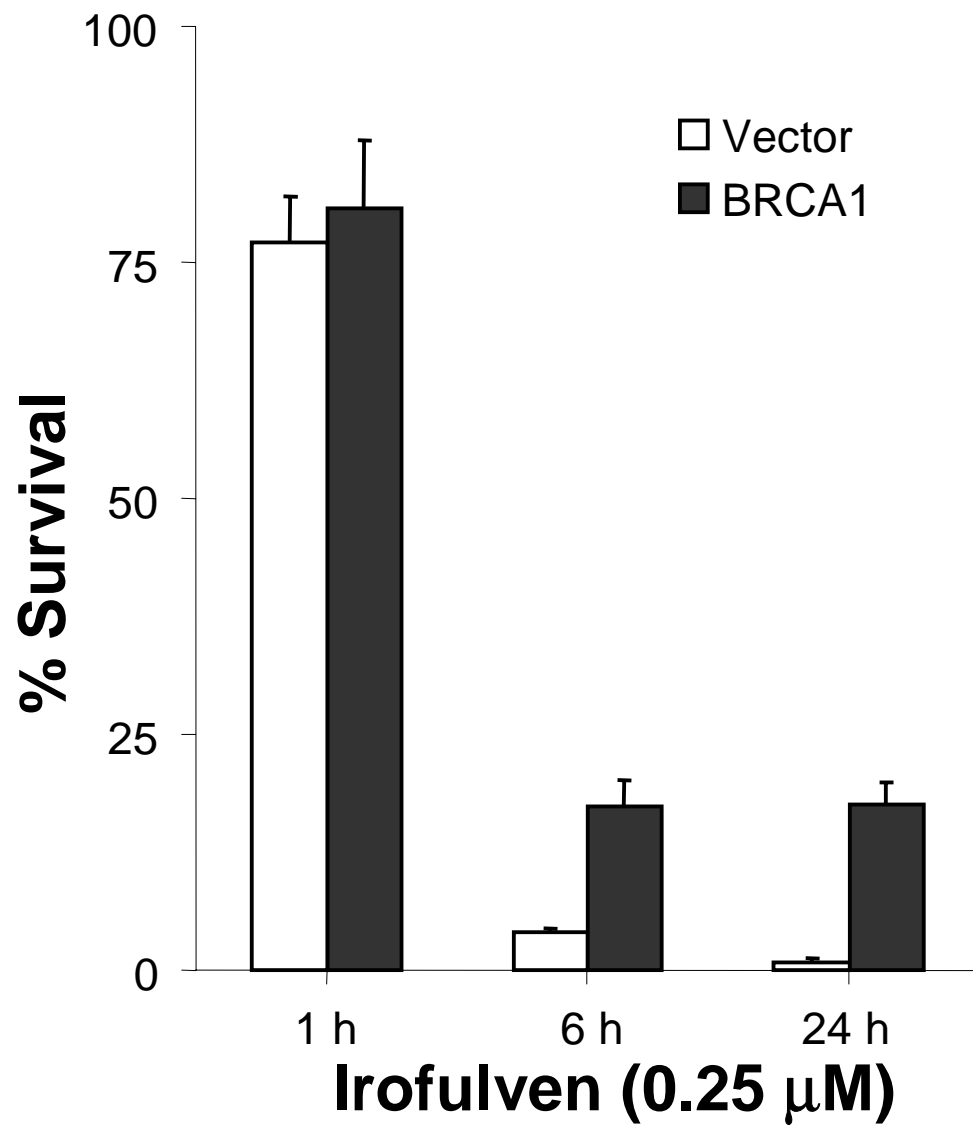


Figure 6C

