BRCA1 Loss Induces GADD153-Mediated Doxorubicin Resistance in Prostate Cancer

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Abstract

BRCA1 plays numerous roles in the regulation of genome integrity and chemoresistance. Although BRCA1 interaction with key proteins involved in DNA repair is well known, its role as a coregulator in the transcriptional response to DNA damage remains poorly understood. In this study, we show that BRCA1 plays a central role in the transcriptional response to genotoxic stress in prostate cancer. BRCA1 expression mediates apoptosis, cell-cycle arrest, and decreased viability in response to doxorubicin treatment. Xenograft studies using human prostate carcinoma PC3 cells show that BRCA1 depletion results in increased tumor growth. A focused survey of BRCA1-regulated genes in prostate carcinoma reveals that multiple regulators of genome stability and cell-cycle control, including *BLM, FEN1, DDB2, H3F3B, BRCA2, CCNB2, MAD2L1,* and *GADD153*, are direct transcriptional targets of BRCA1. Furthermore, we show that BRCA1 targets *GADD153* promoter to increase its transcription in response to DNA damage. Finally, GADD153 depletion significantly abrogates BRCA1 influence on cell-cycle progression and cell death in response to doxorubicin treatment. These findings define a novel transcriptional pathway through which BRCA1 orchestrates cell fate decisions in response to genotoxic insults, and suggest that BRCA1 status should be considered for new chemotherapeutic treatment strategies in prostate cancer.

Introduction

Germline mutations in the tumor suppressor gene *BRCA1* (breast cancer susceptibility gene 1) are responsible for a large percentage of the inherited predisposition to breast and ovarian cancers (1) and are associated with a more aggressive clinical course for prostate cancer (2–4). BRCA1 loss induces genomic instability (5, 6), an almost universal characteristic of solid tumors in humans (7). Cells with dysfunctional BRCA1 show defects in survival and

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proliferation, increased radiosensitivity, chromosomal abnormalities, G_2/M checkpoint loss, and impaired homologous recombination (HR) repair (8).

Defining parameters important for treatment and prognosis are a key objectives in oncologic care, thus an important concern for patients may be how BRCA1 mutation will impacts therapy design and clinical outcome. BRCA1- and p53-deficient mouse mammary tumors are highly sensitive to drugs that induce double-strand breaks, but cannot be eradicated and readily acquire resistance to treatment with docetaxel, doxorubicin, olaparib, or topotecan (9–11). Moreover, though BRCA1-deficient human cancers show a good response to platinum analogues, such as cisplatin, and to PARP inhibitors (12, 13), over time the majority of these cancers become resistant and patients die from refractory diseases. One recent study showed that acquired resistance to platinum analogues or PARP inhibitors in tumors carrying frame-shift BRCA1 mutations came from restored BRCA1 expression and HR function due to secondary intragenic mutations that corrected the open reading frames of mutated BRCA1 (14, 15). Hence, new drugs or drug combinations that selectively kill BRCA1-deficient tumor cells are required to achieve complete eradication of BRCA1-mutated and BRCA1-like cancers. Understanding the mechanisms of resistance may therefore lead to improved treatment.

The mechanism whereby BRCA1 modulates the therapeutic response to DNA-damaging agents is in part mediated by transcriptional regulation. Finding target genes regulated by BRCA1 overexpression in human cell lines might give insight into genetic pathways disrupted in cells that have mutated BRCA1. It had been published that BRCA1 controls the transcription of several genes (16–18) including its own transcription (19, 20). Understanding BRCA1 transcriptional targets and their functions will better explain the diverse role that BRCA1 plays in the control of DNA repair and cell-cycle progression. To accomplish this, an important goal will be to define how many of these BRCA1-controlled genes are targeted directly through gene-specific BRCA1 recruitment.

In this study, we showed that BRCA1 plays a central role in modulating doxorubicin resistance in prostate cancer. We identified several BRCA1 target genes involved in cell-cycle regulation and DNA damage response, and focus on GADD153 as an important mediator of the increased sensitivity to genotoxic stress in BRCA1-expressing cells. Finally, using a prostate cancer xenograft model in *nu/nu* mice, we show that BRCA1 silencing influences prostate tumor growth *in vivo*.

Materials and Methods

Cell culture and treatments

PC3 (ATCC: CRL-1435), LNCaP (ATCC: CRL-1740), 22Rv1 (ATCC: CRL-2505), and C4-2 (derived from LNCaP) cells were grown in RPMI with 10% FBS. PC3 stable cell lines [pcDNA3, pcDNA3 BRCA1, short hairpin RNA (shRNA) scramble, and shRNA BRCA1] were previously described (19). Doxorubicin (Rontag S.A.) was prepared in dimethyl sulfoxide (DMSO). UV treatment was previously described (21).

Plasmids

GADD153 promoter luciferase reporter plasmid has been previously described (22). BRCA1 expression vector (pcDNA3 BRCA1) has also been previously described (23). shRNA scramble control and shRNA BRCA1 were from Upstate. siRNA control and siRNA GADD153 were from Santa Cruz Biotechnology.

Western blot

PC3 cells were exposed to different treatments, lysed, and immunoblotted as previously described (24), using antibodies against GADD153 (R20), BRCA1 (19), p21[WAF1/CIP1] (C-19), cyclin D1 (HD11), cyclin E (HE12), cyclin A (C-19), cyclin B1 (GNS1), Lamin A/C (636), and actin B (I19) proteins from Santa Cruz Biotechnology. Protein quantitation was determined by Image J 1.41 software.

RNA isolation, cDNA synthesis, and quantitative PCR

PC3 cells were exposed to different treatments and total RNA was isolated by using RNeasy MINI kit (Qiagen). cDNA was synthesized from 2 μg of RNA by using RevertAid First Strand (Fermentas). Quantitative PCR (qPCR) was carried out as previously described (19, 25), using Taq Polimerase (Fermentas) in a DNA Engine Opticon (MJ Research; BioRad). Three biological independent replicates were carried out. Figures show media and SD from 3 independent experiments. Primer sequences were as follows: FEN1, 5′-GGCAACCCCGAACCAAGC-3′ and 5′-GCTCATAGAGGCATCAATGGC-3′; GADD153, 5′-AAGGCACTGAGCGTATCATGTTAAA-3′ and 5′-TTTCAGGTGTGGTGATGTATGAAGA-3′; BLM, 5′-GAATGGTTAAGCAGCGATG-3′ and 5′-TCAATACATGGAACTTTCTCAG-3′; H3F3B, 5′-AAAGCCGCCAGGAAAAGC-3′ and 5′-CAGACCCACCAGGTACGC-3′; BRCA2, 5′-AAGCATTGGAGGAATATCGTAGG-3′ and 5′-CAGGTTCAGAATTATAGGGTGGAG-3′; CCNB2, 5′-TTCTGATGCCTTGCTCTGC-3′ and 5′-ATGCGTCCATTTATATCTCTTCC-3′; DDB2, 5′-TCACTTCCAGCACCTCACAC-3′ and 5′-ACGTCGATCGTCCTCAATTC-3′; MAD2L1, 5′-AATACGGACTCACCTTGCTTGTAACTAC-3′ and 5′-TGCCATCTTTCCAGGACCTCACC-3′; ACTB (actin B), 5′-AAGATCATTGCTCCTCCTGAGC-3′ and 5′-CATACTCCTGCTTGCTGAT CCA-3′.

Reporters

PC3 cells were transiently transfected by using lipofectamine 2000 (Invitrogen) with 1 μg of GADD153 luciferase plasmid or cotransfected with 1 μg of pcDNA3. After 24 hours, cells were exposed to genotoxic agents as indicated (50 J/m^2 UV or 1 μmol/L doxorubicin), harvested, and lysed with 40 μL of Steady Glomax Luciferase System (Promega). Luciferase activity was measured in Luminometer (Glomax Multi Detection System; Promega). Data were normalized to total protein.

Chromatin immunoprecipitation

BRCA1 chromatin immunoprecipitation (ChIP) experiments were conducted as previously described (19) from PC3 cells exposed or nonexposed to doxorubicin (1 μmol/L, 24 hours),

using BRCA1 antibody and nonspecific antibody control (GAL4 or IgG). ChIP-DNA was amplified by qPCR by using primers mapping at 1.9 or 0.2 kb upstream or 0.3 kb downstream from GADD153 transcription start site (TSS) or the proximal promoter region for the other targets. Primer sequences were as follows: FEN1, 5′-CCTCTCGCCCTTAGAAATCG-3′ and 5′-TAGACGCTCCTGGAACC TC-3′; BLM, 5′-CATGGAGGCATCTGAGTGTG-3′ and 5′–TCTCTGTTTCACCCGTACCC-3′; H3F3B, 5′-GGGTAAACCTACGAACGCCTA-3′ and 5′-GTCGGAGAAGTGGCCTAAAAC-3′; BRCA2, 5′-CCCGCCCACTACCACTAAG-3′ and 5′-GATGTTCTCTCAAGACCTGTTCC-3′; CCNB2, 5′-AACACACCAGAAGAGGAATAAAGG-3′ and 5′-GGAACTGTCTCATTGGGAAAGC-3′; DDB2, 5′-GAGGCTGAGGTGGGAGGATCGCTTG-3′ and 5′-GGTCTGGCTCTGTCGCTCACTCTGG-3′; MAD2L1, 5′-ATCACAGGAAGGCTGAGAGAAAG-3′ and 5′-CGTCGTCAGGTCCTTTGCG-3′; and GADD153 1.9 kb upstream, 5′-CCAATCCCAGCCCTCCTCAG-3′ and 5′-TCAAGTCCCAGCACACTACCC-3′; 0.2 kb upstream, 5′-CCTCCGTGAAGCCTCGTGAC-3′ and 5′-CTCGCATCCGCCACTCAGG-3′; and 0.3 kb downstream, 5′-TTTCTTCTTGCCTTTAGGG-3′ and 5′-TCTTGCCTCACTGTTACC-3′. Fold enrichment was calculated normalizing data to input and nonspecific binding as previously described (25).

Binding assay

Nuclear extract isolation was done from PC3 cells exposed to doxorubicin as previously described (19). Probe–protein complexes were analyzed by Western blot (WB) by using anti-BRCA1 antibody. GADD153–biotin probe was obtained by PCR by using Taq HotStart (Qiagen). Primer sequences were 5′-biotin-CCACTGAGCCCGGCCAGGAGACCTC-3′ and 5′-biotin-GCTCTGTCGCTGCCACCCGCTCA-3′.

Cell-cycle analysis

PC3 stable cell lines were exposed to doxorubicin, stained with propidium iodide (PI), and analyzed by fluorescence-activated cell sorting (FACS) as previously described (26). Alternatively, cells were transfected with 50 pmol of siRNA GADD153 or siRNA scramble by using lipofectamine 2000 (Invitrogen), and cells were treated with doxorubicin and analyzed by FACS 48 hours posttransfection.

Apoptotic cell measurement by Annexin V–FITC and PI staining

After exposure to doxorubicin, PC3 stable cell lines were stained with Annexin V–FITC/PI and analyzed by FACS as described (26). Alternatively, PC3 pcDNA3 and pcDNA3 BRCA1 were transfected with 50 pmol of siRNA GADD153 by using lipofectamine 2000, treated with doxorubicin, stained, and analyzed by FACS.

Cell viability (MTS)

Cell viability was assayed by MTS (Cell Titer 96 wells Aqueous non-Radioactive Cell Proliferation Assay; Promega) following the manufacture instructions.

Xenografts

Six-week-old male *nu/nu* mice, each weighting at least 20 g were housed under pathogen-free conditions following the University of Buenos Aires animal care guidelines. Mice were randomized into 2 groups: shRNA scramble or shRNA BRCA1. Tumor cells (4.8×10^6 cells) were inoculated s.c. into the right flank of the mice. After 14 days, when the tumors reached approximately 50 mm^3, mice were randomly distributed into 2 groups (5 mice per group) and injected intraperitoneally (i.p.) with doxorubicin (8 mg/kg mouse). A second injection was administered after 9 days and the mice were sacrificed 24 hours after the last injection. RNA was isolated from tumors as described (24). Tumor size was measured 3 times per week by using digital caliper and the volume was calculated by using the formula: $0.523 \times$ width$^2 \times$ length, where width is the smallest side of the tumor.

Statistical analysis

All results are given as mean ± SD of "*n*" separate independent experiments, unless stated otherwise. Student's *t* tests were used to ascertain statistical significance with a threshold of $P < 0.05$. Comparisons for *in vivo* experiments were made with 1-way ANOVA followed by Dunnett's test, with $P < 0.05$ as the criterion for statistical significance.

Results

BRCA1 is required for doxorubicin sensitivity in prostate cancer cells

Despite many studies in diverse cell types, the transcriptional role of BRCA1 in the DNA damage response in human prostatic cancer cell lines has remained relatively unexplored (27). In this study, we first assessed BRCA1 expression levels in different human prostate cancer cell lines: PC3 (androgen insensitive and p53 deficient), LNCaP (androgen sensitive and p53 wild type), 22Rv1 (androgen insensitive and p53 wild type), and C4-2 (androgen insensitive and p53 wild type). LNCaP cells showed the highest levels of BRCA1, whereas the lowest expression was found in PC3 cells (Fig. 1A).

To investigate BRCA1 influence on DNA damage sensitivity in prostate cancer, we next generated stable PC3 cells with both BRCA1-depleted and -overexpressed levels, and LNCaP cells with stable depletion of BRCA1 expression (Fig. 1B). We analyzed viability of these cells after doxorubicin or etoposide treatment as DNA-damaging agents by MTS assay. We found that BRCA1 overexpression in PC3 cells significantly decreased cell viability after doxorubicin treatment (Fig. 1C) showing a cell growth inhibition 50 (GI50) at 2 µmol/L doxorubicin for the pcDNA3 BRCA1 compared with 9 µmol/L for control (PC3 pcDNA3) at 24 hours. In addition, BRCA1 knockdown increased doxorubicin resistance in PC3 (data not shown) and LNCaP cells (Fig. 1C). All cell lines were resistant to etoposide treatment suggesting that the sensitivity to this agent is independent of BRCA1 status (Fig. 1C). On the basis of these results and due to the lack of good chemotherapeutic options for advanced prostate cancer, we explored further the role of BRCA1 in the doxorubicin-induced cytotoxicity in PC3 cells because these cells represent a more aggressive phenotype.

We assessed the effect of BRCA1 modulation over doxorubicin-dependent apoptosis by Annexin V/PI double staining and FACS analysis. Consistent with the reduced viability in

the BRCA1-overexpressing cells, there was a significant increase of early apoptotic cells under doxorubicin treatment (Fig. 1D). Accordingly, BRCA1 silencing markedly decreased the percentage of early apoptotic cells induced by this agent (Fig. 1D). Caspase-3 activity was significantly diminished on BRCA1 silencing PC3 cells after doxorubicin exposure confirming the BRCA1 role on doxorubicin-mediated apoptosis (data not shown).

We also investigated the requirement for BRCA1 in cell-cycle regulation after doxorubicin treatment in PC3 cells. We determined DNA content by PI staining and FACS in BRCA1-overexpressing cells, BRCA1 knockdown cells, and controls, exposed to doxorubicin or vehicle. Doxorubicin treatment increased the percentage of S-phase cells ($P = 0.02$; Fig. 1E) which correlates with an increase of p21$^{\text{WAF1/CIP1}}$ expression (Fig. 1F) suggesting that doxorubicin induces cell-cycle arrest. Notably, BRCA1 overexpression significantly induced G_2/M cells accumulation after doxorubicin treatment ($P = 0.02$; Fig. 1E). In addition, BRCA1 overexpression and doxorubicin exposure clearly induced p21$^{\text{WAF1/CIP1}}$ with a marked diminution of cyclin D1, E, A, and B1 expression (Fig. 1F) indicating a loss of G_1 cells due to an arrest of cells in G_2/M checkpoint. Consistently, BRCA1 silencing abolished the cell-cycle arrest induced by doxorubicin (Fig. 1E).

Altogether, these results show that BRCA1 is required for doxorubicin sensitivity in prostate cancer cells.

BRCA1 binds and regulates several targets involved in cell-cycle and DNA damage response *in vitro* and *in vivo*

To identify new candidates for BRCA1-induced sensitivity in doxorubicin-treated PC3 cells, we selected several BRCA1 targets obtained from a previously reported BRCA1 ChIP-chip experiment (19): *FEN1* (flap structure-specific endonuclease 1), *GADD153* (growth arrest and DNA damage gene 153), *BLM* (Bloom syndrome), *H3F3B* (histone 3 family 3B), *BRCA2*, *CCNB2* (cyclin B2), *DDB2* (DNA damage-binding protein 2), and *MAD2L1* (mitotic arrest-deficient 2 homologue-like 1). These genes were selected because of their role in DNA damage and cell-cycle regulation. We first validated BRCA1 binding to the promoters of these genes by anti-BRCA1 ChIP-qPCR in PC3 cells exposed to doxorubicin or vehicle. As shown in Figure 2, BRCA1 binds to all the selected promoters. On the basis of BRCA1 binding to the promoters, 3 different clusters of genes were identified: cluster 1, genes that showed enrichment for BRCA1 when the cells were exposed to doxorubicin (*FEN1* and *GADD153*); cluster 2, genes that showed enrichment for BRCA1 when the cells were untreated (*BLM, H3F3B, BRCA2,* and *CCNB2*); and cluster 3, genes that showed enrichment for BRCA1 when the cells were exposed or not to doxorubicin (*DDB2* and *MAD2L1*).

To further investigate whether the 8 BRCA1-bound genes were directly regulated by BRCA1, RNA was isolated from doxorubicin exposed pcDNA3 BRCA1 or control stable cell lines, and gene specific expression was analyzed by reverse transcriptase quantitative PCR (RT-qPCR). As shown in Figure 3A, the expression of all 8 genes was modulated by BRCA1. Furthermore, BRCA1 silencing also significantly affected the expression of all these genes (Fig. 3B). All the cluster 1 genes were induced by BRCA1 overexpression and the cluster 2 genes were repressed (Fig. 3A). These findings were validated in BRCA1

knockdown cells (Fig. 3B). In addition, BRCA1 modulation in PC3 cells also modified these genes expression after doxorubicin treatment (Fig. 3). Although cluster 3 expression genes were sensitive to BRCA1 and/or doxorubicin treatment, further studies should be done to understand the mechanism of these genes regulation and to identify other factors involved in this response.

To elucidate whether the 8 BRCA1 targets are regulated by doxorubicin *in vivo*, in this study we injected BRCA1 knockdown PC3 or control cells in athymic *nu/nu* mice. Tumor volume was measured every 2 days during 25 days. After 14 days, mice were randomly distributed into 2 groups (5 mice per group) and injected i.p. with doxorubicin as described in methods. No significant difference was observed in the body weight of mice throughout the experiment (data not shown). Tumor growth was increased (3-fold; $P < 0.05$) in shRNA BRCA1 xenografts compared with controls. BRCA1 knockdown in the xenografts was confirmed after the mice were sacrificed by RT-qPCR (data not shown). In addition, as shown in Figure 4, *GADD153, BLM, BRCA2*, and *DDB2* genes were modulated by BRCA1 and/or doxorubicin treatment in the xenografts at 25 days after inoculation.

In summary, BRCA1 regulates several important targets involved in cell-cycle progression and/or DNA damage response in prostate cancer *in vitro* and *in vivo*.

DNA damage induces GADD153 expression in prostate cancer cells

There is a need to develop new therapies for treating prostate cancer recurrence after the failure of antiandrogen therapy. In this context, to identify new pathways regulated in prostate cancer, it is crucial to test new treatment strategies that target different pathways to improve therapeutic response. In addition, a better understanding of drug resistance mechanisms in the BRCA1 mutated tumors will help to develop more effective treatment options for prostate cancer. In this study, we found that BRCA1 targeted GADD153. This gene is a proapoptotic transcription factor regulated by DNA damage and endoplasmic reticulum (ER) stress, both pathways highly induced by cytotoxic therapies (28–30). Thus, GADD153 is likely to be a major functional target of BRCA1 in the transcriptional response to genotoxic insult.

We first assessed GADD153 regulation after DNA damage. We exposed PC3 cells to genotoxic stress produced by different UV doses and examined GADD153 expression by immunoblotting. As shown in Figure 5A, UV treatment elevated GADD153 protein levels (2-fold). This induction is due to an increase of transcription because both GADD153 mRNA levels (Fig. 5B) and GADD153 promoter transcriptional activity (Fig. 5C) are elevated in response to DNA damage produced by either UV or doxorubicin.

BRCA1 binds and induces *GADD153* transcription after DNA damage

To further investigate the mechanisms of GADD153 regulation by DNA damage we examined BRCA1 binding region within *GADD153* promoter by measuring the enrichment of the BRCA1 ChIP at several positions (primers located at 1,900 or 200 bp upstream, or 300 bp downstream from the TSS of the *GADD153* gene) by real-time PCR. We found that BRCA1 binds to *GADD153* promoter in PC3 cells with higher affinity in the region of 200 bp upstream from the TSS after doxorubicin exposure (Fig. 5D). To further confirm this

interaction, the binding of endogenous BRCA1 to *GADD153* promoter probe was measured *in vitro* by a pull-down assay by using a biotinylated fragment mapping 0.7 kb upstream of the GADD153 TSS. As shown in Figure 5E, BRCA1 binds with higher affinity after doxorubicin treatment to biotinylated *GADD153* promoter sequences and not to the control without probe. Both *in vivo* ChIP and *in vitro* binding assay results indicate that BRCA1 is associated to the *GADD153* proximal promoter region following exposure to genotoxic agents.

To show the direct role of BRCA1 as a regulator of *GADD153* transcription in response to DNA damage, PC3 cells were cotransfected with GADD153 reporter construct containing 1 kb of the *GADD153* upstream regulatory region and BRCA1 wild type expression vector or shRNA BRCA1 silencing vector (Fig. 5F). The transcriptional activity of *GADD153* promoter was again increased after doxorubicin exposure and this activity was significantly induced by BRCA1 overexpression. Accordingly, BRCA1 depletion with shRNA significantly decreased *GADD153* promoter activity in the presence of DNA damage (Fig. 5F).

These data revealed that BRCA1 directly regulates *GADD153* transcription in response to DNA damage.

Doxorubicin sensitivity induced by BRCA1 is mediated by GADD153

GADD153 induction leads to the activation of caspase-3 and the proapoptotic molecules Bax and Bak (31). To further test the linkage between BRCA1 and GADD153 in PC3 cells, we estimated doxorubicin-induced apoptosis and the percentage of cells in the different cell-cycle phases after BRCA1 overexpression and/or GADD153 depletion. Interestingly, we found that the levels of apoptosis following doxorubicin treatment were significantly reduced from 39.1% to 28.2% (25%) after BRCA1 overexpression and GADD153 depletion ($P < 0.05$; Fig. 6B). These findings suggest that BRCA1 regulates apoptosis at least in part through GADD153 transcriptional modulation. GADD153 expression was significantly reduced after siRNA GADD153 transfection (Fig. 6A).

Similarly, cell-cycle analysis showed that GADD153 silencing decreased the percentage of G_2/M blocked cells induced by doxorubicin ($P < 0.05$; Fig. 6C, bars 2 and 4), independent of BRCA1 expression (Fig. 6C, bars 4 and 8). These data reveal that GADD153 depletion significantly abrogates BRCA1 influence on cell-cycle progression in response to doxorubicin treatment.

In summary, BRCA1 plays a central role in the transcriptional pathways mediating PC3 sensitivity to doxorubicin treatment.

Discussion

In this study we showed that BRCA1 induces doxorubicin sensitivity through the transcriptional regulation of several genes. A major pathway in this response is the direct control of GADD153 transcription. We showed that this interaction governs the ability of

BRCA1 to induce GADD153-mediated apoptosis and cell-cycle arrest in response to genotoxic stress.

Previous studies indicate that both DNA damage-induced and growth-related signals enhanced *GADD153* transcription (28). However, the transcriptional and posttranscriptional mechanisms responsible for controlling GADD153 expression in response to DNA damage are poorly understood. Conflicting results on the role of BRCA1 in the control of GADD153 have previously been reported. Although gene-depletion studies have shown that BRCA1 represses GADD153 expression (17, 32), other studies showed that BRCA1 induces GADD153 in response to DNA damage (33). Furthermore, MacLachlan and colleagues showed that GADD153 induction by BRCA1 following DNA damage is cell specific, occurring primarily in cells with mutant p53 (33). These finding are consistent with the data presented here, as PC3 cells have mutant p53. Notably, we found that GADD153 regulation by BRCA1 is diminished in LNCaP, a prostate cancer cell line that expresses wild-type p53. In addition, restored expression of p53 in PC3 cells abolishes the induction of GADD153 by BRCA1 under genotoxic stress (unpublished findings not shown).

Several studies indicate that GADD153 expression is induced in response to a variety of cellular stresses. It was reported that a balance between the expressions of GADD153 versus GRP78 determines the cell fate decisions in response to the unfolded protein response (UPR); with the former protein favoring apoptosis and the later favoring survival (34). Recently, BRCA1 was shown to play a regulatory role in the UPR through its ability to represses expression of GRP78 tipping the scale toward apoptosis (32). Though inhibition of BRCA1 expression resulted in both elevated levels of GRP78 and GADD153, it is tempting to speculate that this level of titration will also be cell specific and dependent on the cellular p53 status. Future studies will have to focus on how BRCA1 may influence the direct transcriptional regulation of *GADD153* following UPR activation and how p53 status is involved in this regulation.

Recently, Wu and colleagues (35) tested the hypothesis that proapoptotic ER stress response could be exploited as a mechanism to sensitize cancer cells to chemotherapeutic drugs. They showed that ER stress inducers, such as thapsigargin and methylseleninic acid (MSA) increased the cell killing potency of paclitaxel or docetaxel. Furthermore, knocking down GADD153 by siRNA also reduced the cell killing effect of MSA triggering apoptosis. Our study may support the hypothesis that ER stress apoptotic response contributes to chemotherapeutic sensitization as an alternative for cancer therapy.

Severe ER stress can cause cell death, usually by activating intrinsic apoptosis (36). Moreover, the UPR may upregulate the autophagy machinery (37). Although autophagy is mainly activated to protect cells against death (38), in the case of the UPR, stimulation of autophagy can be required to activate the cell death machinery (39). Certain promising anticancer regimens have been shown to activate concomitantly ER stress and autophagy in cancer cells (40). In addition, genetic studies have shown that GADD153 loss of function results in cytoprotection, whereas GADD153 gain of function enhances sensitivity to a variety of stresses (41, 42). We propose here that when the tumor cells are exposed to doxorubicin, BRCA1 induces apoptosis and cell-cycle arrest mainly through

GADD153 regulation. However, as shown, the effects of doxorubicin on cell viability are much greater than the effects on apoptosis or cell cycle. These findings and the mentioned previous reports suggest that it is plausible that other additional effects are involved as autophagy activation. Further studies are needed to understand the precise molecular mechanisms that regulate the extent of autophagy activation in response to doxorubicin in connection with BRCA1/GADD153 pathway.

Mutations in BRCA1 have been associated with increased risk of breast, ovarian, and more recently, with high grade prostate cancer (3, 4). However, whereas mutations in BRCA1 may influence familial prostate cancer progression, few studies have examined BRCA1 protein expression in prostate cancer tumor tissue. Fiorentino and colleagues (43) had addressed this important point and they found that human prostate tumor samples stained positive for BRCA1 had significantly higher Gleason score, PSA at diagnosis, and tumor proliferation as well as significantly worse prognosis than those with negative BRCA1 staining. Additionally, our results showing that BRCA1 loss of function is also involved in the differential response to DNA damage agents can also explain why the evolution of the disease could be favored or not by the presence of BRCA1 mutation.

Preclinical and clinical data indicate that BRCA1 can modulate the response to chemotherapeutic agents. More specifically, loss of BRCA1 function leads to increased sensitivity of tumor cells to DNA-damaging chemotherapeutic agents such as cisplatin, bleomycin, and etoposide (44–48). However, other studies show that BRCA1 defective cells are resistant to doxorubicin (49). Our findings extend these finding by showing BRCA1 transcriptional role in doxorubicin sensitivity showing for the first time several BRCA1 targets involved in this response.

Therapeutic agents commonly used in breast and ovarian cancer treatments can damage DNA through various mechanisms. There is a group of agents, including etoposide, mitoxantrone, irinotecan, and topotecan, which inhibits topoisomerases I and II and introduce temporary breaks in the DNA strands. Inhibition of topoisomerases stabilizes the topoisomerase–DNA complex and causes the arrest of DNA replication forks and double-strand DNA breaks (50). This group of agents also includes anthracyclines, such as doxorubicin and epirubicin, that inhibit topoisomerase II, but these drugs have additional mechanisms of action, such as DNA interstrand cross-linking and generation of oxygen free radicals (50). In this context, prostate cancer is a heterogeneous disease with a variable natural history that is not accurately predicted by currently used prognostic tools. Here, we suggest that BRCA1-associated prostate cancer affect chemotherapy response, establishing a new subset of patients that may require alternative therapies. There has been little progress in the systemic management of prostate cancer beyond androgen deprivation therapy, and a novel therapeutic approach is urgently needed. Thus, BRCA1 mutations might confer a more aggressive clinical course for men with localized prostate cancer and personalized interventions may be warranted. In addition, due to p53 gene mutation is a late event in prostate cancer progression and is associated with advanced stage, loss of differentiation, and the transition from androgen-dependent to androgen-independent growth (51), testing patients for p53/BRCA1 genes mutations may provide useful prognostic information and could influence the recommended course of treatment.

In this study we found that BRCA1 directly modulates several important targets in DNA damage response and cell-cycle regulation processes, such as *FEN1, GADD153, BLM, H3F3B, BRCA2, CCNB2, DDB2*, and *MAD2L1*. Specifically, *BLM* and *BRCA2* were significantly repressed by BRCA1 *in vivo*. It was reported that mutations in the *BLM* gene are responsible for Bloom syndrome, an autosomal recessive disorder characterized by chromosomal instability; and *BLM* is also implicated in gastrointestinal tumorigenesis (52). BRCA2 is induced by regulatory pathways involved in the control of cell proliferation and differentiation (53). Mutations in the DNA damage-binding protein 2 (*DDB2*) gene give rise to xeroderma pigmentosum group E, a disease characterized by increased skin tumorigenesis in response to UV irradiation. We found that doxorubicin induces *DDB2* expression *in vivo*, and BRCA1 silencing abolishes that regulation. Similar results were observed for GADD153, and because of its role in cell-cycle regulation we focus on that gene to understand the BRCA1 role in DNA damage response regulation. Further studies will be directed at to deciphering the how integrated regulation of these genes by BRCA1 impacts cellular function under diverse conditions.

In summary, breast and ovarian cancer risk is higher if BRCA1 is mutated. Because most chemotherapeutic agents function by damaging DNA, the role of BRCA1 as a regulator of chemotherapy-induced DNA damage has been the subject of several investigations. This work established for the first time that BRCA1 status plays a central role in doxorubicin resistance in prostate cancer. We found that BRCA1 directly modulates several targets in DNA damage response and cell-cycle regulation processes *in vitro* and *in vivo*, and focused on GADD153 as an important mediator in this response. Because BRCA1 mutations confer a more aggressive clinical course for men with prostate cancer, and p53 gene mutation is associated with advanced stage of this disease, testing patients for p53/BRCA1 gene mutations may influence the recommended course of treatment. Hence, the linkage between BRCA1/GADD153 and p53 arises as an attractive target for the development of new chemotherapeutic strategies for advance prostate cancer.
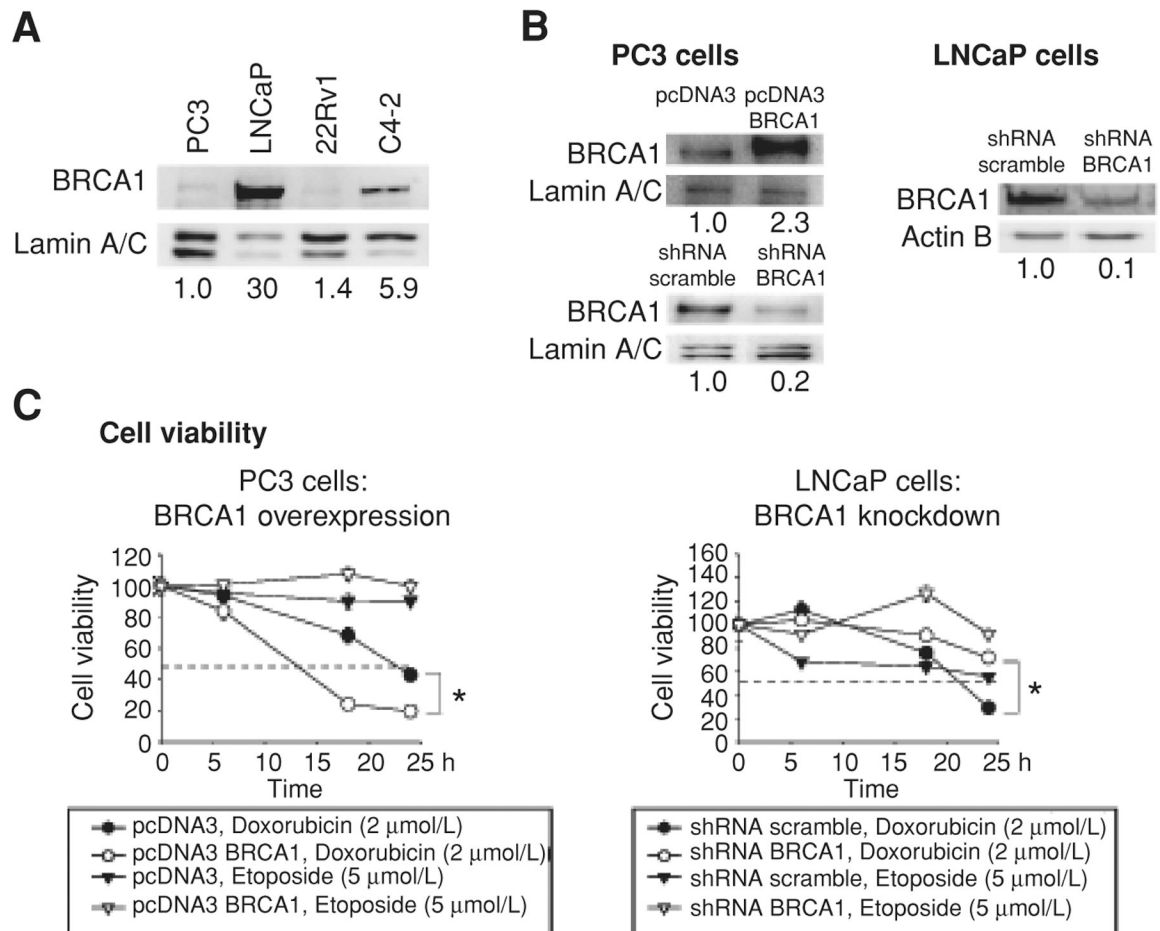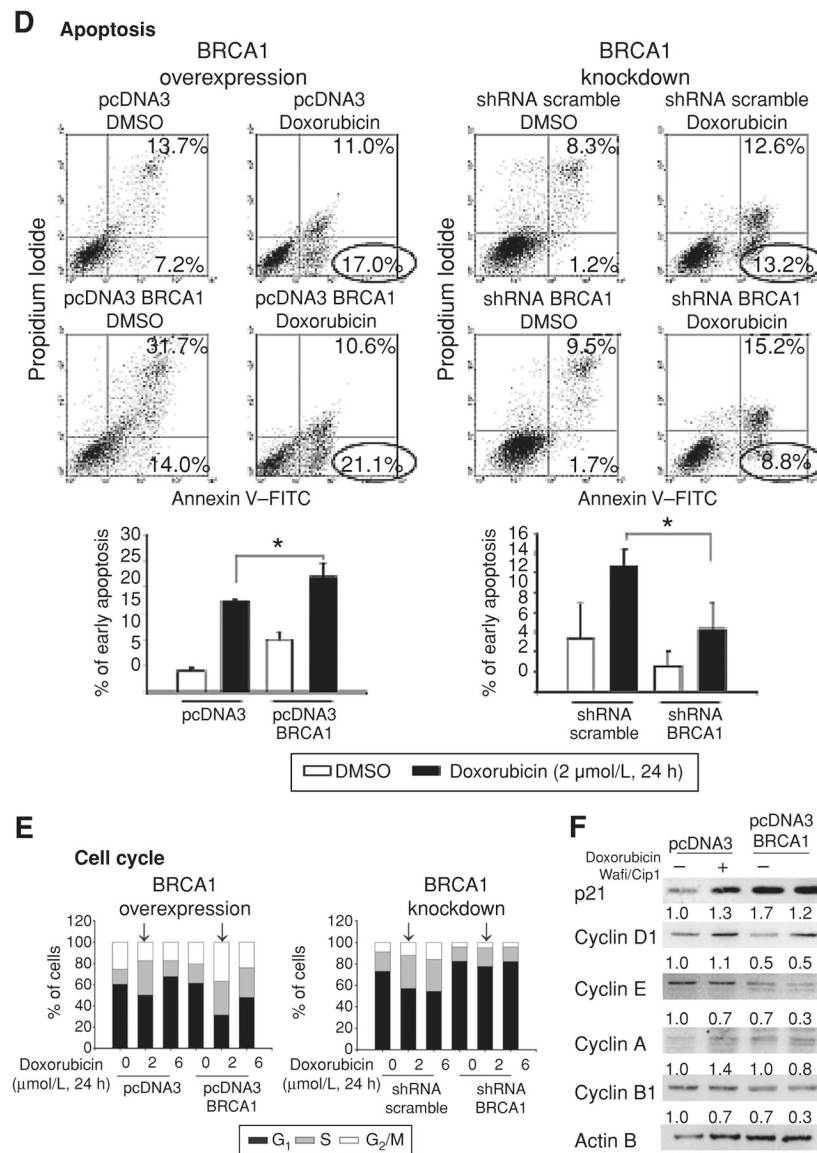
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A

B

PC3 cells

LNCaP cells

C

Cell viability

PC3 cells:
BRCA1 overexpression

LNCaP cells:
BRCA1 knockdown

Figure 1.
BRCA1 increases doxorubicin sensitivity in prostate cancer. A, BRCA1 expression was determined by WB in human prostate cancer cell lines. B, PC3 (pcDNA3, pcDNA3 BRCA1, shRNA scramble, and shRNA BRCA1) and LNCaP (shRNA scramble and shRNA BRCA1) stable cell lines were generated and BRCA1 expression was determined by WB. C, cells were exposed to doxorubicin or etoposide and viability was determined by MTS. Each sample was assayed in triplicate in 2 biological independent experiments. D, cells were stained with Annexin V–FITC and PI and analyzed by FACS after doxorubicin treatment (2 μmol/L, 24 hours). The average from 3 biological independent experiments (bottom) or 1 representative experiment (top) is shown. E, cells were treated with doxorubicin, fixed, and stained with PI. DNA content was determined by FACS. Histogram shows the percentage of cells in G_1, S, and G_2/M phases. The figure depicts the result of 3 biological independent experiments. F, cells were exposed to doxorubicin and WB analysis was carried out by using
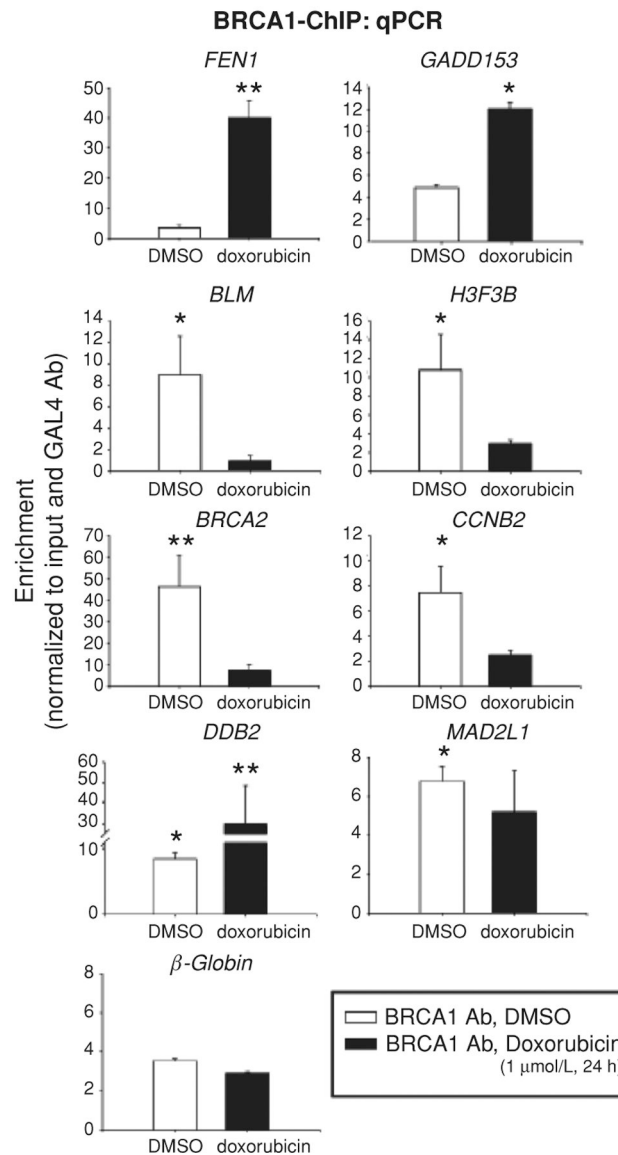
anti-p21$^{\text{Waf1/Cip1}}$, -lamin A/C, or -cyclin D1, E, A, and B1 antibodies. In the Western blots, the numbers under the bands indicate BRCA1 quantitation normalized to actin B and PC3 cells (A) or control (B, F).*, $P < 0.05$.

Figure 2.
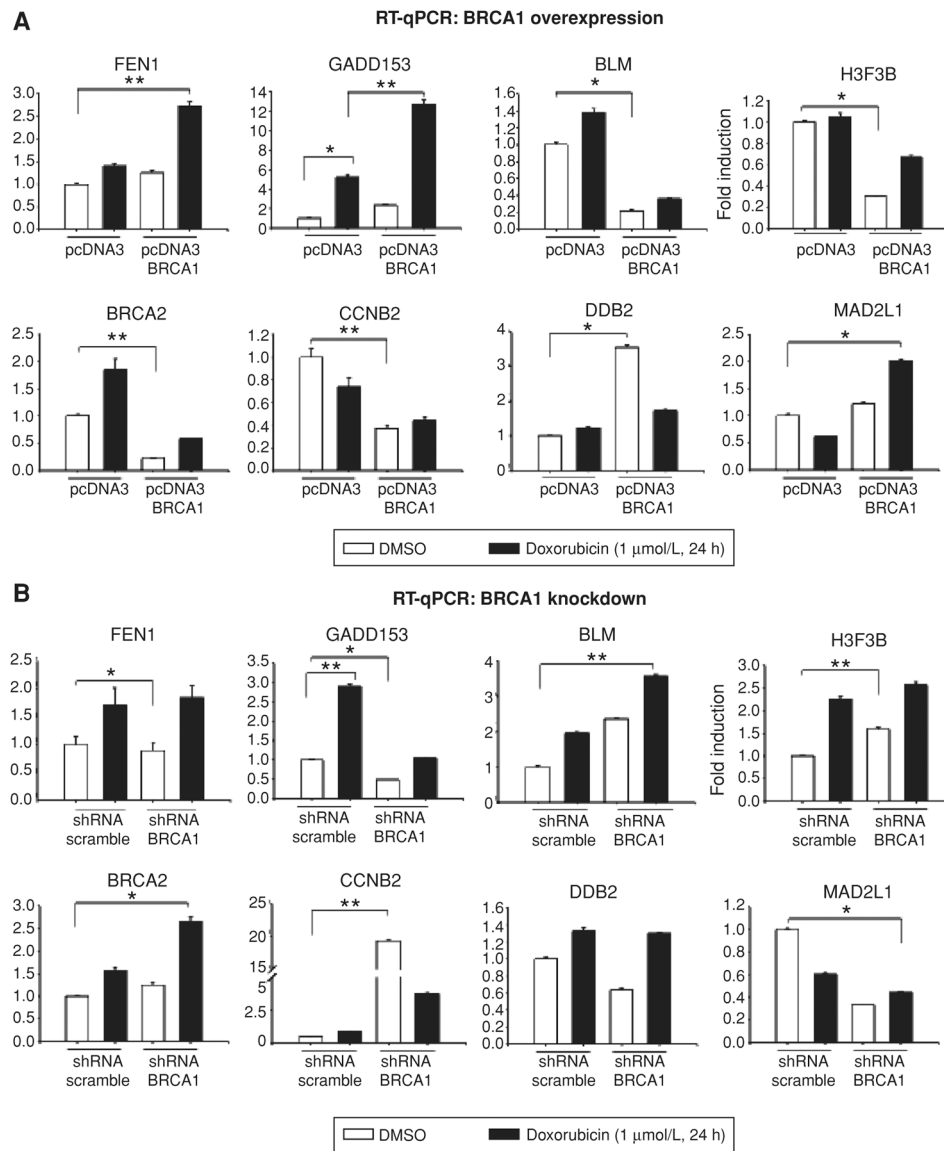BRCA1 protein binds several genes involved in cell-cycle and DNA damage response.
BRCA1-ChIP was conducted from PC3 cells exposed to doxorubicin. DNA-ChIP was
analyzed by qPCR by using primers located at the proximal promoter region of *BLM,
FEN1, DDB2, H3F3B, BRCA2, CCNB2, GADD153*, and *MAD2L1* genes or β-*Globin* as
negative control. Fold enrichment was calculated normalizing data to input and GAL4
antibody. *, $P < 0.05$; **, $P < 0.01$.
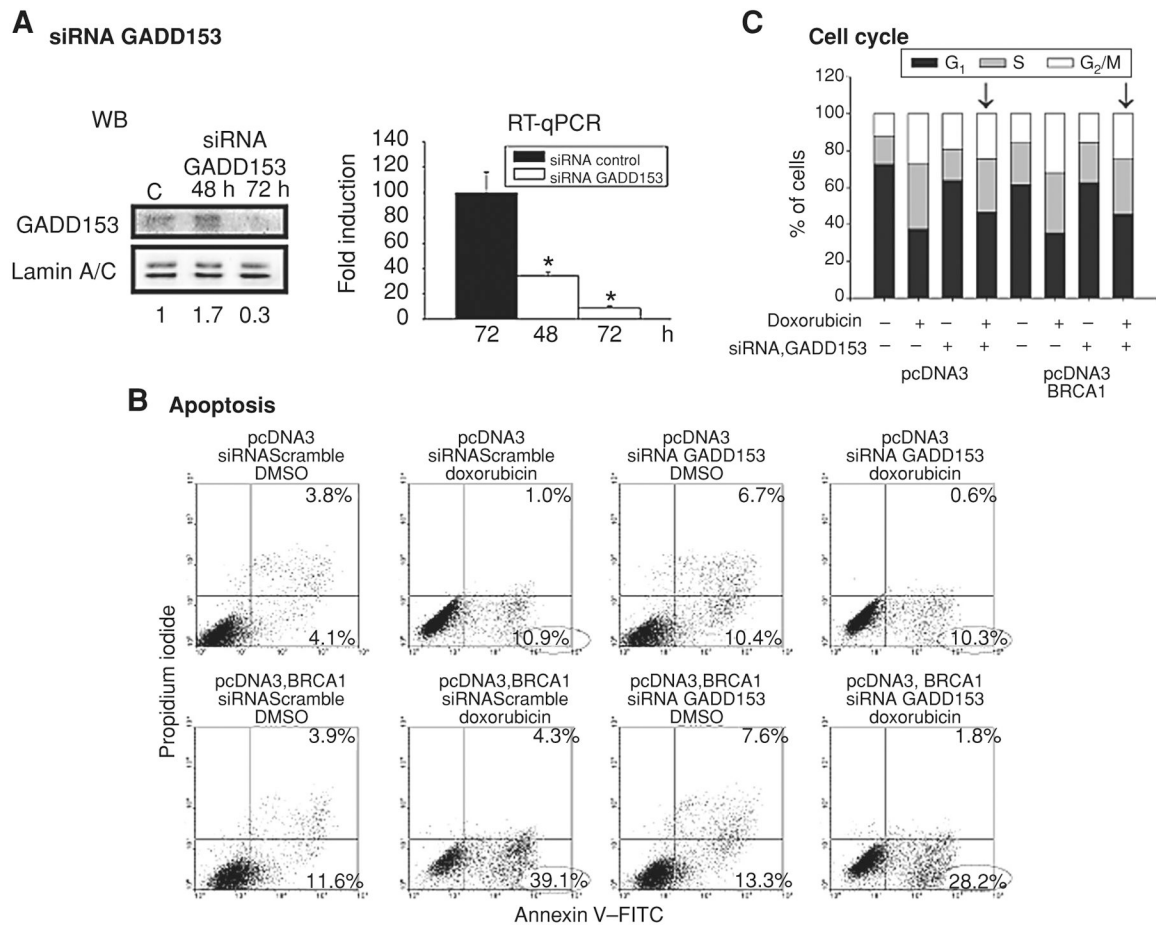
Figure 3.
BRCA1 protein regulates several genes involved in cell-cycle and DNA damage response. pcDNA3 and pcDNA3 BRCA1 (A) or shRNA scramble and shRNA BRCA1 (B) stable cell lines were exposed to doxorubicin (1 μmol/L; 24 hours). mRNA expression levels for the indicated genes were analyzed by RT-qPCR. Data were normalized to actin B. Media and SD from 3 biological independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$.
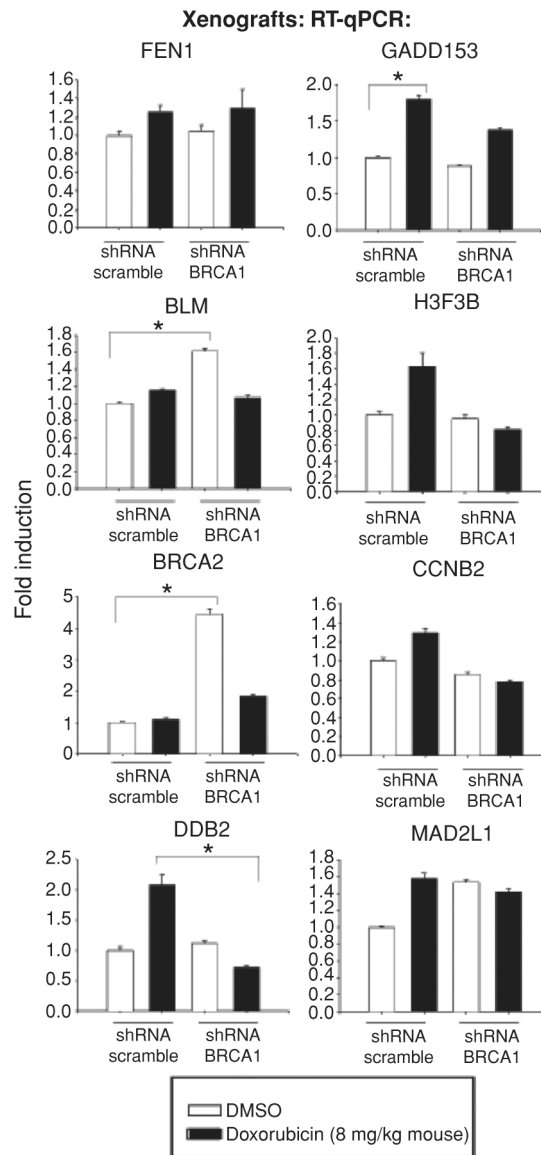
Figure 4.
BRCA1 induces several target genes expression in response to doxorubicin in prostate cancer xenografts. *Nu/nu* mice were inoculated with PC3 stable cells. Mice were injected i.p. with doxorubicin (8 mg doxorubicin/kg mouse) or vehicle (DMSO) on days 14 and 24. Mice were sacrificed 24 hours after the last injection and RT-qPCR analysis of candidate genes was carried out. Graph bar represents the average and SD from 5 tumors. Data were normalized to actin B. *, $P < 0.05$.
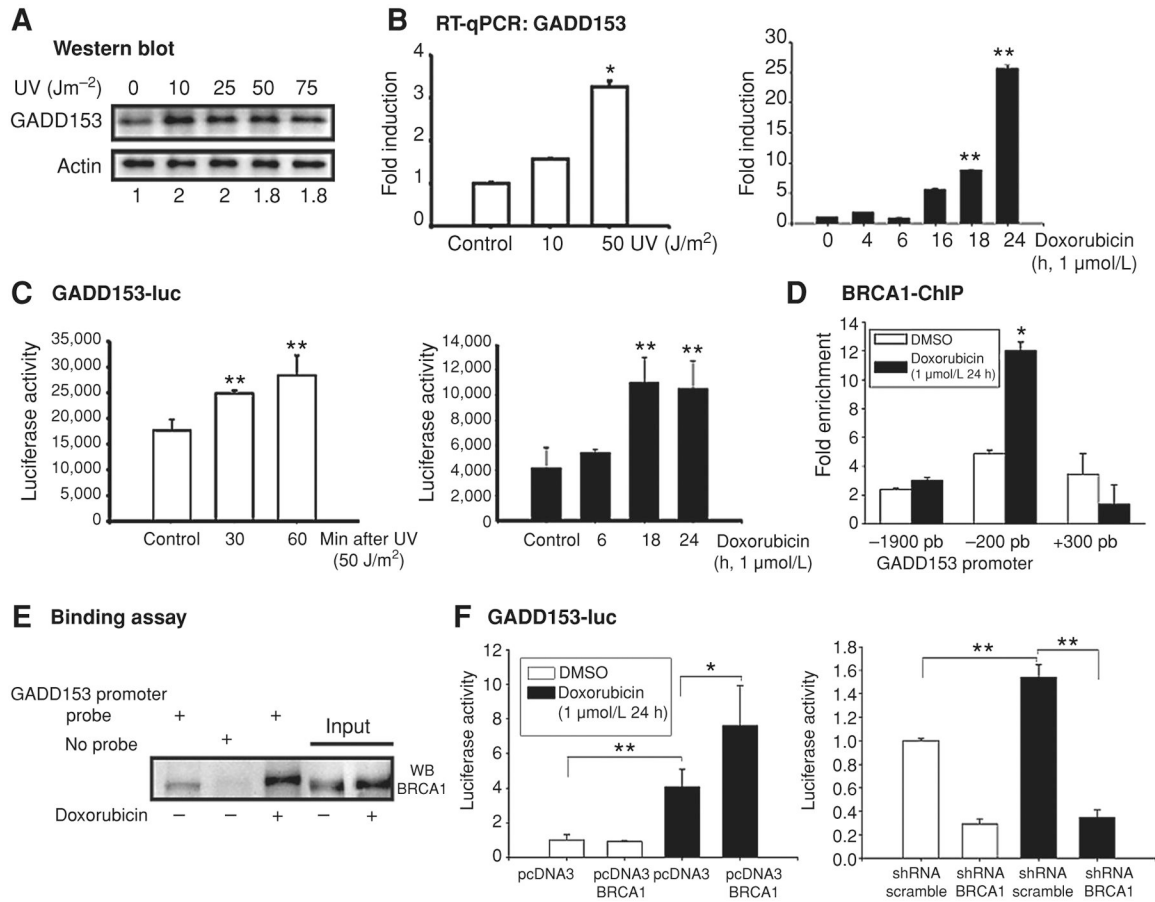
Figure 5.
BRCA1 binds and regulates GADD153 promoter in prostate cancer cells. A, PC3 cells were exposed to different UV doses, incubated for 1 hour, and analyzed by WB by using anti-GADD153 and -actin antibodies. The numbers under the bands indicate GADD153 quantitation normalized to actin B and control. B, PC3 cells were exposed to different UV doses or doxorubicin and GADD153 mRNA expression levels were determined by RT-qPCR. Data were normalized to actin B (ACTB). One result from 3 biological independent experiments is shown. C, PC3 cells were transiently transfected with GADD153 luciferase plasmid, after 24 hours cells were treated as before, harvested, and luciferase activity was quantified. Data were normalized to total protein. All transfections were done in triplicate and each experiment was repeated 3 times. D, BRCA1-ChIP experiments were conducted from PC3 cells untreated or treated with doxorubicin as was described in Materials and Methods. qPCR was carried out with primers located at 1,900 or 200 bp upstream or 300 bp downstream from the TSS of the *GADD153* gene. Fold enrichment was calculated normalizing data to input and IgG. E, binding assay was carried out from PC3 cells exposed or not to doxorubicin (1 μmol/L; 24 hours). F, PC3 stable cells were cotransfected with GADD153 luciferase plasmid and treated with doxorubicin (1 μmol/L; 24 hours) and harvested 48 hours posttransfection. Luciferase activity was measured. Transfections were done in triplicate in 3 biological independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Figure 6.
BRCA1 regulates doxorubicin-induced apoptosis and cell-cycle arrest through GADD153.
A, PC3 cells were transfected with 50 pmol siRNA GADD153 and 72 hours posttransfection cells were harvest and GADD153 expression was determined by WB and RT-qPCR. The numbers under the bands indicate GADD153 quantitation normalized to actin B and control. B, PC3 stable cell lines (pcDNA3 or pcDNA3BRCA1) were transiently transfected with siRNA scramble or siRNA GADD153, exposed to doxorubicin (6 μmol/L) or vehicle (DMSO) during 24 hours, and double stained with Annexin-FITC and PI for FACS analysis. One representative experiment of 3 biological independent experiments is shown. C, alternatively, cells were exposed to doxorubicin (2 μmol/L; 24 hours) and then stained with PI for FACS analysis. Histogram shows the percentage of G_1, S, and G_2/M cells from 1 representative experiment from 3 biological independent experiments. *, $P < 0.05$.