Transcriptional Autoregulation by BRCA1

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Abstract

The *BRCA1* gene product plays numerous roles in regulating genome integrity. Its role in assembling supermolecular complexes in response to DNA damage has been extensively studied; however, much less is understood about its role as a transcriptional coregulator. Loss or mutation is associated with hereditary breast and ovarian cancers, whereas altered expression occurs frequently in sporadic forms of breast cancer, suggesting that the control of *BRCA1* transcription might be important to tumorigenesis. Here, we provide evidence of a striking linkage between the roles for BRCA1 as a transcriptional coregulator with control of its expression via an autoregulatory transcriptional loop. BRCA1 assembles with complexes containing E2F-1 and RB to form a repressive multicomponent transcriptional complex that inhibits *BRCA1* promoter transcription. This complex is disrupted by genotoxic stress, resulting in the displacement of BRCA1 protein from the *BRCA1* promoter and subsequent upregulation of *BRCA1* transcription. Cells depleted of BRCA1 respond by upregulating *BRCA1* transcripts, whereas cells overexpressing BRCA1 respond by downregulating *BRCA1* transcripts. Tandem chromatin immmunoprecipitation studies show that *BRCA1* is regulated by a dynamic coregulatory complex containing BRCA1, E2F1, and Rb at the *BRCA1* promoter that is disrupted by DNA-damaging agents to increase its transcription. These results define a novel transcriptional mechanism of autoregulated homeostasis of BRCA1 that selectively titrates its levels to maintain genome integrity in response to genotoxic insult. *Cancer Res; 70(2); 532–42. ©2010 AACR.*

Introduction

Loss or mutation of the *BRCA1* gene is associated with nearly 5% of all breast cancers and greater than 80% of all cases of hereditary ovarian/breast cancer syndromes (1). Cells carrying absent or mutated alleles of BRCA1 show many features characteristic of reduced genome stability, including impaired cell cycle checkpoints, reduced efficiency in homologous recombination, and defective DNA repair following genotoxic insults (2). Efforts to elucidate this genomic "caretaker" function have led to the identification of multiple roles for BRCA1 in a variety of cellular processes, including cell cycle checkpoint control, homologous recombination, centrosome replication, response to DNA damage, and transcriptional control (3, 4). The major BRCA1 gene product is a 1,863-amino-acid protein containing multiple functional domains and protein interaction surfaces. An NH_2-terminal ring finger domain dimerizes with BARD1 to provide E3 ubiquitin ligase activity. The central portion interacts with many factors involved in DNA repair. Two tandem COOH-terminal BRCT motifs interact with protein complexes that control transcription and mediate the DNA damage response (reviewed in refs. 3–5).

Although BRCA1 was originally predicted to function as a transcription factor when it was first discovered in 1994 (1), subsequent studies primarily focused on defining characteristics of BRCA1-containing complexes induced by DNA damage (3, 6). Evidence of a transcriptional role for BRCA1 was first provided by reporter assays using hybrid fusions of BRCA1 with the DNA-binding domain of Gal4 (7, 8). BRCA1 was subsequently found to form direct complexes with the RNA polymerase II holoenzyme and a variety of transcription factors, including p53, c-Myc, Stat1, c-jun, estrogen receptors, p300, E2F, retinoblastoma protein (Rb), TRAPP220, and CtIP-CtBP (reviewed in refs. 4, 5). Several genes were later found to be regulated by BRCA1. Many of these genes regulate cell cycle progression and the response to DNA damage, including p21, p27, GADD45a, GADD153, DDB2, 14-3-3σ, hTERT, and several estrogen-responsive genes (reviewed in refs. 9, 5). However, it remains unclear how many of these BRCA1-regulated genes are directly targeted through gene-specific BRCA1 recruitment, as has been shown for MAD2 and ANG1 (10, 11).

Reports that many cases of sporadic breast cancer show decreased expression of BRCA1 in the absence of *BRCA1* mutation (12) and loss of BRCA1 expression is associated with higher-grade noninherited breast cancer (13–15) created

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significant interest in defining mechanisms of BRCA1 regulation at the level of transcription. BRCA1 transcription is regulated by a highly active bidirectional promoter (16, 17). Transcriptional regulation of the BRCA1 promoter is complex and modulated by multiple different components, including ETS factor complexes, E2F factors, cAMP-responsive element binding protein (CREB), Rb, p53BP1, and the SWI/SNF complex (reviewed in refs. 4, 5).

In this report, we describe a new and unexpected transcriptional target of BRCA1: the *BRCA1* promoter. We show that BRCA1 binds directly to its own promoter and this association downregulates expression. Furthermore, we show that this negative autoregulation by BRCA1 is modulated in response to genotoxic stress through an intricate and dynamic assembly of transcription factors and transcriptional coactivators that titrates the level of BRCA1 in response to environmental stress. This is the first description of an autoregulatory loop for *BRCA1* gene regulation and provides a novel mechanistic framework to study the transcriptional regulation of other BRCA1-dependent genes.

Materials and Methods

Cell culture, transfections, and reporters. Jurkat T cells, HEK 293, HEK 293 LTV, PC3, and LNCaP prostate cancer cells were maintained in RPMI 1640 with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. UV treatment was performed by irradiating 1×10^8 Jurkat T cells in a Stratalinker 800 (Stratagene) followed by incubation in fresh medium for the indicated times. Doxorubicin hydrochloride (Sigma) was prepared in DMSO. G418 concentration for selection (100 μg/mL) or maintenance (50 μg/mL) was determined by performing a concentration curve in PC3 cells between 10 and 500 μg/mL. PC3 stable cell lines were generated by transfecting with 10 μg of pcDNA3 BRCA1 wild-type (WT) or pcDNA3 empty vector by the calcium phosphate method in a 100-mm plate. Twenty-four hours posttransfection, G418 was added at the concentration for selection. After 10 d, single clones were amplified and BRCA1 expression was determined by Western blot and quantitative reverse transcription-PCR (RT-qPCR). Clones with the highest BRCA1 expression were used. PC3 with BRCA1-silenced expression in 100-mm plates were transfected with 10 μg of pKD or shRNA BRCA1 plasmids and 2 μg of pcDNA3 to introduce G418 resistance. The same G418 concentrations were used for selection and maintenance. Jurkat T-cell transfections were carried out as described previously (18). Briefly, transfections were performed by 96-well-format electroporation using a BTX ECM830 electroporator (Genetronics, Inc.) in 100 μL of RPMI 1640 for 50 ms at 260 V. Jurkat T cells (5×10^6) were transfected with 4 μg of each reporter and the indicated amount of expression vectors or siRNA constructs. Cells were then immediately transferred to 10 mL of RPMI 1640 and incubated 16 or 48 h at 37°C for experiments including siRNA. All transfections were carried out in triplicate and data shown are representative of at least three independent biological replicates.

Plasmids and RNAi reagents. The pGL12 BRCA1 promoter luciferase reporter plasmid was previously described (19). The pGL35 BRCA1 luciferase reporter plasmid was generated by cloning a PCR-amplified 900-bp fragment of the promoter upstream of the transcription start site (TSS) into the *Hin*dIII site of pGL3 Basic (Promega). BRCA1 promoter–specific primers used were 5′-AGC*AAGCTT*AACGACCACCCCATTGACTG-3′ (forward) and 5′-GCT*AAGCTT*TCCAGGAAGTCTCAGCGAGCTCA-3′ (reverse). BRCA1 expression vectors (pcDNA3 BRCA1 and pcDNA3 BRCA1ΔBRCT) have been previously described (20). BARD1 expression vector (pcDNA3 BARD1) was purchased from Origene Technologies. shRNA control and shRNA BRCA1 were from Upstate. siRNA control and siRNA BARD1 (AACAGUAACAUGUCCGAUGAAdTdT) were from Dharmacon, Inc. shRNA E2F1 A, shRNA E2F1 B, and pGIPZ control plasmids were obtained from OpenBiosystems. Plasmids expressing E2F-1 (E132) and E2F-1 (1–363) were generously provided by Doron Ginsberg (The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel). pGL3.BRCA1luc full-length (1,312–1,627), pGL3.BRCA1luc-M2 (E2F1 "A" mutation), pGL3.BRCA1luc-M3 (E2F1 "B" mutation), and pGL3.BRCA1-luc-M23 (both sites mutated) was described previously and was generously provided by P.M. Glazer (Department of Therapeutic Radiology, Hunter Radiation Therapy Center, Yale University School of Medicine, New Haven, CT; refs. 21, 22).

Chromatin immunoprecipitation and immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as previously described (23, 24) using anti-Rb, anti-Gal4, anti-E2F1 (Santa Cruz), and anti-BRCA1 antibody. Anti-Gal4 and/or purified IgG polyclonal antibodies were used as nonspecific controls. The anti-BRCA1 antibody was affinity purified from the sera of rabbits inoculated with a glutathione *S*-transferase fusion containing BRCA1 amino acids 304 to 772. Primer sequences are as follows: *BRCA1 55KB UP*, 5′-AAAGAGATGGGACTGTAACTGAGAAGGACC-3′ and 5′-TGTTTATAGGGAGACTGATGAATGGGC-3′; *BRCA1 1KB UP*, 5′-CAGCTTCCCG-CCCCCTGGGGA-3′ and 5′-CGTCGACG-CAATCGCCACCA-3′; *BRCA1 0.4KB UP*, 5′-TTCCCTC-CACCCCCCCAACAATC-3′ and 5′-CCCAATCCCC-CACTCTTTCCGCC-3′; *BRCA1 0KB UP*, 5′-CGACTGCTTTG-GACAATAGGTAGCG-3′ and 5′-AGTCTGCCCCCGGA-TGACGTAA-3′; and *BRCA1 62 KB DOWN*, 5′-GCGGGAG-GAAAATGGGTAGTTAGC-3′ and 5′-CCATTTTCCCAGCAT-CACCAGC-3′. Immunoprecipitation and Western blot analysis from isolated nuclear extracts were performed as previously described (25).

ChIP-chip. To identify BRCA1 binding sites, Jurkat cells were not treated or treated with UV before cross-linking with 1% formaldehyde and subjecting to ChIP. ChIP-chip, DNA purification, and ligation-mediated PCR were performed as described previously (23) using antibody against BRCA1. ChIP-chip samples were labeled and hybridized to NimbleGen HG17 proximal promoter microarrays (Roche NimbleGen) as previously described (23). For peak detection, a modification of the sliding window algorithm described by Buck and
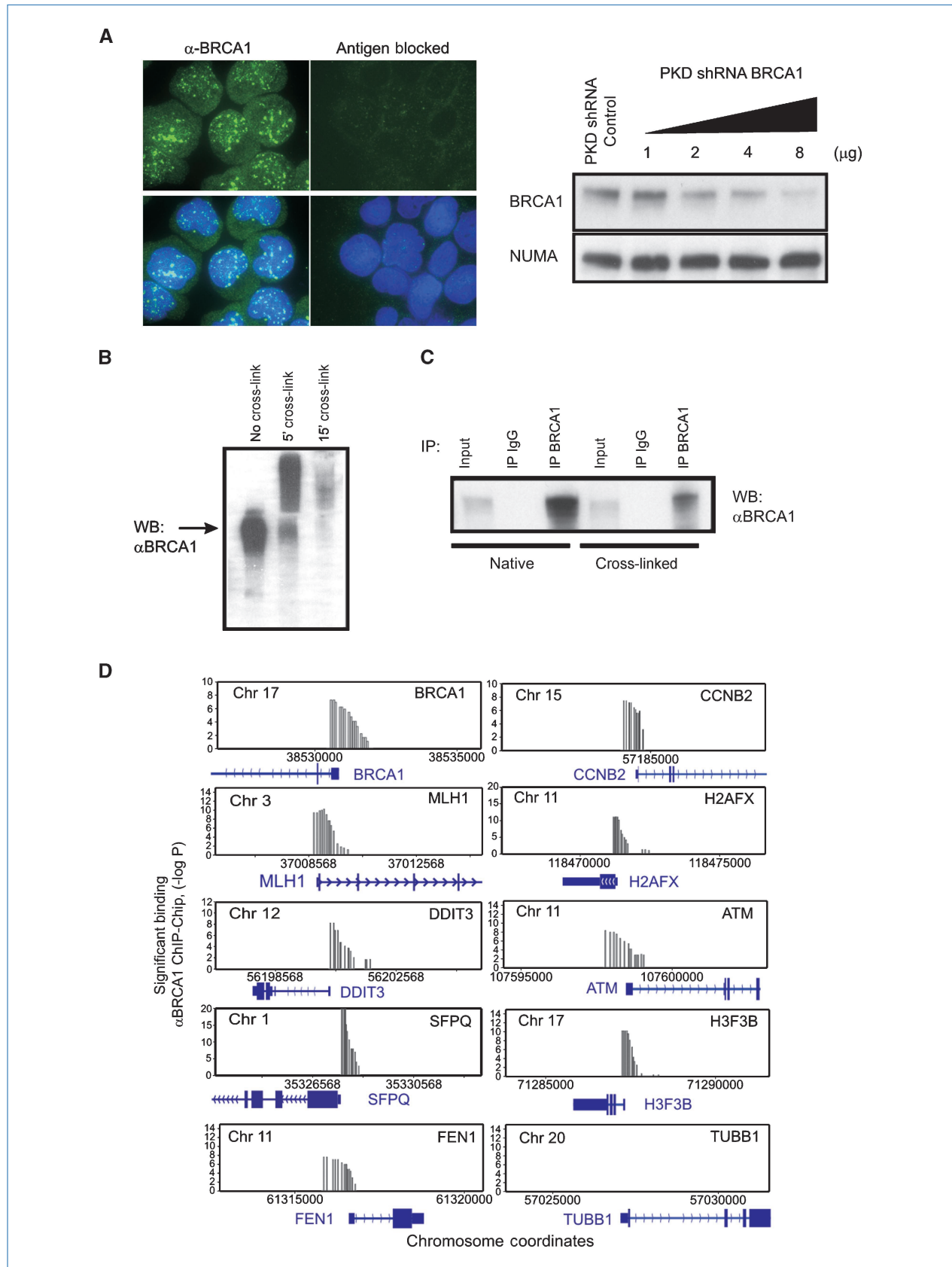
colleagues (26) was used, in which each probe on the array was assigned a P value based on a 350-bp probe-centered window advanced across each promoter region. P value calculations and Bonferroni corrections were done using the standard error function described by Buck and colleagues (26). Each promoter was assigned the value of the probe with the lowest determined P value. A potential binding site was identified if its P value was less than the arbitrarily set (cutoff of $P = 0.05$). P values were calculated averages from two independent biological replicates.

RNA isolation and RT-qPCR. RNA isolation and RT-qPCR were performed as previously described (24) and normalized to *ACTB* (actin B). BRCA1 pre-mRNA expression was assayed using QuantiTect Reverse Transcription kit (Qiagen) and primers were designed to amplify an amplicon that spans the exon 2/intron 2 junction. Primer sequences are as follows: *ACTB*, 5′-AAGATCATTGCTCCTCCTGAGC-3′ and 5′-CATACTCCTGCTTGCTGATCCA-3′; *BRCA1*, 5′-TGAAATCAGTTTGGATTCTGC-3′ and 5′-CATGCAAGTTT-GAAACAGAAC-3′; *E2F1*, 5′-GGCCAGGTACTGATGGTCA-3′ and 5′-GACCCTGACCTGCTGCTCT-3′; *EP300*, 5′-TCTGG-TAAGTCGTGCTCCAA-3′ and 5′-GCGGCCTAAACTCT-CATCTC-3′; and *BRCA1 pre-mRNA*, 5′-TGGAACAGAAAGA-AATGGATTTATCTGC-3′ and 5′-GGAATCCCAAATTAATA-CACTCTTGTGC-3′.

Immunoblot analysis and immunofluorescence. Nuclear extract and Western blot analysis were previously described (27, 28). Slides of Jurkat cells were prepared by cytospin and fixed with 3.5% paraformaldehyde for 15 min at room temperature. After fixation, cells were permeabilized for 10 min with 0.5% Triton X-100 in PBS and washed thrice in PBS for 10 min each. Cells were incubated overnight with the primary anti-BRCA1 rabbit polyclonal antibody diluted in PBS with 4% bovine serum albumin and 0.1% Tween 20. After incubation, cells were washed three times for 10 min each in PBS and then incubated for 1 to 2 h with secondary anti-rabbit antibody conjugated to FITC. The cells were then washed three more times in PBS before final mounting in PBS and examination on a Leica DMRA microscope with a Zeiss 510 ×100 1.3 numerical aperture oil immersion objective.

Results

To profile BRCA1 interactions with the genome, we developed an affinity-purified polyclonal antibody against BRCA1 that selectively detects nuclear BRCA1 by immunofluorescence, immunoblot analysis, and immunoprecipitation from both native and formalin cross-linked nuclear extracts (Supplementary Fig. S1; Fig. 1A–C). Specificity of reactivity was demonstrated by antigen blockade of immunofluorescence (Fig. 1A) and gene depletion by RNA interference (Fig. 1B). This antibody was used in a genome-wide screen of 37,365 human proximal promoters by the combination of ChIP and microarray technology (ChIP-chip) using NimbleGen (Roche) tiled proximal promoter arrays. In an initial screen, gene promoters showing a binding significance of $P < 0.000001$ were selected for further examination (Fig. 1D). Not surprisingly, several of the genes identified by this genome location analysis screen (including *CCNB2, MLH1, H2AFX, DDIT3, SFPQ, FEN1*, and *H3F3B*) were previously reported to be regulated by BRCA1 in gene expression studies (5, 29, 30). Remarkably, one of the BRCA1-associated genes was the *BRCA1* promoter itself. We focused on the intriguing possibility that BRCA1 could regulate its own expression for further analysis.

When compared with the nonspecific Gal4 antibody, a peak of BRCA1 binding to the proximal promoter region of the *BRCA1* gene was readily detected by α-BRCA1 ChIP and quantitative real-time PCR (Fig. 2A). Interestingly, this binding was disrupted by 3 hours of treatment with the DNA damage–inducing agent doxorubicin (Fig. 2A). Most notably, loss of BRCA1 from the *BRCA1* promoter correlated with an increase in *BRCA1* transcription, suggesting that BRCA1 may function as a negative regulator of its own transcription (Fig. 2B). This mode of BRCA1 regulation is not cell type specific because it is observed in the PC3 prostate cancer cell line (Fig. 2B and C). BRCA1 binding is readily detected at multiple gene promoters in different cell types. Similar to Jurkat, significant binding by BRCA1 is also shown in LNCaP prostate cells at the *ATM* and *PSA* promoters (Fig. 2D).

The region within 300 bp upstream of the *BRCA1* TSS has high transcriptional activity as previously described (Fig. 3A; ref. 16). This activity is significantly repressed by BRCA1 overexpression; however, a construct lacking the COOH-terminal BRCT domain showed reduced inhibitory activity (Fig. 3A). Similar to previous studies (16), reporter constructs containing additional sequence from −300 to −900 bp showed reduced transcriptional activity (Fig. 3A). Nonetheless, this activity is also repressed by BRCA1 overexpression, although this repression shows little requirement for the COOH-terminal BRCA1 repeat domain (Fig. 3A). In sharp contrast, BRCA1 depletion by *BRCA1*-specific shRNA increased transcription from both fragments of the *BRCA1* regulatory region (Fig. 3B). Similar results were obtained in response to

Figure 1. BRCA1 protein associates with the promoter regions of *BRCA1* and multiple other genes. *A,* characterization of affinity-purified antibodies against BRCA1 (*left*) by immunofluorescence staining of Jurkat T cells before and after antigen blockade. *Green,* α-BRCA1; *blue,* 4′,6-diamidino-2-phenylindole DNA staining (*right*). Characterization of α-BRCA1 antibodies by immunoblot analysis of cell lysates from Jurkat T cells depleted of BRCA1 by transfection with control and increasing amounts of BRCA1 shRNA-producing plasmid. NuMA antibody was used as loading control. *B,* immunoblot characterization of α-BRCA1 antibody reactivity against formalin cross-linked BRCA1 protein in Jurkat cell lysates. *WB,* Western blot. *C,* characterization of α-BRCA1 antibodies by immunoprecipitation (*IP*) of native and formalin cross-linked cell lysates compared with nonspecific IgG. Following immunoprecipitation, formalin cross-links were reversed before SDS-PAGE and immunoblot analysis. *Input,* signal from 10% of lysate. *D,* BRCA1 ChIP profile of *BRCA1* and other genes that show significant association ($P \leq 0.000001$) with BRCA1 by ChIP-chip analysis using the Nimblegen HG17 tiled proximal promoter array (HG17 array 2005-04-18_min_promoter_set) compared with nonspecific control (*TUBB1*). Results are the average of two independent biological replicates. *Y*-axis is shown in −log (P) scale and *X*-axis shows coordinates of the indicated chromosomes.
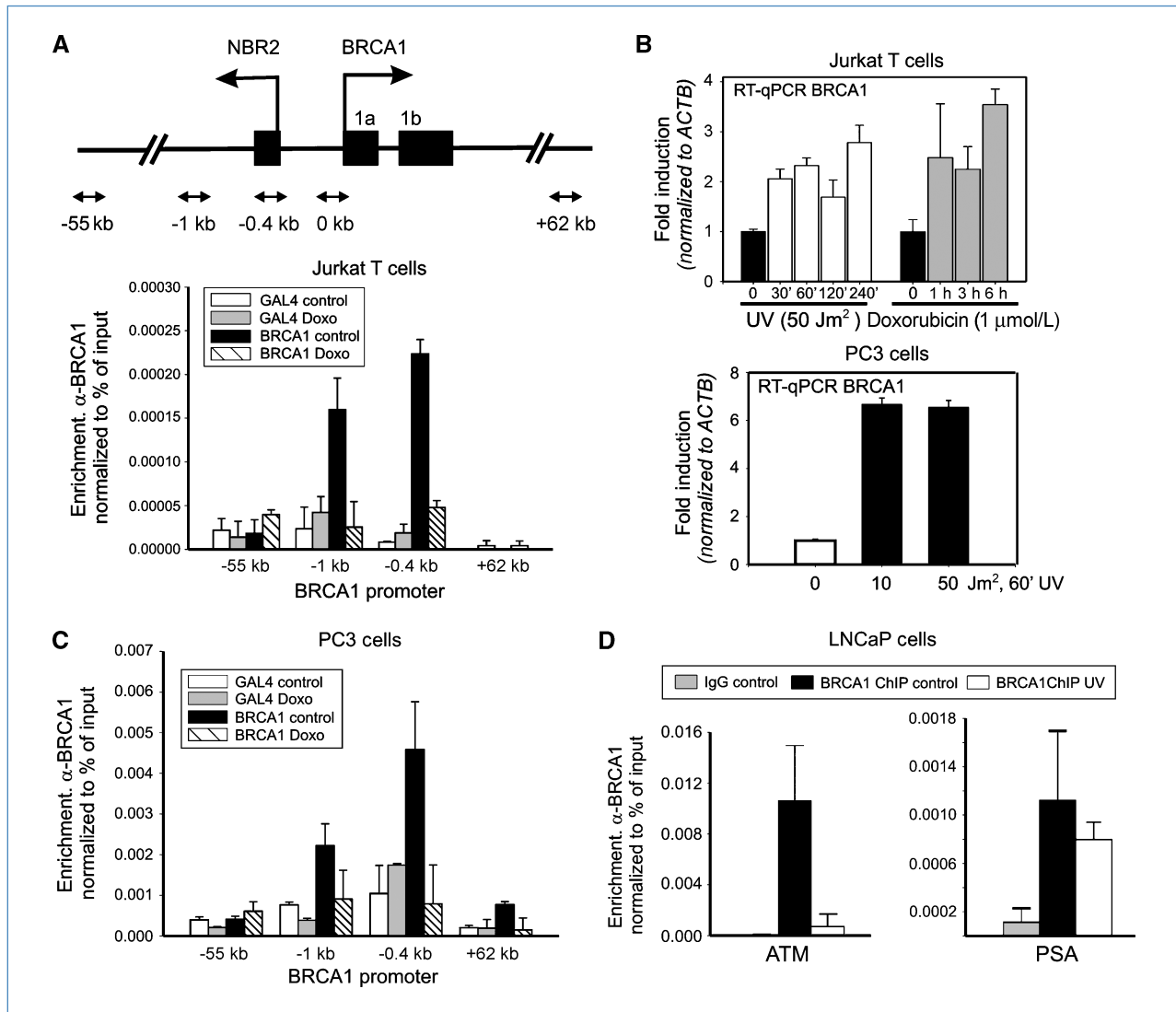
Figure 2. BRCA1 protein association with the *BRCA1* promoter and *BRCA1* expression is regulated by genotoxic stress. *A, inset, top,* schematic diagram of the *BRCA1* promoter shows divergent *NBR2* gene and relative positions of primer sets used in this study. ChIP analysis at the *BRCA1* promoter using BRCA1-specific or nonspecific control IgG (α-Gal4) in untreated Jurkat T cells or cells treated 3 h with doxorubicin (*Doxo*; 1 μmol/L). Shown is the enrichment at positions of the *BRCA1* locus relative to the TSS, presented as percent recovery of input. *B,* Jurkat T cells or PC3 cells were treated with either UV or doxorubicin (1 μmol/L) and harvested at the indicated times before RNA isolation. Mature RNA message was determined by RT-qPCR normalized to *ACTB. C,* ChIP analysis of BRCA1 binding to the *BRCA1* promoter in PC3 human prostate cell lines at indicated positions relative to the TSS in untreated and doxorubicin-treated cells (1 μmol/L, 24 h). IgG against yeast Gal4 (α-Gal4) was used as a nonspecific binding control. *D,* ChIP analysis of BRCA1 binding to the *ATM* and *PSA* promoters in LNCaP cells untreated or after UV treatment. *Columns,* mean of two independent experiments; *bars,* SD.

enforced or depleted expression of the BRCA1 dimerization partner, BARD1 (Fig. 3*C*). In addition, like BRCA1, BARD1 is enriched at the *BRCA1* promoter but is displaced by pretreatment with doxorubicin (Fig. 3*D*). Furthermore, stable overexpression or depletion of BRCA1 in the PC3 prostate cell line causes transcriptional repression and upregulation of the *BRCA1* promoter, respectively (Fig. 4*A* and *B*). Most notably, however, cells that stably overexpress BRCA1 show downregulation of endogenous nascent *BRCA1* RNA expression, whereas cells that have stable depletion of BRCA1 expression show upregulation of nascent *BRCA1* RNA transcripts.

Although BRCA1 does not bind DNA specifically, prior studies have suggested that BRCA1 may be tethered directly or indirectly to sequence-specific DNA-binding proteins. One potential interaction involves the Rb that forms dynamic repressor complexes with the E2F family of transcription factors and is readily detected in complexes with BRCA1 by immunoprecipitation (20, 31). In addition, the E2F family of proteins are well known to bind to both the *BRCA1* promoter (21, 32) and BRCA1 (33) and therefore may serve to recruit Rb/BRCA1 complexes to the *BRCA1* promoter. As shown by BRCA1 and Rb immunoprecipitation in Fig. 5*A*,

a small population of Rb in the nuclear extracts of Jurkat cells is associated with BRCA1 and these levels decrease in cells treated with doxorubicin. Similarly, BRCA1 complexes containing E2F-1 show detectable binding above background in BRCA1 immunoprecipitates, although this is a small population compared with the amount of E2F-1 associated with

Rb (Fig. 5A). Similarly, ChIP analysis shows that both E2F-1 and Rb are associated with *BRCA1* promoter under basal conditions and are significantly decreased in cells treated with doxorubicin (Fig. 5B–C). To further show the linkage between E2F-1 assembly at the *BRCA1* promoter and BRCA1, tandem ChIP was performed at the *BRCA1* promoter to
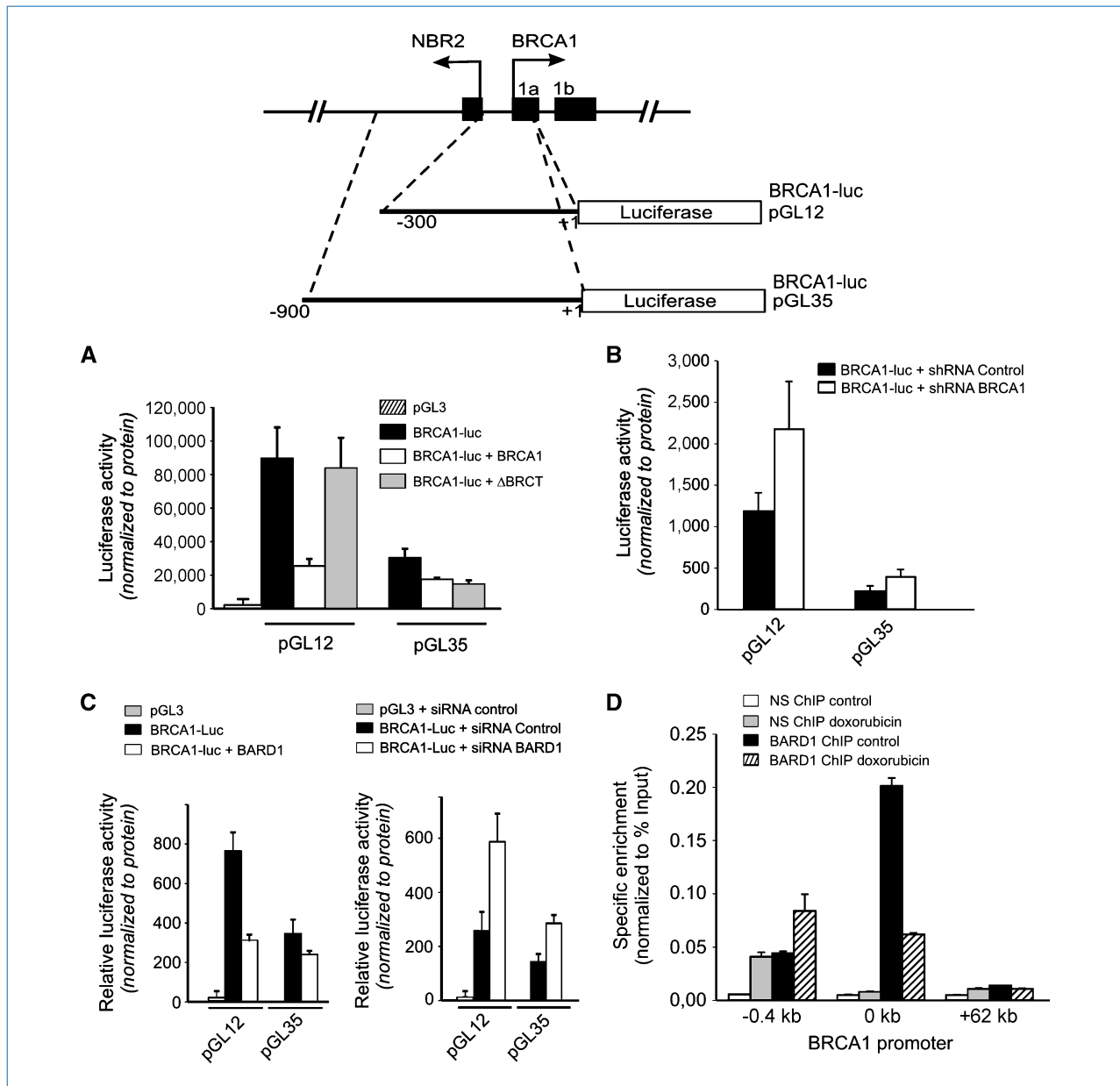
Figure 3. BRCA1 and BARD1 negatively regulate transcription from the *BRCA1* promoter. *A,* Jurkat T cells were cotransfected with luciferase reporter constructs (see schematic above) spanning the indicated lengths of regulatory sequences upstream of the *BRCA1* TSS (pGL12 or pGL35) in combination with either of the BRCA1 WT (pcDNA3 BRCA1), BRCA1 COOH-terminal mutant (pcDNA3 ΔBRCT), or empty (pcDNA3) expression vectors. *B,* Jurkat T cells were cotransfected with the indicated *BRCA1* promoter reporters and either shRNA BRCA1 or control shRNA with scrambled sequence expression vectors. *C,* Jurkat T cells were cotransfected with the indicated *BRCA1* promoter reporters and either WT *BARD1* (BARD1) or empty expression vectors, or *BARD1* siRNA or control duplex siRNA oligonucleotides. *Columns,* mean from at least three independent transfections; *bars,* SEM. *D,* ChIP analysis of BARD1 enrichment at the *BRCA1* promoter with and without pretreatment with 1 μmol/L doxorubicin. *Columns,* mean derived from two independent biological replicates; *bars,* SEM.
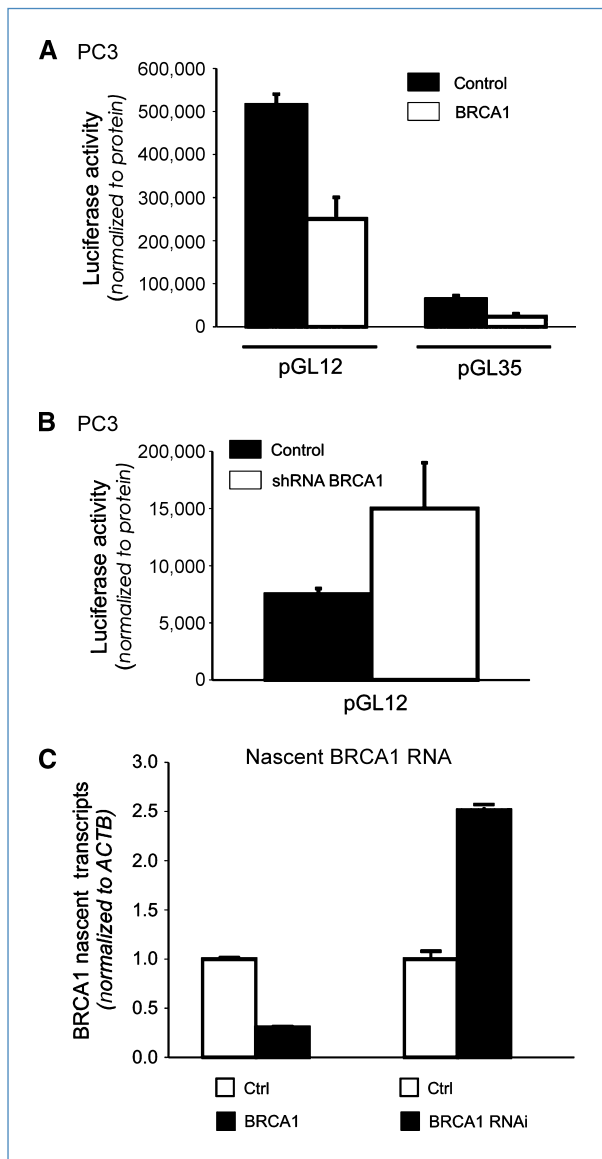
Figure 4. Reciprocal regulation of *BRCA1* promoter activation and transcription in PC3 prostate cell lines by stable overexpression or depletion of BRCA1 protein. *A,* PC3 cells stably transfected with control empty vector (*Control*) or BRCA1 expression vector (*BRCA1*) were transiently transfected with BRCA1-luc reporter constructs (pGL12 or pGL35). *B,* PC3 cells stably expressing shRNA BRCA1 or control shRNA with scrambled sequence shRNA expression vectors were transfected with BRCA1-luc reporter (pGL12). Luciferase activity was normalized to protein. *Columns,* mean from three independent transfections; *bars,* SEM. *C,* PC3 prostate cancer cells that stably overexpress BRCA1 as in *A* were analyzed for nascent BRCA1 RNA levels (unspliced transcript) by qRT-PCR and compared with cells stably expressing empty vector (*Ctrl*). Similarly, cells stably depleted of *BRCA1* as in *B* were analyzed for nascent *BRCA1* RNA levels compared with cells stably producing shRNA containing a scrambled sequence (*Ctrl*).

sequentially enrich for BRCA1-associated complexes (Fig. 5*D*). Compared with nonspecific control (α-Gal4), primary ChIP precipitates of *BRCA1* promoter–containing complexes, isolated with α-BRCA1 antibodies, showed a significant reten-

tion of E2F-1–associated *BRCA1* promoter sequences, and this enrichment was dramatically decreased following genotoxic stress (Fig. 5*D*). These findings further support the existence of a dynamic assembly of complexes containing Rb, E2F-1, and BRCA1 at the *BRCA1* promoter and their displacement and/or disruption by genotoxic stress.

Several studies have reported a complex regulation of the *BRCA1* promoter by members of E2F and Rb factors, which is likely to be determined by the relative abundance of the factors (21, 22, 34). This is shown by the significantly reduced basal formation of BRCA1-containing complexes at the *BRCA1* promoter in the Soas cell line, which does not express Rb (Fig. 6*A*). In addition, transient expression of dominant interfering mutants of E2F1 relieves BRCA1-dependent repression of the *BRCA1* promoter in PC3 cells. As shown in Fig. 6*B*, cotransfection of a mutant containing only the DNA-binding domain of E2F-1 (amino acid 1–363) and, to a lesser extent, a DNA-binding defective point mutant (E132; refs. 35, 36) results in increased *BRCA1* promoter activity compared with control. Notably, this effect is blunted in PC3 cells that stably overexpress BRCA1 (Fig. 6*B*). Prior studies have shown that the manner in which E2F and Rb factors associate with the BRCA1 is significantly complex and differentially regulated by two separate E2F binding sites in the *BRCA1* promoter region (21, 22). Reporter assays using *BRCA1* promoter reporter constructs in which one or both of the E2F sites is mutated (22) show that the ability of BRCA1 to regulate the *BRCA1* promoter is significantly influenced by the integrity of these sites (Fig. 6*C*). Mutation of the most distal site (E2FA) results in decreased transcriptional activity, suggesting that this site may play a more positive role in the activity of the *BRCA1* promoter whereas mutation of the more proximal E2F site (E2FB) results in a slight increase in activity (Fig. 6*C, left*). Both single mutants are repressed by BRCA1 overexpression (Fig. 6*C, left*) and induced by BRCA1 depletion (Fig. 6*C, right*); however, mutation of both sites results in the largest increase in *BRCA1* promoter activity, the most resistance to BRCA1 overexpression (Fig. 6*C, left*), and the largest increase in promoter activity following BRCA1 depletion (Fig. 6*C, right*). The finding that *BRCA1* promoter repression by BRCA1 overexpression and *BRCA1* promoter induction by BRCA1 depletion persist following mutation of both sites suggests that other proteins or sites, in addition to the two E2F binding sites, are likely to be involved in recruitment of BRCA1 to the *BRCA1* promoter.

Discussion

Several groups have made significant contributions to our understanding of the regulation of the *BRCA1* promoter in response to a variety of environmental conditions ranging from genotoxic insult to hypoxia (22, 37–40). The general consensus from these studies is that BRCA1 is regulated by a highly dynamic promoter that adapts rapidly to cellular conditions. Interestingly, several groups have examined the influence of genotoxic stress on BRCA1 expression with conflicting results. Previous studies showed that genotoxic stress repressed BRCA1 expression and showed a significant

role for p53 in the repression (37, 38). Subsequently, it was shown that although BRCA1 expression decreases several hours following DNA damage, there is an initial rapid increase in expression within minutes following exposure to genotoxic stress with a subsequent p53-mediated feedback relay that quenches *BRCA1* transcription (39, 40). Others have found that activation and repression could be seen in the same system depending on the time and dose of genotoxic agents (41). Interestingly, BRCA1 expression also stabilizes p53, which likely contributes to the quenching of its expression (42). The intersection of these feedback loops suggests that BRCA1 participates in an intricate autoregulatory interplay following DNA damage (see schematic in Fig. 6). In the early stages, levels of BRCA1 are elevated to allow it to perform its "caretaker" function by participating in DNA repair. Under these conditions, BRCA1 would be released from its promoter and recruited to sites of DNA repair, allowing the now derepressed *BRCA1* promoter to increase *BRCA1* transcription so that BRCA1 protein, consumed in the repair process, can be replaced. Should repair be insufficient in the ensuing hours and the DNA damage signal persist, increased expression of p53, stabilized by BRCA1, would activate its p53 "gatekeeper" function to shut down transcription of *BRCA1* and other genes and initiate programmed cell death. It should be noted that p53 is not absolutely required for this entire process because the reduction in BRCA1 expression can occur in p53-deficient cells (37).

In retrospect, a role for BRCA1 in regulation of its own expression could have been predicted. BRCA1 has been previously shown to interact with Rb and members of the E2F family of transcription factors (33). Rb, Rb-related proteins, and E2F factors have all been found to bind to the *BRCA1* promoter (21, 32, 34, 43). Thus, a role for BRCA1 in an autoregulatory assembly at its own promoter responsive to environmental changes is an attractive hypothesis.

The role of Rb/E2F in the regulation of *BRCA1* transcription during cell cycle progression and in response to DNA damage has yet to be fully explored. Although exposure to DNA-damaging agents can ultimately disrupt normal cell cycle progression, the time periods used for the treatment of cells with genotoxic agents (1–3 hours) in this study are much too short to influence the cell cycle (see Supplementary Fig. S2), and, therefore, the observed effects are due predominantly to the immediate stress response from genotoxic insult. The expression profiles that correlate with BRCA1 release following DNA damage are likely to reflect a complex integration of multiple events linked to changes in covalent modification and protein-protein interactions involving E2F-1, Rb, and BRCA1 at the *BRCA1* promoter. Presently, the actual nature of the disruption of the E2F-1/Rb/BRCA1 assembly at the *BRCA1* promoter following genotoxic stress remains to be precisely defined. The work of Glazer and colleagues suggests (21) that there may be an intricate rearrangement of the interaction of multiple E2F family members with two distinct E2F binding sites at the *BRCA1* promoter in response to environmental challenge (22). Interacting components include E2F-1 and E2F-4 in addition to the Rb-related factors p107 and p130. Thus, E2F-1 binding

Figure 5. BRCA1 forms a negative regulatory complex with E2F-1 and Rb at the *BRCA1* promoter that is disrupted by genotoxic stress. *A,* coimmunoprecipitation of BRCA, Rb, and E2F-1 using antibodies against nonspecific IgG, α-Rb, and α-BRCA1 antibodies. *IB,* immunoblot. *B,* ChIP-qPCR analysis of Rb enrichment at the indicated positions of the *BRCA1* promoter region in untreated Jurkat T cells and cells treated 3 h with 1 μmol/L doxorubicin compared with nonspecific control (α-Gal4). *C,* α-E2F-1 ChIP-qPCR analysis of E2F-1 enrichment at the indicated positions of the *BRCA1* promoter region in untreated Jurkat T cells and cells treated 3 h with 1 μmol/L doxorubicin compared with nonspecific control (α-Gal4). *D,* untreated Jurkat cells or cells treated with 1 μmol/L doxorubicin were analyzed by tandem BRCA1/E2F-1 ChIP using combinations of first ChIP with either the nonspecific antibody (α-Gal4) or the α-BRCA1 antibody followed by re-ChIP of the immunoprecipitated complexes with α-Gal4, α-BRCA1, or α-E2F-1. *Columns,* mean of at least two independent experiments; *bars,* SD.
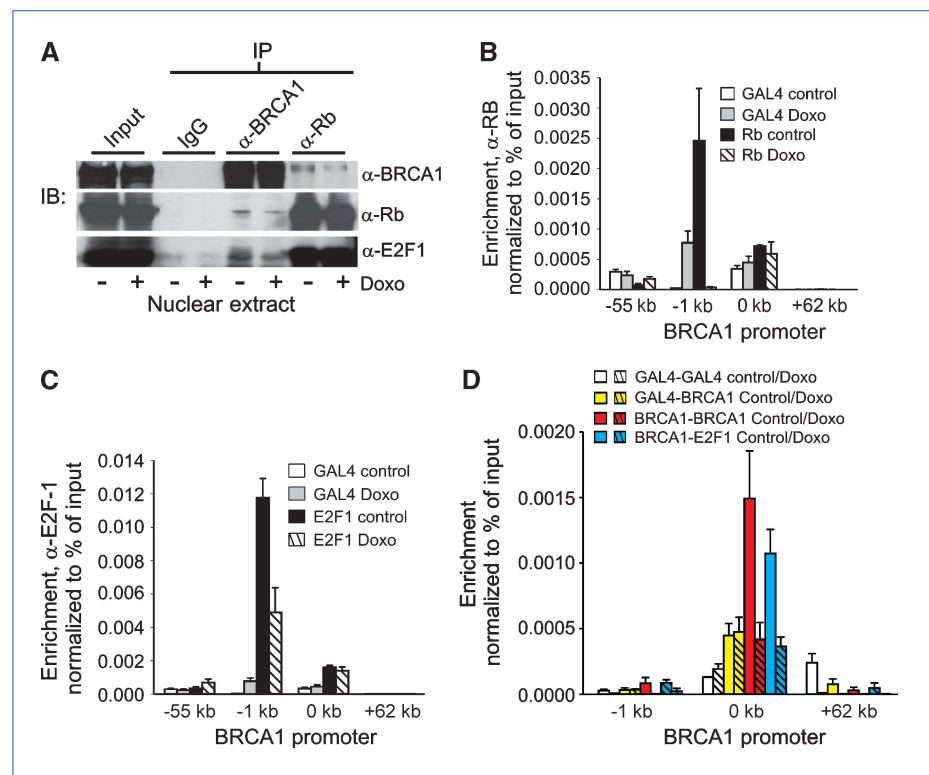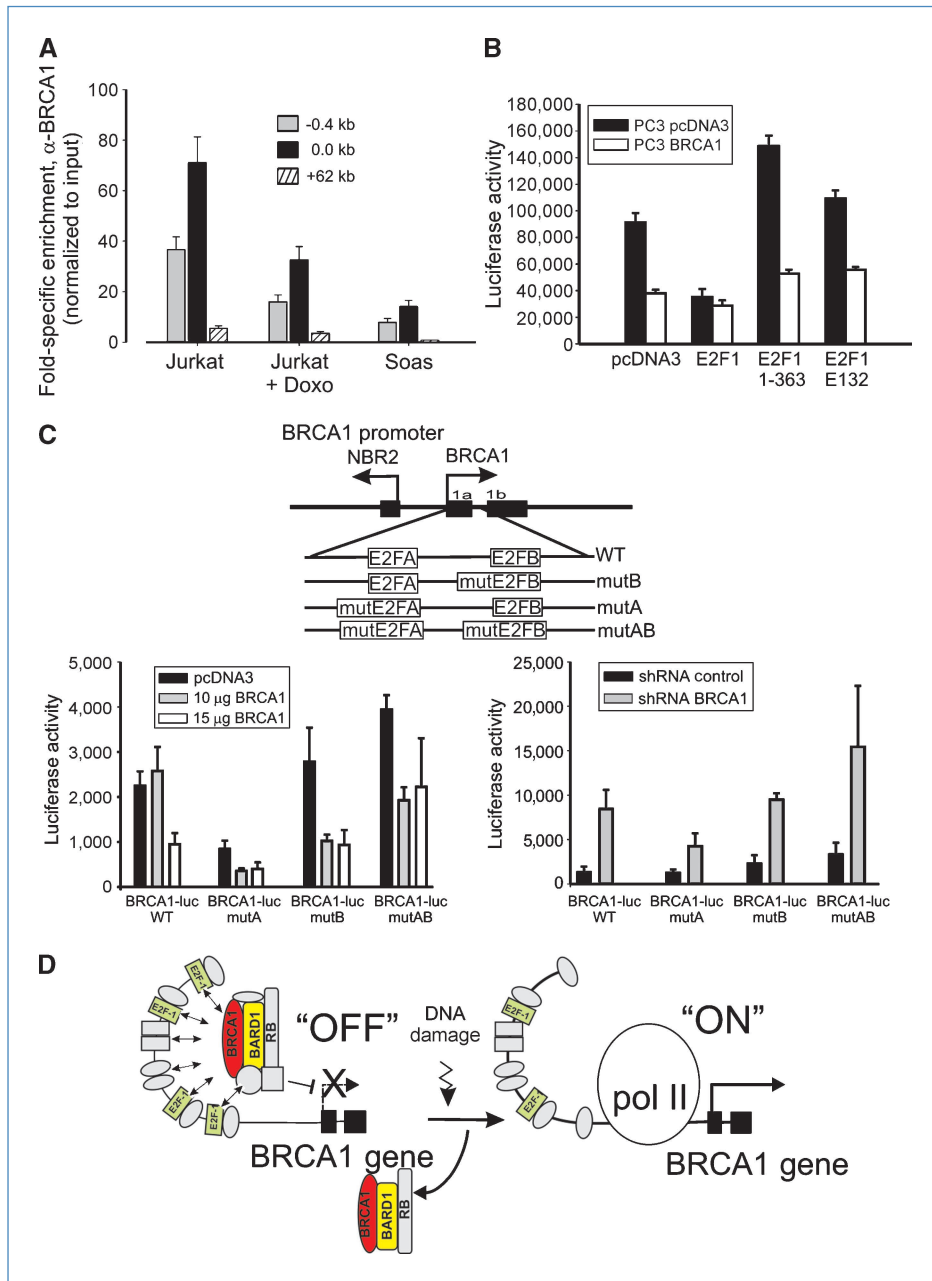
Figure 6. BRCA1 assembles at the *BRCA1* promoter in association with E2F-1 and Rb complexes. *A,* BRCA1 enrichment by ChIP at the *BRCA1* promoter of untreated and doxorubicin-treated Jurkat cells compared with untreated Rb-deficient Saos cells normalized to nonspecific IgG. *Columns,* mean from two independent experiments; *bars,* SEM. *B,* PC3 prostate cancer cells stably overexpressing BRCA1 (PC3 BRCA1) or empty vector (PC3 pcDNA3) were cotransfected with the BRCA1-luc reporter plasmid (pGL12) and WT or the indicated mutant E2F-1 expression vectors (see text). *Columns,* mean from three independent transfections; *bars,* SEM. *C,* BRCA1 regulation of the *BRCA1* promoter depends on the E2F binding sites. BRCA1 was overexpressed (*left*) or depleted (*right*) in cells transfected with reporter plasmid containing WT or mutated E2F transcription factor binding sites within the *BRCA1* promoter sequences (see schematic diagram above). *Columns,* mean from three independent transfections; *bars,* SEM. *D,* hypothetical schematic diagram depicting BRCA1-negative autoregulation by a complex containing BRCA1, E2F-1, and Rb at the *BRCA1* promoter, which is upregulated by displacement/disruption of this complex at the promoter to allow *BRCA1* transcription in response to genotoxic stress. Note that E2F-1 complexes are rearranged but not completely lost from the promoter.

at the *BRCA1* promoter is probably multivalent and may change, but may not be absent from the promoter, following DNA damage (Fig. 6), and is consistent with the observation that E2F-1 plays a role in both the activation and repression of *BRCA1* promoter activity through its interaction with Rb. Furthermore, this interplay may be highly influenced by tissue-specific ratios of individual Rb- and E2F-related factors (Fig. 6*A*; refs. 21, 22). The intricacy of this complex may also explain why peak ChIP enrichment for the BRCA1, E2F-1, and Rb components of these complexes spans a distance between 0 and −1 kb relative to the TSS (Figs. 2 and 5).

Rb interacts with a common set of factors involved in transcriptional repression, including CtIP, CtBP, and RbAP46/48 (20, 31, 44). These components are also known to associate with certain histone deacetylases and chromatin remodelers. Detailed analyses of the acute changes in chromatin structure and covalent modification at the *BRCA1* promoter in response to genotoxic insult will also be important future objectives. Although the E2F transcription factors are primary candidates in BRCA1 recruitment, the possibility of a role for other sequence-specific DNA binding factors such as CREB, ZBRK1, and ETS must be considered. This is particularly important because the

site-specific alteration of the two characterized E2F sites does not completely abolish BRCA1 regulation of the *BRCA1* promoter (Fig. 6*C*). The recent finding that BRCA1 can re-press transcription by ubiquitylation of preinitiation complex components suggests that genotoxic stress may induce dynamic changes in protein ubiquitylation at the *BRCA1* promoter through the E3-ligase activity of BRCA1 in complex with BARD1 (45). Future experiments will have to assess if and how such modifications occur at the *BRCA1* promoter *in vivo*.

Earlier clues that BRCA1 may have a regulatory role in its own expression come from mouse embryonic tissue expression studies in which exon 11 has been deleted. Deletion of exon 11 produces a gene product with significantly reduced nuclear localization (46). RNA isolated from this tissue show a >2-fold increase in transcription from the mutant *BRCA1* alleles, consistent with loss of repression due to impaired nuclear entry of BRCA1 protein (47) and is highly consistent with the *BRCA1* gene depletion data presented in this study (Figs. 3 and 4).

Our current understanding of the role of BRCA1 as a regulator of transcription is still in its infancy. The list of direct transcriptional targets of BRCA1 now includes *BRCA1* itself but still remains quite small despite the implications from several gene expression studies. Expanded efforts to identify those genes that represent direct targets of BRCA1 will be of central importance in improving our understanding of the function of BRCA1 as a transcriptional coregulator.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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