Decreased BRCA1 Confers Tamoxifen Resistance in Breast Cancer Cells by Altering Estrogen Receptor-Coregulator Interactions

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Abstract

The breast cancer susceptibility gene 1 (*BRCA1*) is mutated in approximately 50% of hereditary breast cancers, and its expression is decreased in 30–40% of sporadic breast cancers, suggesting a general role in breast cancer development. BRCA1 physically and functionally interacts with estrogen receptor-alpha (ERα) and several transcriptional regulators. We investigated the relationship between cellular BRCA1 levels and tamoxifen sensitivity. Decreasing BRCA1 expression in breast cancer cells by small interfering RNA alleviated tamoxifen-mediated growth inhibition and abolished tamoxifen suppression of several endogenous ER-targeted genes. ER-stimulated transcription and cytoplasmic signaling was increased without detectable changes in ER or ER coregulator expression. Co-immunoprecipitation studies showed that with BRCA1 knockdown, tamoxifen-bound ERα was inappropriately associated with coactivators, and not effectively with corepressors. Chromatin immunoprecipitation studies demonstrated that with tamoxifen, BRCA1 knockdown did not change ERα promoter occupancy, but resulted in increased coactivator and decreased corepressor recruitment onto the endogenous cyclin D1 promoter. Our results suggest that decreased BRCA1 levels modify ERα-mediated transcription and regulation of cell proliferation in part by altering ERα-coregulator association. In the presence of tamoxifen, decreased BRCA1 expression results in increased coactivator and decreased corepressor recruitment on ER-regulated gene promoters.

Keywords

tamoxifen resistance; BRCA1, ERα; coactivators; corepressors

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Introduction

The steroid hormone 17β-estradiol (E2), acting through estrogen receptors (ERα and ERβ), plays an important role in development and progression of breast cancer (Doisneau-Sixou et al, 2003). Breast cancer occurrence is linked to E2 exposure, and approximately 75% of breast tumors express ERs (Dowsett et al, 2006). E2 induces cell proliferation at least partially by stimulating progression through the G1 phase of the cell cycle (Doisneau-Sixou et al, 2003). Acting through ERs, E2 regulates transcription of responsive genes, some of which control cell proliferation and apoptosis (Basu and Rowan, 2005). Both ER subtypes are members of the nuclear receptor superfamily and present in normal mammary cells, but ERα is expressed at higher levels in tumors (Doisneau-Sixou et al, 2003). ER-mediated transcription can be inhibited by anti-estrogens, which suppress ER activity and cell proliferation. Tamoxifen (Tam), an anti-estrogen, reduces breast cancer recurrence and mortality in women with ER-positive tumors (Fink, 2006). However, 30–50% of women with ER-positive tumors do not initially respond to Tam, exhibiting intrinsic resistance, and patients with initially positive responses may suffer a recurrence, displaying acquired resistance (Clarke et al, 2003). Thus, it is important to identify molecular mechanisms of Tam resistance to better predict therapeutic responses.

In the presence of ligand ER undergoes conformational changes, dimerizes, and binds to DNA sequences such as estrogen response elements (EREs), or forms complexes with other transcription factors bound to target gene promoters (Hall et al, 2001). E2-bound ER recruits coactivators, which serve as a bridge between ER and general transcription machinery, or are associated with histone acetyltransferases to stimulate transcription (Shang et al, 2000). Tam-bound ER does not bind coactivators, but instead binds corepressors, which inhibit gene transcription by recruiting histone deacetylases (HDAC) (Kurtev et al, 2004). Therefore, ER coregulators play a critical role in regulating ER transcription. Changes in coregulator activity or expression may alter ER activity and response to different ligands (Smith and O'Malley, 2004).

The protein product of the breast cancer susceptibility gene1 (*BRCA1*) directly interacts with ERα, primarily through its N-terminus (Ma et al, 2005), and inhibits ERα transcription (Fan et al, 1999). *BRCA1* germ line mutations have been identified in nearly 50% of hereditary breast cancers and 80% of cases with both hereditary breast- and ovarian cancers (Narod and Foulkes, 2004). However, most breast cancers are sporadic, with only 5–10% due to inherited susceptibility (Bissonauth et al, 2008). Decreased BRCA1 expression due to promoter hyper-methylation or loss of one BRCA1 allele (Magdinier et al, 1998) was shown in 30–40% of sporadic breast cancers (Birgisdottir et al, 2006). Thus, BRCA1 may generally play a role in breast cancer development.

BRCA1 is a predominantly nuclear phosphoprotein with 1863 amino acids (aa) (Chen et al, 1996). It has several functional domains, including an N-terminal RING finger, central region nuclear localization signals, and two BRCA1 C-terminal (BRCT) domains. The RING finger domain is important for association with several proteins, particularly BARD1 (Wu et al, 1996). BRCA1-BARD1 complexes display ubiquitin E3 ligase activity and are involved in protein ubiquitination (Hashizume et al, 2001). The BRCT domains are involved

in DNA damage repair (Glover et al, 2004) and association with components of basal transcription machinery such as RNA polymerase II (Krum et al, 2003), ER coregulators such as p300/CBP (Fan et al, 2002), and chromatin modification proteins such as HDAC1/2 (Yarden and Brody, 1999).

In this study, we investigated potential links between decreased BRCA1 levels and responses to Tam in ER-positive human breast cancer cell lines (T47D and ZR-75-1). We showed that BRCA1 knockdown abolished Tam suppression of cell proliferation and ERα transcriptional activity. This occurred not through altered protein expression of ERs or ER coregulators, but by promoting ER-coactivator interactions and decreasing ER-corepressor association in the presence of Tam. Based on these findings, we suggest decreased BRCA1 levels alter ER-coregulator interactions to make ERα– mediated transcription less responsive to Tam, thus contributing to Tam-resistant phenotypes.

Results

BRCA1 knockdown alters proliferation responses of breast cancer cells to Tam

To investigate effects of decreased BRCA1 expression, BRCA1 small interfering RNA (siRNA) oligonucleotides (DO3 or DO7) were used to knockdown endogenous BRCA1 in T47D (Hu et al, 2005) and ZR-75-1 ER-positive breast cancer cells. Figure 1A shows BRCA1 protein expression was efficiently decreased in both DO3- and DO7-transfected T47D cells. BRCA1 in parental T47D cells is present predominantly as the full-length (220kD) protein, with only a minor fraction as shorter isoforms. All isoforms were efficiently eliminated by siBRCA1 (not shown). To determine if decreased BRCA1 expression altered DNA synthesis, a measure of cell proliferation, BrdU incorporation was analyzed. In cells transfected with control siRNA (siCon), BrdU incorporation was significantly stimulated by 17β-estradiol (E2, 10nM) and suppressed by 4-hydroxytamoxifen (Tam, 1μM or 10μM). In BRCA1 knockdown cells with either siRNA (DO3 or DO7), E2 remained stimulatory, but Tam was no longer suppressive (compare checkered and hatched bars with siCon). However, lentivirus re-expression of silent mutant BRCA1 protein (silent mut.) rescued Tam suppression of DNA synthesis (Fig. 1B). BRCA1 protein was efficiently decreased in DO7-transfected ZR-75-1 cells compared with siCon-transfected cells, and Tam-induced growth inhibition was abolished in BRCA1 knockdown cells (Fig. 1C). These data indicated that BRCA1 protein levels can regulate cell sensitivity to Tam.

These results could not be explained by altered levels of ERα,ERβ,ER coactivators (SRC1 and SRC3), or corepressors (SMRT and REA) (Fig. 2A). BRCA1 knockdown also did not change normalized ERα or ERβ protein levels in the presence of ligand (Fig. 2B and 2C). Consistent with published results (Horner-Glister et al, 2005), E2 treatment decreased ERα to 20% and ERβ to 70% of untreated levels within 24h in both siCon- and siBRCA1-transfected cells (Fig. 2B). Tam treatment did not appreciably decrease ER protein levels even after 24h in cells with endogenous or decreased BRCA1 (Fig. 2C).

BRCA1 knockdown alleviates Tam suppression of ER-regulated gene transcription

To assess mechanisms by which BRCA1 knockdown rendered cell proliferation less responsive to Tam suppression, we first investigated ER transcriptional activity with a transfected luciferase reporter (pGL3-2ERE) containing two tandem EREs. E2-stimulated ERE-dependent transcription was significantly enhanced in BRCA1 knockdown cells (Fig. 3A). Tam treatment slightly increased transcription in BRCA1 knockdown cells, and responses to E2+Tam were significantly enhanced compared to control cells, indicating that Tam suppression of ERE activity was also decreased. Co-transfection of ERα siRNA dramatically decreased ERE activity. In BRCA1 and ERα double knockdown cells, no stimulation was observed with Tam or E2+Tam, indicating that BRCA1 knockdown-induced loss of Tam suppression required ERα protein.

We next tested ER activity on endogenous genes, including progesterone receptor (PR), c-Myc and cyclin D1, which are E2-stimulated and involved in either proliferation or cell cycle progression (Dubik and Shiu, 1992; Graham et al, 1995; Kenny et al, 1999). In control cells, mRNAs for PR (Fig. 3B), c-Myc (Fig. 3C) and cyclin D1 (Fig. 3D) were decreased 40–50% by Tam, and were either significantly (c-Myc) or moderately increased (PR and cyclin D1) by E2 at these time points. However, in cells transfected with siBRCA1, Tam suppression of all three genes was eliminated and E2 stimulation was enhanced at the same time point. Stimulation by E2+Tam for these genes was also significantly enhanced in BRCA1 knockdown cells, indicating that Tam suppression of E2-stimulated transcription was decreased. There was no Tam stimulation of any endogenous gene. Similar results were observed for cyclin D1 mRNA in ZR-75-1 cells (Fig. 3E). Together, these results show that decreased expression of BRCA1 modulates ER-mediated transcription and abrogated Tam suppression of endogenous ER target genes.

Decreased BRCA1 expression alters ERα–coregualtor association

Because knockdown of endogenous BRCA1 does not affect protein levels of ER coregulators (Fig. 2A), or ERα and ERβ in the presence of ligand (Fig. 2B and 2C), other mechanisms must regulate ER transcriptional activity. BRCA1 can associate not only with ERα, but also with many ER coregulators and general transcription factors, and thus may alter interactions between ERα and regulatory proteins. To evaluate effects of decreased BRCA1 expression on ERα-coregulator interactions, immunoprecipitation assays were performed. Figure 4A shows that in control T47D cells, E2 but not Tam treatment increased association between ERα and the coactivator SRC3 approximately 2.6-fold compared with vehicle-treated cells. In BRCA1 knockdown cells, E2-stimulated ERα-SRC3 association increased 4.1-fold compared with vehicle. Strikingly, Tam treatment also stimulated ERα-SRC3 association by 2.4-fold in BRCA1 knockdown cells. Similar results were observed for ERα-SRC1 interactions in vehicle- and Tam-treated cells, indicating that BRCA1 knockdown allows ERα to associate with coactivators, such as SRC1 and SRC3, in the presence of Tam.

ERα association with corepressors, such as repressor of estrogen receptor activity (REA) (Lin et al, 2003) and SMRT (Fleming et al, 2004) were also tested. Figure 4B shows that in control cells, ERα-REA association decreased by 45% with E2, and increased 2.4-fold with

Tam compared with vehicle. However, in BRCA1 knockdown cells, association between ERα and REA was dramatically decreased in both vehicle- and Tam-treated cells compared with control cells. Similarly, Tam-induced ERα-SMRT association was also decreased in BRCA1 knockdown cells. Taken together, these data suggest that in the absence of BRCA1, Tam-bound ERα aberrantly recruits coactivators, but does not recruit corepressors as efficiently as in control cells.

BRCA1 knockdown alters association of ER coregulators with the cyclin D1 promoter

We next determined if BRCA1 knockdown affects recruitment of ERα and/or its coregulators onto the promoter of an endogenous E2-responsive gene. We chose the cyclin D1 promoter because it plays a critical role in cell cycle progression. Control and BRCA1 knockdown T47D cells were exposed to either 10nM E2 or 1μM Tam for 0–60min. In Figure 5, open squares represent protein association with the promoter in control cells, and solid triangles represent associations in BRCA1 knockdown cells. In the presence of E2 (Fig. 5A), ERα promoter binding exhibited a cyclical pattern in both control and BRCA1 knockdown cells, as observed for other E2-responsive genes (Reid et al, 2003). Overall, E2-ERα recruitment was not significantly changed by decreased BRCA1 expression. Similarly, in the presence of Tam, ERα promoter occupancy was comparable in control and BRCA1 knockdown cells. BRCA1 was significantly associated with the promoter in Tam-treated siCon cells (Fig. 5B); but not in BRCA1 knockdown cells. With Tam treatment, the corepressor SMRT was significantly associated with the promoter in siCon-transfected cells, but not in siBRCA-transfected cells. In contrast, in Tam-treated siCon-transfected cells no significant association of SRC3 or CBP with the promoter was observed (Fig. 5C); whereas, in cells with decreased BRCA1, association of both SRC3 and CBP was dramatically increased. Taken together, chromatin immunoprecipitation results showed that with Tam treatment decreased BRCA1 expression did not significantly alter ERα recruitment on a target gene promoter, but increased recruitment of ER coactivators and decreased recruitment of ER corepressors. This would contribute to lack of suppression of ER-regulated gene transcription, and ultimately cell proliferation, in the presence of Tam.

BRCA1 knockdown upregulates E2-induced MAPK (ERK1/2) and Akt activity

Previous studies showed that BRCA1 overexpression inhibited membrane ER and EGFR signaling in breast cancer cells (Razandi et al, 2004). To explore effects of decreased BRCA1 expression on ER cytoplasmic signaling, we examined control and BRCA1 knockdown cells for MAPK (ERK1/2) and Akt activation in response to E2 or Tam. In control cells, MAPK phosphorylation was stimulated by E2 but not Tam by 15min (Fig. 6A). BRCA1 knockdown significantly enhanced only E2-stimulated MAPK activation in terms of both kinetics and magnitude. E2, but not Tam-induced Akt phosphorylation (S473) was also enhanced in BRCA1 knockdown cells, with E2 activation occurring earlier and to a greater extent (Fig. 6B). These data suggest that endogenous BRCA1 has inhibitory effects on E2-stimulated MAPK and Akt activation. Elevated MAPK or Akt activity could stimulate ERα activity by increasing phosphorylation of serine residues in ERα AF-1 domain, including the well-characterized MAPK-sensitive ERα serine118 (S118) site (Murphy et al, 2004). Figure 6C shows that ERα S118 phosphorylation was stimulated by E2 but not Tam in both control and BRCA1 knockdown cells, but there was no significant

difference with BRCA1 knockdown. The growth factor receptor, HER2, was also examined. No E2- or Tam-induced HER2 phosphorylation was detected in our siCon- or siBRCA1-tranfected cells (Fig. 6D). However, EGF stimulated HER2 phosphorylation in control cells, which was further enhanced in BRCA1 knockdown cells. This is consistent with previous work showing BRCA1 overexpression inhibited EGF-stimulated signaling (Razandi et al, 2004).

Discussion

The development of intrinsic or acquired resistance to ER-directed agents in breast cancer therapy is of great therapeutic interest (Clarke et al, 2001). In this report we found that decreased BRCA1 expression in T47D and ZR-75-1 breast cancer cells results in decreased responsiveness to the anti-estrogen Tam. Previous work showed that Tam promoted mammary epithelial cell proliferation and cancer development in a mouse model of mammary gland Brca1-deficiency (Jones et al, 2005), and defective BRCA1 levels in ovarian cancer cells resulted in differential altered responses to several chemotherapeutic anti-estrogen drugs (Srinivas et al, 2004; Thasni et al, 2008). These data, and studies demonstrating direct suppressive effects of BRCA1 on ER function (Fan et al, 2001), suggest that cellular levels of BRCA1 play a role in breast cancer development, cell proliferation in response to ER ligands, and sensitivity to Tam.

Our data demonstrate that decreasing BRCA1 expression with two separate siRNAs in T47D or ZR-75-1 cells abolished Tam-induced growth inhibition (Fig. 1). Inhibition could be restored after re-expression of BRCA1 protein resistant to siRNA. BRCA1 has a RING finger domain, and may serve as an ubiquitin ligase (Hashizume et al, 2001). Although in vitro studies suggested ERα monoubiqutination, which is not associated with protein degradation, may occur in the presence of BRCA1 (Eakin et al, 2007), we observed no effect of BRCA1 knockdown on protein levels of ERα, ERβ, or ER coregulators (Fig. 2A), or in steady-state ER levels with E2 or Tam (Fig. 2B–C). These data suggest BRCA1 does not directly regulate ER turnover in breast cancer cells.

In the absence of BRCA1, we observed increased ERα transcriptional activity with a luciferase reporter gene (Fig. 3A). This is consistent with previous work showing that ERα transcription on reporter genes was decreased by BRCA1 overexpression (Fan et al, 1999; Zheng et al, 2001). Similarly, BRCA1 globally inhibits the E2 effects on gene expression in breast cancer cells (Xu et al, 2005). Knockdown of endogenous ERα significantly decreased ERE activity and inhibited the stimulatory effect of Tam in the BRCA1 and ERα double knockdown cells, showing that these BRCA1 knockdown-induced Tam effects are ERα-dependent. We examined the effect of BRCA1 knockdown on the transcription of endogenous E2-responsive genes, including PR, c-Myc and cyclin D1 (Fig. 3B–E). These genes are important for mammary gland differentiation and ductal branching throughout the menstrual cycle and pregnancy (PR), or for cell proliferation (c-myc and cyclin D1), and are stimulated by E2-ER through a variety of tethered transcription factors (Dubik and Shiu, 1992; Petz et al, 2004; Sabbah et al, 1999). Overexpression of c-Myc occurs in 20–30% of human breast cancers (Dubik and Shiu, 1992), and cyclin D1 is required for Tam-induced cell cycle progression in human breast cancer cells (Kilker and Planas-Silva, 2006). In T47D

BRCA1 knockdown cells, E2 stimulation of all three genes is enhanced compared to control cells, Tam suppression was eliminated, and Tam suppression of E2-stimulated gene transcription was decreased. Similar results were observed for cyclin D1 in ZR-75-1 cells (Fig. 3E), indicating this is not a cell-specific phenomenon. These data suggest the Tam-resistant phenotype induced by decreased BRCA1 expression may result as least partially from loss of Tam suppression of ERα transcriptional activity. In contrast to the ERE reporter, no Tam stimulation was observed with endogenous genes. Because ERE-luciferase is only a synthetic reporter, it may not faithfully recapitulate all aspects of ER function on cell proliferation. Thus, Tamoxifen-alone stimulation of the reporter can differ from results with BrdU incorporation, or on endogenous mRNAs that are regulated through complex response elements rather than simple EREs (Fig 1 and Fig 3).

Appropriate ERα responses to Tam depend on appropriate function and expression of ER coregulators (de Mora and Brown, 2000; Kurtev et al, 2004). E2-bound ERα stimulates transcription via coactivator recruitment, (de Mora and Brown, 2000), whereas Tam-bound ERα inhibits transcription by recruiting corepressors and preventing coactivator binding (Liu and Bagchi, 2004). Elevated expression or enhanced activity of coactivators and/or decreased expression of corepressors have all been reported to alter Tam responses in tissue culture or human tumors (Girault et al, 2003; Murphy et al, 2000; Shang and Brown, 2002). In our studies, altered ER coregulator expression cannot account for Tam resistance induced by decreased BRCA1, since there were no detectable changes in levels of any tested coactivators or corepressors (Fig. 2A). Instead, our co-immunoprecipitation assays showed that BRCA1 knockdown increased ERα-coactivator (SRC3 and SRC1) association and decreased ERα-corepressor (REA and SMRT) association in the presence of Tam (Fig. 4). We next examined whether recruitment of ERα and its coregulators to the cyclin D1 gene promoter varied with BRCA1 levels. BRCA1 associates with ERα at the promoter of endogenous E2-responsive genes (Zheng et al, 2001), and we observed that Tam treatment resulted in BRCA1 association with the cyclin D1 promoter in control cells, but not in BRCA1 knockdown cells (Fig. 5B). Chromatin immunoprecipitation assays showed that in the absence of BRCA1, Tam treatment significantly increased association of ER coactivators (SRC3 and CBP) but decreased association of the corepressor (SMRT) with the promoter (Figure 5B and 5C). Decreased BRCA1 expression did not significantly alter recruitment of ERα to the cyclin D1 promoter with either E2 or Tam (Fig. 5A), suggesting that it is the altered association of ERα with coregulators that contributes to the inappropriate Tam response.

BRCA1 is a predominantly nuclear protein, but may also play a role in cytoplasmic signaling transduction. Overexpression of wild-type, but not mutant, BRCA1 in MCF-7 and ZR-75-1 cells inhibited MAPK activation and cell proliferation in response to E2, EGF and IGF-1 (Razandi et al, 2004), and crosstalk between ER and growth factor pathways plays an important role in breast cancer cell proliferation (Shupnik, 2004). In addition, upregulation of growth factor signaling modulated BRCA1 suppression of ERα activity, in part through serine phosphorylation of the ERα AF-1 domain (Ma et al, 2007). In this study, BRCA1 knockdown resulted in higher and more rapid E2-stimulated MAPK (ERK1/2) and Akt activation (Fig. 6A and B). This is consistent with a previous report that BRCA1 knockdown in MCF-7 cells increased E2/ER stimulation of MAPK (Razandi et al, 2004). ERα-S118 is a

MAPK substrate (Murphy et al, 2004), and its phosphorylation was detected after E2 treatment (Fig. 6C), but with no significant difference between control and BRCA1 knockdown cells. However, additional targets of MAPK or Akt, such as coregulators, may have increased phosphorylation in BRCA1 knockdown cells, and contribute to altered activity or protein-protein associations between ER and coregulators and/or the transcriptional machinery. HER2 phosphorylation was not stimulated by either E2 or Tam in control or BRCA1 knockdown cells (Fig. 6D), indicating that it was not significantly regulated by ER cytoplasmic signaling in these cells, although other EGFR family members may play a role in this pathway. Tam alone had no effects seen only in BRCA1 knockdown cells.

Because BRCA1 associates with ERα (Fan et al, 2001) and proteins that alter ER function such as CBP/p300 (Fan et al, 2002), RNA polymerase II (Krum et al, 2003; Starita et al, 2005) and histone modification enzymes (Yarden and Brody, 1999), BRCA1 may play a functional role in associations between these molecules. With BRCA1 knockdown, functional proximity between ERα and its coregulators or the physical sites of protein interaction may be altered, leading to inappropriate recruitment of coregulators by ERα onto target gene promoters. In the presence of Tam, these inappropriate protein-protein associations on the promoter would lead to altered transcriptional activity of ERα and gene transcription that ultimately leads to upregulated cell proliferation, or failure to be suppressed by Tam. Thus, intrinsic resistance to Tam in some ER-positive breast cancers may be due to reduced BRCA1 in these tumors. It will be interesting for future studies to test similar pathways for additional SERMS or therapies that target ER. Based on these findings, BRCA1 may serve as a potential additional biomarker for prediction of hormonal sensitivity of ER-positive breast cancer.

Materials and Methods

Cell Culture

T47D human breast carcinoma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (Life Technologies, Grand Island, NY). ZR-75-1 human breast carcinoma cells were cultured in RPMI-1640 (Invitrogen, Grand Island, NY) with 10% FBS, 1% penicillin/streptomycin (Invitrogen). For all experiments, cells were plated and treated in phenol red-free medium supplemented with charcoal-stripped serum. Cells were maintained at 37°C in 5% CO_2/95% air.

Antibodies and Reagents

Anti-ERα antibody (HC-20) was from Santa Cruz Biotechnology (Santa Cruz, CA) or (1D5) Dako (Carpinteria, CA), anti-ERβ was from Upstate (Lake Placid, NY). Anti-BRCA1 (Ab-1) was from CalBiochem (San Diego, CA). Anti-SRC3 and anti-CBP were from Santa Cruz Biotechnology. Anti-SRC1 was from Affinity BioReagents (Golden, CO), anti-SMRT from BD Biosciences (San Jose, CA) and anti-REA from Novus Biologicals (Littleton, CO). Antibodies for phospho-ERα (S118), total and phospho-MAPK (p44/42), total and phospho-Akt (S473) were from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies for total

and phospho-HER2 (Y1248) were from Santa Cruz and Invitrogen. 5-Bromo-2'Deoxyuridine was from Sigma-Aldrich Corp (Saint Louis, MO) and anti-BrdU antibody was from Molecular Probes (Eugene, OR). 17β-estradiol and 4-hydroxytamoxifen were from Sigma-Aldrich Corp.

siRNA Transfection and Immunoblotting

To knockdown BRCA1 expression, siGENOME siRNA reagents, D-003461-03 (DO3) and D-003461-07 (DO7) (Dharmacon Inc. Chicago, IL), targeting human BRCA1 mRNA were used as described (Hu et al, 2005). ERα siRNA, siERα (003401) and non-targeting siRNA, siCon (D-001210-01-20) were from Dharmacon Inc. T47D and ZR-75-1 cells (4×10^6) were transfected with 2μg of either siCon or siBRCA1 by nucleofection and plated into 35mm wells (Fox et al, 2008). Forty-eight hours post-transfection, cells were collected in lysis buffer (31.25mM Tris, pH=6.8, 12.5% glycerol, 1% SDS) plus protease inhibitors. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Protein lysates (50μg) were separated by gradient (5–10%) SDS-PAGE and transferred to nitrocellulose membranes. Specific proteins were detected as described (Fox et al, 2008).

Bromodeoxyuridine Incorporation

T47D and ZR-75-1 cells (4×10^6) were nucleofected with 2μg of either siCon or siBRCA1 and 2μg of green fluorescent protein (GFP) as a transfection marker. Cells were grown on Poly-L-lysine (Sigma-Aldrich)-coated cover slips, and allowed to proliferate for 36h followed by serum starvation overnight. Cells were then treated with ethanol vehicle, 10nM E2, 1μM or 10μM Tam for 24h. Bromo-deoxyuridine (BrdU) was added during the last 4h, and cells were fixed, stained and scored for BrdU incorporation as described (Fox et al, 2008). Experiments were repeated three times.

Lentiviral Gene Expression

Lentiviral stocks were produced in 293T cells (provided by Dr. Anindya Dutta) by transfecting constructs (vector and BRCA1-DO7 silent mutant), packaging plasmid (pMD2.G), and envelope plasmid (psPAX2) using Lipofectamine-2000 (Invitrogen). Virus was harvested 48h post-transfection, filtered through a 0.45μm filter, and stored at 4°C. Before infection, 4×10^6 T47D or ZR-751 cells were nucleofected with siRNA and GFP, and plated onto coated cover slips for measuring BrdU incorporation or into 35mm wells for protein expression. Titering experiments were performed to determine virus amounts needed to rescue BRCA1 expression. Twenty-four hours after nucleofection, virus was applied to cells grown in phenol-red free DMEM containing stripped serum and polybrene (8μg/ml) (Millipore, Phillipsburg, NJ). Cells were incubated at 37°C for 16h before serum starvation overnight and subsequent treatment and BrdU incorporation.

Luciferase Assay

T47D cells (4×10^6) were nucleofected with 2μg of total siRNA and 1μg of pGL3-2ERE luciferase reporter plasmid (Schreihofer et al, 2001), then plated into 22mm wells. After 24h, cells were treated with ethanol vehicle, 10nM E2, 1μM Tam or E2+Tam for 24h, then

collected and assayed for luciferase activity (Tsai et al, 2004). Each treatment was in triplicate, for three experiments.

Real-time RT-PCR

T47D and ZR-75-1 cells (4×10^6) were nucleofected with 2μg of siCon or siBRCA1, then plated into 60mm wells and incubated for 24h followed by serum starvation overnight. Time course experiments were performed with ethanol vehicle, 10nM E2, 1μM Tam or E2+Tam for 1h, 4h and 24h. Time points were chosen for each gene based on maximum Tam suppressive effects. RNA was extracted and reverse-transcribed into cDNA, which was used for real-time RT-PCR (Fox et al, 2008). β-actin was measured as a control and was unaffected by BRCA1 knockdown or treatments. PCR conditions and cycle numbers were adjusted so each reaction fell within the linear range of product amplification. Primers for cyclin D1, c-Myc and β-actin were described (Fox et al, 2008). PR primers are: forward: 5'-ACAGGACCCCTCCGACGAAAA-3'; reverse: 5'-GGTGCAAGGTTGGAGACAGCT-3'.

Immunoprecipitation and phosphorylation studies

Cells (4×10^6) were nucleofected with 2μg of siCon or siBRCA1 then plated into 10cm dishes. After 48h, cells were treated with ethanol vehicle, 10nM E2 or 1μM Tam for 45min, resulting in maximal association of ER and coregulators with least amount of ER degradation in the presence of E2. Cells were collected in 250μl Mammalian Protein Extraction Reagent (Pierce Chemical Co.) plus protease inhibitors. Protein concentration was measured using the bicinchoninic acid protein assay. Immunoprecipitation was performed by overnight incubation of anti-ERα (HC20) with lysates containing equivalent amounts of ERα protein determined by western blot, then adding protein G-agarose (Santa Cruz) and incubation at 4°C for 1h. Agarose pellets were washed three times with RIPA buffer, and bound proteins eluted by boiling for 5min in buffer (250mM Tris-HCl pH=6.8, 4% SDS, 10% β-ME, 20% glycerol). Eluted proteins were separated on a 5–10% gradient SDS-PAGE and transferred onto nitrocellulose membranes for immunoblotting.

For phosphorylation studies, cells were collected and lysed in gel loading buffer plus protease inhibitors and sodium orthovanadate, then immediately boiled at 95°C for 5min. Protein lysate (20μg) was separated by 10% SDS-PAGE (for MAPK, Akt and ERα), or (for HER2, 80 μg) by 5% SDS-PAGE, then immunoblotted for total and phosphorylated proteins.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed as described (Curtin et al, 2004). Cross-linked chromatin lysates were sonicated and diluted with ChIP sonication buffer plus protease inhibitors (0.2μg/μl Leupeptin and aprotinin, 20mM PMSF), divided and incubated with normal rabbit serum, antibodies to ERα (HC20), BRCA1, SMRT, SRC3 or CBP at 4°C overnight, then precipitated with protein G-agarose. Cross-linking was reversed by overnight 65°C incubation. DNA fragments were extracted with a PCR purification kit (Qiagen) and quantitated by real-time PCR as described (Ferris et al, 2007). Cyclin D1 promoter primers were: forward 5'-TCTTGGGCTGCTGCTGCTGGAAT-3'; reverse 5'-GTCGTTGCAAATGCCCAAGG-3'. Experiments were performed in duplicate, three times.

Statistical Analyses

Statistical significance was determined by 2-way ANOVA using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA) and Bonferroni post-hoc analysis, with $P < 0.05$ considered significant.

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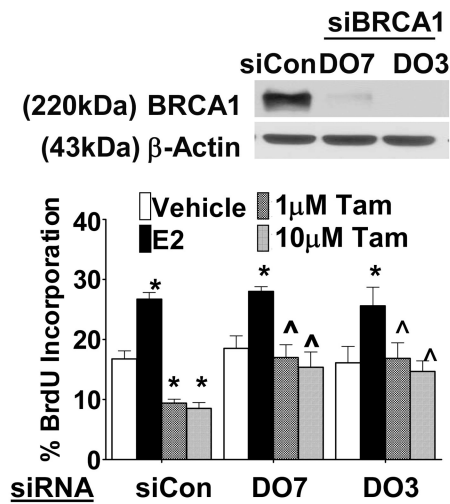
A T47D Cells

B T47D Cells
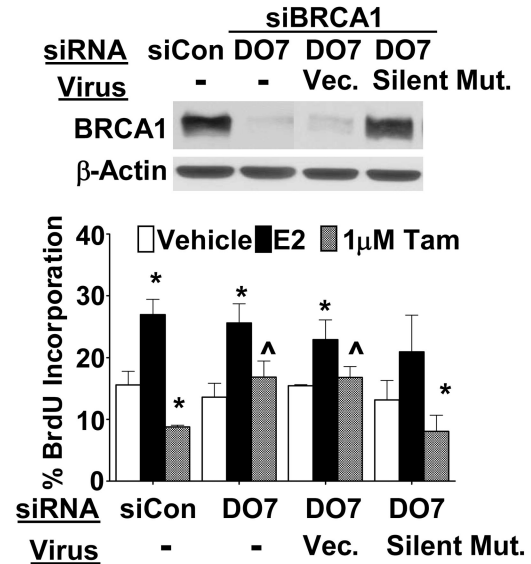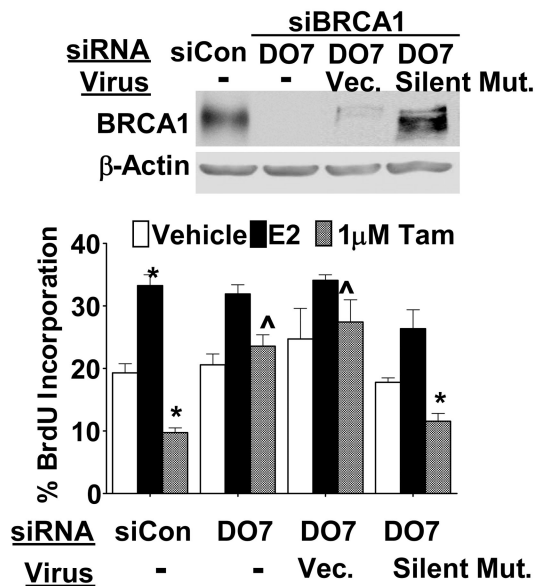

C ZR-75-1 Cells

Figure 1. BRCA1 siRNA knockdown alleviates Tam suppression of cell proliferation
(A) T47D cells (4×10^6 cells) were nucleofected with 2μg of control siRNA (siCon) or BRCA1 siRNA (siBRCA1, DO3 or DO7 oligonucleotides) together with 2μg of GFP expression vector. After 36h, cells were serum starved overnight then treated with ethanol vehicle (V), 10nM E2, 1μM or 10μM Tam for 24h. BrdU was added during the last 4h of treatment. BRCA1 protein levels are shown in western blots insets. (B) T47D cells (4×10^6 cells) were transfected as in (A). Twenty-four hours later, DO7-transfected cells were infected with Lentivirus containing either empty vector (Vec) or the BRCA1 DO7 silent

mutation (silent mut). Sixteen hours after infection, cells were serum starved overnight then treated with vehicle, 10nM E2 or 1μM Tam for 24h and scored for BrdU incorporation. (C) ZR-75-1 cells (4×10^6 cells) were transfected as in (A). Cells were then infected with Lentivirus and BrdU incorporation was measured as described in (B). All BrdU results are the mean of 3 experiments; a representative blot is shown. Two-way ANOVA was used to determine statistical significance. *, P <0.05 treatment vs. vehicle; ^, P<0.05 siBRCA1 vs. siCon group for the same treatment.
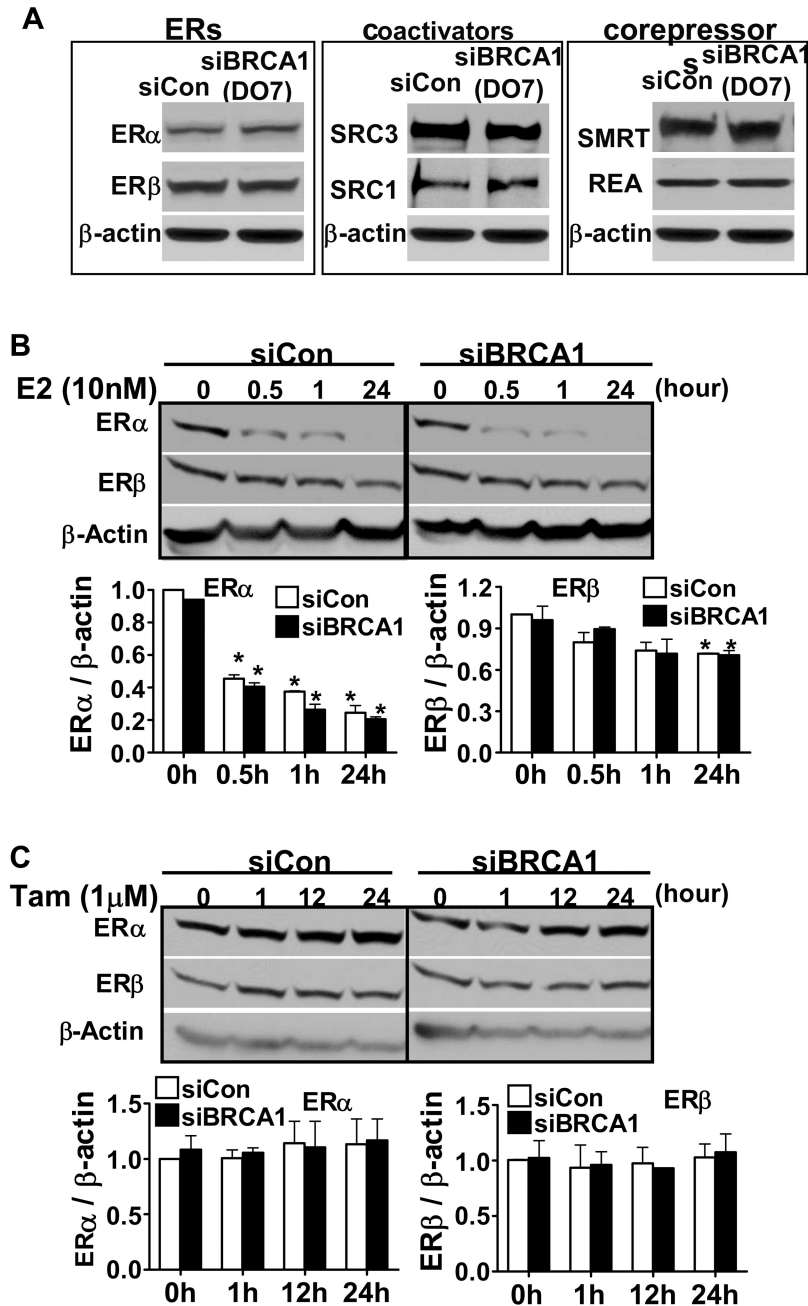
Figure 2. BRCA1 knockdown did not alter ER coregulator protein levels or ER levels in the presence of ligand

(A) T47D cells (4×10^6 cells) were nucleofected with 2μg of either siCon or siBRCA1 (DO7). After 48h, cells were collected and lysed in SDS lysis buffer plus protease inhibitors. Total protein lysate (50μg) was separated by 5–10% gradient SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with indicated antibodies. (B) T47D cells were nucleofected with siCon or siBRCA1 (DO7). Forty-eight hours later, cells were treated with 10nM E2 for 0h, 0.5h, 1h and 24h, or treated with 1μM Tam for 0h, 1h, 12h and 24h and then lysed in SDS lysis buffer plus protease inhibitors. Protein lysate (20μg) was separated

by 8% SDS-PAGE, and probed for ERα, ERβ and β-actin. Intensities of ERα, ERβ and β-actin bands on individual films were measured by densitometry and analyzed with ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA). ERα and ERβ protein levels were normalized to β-actin in each sample, and ERα and ERβ protein levels in each sample were expressed relative to the vehicle-treated siCon-transfected cells, which were set at 1.0. Each experiment was repeated at least three times. Representative blots from one of three independent experiments are shown. Two-way ANOVA was used to determine statistical significance. *, $P < 0.05$ treatment vs. vehicle control.
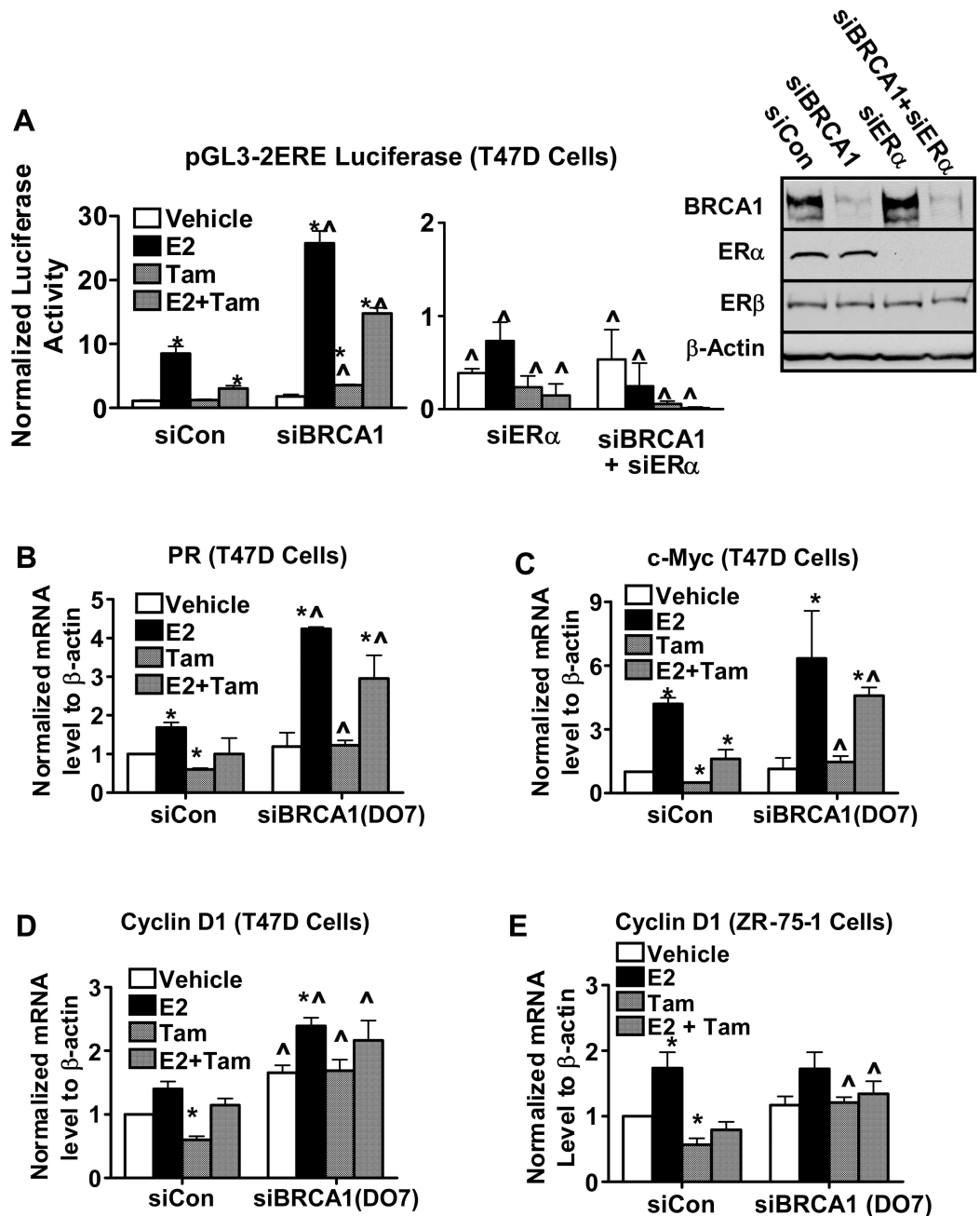
Figure 3. BRCA1 knockdown alters ERα transcriptional activity
(A) T47D cells (4×10^6 cells) were nucleofected with 2μg total siRNA (Either 2μg of siCon, 1μg of siCon + 1μg of siBRCA1 (DO7), 1μg of siCon + 1μg of siERα, or 1μg of siBRCA1 + 1μg of ERα) along with 1μg of pGL3-2ERE luciferase reporter. Twenty-four hours after transfection, cells were treated with ethanol vehicle (V), 10nM E2, 1μM Tam or E2+Tam for 24h, then collected and assayed for luciferase activity. BRCA1, ERα, ERβ and β-actin protein levels are shown in western blots insets. (B–D) The mRNA levels of endogenous E2-responsive genes PR, c-Myc and cyclin D1 were measured in T47D cells

using real-time RT-PCR. (E) Cyclin D1 mRNA measured in ZR-75-1 cells. T47D and ZR-75-1 cells (4×10^6 cells) were nucleofected with 2µg of either siCon or siBRCA1 (DO7). Forty-eight hours after transfection, cells were serum starved overnight and treated with ethanol vehicle (V), 10nM E2, 1µM Tam or E2+Tam for 1h (c-Myc mRNA) or 4h (PR and cyclin D1 mRNA). Values were normalized for β-actin mRNA level measured by real-time RT-PCR in the same samples. Two-way ANOVA was used to determine statistical significance. *, $P < 0.05$ treatment vs. vehicle, ^, $P < 0.05$ siBRCA1 vs. siCon group for the same treatment.
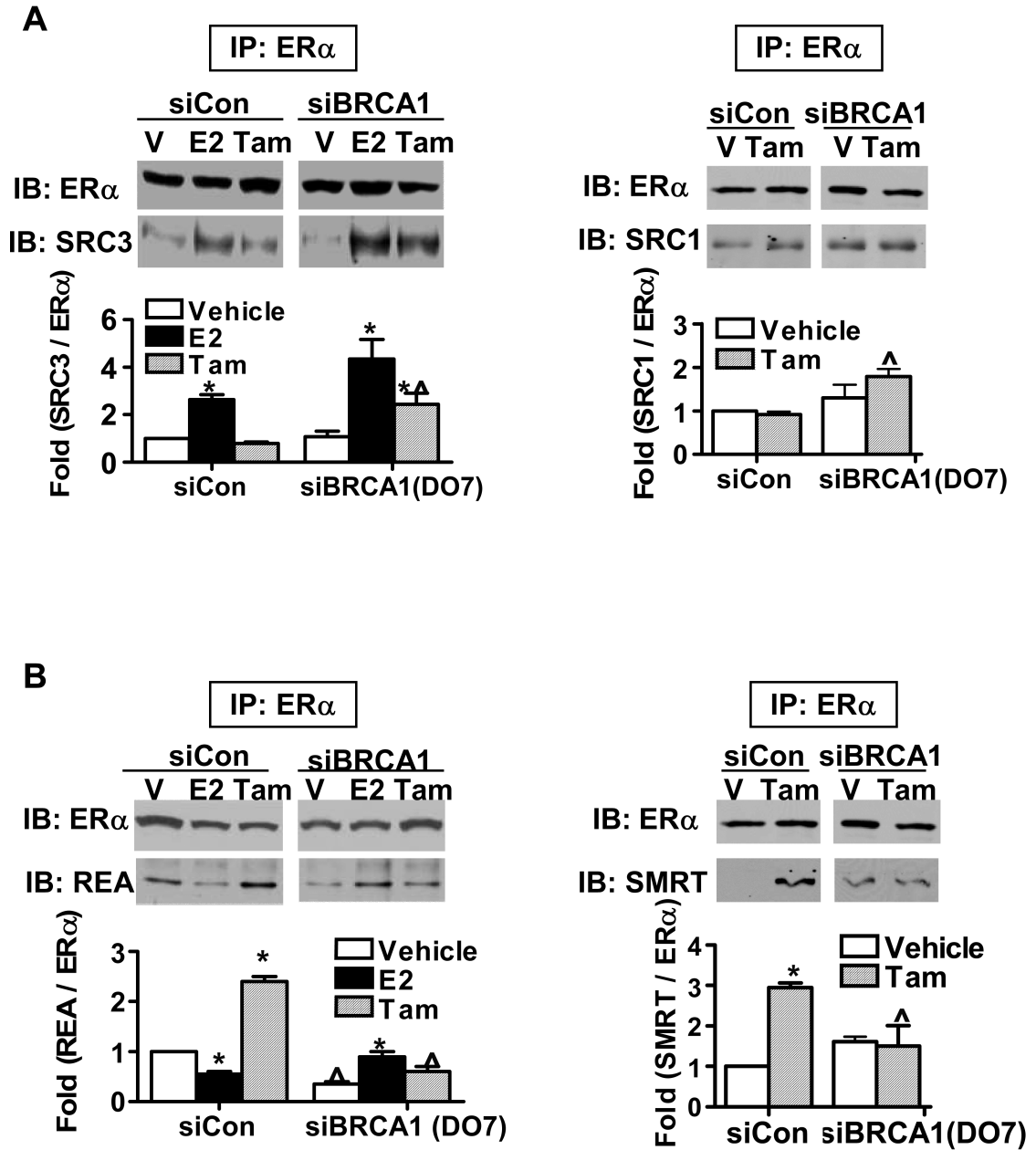
Figure 4. BRCA1 knockdown alters ER-coregulator interaction in the presence of Tam
T47D cells (4×10^6 cells) were nucleofected with 2µg of either siCon or siBRCA1 (DO7) and incubated for 48h, followed by treatment with ethanol vehicle (V), 10nM E2 or 1µM Tam for 45min. Lysates were immunoprecipitated (IP) with polyclonal anti-ERα antibody (HC20) and immunoblotted (IB) with antibodies against ERα (monoclonal antibody: 1D5), ER coactivators SRC3 or SRC1 (A) or ER corepressors REA and SMRT (B). Blots shown are representative of 3 experiments; densitometric results (bars) were normalized from at least three experiments. Two-way ANOVA was used to determine statistical significance. *, $P<0.05$ treatment vs. vehicle, ^, siBRCA1 vs. siCon group for the same treatment.
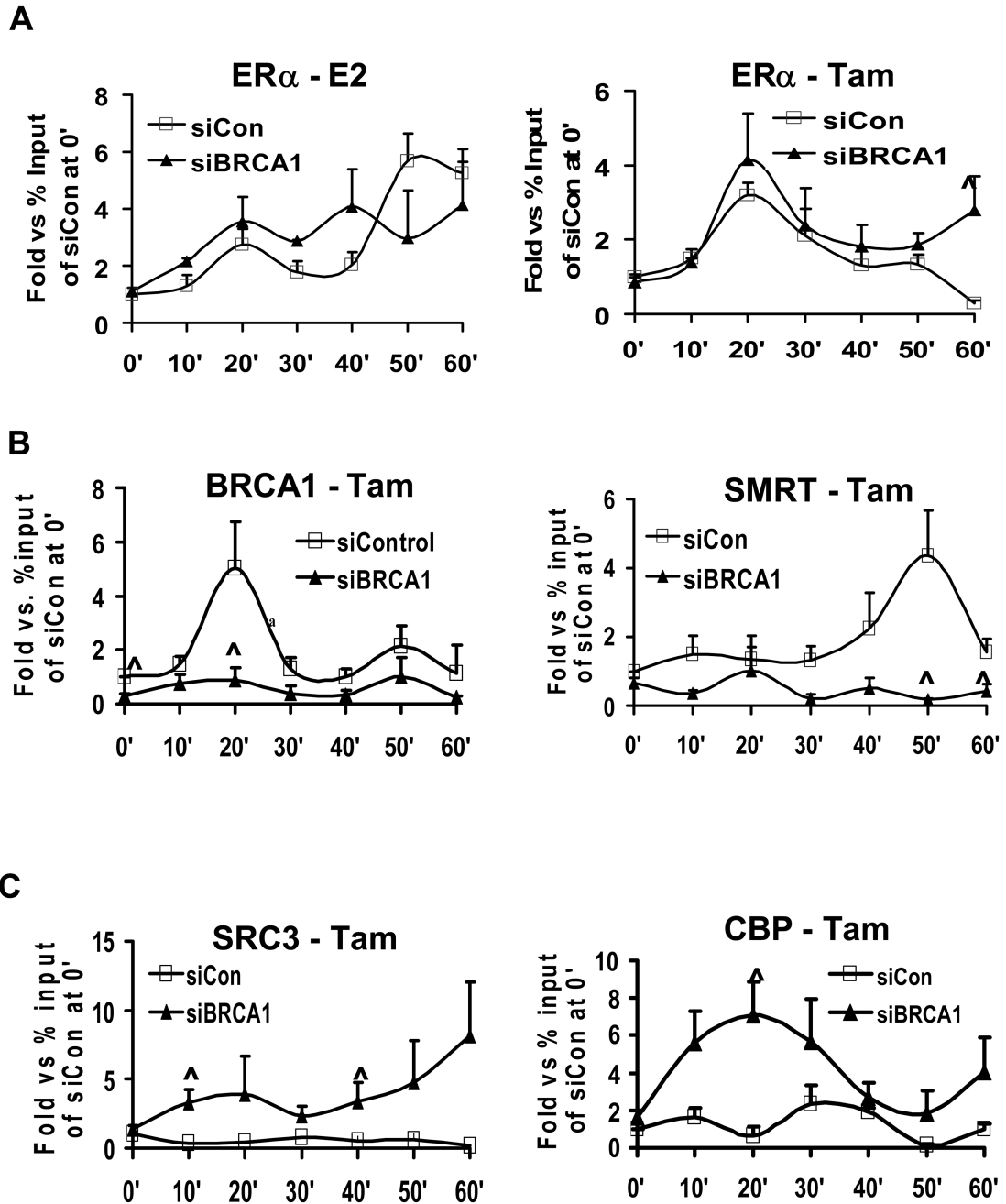
A

B

C

Figure 5. BRCA1 knockdown alters recruitment of ER coregulators to the cyclin D1 promoter
T47D cells (4×10^6 cells) were nucleofected with 2ug of siCon or siBRCA1 (DO7) and incubated for 48h, followed by treatment with ethanol vehicle (V), 10nM E2 or 1μM Tam for 0 to 60min. Chromatin immunoprecipitation was performed using antibodies for ERα (A), BRCA1 or SMRT (B), and SRC3 or CBP (C). Immunoprecipitated DNA was quantitated by real-time PCR. PCR results were analyzed by standard curve generated from a series of input dilutions, and expressed as the fold change of the percentage of total input DNA vs. that of siCon at 0min. Experiments were performed with duplicate samples in a

minimum of three independent experiments, and values represent the mean ± SEM of 3 independent experiments. Two-way ANOVA was used to determine statistical significance. *, $P < 0.05$ treatment vs. vehicle, ^, $P < 0.05$ siBRCA1 vs. siCon group for the same treatment.
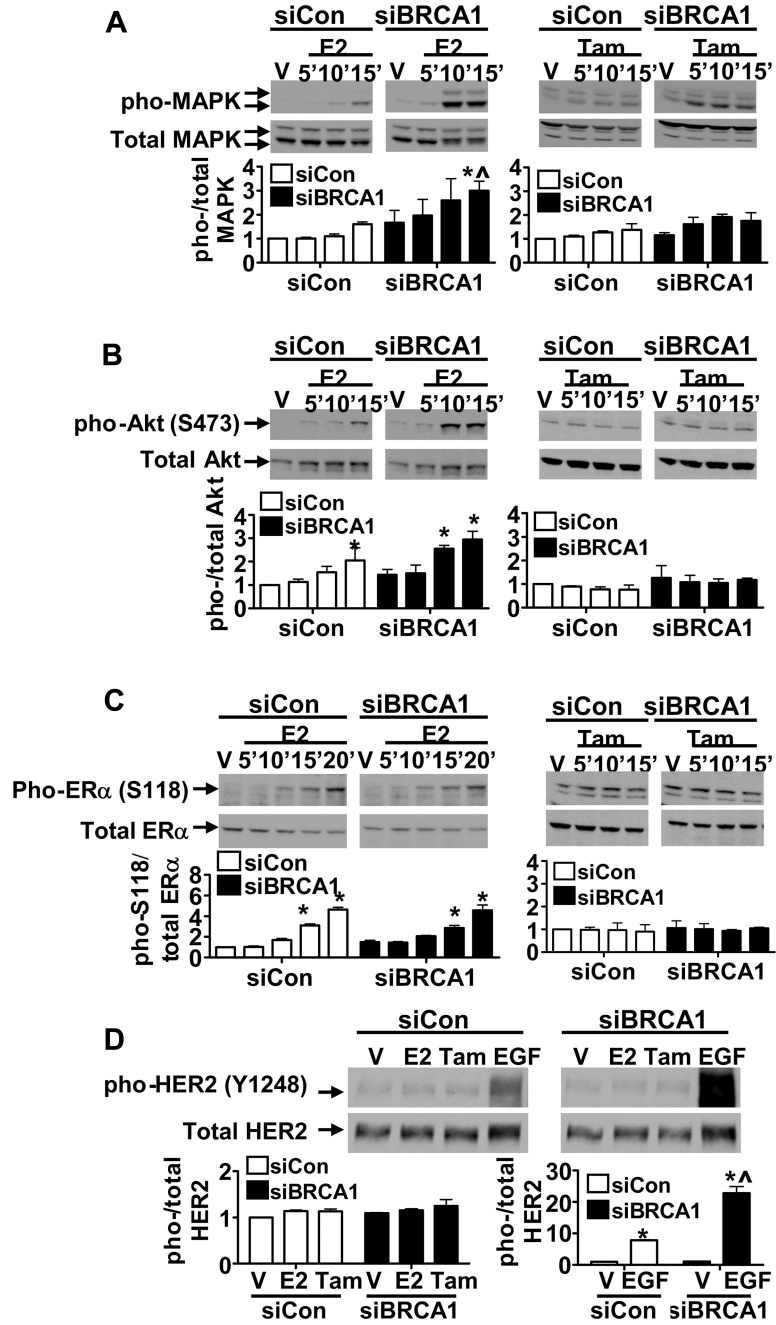
Figure 6. BRCA1 knockdown upregulates E2-induced MAPK (ERK1/2) and Akt activation
T47D cells (4×10^6 cells) were nucleofected with 2μg of either siCon or siBRCA1 (DO7) and incubated for 48h, followed by treatment with ethanol vehicle (V), 10nM E2, or 1μM Tam for 5, 10 or 15min as shown. Protein lysate (20μg) was separated by 10% SDS-PAGE and immunoblotted for total and phosphorylated MAPK (ERK1/2) (A), total and phosphorylated Akt (S473) (B), total and phosphorylated ERα (S118) (C) and β-actin as a loading control (not shown). (D) Forty-eight hours after nucleofection, both control and BRCA1 knockdown cells were treated with ethanol vehicle (V), 10nM E2 or 1μM Tam for

15min, or 100ng/ml hEGF for 3min. Protein lysate (80μg) was separated by 5% SDS-PAGE and immunoblotted for total and phosphorylated HER2 (Y1248). Blots shown are representative examples of three individual experiments. Bars represent densitometric analysis of bands expressed as phosphorylated protein levels normalized for total immunopositive protein (kinase, ERα, or HER2 protein levels) from three experiments, and expressed relative to siCon, vehicle-treated cells (1.0). Two-way ANOVA was used to determine statistical significance. *, $P < 0.05$ treatment vs. vehicle, ^, $P < 0.05$ siBRCA1 vs. siCon group for the same treatment.