

Breast Cancer Susceptibility Gene 1 (*BRCA1*) Is a Coactivator of the Androgen Receptor¹

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Abstract

In the present study, the role of *BRCA1* in ligand-dependent androgen receptor (AR) signaling was assessed. In transfected prostate and breast cancer cell lines, *BRCA1* enhanced AR-dependent transactivation of a probasin-derived reporter gene. The effects of *BRCA1* were mediated through the NH₂-terminal activation function (AF-1) of the receptor. Cotransfection of p160 coactivators markedly potentiated *BRCA1*-mediated enhancement of AR signaling. In addition, *BRCA1* was shown to interact physically with both the AR and the p160 coactivator, glucocorticoid receptor interacting protein 1. These findings suggest that *BRCA1* may directly modulate AR signaling and, therefore, may have implications regarding the proliferation of normal and malignant androgen-regulated tissues.

Introduction

Women who inherit loss-of-function germ-line mutations in *BRCA1*⁵ have an increased lifetime risk of developing breast and/or ovarian tumors (1). The *BRCA1* gene product is a nuclear phosphoprotein with putative roles in DNA repair, cell cycle control, and transcriptional regulation (2). There is some evidence to suggest that wild-type *BRCA1* functions indirectly or directly in the regulation of endocrine signaling pathways: (a) although every cell in an affected individual possesses the same germ-line *BRCA1* mutation, tumors arise exclusively in the breast and ovary, two hormone-regulated tissues; (b) wild-type *BRCA1* inhibits estrogen receptor α signaling in transfected breast and prostate cancer cell lines (3); and (c) breast cancer penetrance among *BRCA1* mutation carriers is modified by allele variation at the *AR* locus (4). Because of the importance of AR signaling in the regulation of prostate and mammary epithelial cell proliferation, we investigated the potential role of *BRCA1* in ligand-

dependent AR transactivation. Herein, we show that *BRCA1* enhances AR signaling in both prostate and breast cancer cell lines, especially in the presence of exogenous p160 coactivator. We further present *in vitro* evidence that *BRCA1* makes direct contacts with the AR and with the p160 coactivator, GRIP1.

Materials and Methods

Plasmids. Mammalian expression or reporter plasmids pCMV-hAR (5), pSG5-GRIP1 and pSG5-SRC-1a (6), pcDNA3.1-AIB1 (7), ARR₃tk-CAT (8), and pcDNA-hAR(Q)_n (9) were described previously. To construct vector pcDNA-AR (NTD-DBD), an *NheI*-*Bam*HI fragment was PCR amplified from pcDNA-hAR (9) plasmid DNA using primer pairs S1 (5'-GTGGGCAGCTAGCTGCAGCGACTAC-3') and AS1 (5'-ATGGAGG-GATCCTCAGGTGCTGGAAGCCTCTCCTC-3') and inserted into the reciprocal restriction sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Vector pcDNA-AR (DBD-LBD) was constructed in sequential cloning steps: (a) an *NheI*-*KpnI* PCR fragment containing the AR Kozak sequence was amplified using primers S1 and AS2 (5'-ACCTAAGGTACCCCTAACTGCACTTC-CATCCT-3') and inserted into the corresponding sites of pcDNA3.1(+); (b) a *KpnI*-*Eco*RI PCR fragment was amplified using primers S2 (5'-AATCGCG-GTACCCGTTTGGAGACTGCCAGGGACCAT-3') and AS3 (5'-GGAAAT-GAATTCG-GGGAAATAGGGTTTCCAAT-3') and inserted into the restored *KpnI* site and the downstream *Eco*RI site of the pcDNA3.1(+) multiple cloning site. *BRCA1* mammalian expression vector pcDNA-*BRCA1* was constructed by inserting a *NotI*-*XhoI* treated *BRCA1* fragment derived from pBSK-1hFL plasmid (10) into the corresponding restriction sites of pcDNA3.1/mycHisC(-) vector (Invitrogen). Bacterial expression plasmids encoding GST, GST-AR, and GST-GRIP1 fragments were described previously (6, 11).

Tissue Culture and Transfections. Cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in RPMI (PC-3, DU-145, and HBL-100 cells) or DMEM (MCF-7 cells) medium that contained 10% FBS. Approximately 24 h prior to transfection, 10⁶ (PC-3, DU-145, and HBL-100) or 5 × 10⁵ (MCF-7) cells were seeded into each 60-mm dish. Cells were transfected in serum-free conditions with Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's protocol. In each experiment, the total amount of DNA per dish was held constant by the addition of pcDNA3.1(+) vector when appropriate. After transfection, cells were grown for 24 h (DU-145, HBL-100, and MCF-7) or 48 h (PC-3) in RPMI 1640 (without phenol red) that contained 5% charcoal/dextran-stripped FBS (Gemini Bio Products, Calabasas, CA) and, where indicated, DHT (1 or 10 nM) for the last 24 h of growth. Whole-cell extracts were prepared in 0.25 M Tris-HCl (pH 8.0) by repeated freezing and thawing. CAT assays were performed using the Quan-T-CAT kit (Amersham Pharmacia Biotech, Piscataway, NJ), and total cellular protein was measured using the Bio-Rad (Hercules, CA) Protein Assay kit. Relative CAT activities (cpm/A₆₀₀) are reported as the mean + SE of three independent dishes.

GST Pull-Downs. GST and GST-fusion proteins were expressed and purified as described previously (12). Glutathione-Sepharose-bound GST protein, GST-AR (1–555), or GST-GRIP1 fragments (5–765, 563–1121, or 1121–1462) were incubated with ³⁵S-radiolabeled full-length *BRCA1* transcribed and translated *in vitro* from pcDNA3.1 vectors. Associated *BRCA1* was eluted,

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⁵ The abbreviations used are: *BRCA1*, breast cancer susceptibility gene 1; AIB1, amplified in breast cancer-1; AF, activation function; AR, androgen receptor; CAT, chloramphenicol acetyltransferase; DBD, DNA binding domain; DHT, dihydrotestosterone; FBS, fetal bovine serum; GRIP1, glucocorticoid receptor interacting protein 1; GST, glutathione S-transferase; LBD, ligand binding domain; NLS, nuclear localization signal; NR, nuclear receptor; NTD, NH₂-terminal domain; SRC-1a, steroid receptor coactivator 1a.

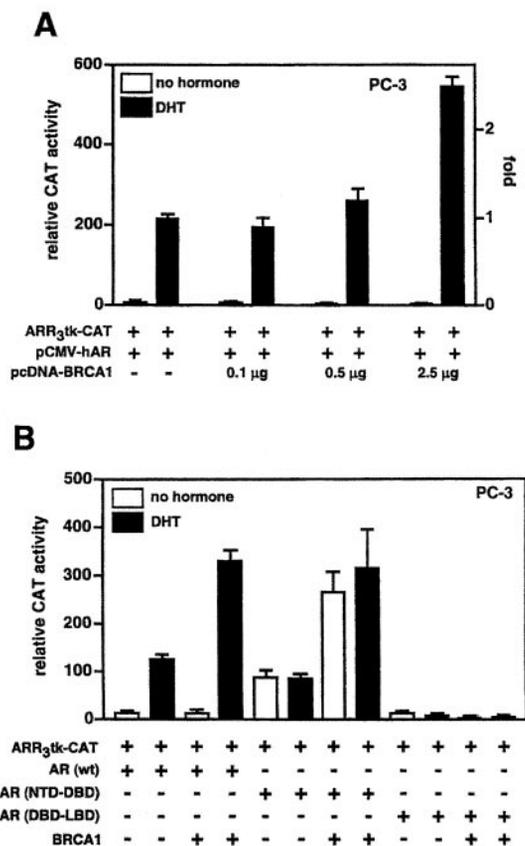


Fig. 1. BRCA1 potentiates AR signaling. *A*, wild-type BRCA1 coactivates AR transactivation in PC-3 prostate cancer cells. Transiently transfected cells were assayed for stimulation of ARR₃tk-CAT reporter activity by DHT. ARR₃tk-CAT is composed of a minimal thymidine kinase (*tk*) promoter under the control of three identical fragments of the rat probasin promoter (nucleotides -244 to -96), each comprising two androgen receptor binding sites (*i.e.*, ARBS-1 and ARBS-2; Ref. 8). Cells were cotransfected with 2.0 μg of ARR₃tk-CAT, 50 ng of pCMV-hAR, and increasing amounts of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and data presented are the means of three independent dishes; bars, SE. Fold is measured relative to DHT-dependent AR activity with no transfected BRCA1. *B*, BRCA1 works through AR AF-1. PC-3 cells were cotransfected with 50 ng of pCMV-hAR, 10 ng of pcDNA-AR (NTD-DBD), or 0.5 μg of pcDNA-AR (DBD-LBD), 2.0 μg of ARR₃tk-CAT, and 2.5 μg of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and data presented are the means of three independent dishes; bars, SE. Mammalian expression vectors pcDNA-AR (NTD-DBD) and pcDNA-AR (DBD-LBD) encode AR amino acids 1–647 and 538–919, respectively. AR (NTD-DBD) is a constitutive activator of ARR₃tk-CAT, and thus, potentiation of its activity by BRCA1 is ligand independent.

resolved by SDS-PAGE, and analyzed by autoradiography. Ten % of the total labeled BRCA1 incubated in each reaction was loaded for comparison.

Western Analysis. Approximately 24 h prior to transfection, 5×10^5 PC-3 cells were seeded into each 16-mm well. Cells were transfected with the Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. After transfection, cells were grown for 48 h in RPMI 1640 (without phenol red) that contained 5% charcoal/dextran-stripped FBS and, where indicated, 10 nM DHT. Transfected cells were harvested in RIPA buffer that contained mammalian protease inhibitors. Total cellular protein was measured by the BCA Protein Quantification Assay (Pierce, Rockford, IL), and equal amounts of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham-Pharmacia Biotech, Piscataway, NJ) and probed with rabbit polyclonal anti-AR antibody N20 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml.

Results

BRCA1 Enhances AR Signaling. To assess the role of BRCA1 in AR signaling, prostatic carcinoma cells (*i.e.*, PC-3 cells) were cotransfected with AR and BRCA1 expression vectors, as well as with the ARR₃tk-CAT probasin reporter. A 2.5-fold DHT-dependent potenti-

ation of AR transactivation activity was observed with 2.5 μg of transfected pcDNA-BRCA1 vector (Fig. 1A). BRCA1 had no effect on AR signaling in the absence of DHT (Fig. 1A), and it failed to activate the reporter gene in the absence of exogenous AR (data not shown).

BRCA1 Works through AR AF-1. The AR, like all NRs, comprises three structural/functional domains: a poorly conserved NTD; a highly conserved DBD; and a COOH-terminal LBD (13). Both the NTD and LBD contain activation functions (*i.e.*, AF-1 and AF-2, respectively) that mediate the transcriptional activation potential of the receptor. To determine which AF primarily is involved in BRCA1-mediated coactivation of the AR, AR constructs containing either AF-1 (NTD-DBD) or AF-2 (DBD-LBD) were coexpressed in PC-3 cells with BRCA1. BRCA1 enhanced the constitutive transactivation activity of AR (NTD-DBD) nearly 3-fold, but failed to enhance AR (DBD-LBD) activity in either the presence or absence of DHT (Fig. 1B). Thus, BRCA1 can potentiate AR signaling through functional interactions with AR AF-1. It is important to note that BRCA1-dependent potentiation of AR signaling is not attributable to increased AR protein expression in the presence of coexpressed BRCA1 (Fig. 2).

BRCA1 and the p160 Coactivators Synergistically Potentiate AR Signaling in Prostate and Breast Cancer Cell Lines. The p160 coactivators are a family of M_r 160,000 nuclear proteins that bind to NRs and potentiate ligand-dependent receptor signaling by recruiting to the target promoter a large, multisubunit coactivator complex that possesses histone acetyltransferase activity (14). The p160 coactivators interact with and coactivate the AR through both of its AFs (6). To determine whether BRCA1 plays a role in p160-mediated coactivation of the AR, PC-3 cells were cotransfected with expression vectors for the AR, BRCA1, and/or the p160 coactivators GRIP1, SRC-1a, and AIB1. As expected, BRCA1 and GRIP1 individually enhanced AR transactivation of the ARR₃tk-CAT reporter about 2- and 3-fold, respectively (Fig. 3A). When coexpressed, however, AR transactivation activity was enhanced 12-fold. This combined BRCA1-GRIP1 coactivation of AR signaling was synergistic because it was greater than the additive effects of BRCA1 and GRIP1 measured independently. Similar results were obtained when either SRC-1a or AIB1 were used, suggesting a generic BRCA1-p160 functional interaction (Fig. 3A).

To rule out the possibility that the observed BRCA1 effects on AR signaling were specific to PC-3 cells, an additional prostate carcinoma cell line (*i.e.*, DU-145), an SV40-transformed breast epithelial cell line (*i.e.*, HBL-100), and a breast cancer cell line (*i.e.*, MCF-7), were used in cotransfection experiments (Fig. 3B). In DU-145 cells, as in PC-3 cells, BRCA1 and GRIP1 individually enhanced DHT-depend-

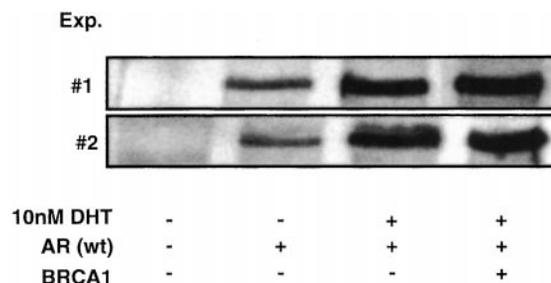


Fig. 2. AR protein expression is not stabilized by BRCA1 in transfected cells. PC-3 cells were cotransfected with 50 ng of pCMV-AR and 250 ng of pcDNA-BRCA1 as indicated. Forty-eight h after transfection, whole-cell extracts were prepared, and equivalent amounts of each extract were probed with anti-AR antibody. Autoradiograms from two independent experiments are presented. Radiographic bands were analyzed by scanning densitometry using a Bio-Rad Model GS-710 imaging densitometer. No significant increase in AR band density was observed in the presence of BRCA1 (data not shown).

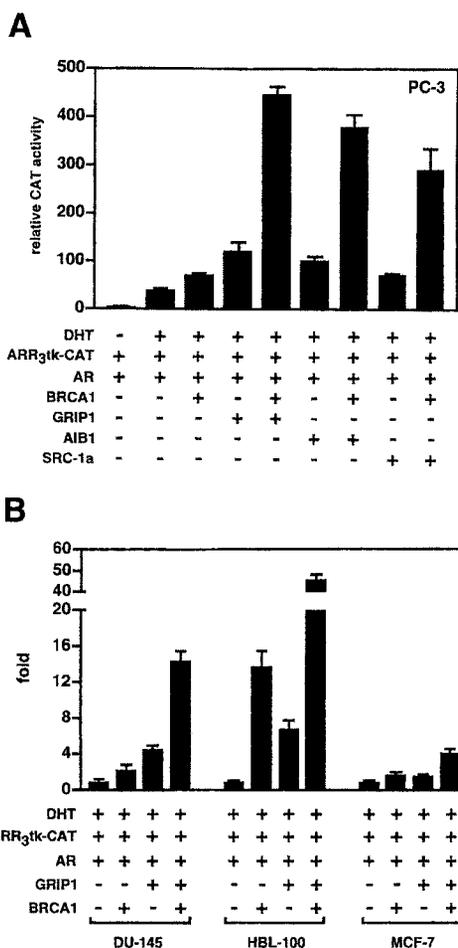


Fig. 3. Coactivation of AR signaling by BRCA1 and members of the p160 family of nuclear receptor coactivators is synergistic. *A*, PC-3 cells were cotransfected with 2.0 μ g of pSG5-GRIP1, pcDNA3.1-AIB1, or pSG5-SRC-1a, 2.0 μ g of ARR₃tk-CAT, 25 ng of pCMV-hAR, and 2.5 μ g of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and data presented are the means of three independent dishes; bars, SE. *B*, potentiation of AR signaling by BRCA1 occurs in both prostate and breast cancer cell lines. Prostate cell line DU-145 and breast cell lines HBL-100 and MCF-7 were cotransfected with 2.0 μ g of ARR₃tk-CAT, 25 ng of pCMV-hAR, 2.0 μ g of pSG5-GRIP1, and 2.5 μ g of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and the data presented are fold relative to DHT-dependent AR activity with no transfected BRCA1 or GRIP1; bars, SE.

ent AR transactivation of the ARR₃tk-CAT reporter (*i.e.*, 2.5- and 5-fold, respectively). Likewise, when BRCA1 and GRIP1 were coexpressed in this cell line, a 14-fold synergistic coactivation of AR signaling was observed. In HBL-100 cells, the effects of BRCA1 were more dramatic. For example, BRCA1 alone potentiated AR transactivation activity greater than 12-fold. In combination with GRIP1, moreover, BRCA1 resulted in a nearly 45-fold enhancement of AR signaling. In MCF-7 cells, BRCA1 and GRIP1 individually potentiated AR activity <2-fold. Together, however, they did result in a 5-fold coactivation of AR signaling, consistent with observations made in the other cell lines. The relatively small BRCA1 effects seen in MCF-7 cells may be attributable to high endogenous p160 coactivator levels (7).

BRCA1 Interacts with the AR NTD and the GRIP1 COOH-Terminus. To determine whether BRCA1 makes physical contacts with the AR and/or GRIP1, GST pull-down experiments were performed in which *in vitro* translated and ³⁵S-labeled BRCA1 was incubated with immobilized GST-AR (amino acids 1–555) or with various GST-fused fragments of GRIP1 (*i.e.*, amino acids 5–765, 563–1121, or 1122–1462). In these experiments, BRCA1 interacted

with GST-AR (1–555) and with GST-GRIP1 (1122–1462) but not with the other GRIP1 fragments (Fig. 4*B*).

Overexpression of BRCA1 Alleviates the Inhibitory Polyglutamine (Poly-Q) Effect on AR Transactivation Activity. We have shown previously that AR transactivation activity decreases with increasing poly-Q length and that this may be attributable to inhibition of p160-mediated coactivation (9). To assess the impact of AR poly-Q length on BRCA1 and BRCA1/GRIP1 coactivation of AR signaling, ARs with varying poly-Q lengths were expressed in PC-3 cells along with BRCA1 alone or in combination with GRIP1 (Fig. 5). As expected, DHT-dependent AR transactivation of the ARR₃tk-CAT reporter decreased modestly with increasing poly-Q length in PC-3 cells (Fig. 5, *white histograms*). This effect was not, however, observed when BRCA1 was coexpressed (Fig. 5, *gray histograms*). In addition, no inhibition of GRIP1 coactivation with increasing AR poly-Q length was observed in the presence of coexpressed BRCA1 (Fig. 5, *black histograms*).

Discussion

The data presented in this study support a direct role for BRCA1 in AR signaling. In both prostate and breast epithelial cell lines, BRCA1 enhanced ligand-dependent AR transactivation of an androgen-responsive probasin reporter gene. BRCA1, moreover, synergized

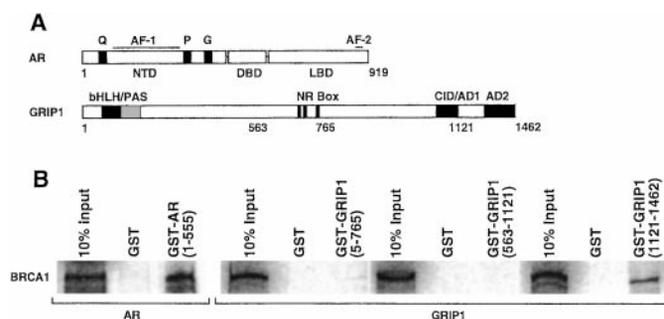


Fig. 4. BRCA1 interacts with the AR and GRIP1. *A*, schematic diagrams of the AR and GRIP1 showing the locations of various functional domains. Domains of AR: AF-1/AF-2, autonomous activation functions 1 and 2; NTD; DBD; LBD; Q/P/G, glutamine/proline/glycine poly-amino acid stretches. Domains of GRIP1: bHLH, basic helix-loop-helix sequence; PAS, Per-Arn-Sim domain; NR boxes, nuclear receptor binding domains (LXXLL motifs); CID, CBP interaction domain; AD1/AD2, activation domains. Numbers represent relative amino acid positions. *B*, full-length BRCA1 binds to the NH₂-terminal domain of the AR and the COOH terminus of GRIP1. Unpurified *in vitro* translated BRCA1 was incubated with GST, GST-AR (1–555), GST-GRIP1 (5–765), GST-GRIP1 (563–1121) or GST-GRIP1 (1121–1462).

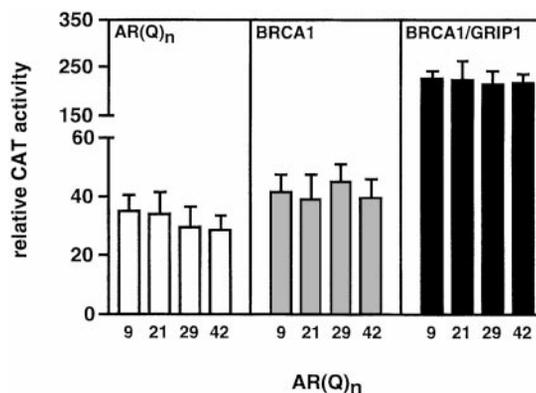


Fig. 5. BRCA1 normalizes the AR poly-Q effect. PC-3 cells were cotransfected with 2.0 μ g of ARR₃tk-CAT, 25 ng of pcDNA-hAR(Q)_n, 2.0 μ g of pSG5-GRIP1, and 2.0 μ g of pcDNA-BRCA1 as indicated. CAT activities in the presence of DHT were normalized to total cellular protein and to AR(Q)_n expression levels as determined by ligand binding assays (9). Data presented are the means of three independent dishes; bars, SE.

with the p160 coactivators to potentiate AR activity. In addition, BRCA1 physically interacted with both the AR and GRIP1, perhaps indicative of the formation *in vivo* of an AR/p160/BRCA1 ternary complex mediated by reciprocal interactions between the AR NTD, the GRIP1 COOH terminus, and BRCA1. In light of this, it is possible that BRCA1 participates in the formation and/or stabilization of the NR coactivator complex. Our data suggest that BRCA1 is directly recruited to androgen-responsive promoters through interactions with the AR NTD or with the GRIP1 COOH terminus. Once localized to the target promoter, BRCA1 may facilitate activated transcription by "bridging" communications between the bound NR coactivator complex and the RNA polymerase II-containing preinitiation complex, which associates with the BRCA1 COOH terminus (15). In this view, BRCA1 plays a critical role in modulating the effects of androgen signals on cells by increasing the efficacy and accuracy of AR-mediated transcriptional events. Loss of cellular BRCA1 function, therefore, perhaps because of mutations causing COOH-terminally truncated forms of the protein, would be expected to reduce the potency of AR-dependent signaling.

Several lines of evidence indicate that androgen signaling in the breast might in fact protect against cancer development and progression: (a) androgens have been used successfully to treat metastatic female breast cancers with comparable efficacy to tamoxifen, but the treatment was not well tolerated because of its masculinizing side effects (16); (b) androgens have been shown to inhibit the proliferation of AR-positive breast cancer cell lines in culture (17); (c) reduced or impaired AR signaling has been implicated in the development of hereditary male breast cancers (18); and (d) Rebbeck *et al.* (4) have reported an association between the polymorphic AR CAG repeat and breast cancer penetrance among *BRCA1* mutation carriers. In their study, women who carried at least one long AR CAG allele (*i.e.*, >27 repeats) had a significantly earlier age at diagnosis than women with only short alleles. Interestingly, breast cancer penetrance increased with increasing AR CAG length. Because of the well-characterized negative effect of increasing poly-Q length (encoded by the CAG repeat) on AR transactivation activity (9), it is tempting to speculate that reduced AR signaling encourages neoplastic transformation in mammary epithelial cells harboring *BRCA1* mutations. Our findings may indirectly support this idea because *BRCA1* overexpression apparently abolishes the inhibitory effect of increasing poly-Q length on p160-mediated coactivation of the AR (Ref. 9; Fig. 5). It may be that in women with germ-line *BRCA1* mutations (and therefore, with reduced functional *BRCA1* protein), breast epithelial cells are under reduced androgen-mediated growth inhibition and tumors develop more rapidly in those women expressing less efficient ARs.

The results of this study, although still at an early stage, suggest a complex interplay between the AR, p160 coactivators, and *BRCA1* that may be important in regulating epithelial cell proliferation and, by implication, cancer risk in certain hormone-regulated tissues like the breast and prostate. In the prostate, loss of *BRCA1* function initially was associated with increased risk for cancer development (19), although more recent studies have failed to find specific *BRCA1* mutations at increased frequencies among prostate cancer patients (20). Decreased AR signaling because of loss of *BRCA1* function might even protect against prostate cancer development and/or progression because most early-stage prostate cancers are androgen dependent. Nevertheless, it is difficult to reconcile the tumor suppressor functions of *BRCA1* (*i.e.*, DNA repair and cell cycle control) with such a proposal. Clearly, future studies are needed to explore these important issues.

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