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9. HEK 293 cells were transfected with plasmids encoding DRα, DRβ, Ii, DMA, and DMB individually. Transfectants were selected in media containing G418, hygromycin, and puromycin. Expression of human leukocyte AG DR (HLA-DR) was evaluated by fluorescence-activated cell sorting analysis with either monoclonal antibodies to HLA-DR1 or HLA-DR4 (One Lambda, Canoga Park, CA). Expression of DMA, DMB, and Ii was detected by protein immunoblot with antibodies to DMA (anti-DMA), anti-DMB, and anti-Ii.
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11. The constructs pHG810, pIHG812, pIHG813, and pIHG814 were made as follows. The Ii fragments (amino acids 1 to 60, 1 to 80, and 1 to 108) were generated by polymerase chain reaction amplification with specific sets of primers and cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, San Diego, CA) to produce the pI60, pI80, and pI108 plasmids, respectively. A 400–base pair DNA fragment containing the HA(307–319) epitope was amplified from a plasmid encoding an influenza virus HA and was inserted into pI60, pI80, and pI108 to generate pIH60, pIH80, and pIH108, respectively. A DNA fragment encoding enhanced green fluorescence protein (EGFP) from pEGFP-N3 (Clontech, Palo Alto, CA) was then fused in-frame to the COOH-terminus of the IH fusion constructs. The resultant plasmids were named pIHG812, pIHG813, and pIHG814. pHG810 was constructed in the same way except for omission of the Ii fragment.
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15. Total RNAs were extracted from 1558mel and 1359mel with Trizol reagent from GIBCO BRL (Gaithersburg, MD). Polyadenylated RNAs were purified from total RNA by using polyATract System (Promega, Madison, WI) and converted to cDNA with a cDNA construction kit (GIBCO BRL) with an oligo-dT primer. The cDNA inserts were cloned into the expression vector pTSX. The cDNA libraries were electroporated into DH10B bacteria cells (GIBCO BRL). Plasmid DNA pools were prepared from bacteria, each consisting of about 100 cDNA clones.
16. The cDNA library from 1359mel was divided into cDNA subpools, each of which consisted of about 100 cDNA clones (*15*). A total of 1.6×10^5 cDNA clones were screened by expression in 293IMDR4 cells (*17*). Two positive pools were identified in the first screening. Individual DNA was prepared from about 800 *Escherichia coli* colonies transformed with the positive-pool DNA, and six positive cDNA clones were isolated by repeating the screening procedure.
17. DNA transfection with GM-CSF assays were performed as previously described [R. F. Wang, P. F. Robbins, Y. Kawakami, X. Q. Kang, S. A. Rosenberg, *J. Exp. Med.* **181**, 799 (1995); R.-F. Wang *et al.*, *ibid.* **184**, 2207 (1996); R.-F. Wang *et al.*, *J. Immunol.* **161**, 3596 (1998)].
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22. Peptides were synthesized by a solid-phase method with an automatic peptide synthesizer (Model AMS 422, Gilson, Worthington, OH). The masses of some peptides were confirmed by mass spectrometry, and peptides were purified by high-pressure liquid chromatography as needed to obtain purities greater than 98%.
23. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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25. Cell lysates were prepared by lysis buffer. Equal amounts of total protein were loaded for each sample and separated on a 4 to 16% SDS-polyacrylamide gel by electrophoresis (SDS-PAGE). The proteins were then blotted onto a nitrocellulose membrane and incubated with a murine antibody to human CDC27 (Transduction Lab) or a rabbit polyclonal antibody to human CDC27. After incubation with an anti-mouse or anti-rabbit immunoglobulin G conjugated with horseradish peroxidase, protein was detected with the ECL system (Amersham).
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BRCA1 Inhibition of Estrogen Receptor Signaling in Transfected Cells

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Mutations of the breast cancer susceptibility gene *BRCA1* confer increased risk for breast, ovarian, and prostatic cancers, but it is not clear why the mutations are associated with these particular tumor types. In transient transfection assays, *BRCA1* was found to inhibit signaling by the ligand-activated estrogen receptor (ER-α) through the estrogen-responsive enhancer element and to block the transcriptional activation function AF-2 of ER-α. These results raise the possibility that wild-type BRCA1 suppresses estrogen-dependent transcriptional pathways related to mammary epithelial cell proliferation and that loss of this ability contributes to tumorigenesis.

Germ line mutations of the *BRCA1* gene (17q21) (*1*) account for 40% to 50% of hereditary breast cancers and confer increased risk for ovarian and prostatic cancers (*2*). The *BRCA1* gene encodes an 1863-amino acid protein with a highly conserved NH_2-terminal RING finger domain and a COOH-terminal acidic transcriptional activation domain (*1, 3*). The product of the *BRCA1* gene is a 220-kD nuclear phosphoprotein (*4*) that has been implicated in regulation of cell proliferation, cell cycle progression (*4, 5*), apoptosis induction (*6, 7*), and DNA repair and recombination (*7, 8*). These functions of *BRCA1* have been observed in various human epithelial cancer cell types and mouse fibroblasts and do not explain the association of *BRCA1* mutations with specific tumor types, such as breast cancer. Estrogen stimulation of mammary epithelia is thought to be a major factor in promoting development of

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breast cancer, probably through the expansion of previously initiated mammary epithelial cell clones (*9*). Thus, we hypothesized that BRCA1 protein might function, in part, to regulate the cellular response to estrogen.

Estrogen responses are mediated by two closely related members of the nuclear receptor family of ligand-activated transcription factors, ER-α and ER-β [reviewed in (*10*)]. The 66-kD ER-α, the major estrogen receptor of mammary epithelia, contains NH_2- and COOH-terminal transcriptional activation functions (AF-1 and AF-2, respectively), a DNA binding domain, a ligand binding domain associated with AF-2, and a cofactor binding domain. Estrogens bind and activate cytoplasmic ER-α, which translocates to the nucleus, dimerizes, binds to estrogen-responsive enhancer elements (EREs), and activates transcription. ER-α activation requires a ligand and cofactor-dependent conformational change that allows interaction between AF-1 and AF-2 and recruitment of coactivator proteins. These coactivators link ER-α to the basal transcriptional machinery and convert chromatin to the active state by histone acetylation.

To assess the effect of BRCA1 on estrogen response, we measured the ability of the *BRCA1* gene to modulate the transcriptional activity of ER-α in transient transfection assays. Human prostate cancer cell line DU-145 lacks ER-α, but, when supplied with exogenous ER-α, these cells became competent for estrogenic signaling, as demonstrated by estradiol (E2)-stimulated activation of estrogen-responsive reporter ERE-TK-Luc (Fig. 1A). Cotransfection of a wild-type *BRCA1* gene (*wtBRCA1*) abolished activation of this reporter in a dose-dependent fashion, whereas empty *BRCA1* vector (pcDNA3) had little or no effect on

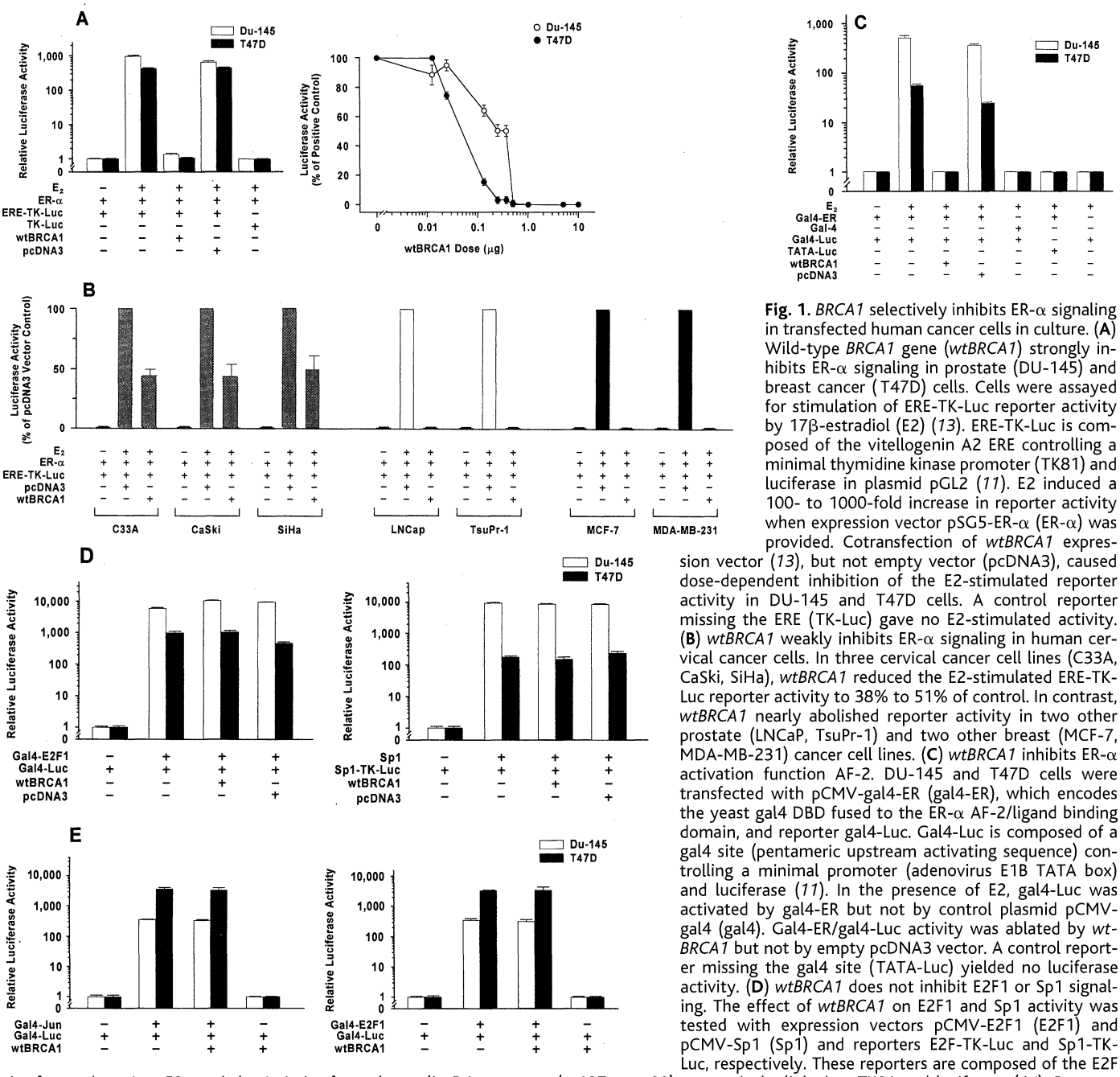

Fig. 1. *BRCA1* selectively inhibits ER-α signaling in transfected human cancer cells in culture. (**A**) Wild-type *BRCA1* gene (*wtBRCA1*) strongly inhibits ER-α signaling in prostate (DU-145) and breast cancer (T47D) cells. Cells were assayed for stimulation of ERE-TK-Luc reporter activity by 17β-estradiol (E2) (*13*). ERE-TK-Luc is composed of the vitellogenin A2 ERE controlling a minimal thymidine kinase promoter (TK81) and luciferase in plasmid pGL2 (*11*). E2 induced a 100- to 1000-fold increase in reporter activity when expression vector pSG5-ER-α (ER-α) was provided. Cotransfection of *wtBRCA1* expression vector (*13*), but not empty vector (pcDNA3), caused dose-dependent inhibition of the E2-stimulated reporter activity in DU-145 and T47D cells. A control reporter missing the ERE (TK-Luc) gave no E2-stimulated activity. (**B**) *wtBRCA1* weakly inhibits ER-α signaling in human cervical cancer cells. In three cervical cancer cell lines (C33A, CaSki, SiHa), *wtBRCA1* reduced the E2-stimulated ERE-TK-Luc reporter activity to 38% to 51% of control. In contrast, *wtBRCA1* nearly abolished reporter activity in two other prostate (LNCaP, TsuPr-1) and two other breast (MCF-7, MDA-MB-231) cancer cell lines. (**C**) *wtBRCA1* inhibits ER-α activation function AF-2. DU-145 and T47D cells were transfected with pCMV-gal4-ER (gal4-ER), which encodes the yeast gal4 DBD fused to the ER-α AF-2/ligand binding domain, and reporter gal4-Luc. Gal4-Luc is composed of a gal4 site (pentameric upstream activating sequence) controlling a minimal promoter (adenovirus E1B TATA box) and luciferase (*11*). In the presence of E2, gal4-Luc was activated by gal4-ER but not by control plasmid pCMV-gal4 (gal4). Gal4-ER/gal4-Luc activity was ablated by *wtBRCA1* but not by empty pcDNA3 vector. A control reporter missing the gal4 site (TATA-Luc) yielded no luciferase activity. (**D**) *wtBRCA1* does not inhibit E2F1 or Sp1 signaling. The effect of *wtBRCA1* on E2F1 and Sp1 activity was tested with expression vectors pCMV-E2F1 (E2F1) and pCMV-Sp1 (Sp1) and reporters E2F-TK-Luc and Sp1-TK-Luc, respectively. These reporters are composed of the E2F site from adenovirus E2a and the Sp1 site from the cyclin D1 promoter (−127 to −99), respectively, linked to TK81 and luciferase (*14*). Reporter activity was stimulated by E2F1 or Sp1 vector. Neither E2F1- nor Sp1-stimulated reporter activity was inhibited by *wtBRCA1*. (**E**) *wtBRCA1* does not inhibit the transcriptional activation functions of c-Jun and E2F1. Expression plasmids encoding gal4 DBD linked to the activation domains of c-Jun and E2F1 (*15*) activated the gal4-Luc reporter, but *wtBRCA1* failed to inhibit gal4-jun and gal4-E2F1 stimulated reporter activity.

reporter activity (Fig. 1A). Equally strong inhibition of ER-α signaling by *wtBRCA1* was observed in breast cancer cell line T47D, in two other human breast cancer cell lines (MCF-7 and MDA-MB-231), and in two other human prostate cancer cell lines (LNCaP and TsuPr-1) (Fig. 1, A and B). However, three human cervical cancer cell lines showed relatively weak inhibition of ER-α signaling by *wtBRCA1*: E2-stimulated reporter activity in the presence of *wtBRCA1* was about 40% to 50% that in the presence of empty vector (Fig. 1B).

We used the gal4-ER/gal4-Luc assay system (*11*) to determine whether BRCA1 directly inhibits the highly conserved COOH-terminal activation function (AF-2) of ER-α, independently of any effect it might have on the DNA binding activity of the receptor. When linked to the yeast gal4 DNA binding domain (DBD), the AF-2 domain of ER-α stimulated activity of a reporter containing a multimeric gal4 binding site in an E2-dependent fashion (Fig. 1C). In both DU-145 and T47D cells, *wtBRCA1* reduced gal4-ER/gal4-Luc activity to background levels, whereas pcDNA3 vector had little or no effect on reporter activity. These findings suggest that BRCA1 inhibits the AF-2 function of ER-α, but they do not rule out the possibility that BRCA1 targets additional steps in ER-α activation.

In both the ER-α and gal4-ER assays, basal reporter activity was very low, so it was not possible to determine whether BRCA1 acted as a nonspecific transcriptional repressor. We tested the ability of *wtBRCA1* to regulate the activity of other transcription factors. Expression vectors for cell cycle–regulated transcription factors E2F1 and Sp1 caused activation of luciferase reporters controlled by the corresponding binding elements, but the *wtBRCA1* gene did not inhibit activation of either reporter in DU-145 or T47D cells (Fig. 1D). *wtBRCA1* also failed to repress activation of the gal4-Luc reporter by vectors encoding the gal4 DBD linked to the transcriptional activation domains of c-Jun and E2F1 (Fig. 1E). In fact, in some of these assays, reporter activity was slightly higher in the presence of *wtBRCA1*. These results suggest that BRCA1 is not an indiscriminate repressor of transcription.

Previous studies have linked expression of the *BRCA1* gene with differentiation of specific cell types, including mammary epithelium (*12*), but the molecular targets of BRCA1 for mammary differentiation have not been identified. Our findings suggest that the wild-type BRCA1 protein may function, in part, to suppress estrogen-dependent mammary epithelial proliferation by inhibiting ER-α-mediated transcriptional pathways related to cell proliferation. Conversely, inactivation of the ability to repress ER-α transcriptional activity by mutation or by loss of *BRCA1* expression may contribute to mammary carcinogenesis. Interestingly, the relatively weak inhibition of ER-α signaling in human cervical cancer cell lines by the *wtBRCA1* gene correlates with the absence of an association of cervical cancer, another estrogen-dependent tumor type, with germ line mutations of *BRCA1*. To establish the validity of these hypotheses, it is necessary to confirm the ability of BRCA1 to regulate the ER-α response in in vivo models.

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13. The wild-type *BRCA1* expression plasmid (*wtBRCA1*) was created by cloning the *BRCA1* cDNA into the pcDNA3 vector (Invitrogen) with artificially engineered 5′ Hin dIII and 3′ Not I sites. For transcription assays, we incubated asynchronously proliferating cells overnight at 50% to 70% of confluency in 24-well dishes with 0.5 μg of each vector (except where otherwise indicated) in serum-free Dulbecco's modified Eagle's medium (DMEM) containing Lipofectamine (Life Technologies). Cells were washed, incubated in serum-free phenolphthalein-free DMEM (0.2 ml per well) with or without 17β-estradiol (1 μM) for another 24 hours, and harvested for luciferase assays. Values are means ± SEM of four replicate wells and are representative of at least three experiments. Results were similar whether ER-α assays were performed in serum-free DMEM or in the presence of 2% charcoal-stripped serum.
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Self-Organization of Microtubule Asters Induced in *Xenopus* Egg Extracts by GTP-Bound Ran

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The nucleotide exchange activity of RCC1, the only known nucleotide exchange factor for Ran, a Ras-like small guanosine triphosphatase, was required for microtubule aster formation with or without demembranated sperm in *Xenopus* egg extracts arrested in meiosis II. Consistently, in the RCC1-depleted egg extracts, Ran guanosine triphosphate (RanGTP), but not Ran guanosine diphosphate (RanGDP), induced self-organization of microtubule asters, and the process required the activity of dynein. Thus, Ran was shown to regulate formation of the microtubule network.

Ran is an abundant Ras-like nuclear small guanosine triphosphatase (GTPase) essential for nucleus-cytosol exchange of macromolecules (*1–4*). Its GTPase activity is activated by RanGAP1 or Rna1p, and its nucleotide is exchanged by RCC1 (*1*, *2*). Although all of the yeast *ran* mutants thus far isolated show nuclear protein import defects (*3*), hamster and yeast *rcc1* mutants show diverse phenotypes, such as suppression of receptor-less mating process, chromosome instability, premature chromatin condensation, lack of chromosome decondensation, abnormal mRNA metabolism, and mRNA export defects (*1*). How Ran regulates these processes is controversial. In addition to the RanBP1 and importin β families, which are required for nucleus-cytosol exchange of macromolecules (*4*), Dis3p (*5*) and RanBPM (*6*) are reported to bind to RanGTP. Although Dis3p is required for ribosomal RNA processing (*7*), RanBPM is localized in the centrosomes and the antibodies to RanBPM inhibit microtubule aster formation in vitro (*6*).

The centrosome consists of a pair of centrioles surrounded by pericentriolar material (PCM) (*8*). *Xenopus* γ-tubulin ring complex-

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