BRCA1 Associates with Processive RNA Polymerase II.

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Running title: Minimal transcriptional activation activity in mammalian BRCA1.

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Summary:

The human BRCA1 tumor suppressor interacts with transcriptional machinery, including RNA polymerase II (RNA pol II). We demonstrated that interaction with RNA pol II is a conserved feature of BRCA1 proteins from several species. We found that full-length BRCA1 proteins universally fail to activate transcription in classic GAL4-UAS one-hybrid assays, and that the activity associated with the human BRCA1 C-terminus was poorly conserved in closely related homologs of the gene. Fractionation studies demonstrated that BRCA1 proteins from all species tested interacted specifically with hyper-phosphorylated pol II (IIO), in preference to hypo-phosphorylated RNA pol II (IIA) expected at promoters. BRCA1-RNA pol II complexes showed evidence of a multiply-phosphorylated heptad repeat domain in the catalytic subunit (p220) of RNA pol II, and the complex was highly functional in transcriptional runoff assays. Interestingly, endogenous BRCA1 associated with a large fraction of the processive RNA pol II activity present in undamaged cells and the interaction was disrupted by DNA damaging agents. Preferential interaction with processive RNA pol II in undamaged cells places BRCA1 in position to link late events in transcription with repair processes in eukaryotic cells.
Introduction:

Mutations in the BRCA1 tumor suppressor gene are associated with an increased risk of breast and ovarian cancer and an elevated incidence of certain other cancers (1-3). Genetic and biochemical data place BRCA1 as a downstream target of ATM/ATR in cellular responses to genotoxic stress, and the BRCA1 protein has been implicated in chromatin remodeling and homologous recombination functions in mitotic and meiotic cells (4-8). BRCA1 complexes contain E3-ubiquitin ligase activity, but the precise target(s) remains an avenue of active study (9,10). Though BRCA1 proteins show surprisingly low sequence identity, human BRCA1 can replace the mouse gene in genetically engineered animals (11,12), a result which implies general conservation of important functional motifs. Comparative functional studies are thus likely to play an important role in identifying critical targets of BRCA1 in human cells.

A distinct literature has evolved linking BRCA1 to roles in transcription. BRCA1 protein has been shown to associate with RNA polymerase II (RNA pol II) and various transcriptional regulators (13). Early on, several groups demonstrated that the C-terminal domain (CTD, amino acids 1380-1863) of human BRCA1 scored positively in transcriptional-activator trap experiments using various forms of the so called “one-hybrid” assay (14-18). While not elucidating a specific function, ectopic expression of full-length human BRCA1 was then shown to increase expression of stress-responsive genes including p21 (19), GADD45 (20), and p27 (21), and decreased expression of other genes, including c-myc regulated genes (22) and certain estrogen regulated genes (23). However, it remains an open question as to whether endogenous BRCA1 directly mediates recruitment of RNA pol II to promoters of these genes in unmanipulated cells (24-26).

Previous experiments indicate that BRCA1 associates with RNA pol II through its acidic
C-terminus (27), and may do so by binding RNA helicase A (13,28). Current models of BRCA1 function(s) in transcription are poorly defined, but favor regulation of various pre-initiation steps, including, but not limited to: recruitment of RNA pol II to promoters, transcriptional activation (29,30), transcriptional co-activation (31,32), and transcriptional inhibition (23). A distinct, but not mutually exclusive, model would suggest that BRCA1 interacts with elements of the RNA pol II holoenzyme linked to post-promoter activities of the complex. Evidence for this model include interactions of BRCA1 with the elongation factor NELF-B/COBRA1 (33) and the CstF-50 component of the polyadenylation complex (34,35).

Negative charge can lead to false positives in one-hybrid assays (36-38). Following our observation that the CTD of bovine BRCA1 had less charge and less one-hybrid activity than the same region of human BRCA1(39), we decided to search for structural and functional features of the BRCA1-RNA pol II complex that are more broadly conserved in mammalian cells. In the present study, we compared full-length and truncated BRCA1 proteins from four species for their ability to activate transcription in one-hybrid transcription assays, and then assessed their association with cellular RNA pol II complexes. While BRCA1 proteins from all four species associated strongly with the hyper-phosphorylated (IIO form) of RNA pol II, none of the full-length BRCA1 proteins displayed ability to correctly initiate pol II-dependent transcription. Importantly, we observed that BRCA1 co-purified with a large percentage of hyper-phosphorylated (IIO) RNA pol II found in cycling epithelial cells, but interacted minimally with hypo-phosphorylated (IIA) forms of the enzyme, which are more abundant. This distinction, based on fractionation of the endogenous proteins, suggested that a major role of BRCA1 in undamaged cells is directed toward post-initiation functions of RNA pol II. These results provide evidence that BRCA1 is not a transcriptional activator *per se*, and suggest that roles in
transcriptional regulation would likely involve post-initiation functions, or aspects of genomic surveillance, that are linked to RNA pol II transcriptional machinery.

**Experimental Procedures:**

*Cell culture:* MCF-7 and T47D (human mammary epithelial cell lines), MNuMg and C57MG (mouse mammary epithelial cell lines), BAEC and MDBK (bovine epithelial cells), 293T (human fibroblasts), and NIH-3T3 (mouse fibroblasts), were maintained in a humidified atmosphere with 5% CO\(_2\) in DMEM supplemented with 10% fetal calf serum, 100 units/mL penicillin and 100 µg/mL streptomycin. HBL100 (human epithelial cell line) were maintained in RPMI with 10% fetal calf serum, 100 units/mL penicillin and 100 µg/mL streptomycin.

*PCR:* All PCR reactions were done with the following conditions: 0.5 µL cDNA, 1X Dynazyme EXT (Finnzymes, Finland) buffer, 0.5 U Dynazyme EXT (Finnzymes), 250 µM dNTPs, and 1 µM each oligo. PCR conditions were as follows: 1 cycle of 95°C for 5 minutes; 35 cycles of 94°C for 20 seconds, 50°C for 20 seconds, 72°C for 2 minutes; and 1 cycle of 72°C for 5 minutes.

*Plasmid constructs:* The human BRCA1 C-terminal domains (CTD), representing amino acids 1380-1863 previously shown to have the highest activity in GAL4 transcriptional activation assays (16), was generated by PCR using full-length human BRCA1 cDNA as the template (a gift of E. Solomon, Guy’s Hospital, London, UK) and the primers 5’-CCAAGAATTCTGAAGACTGCTCAGGGCTATCC-3’ and 5’-CTCATTCTTGGGGTCTGTGG-3’. The canine BRCA1-CTD (codons 1380-1879) was generated from canine first strand cDNA with the following primers: 5’-AAACGAGCCTCTCTGAAGACTGTTCC-3’ and 5’-GGATCCTTACACGCATGGCTGGCTTG-3’
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(based on the sequence of accession number U50709). Full-length and D11 mouse BRCA1 were generated by RT-PCR using first strand cDNA from a mouse mammary epithelial cell line (MNuMg). The murine BRCA1-CTD (codons 1334-1813) was then amplified with the primers 5'-CCAAGAATTCCCGGATACGAGAGTGAAACAA-3' and 5'-AAGCACCAAAGACTGAGA-3' (40). Bovine BRCA1-CTD and full-length constructs were described previously (39). All PCR fragments were ligated into pCR-blunt II TOPO (Invitrogen) and sequenced in both directions to confirm identity with previously reported sequences. The pCR-CTD vectors were digested with EcoRI and BamHI, and the CTDs were ligated into the GAL4 vector pFA-CMV (Stratagene). Full-length GAL4-human BRCA1 and a fusion of the GAL4-DBD to the transactivation domain of herpes virus VP16 were a gift of Alvaro Monteiro (Rockefeller University). CTDs from two human disease-associated BRCA1 mutations, BRCA1 1853term-CTD and BRCA1 5382insertC-CTD were generated by PCR. The 5382insertC mutation was the kind gift of Dr. Charlie Chan and Dr. Charles Sawyers, UCLA. All other constructs were generated by restriction digestion of the constructs described above. All fusions were verified by sequencing in both directions and expression was verified by transfection into 293T cells and immunoblotting with appropriate antiseras.

To create constructs tagged with green fluorescence protein (GFP) (GFP-CTD constructs), the CTDs were cut out of pCR-blunt II TOPO with EcoRI and ligated in-frame into pQBI25-fc2 (Qbiogene, Carlsbad, CA). Nuclear localization signals were introduced by ligating oligonucleotides encoding the SV40 nuclear localization signal at the 5’ end of the CTD. To create constructs tagged with glutathione S-transferase (GST) the CTDs were cut out of pCR-blunt II TOPO with EcoRI and ligated in-frame into pGEX2TK (Pharmacia).

Expression analysis of fusion vectors: 3 µg of purified (Qiagen) plasmid DNA was
transfected into 293T cells using calcium phosphate precipitation. After 24 hours, the cells were lysed in SDS-PAGE loading buffer and extracts were run on a 10% SDS-PAGE gel. Proteins were then transferred to PVDF membranes, blocked in 5% nonfat dry milk and incubated with a primary antibody generated against the GAL4 DNA binding domain (Upstate Biotechnology, Charlottesville, VA), followed by a goat anti-rabbit horse radish peroxidase conjugated secondary antibody (Pierce, Rockford, IL). Blots were developed with a chemiluminescence detection substrate (SuperSignal™, Pierce). The GAL4 fusions encoded the GAL4 DBD plus 483 amino acids of the human CTD, 499 amino acids for the dog, 485 amino acids for the cow and 479 amino acids for the mouse CTD. The GST fusions used the same CTDs, except in the case of the GST-dog CTD, which included 60 additional amino acids of the canine BRCA1 protein, which had no affect on any of the assays.

**Immunofluorescence:** A Leica DM IRBE fluorescent microscope equipped with a 40x objective (Leica Microsystems, Germany) and a Hamamatsu C4742-95 digital camera was used for image acquisition. Images were stored and processed with an Openlab 3.1 software package (Improvision, Lexington MA).

**Mammalian one-hybrid (luciferase) assays:** 25,000-50,000 cells were plated in 24-well tissue culture dishes and transfected with 1 µg of the firefly luciferase reporter vector, pFR-luc (Stratagene) and the indicated pFA-GAL4-fusion vector (referred to as GAL-BRCA1 or GAL4-BRCA1-CTD, etc). A dosage-titration experiment was performed with all the key plasmid constructs and optimal activation was seen when 100 ng of the GAL4-BRCA1-CTD fusion plasmids were used (data not shown). All subsequent transfections were done with identical quantities. Firefly luciferase transfections also included 100 ng of a Renilla luciferase construct (pRL-SV40, Promega, Madison, WI), which served as an internal control for analysis using the
Dual-Reporter™ Assay Kit (Promega). Each transfection was done in triplicate and each
construct was retested in at least three separate assays. 24 hours after transfection, cells were
lysed in 100 µL of lysis buffer and luciferase assays were performed according to the
manufacturer’s protocol. The parental GAL4 vector (pFA-CMV), without additional fusion
sequence, served as a control value used to calculate induction. To obtain the values reported as
“fold-induction”, all luciferase results were normalized as follows: the raw firefly luciferase
value was divided by the internal Renilla Luciferase value (pRL-SV40). Subsequently, all
results were divided by the value obtained for the pFA-CMV vector control (averaged over
triplicate samples), which expressed the GAL4 DNA-binding domain alone.

The choice of pFR-luc as the reporter vector followed examination of a series of
additional GAL4 responsive vectors with variant promoters and TATA boxes, including:
pWHGG (41), M1, and M2 (42). These were the kind gift of Arnie J. Berk (UCLA, Los
Angeles, CA. pFR-luc was selected since it gave the highest GAL4 response to the human
BRCA1-CTD in all cells tested (see Supplemental Fig. #1). MCF7 cells gave the highest values
for the human BRCA1-CTD and were thus used for the initial comparison of CTD activity.

*GST Pull-down assay:* GST fusion proteins were purified on glutathione affinity
chromatography following expression in BL21 bacteria using standard methods. To identify
BRCA1 binding proteins, nuclear extracts were prepared from several distinct mammalian cell
lines. Cells were initially lysed in low salt buffer (20 mM Hepes pH 7.4, 0.5% NP-40, and 100
mM NaCl) supplemented with phosphatase inhibitors (10 mM NaF, 100 µM Na₃VO₄) and a
protease inhibitor cocktail (Complete™ without EDTA, Roche), for 30 minutes on ice. The
nuclei were then pelleted at 5000 RPM for 5 minutes and extracted in a high salt buffer (20 mM
Hepes, 0.5% NP-40, 1.5 mM MgCl₂ and 0.5 M NaCl) for 30 minutes, prior to dilution to a final
NaCl concentration of 150 mM. 5 µg of GST fusion protein bound to glutathione-agarose beads were incubated overnight at 4°C with nuclear extract from 5 x 10^6 cells. Bound complexes were washed three times in lysis buffer before elution with SDS-PAGE loading buffer. For some experiments, the bound proteins were treated with Calf Intestinal Phosphatase (CIP, New England Biolabs, Beverly, MA) prior to elution with SDS-PAGE loading buffer. Eluted proteins were subjected to SDS-PAGE and immunoblotting with antisera to the 220 kDa subunit of RNA pol II: 1) 8WG16 (QED Biosciences, San Diego, CA), or 2) N-20 (Santa Cruz Biotechnology, Santa Cruz, CA). Additional antisera to p220 included 3) an antibody to phosphorylated heptad serine 5 (S5) (clone CTD4H8, Upstate Biotechnology), and 4) an antibody to phosphorylated heptad serine 2 (S2) (H5, Covance, Inc., Princeton, NJ). BRCA1 from human cell extracts were identified with either Ab4 (Oncogene Research Products, San Diego, CA) or C-20 (Santa Cruz Biotechnology, Santa Cruz, CA). BRCA1 from bovine cells was detected with C3F (39). Antisera to FCP1 were generously provided by Dr. Michael E. Dahmus (UC Davis). GST fusions were detected with anti-GST (Roche).

**Immunoprecipitations:** Nuclear extracts were prepared as described above. For some experiments, whole cell extracts were made using EBC buffer (50 mM Tris pH 8, 120 mM NaCl, 0.5% NP-40) supplemented with phosphatase inhibitors (10 mM NaF, 100 µM Na_3 VO_4) and a protease inhibitor cocktail (Complete™ without EDTA, Roche), for 30 minutes on ice. Lysate was pre-cleared with protein A or G beads and proteins were incubated overnight with primary antibodies: RNA pol II p220 (N-20 or 8WG16), phospho p220 (S5 or S2) or BRCA1 (either Ab4 or C-20). To remove phosphate from precipitated proteins, immunoprecipitated proteins were treated with calf intestinal phosphatase (CIP, New England Biolabs) prior to elution with SDS-PAGE loading buffer. To test for protein-DNA interactions, some immunoprecipitations
were performed in the presence of 50 μM ethidium bromide (EtBr). Eluted proteins were subjected to SDS-PAGE and immunoblotting as described above.

**DNA damage:** Randomly cycling HBL100 cells were treated with 4 μM adriamycin (adr), 2 μM camptothecin (cpt), or 1 μM hydroxyurea (HU). Alternatively, cells were exposed to the indicated dose of ionizing radiation (IR) (0.5-10 Gy) using a 137Cs source (Mark 1 irradiator; Shepherd and Assoc., San Fernando, CA). Following treatments, cells were returned to a 5% CO₂ incubator for 1 hour, then lysed and immune complexes prepared as above.

**p220 ELISA:** To quantify the amount of p220 catalytic subunit present in various immunoprecipitates, an immobilized substrate assays were developed. Cell lysates were immunoprecipitated with rabbit polyclonal antisera to either p220 subunit of RNA pol II (N-20) or BRCA1 (C-20), and volumes were normalized to cell number. Equivalent volumes of normalized cell extract were then diluted in plating buffer (50 mM carbonate buffer, pH 9.5), and adsorbed to 96-well plates (EIA8 plates, Costar). Plates were washed, blocked (0.1% BSA in PBS), and incubated with mouse monoclonal antibodies to p220 catalytic subunit (8WG16). Bound monoclonal antibodies were detected by incubation with peroxidase-conjugated goat anti-mouse antibody (Pierce) followed by addition of color producing 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO). The reaction was stopped after 10 minutes with 10 mM (final) H₂SO₄ and the reaction product was read at 450 nm on a 96-well plate reader (Molecular Devices Corp. Sunnyvale, CA). Values were used from dilutions that were within the linear range of the assay as established from a standard curve created with immunopurified p220.

**Runoff (C-tailed) transcription assays:** The runoff transcription assay used was a modification of previously reported C-tail assay (43,44). Oligonucleotides were purchased from
MWG (Highpoint, NC). The C-tailed oligo was a 65-mer (5’-
ATTGGTAAAGGAGAGTTTGAAGCAGGACAGTACTCCGGGTCCTCCCC
CCCCCCCCCCCC-3’) that was annealed to a complementary 45-mer (5’-
GACCCGGAGTACTGTCTCCTCGC TCTTTTACTCTCCTTTACCCAAT-3’). The runoff
template was created by annealing 50 pmol of each oligonucleotide in a 200 µL annealing
mixture containing 20 mM Tris (pH 7.4), 1 mM EDTA, and 0.2 M NaCl. Runoff transcription
reactions contained 8.25 mM MgCl$_2$, 250 µg BSA, 250 nM NTPs, 5 U RNAse inhibitor, 50 ng of
poly (dI-dC), 0.05% Nonidet P-40, 1 pmol of annealed oligonucleotides and 0.5 mCi of [$^{32}$P]
CTP. Following addition of test immunoprecipitations (containing nuclear extracts from
equivalent numbers of cells), reactions (20 µL) were incubated for 40 minutes at 30°C, then
stopped with 50 µL of PK buffer (300 mM sodium acetate, 0.2% SDS, 10 mM EDTA, 100 ng of
tRNA and 10 µg of proteinase K). Transcription reactions were then incubated at 55°C for 20
minutes, extracted with phenol-chloroform, and ethanol precipitated. Single stranded RNA
transcripts were resolved under denaturing conditions on 12% polyacrylamide-urea gels,
electrophoresed for 1.5 hours at 300 V, dried, and visualized by autoradiography on X-ray film,
as described (43,44). Dried gels were also exposed to phosphoimager plates and the signals
quantified on a Storm™ Phosphoimager (Molecular Dynamics Inc., Sunnyvale, CA).

**Results:**

*The C-terminal domain (CTD) of mouse BRCA1 lacks one-hybrid transcriptional
activation activity and the limited activity associated with CTDs from human, dog and cow
BRCA1 correlate in part with their respective charge.*

To determine if classic one-hybrid activation activity is a conserved feature of BRCA1,
we created a panel of GAL4 fusion proteins with BRCA1-CTDs from human, mouse, cow and dog. This panel is informative since mouse and human BRCA1 genes share only 60% amino acid identity (40), while cow and dog are approximately 80% identical to each other and to both mouse and human proteins (39). Initially, regions homologous to amino acids 1380-1863 of human BRCA1 were tested in the MCF7 one-hybrid assay (Fig. 1C and Fig. 2A), as this region had been shown to be the most active in previous reports (16).

When tested in human MCF-7 breast cancer cells, the human GAL4-BRCA1-CTD fusion activated reporter activity 20.8 fold over vector alone, similar to values previously reported for this domain (16), while the mouse BRCA1-CTD produced baseline responses (1.7 fold over vector alone) (Fig. 1C and Fig. 2B). Homologous regions of cow and dog BRCA1 showed intermediate activities. The level of activation achieved by the human BRCA1-CTD was 300-fold lower than that observed for the classic activation domain of VP16 (Fig. 1C). Interestingly, exposing the cells to rates of DNA damage (1-10 Gy IR) known to increase phosphorylation of the BRCA1-CTD, did not affect readings in these assays (data not shown).

The sequence of each BRCA1-CTD was analyzed for total charge and compared with reporter activation (Fig. 1C, inset). Total charge was assessed by subtracting the number of negatively charged amino acids (D, E) from positive amino acids (K, R). Inclusion of mildly basic histidines in the equation had no significant effect on the slope of the curve since there are similar numbers of this amino acid in all BRCA1-CTDs analyzed. The human and dog BRCA1-CTDs had the largest net negative charge (-28.9 and -24 respectively) and the highest level of activity in all cell types tested. In contrast, the cow and mouse BRCA1-CTDs had lower net negative charge (-14.6 and -15.4 respectively) and scored poorly in this assay, suggesting some correlation between charge and one-hybrid activity.
One-hybrid transactivation activities for all GAL4-BRCA1-CTD fusions were tested in several additional human cell lines with similar results (MCF-7 (Fig. 1C) and 293T and T47D (Fig. 1D). While the maximal fold-induction varied between human cell lines, the relative order of activity (hCTD - dCTD >> cCTD > mCTD) remained consistent. The highly negative human CTD (12.3-20.8 fold) and dog CTDs (14.4-21.7 fold) always gave higher activity in human cell lines than the less charged cow (2.3-5.3 fold) and mouse sequences (1.7-2.2 fold) (Fig 2).

To test this notion further, we conducted a series of assays on various size fragments of BRCA1 and on disease associated mutant BRCA1 alleles. As previously reported (16), amino acids 1380-1863 from the CTD of human BRCA1 displayed the highest degree of one-hybrid transcriptional activation activity when compared to shorter segments of the C-terminus (Fig. 2A). The drop in activity of shorter fragments of the human BRCA1-CTD correlated with charge. Similar fragments of mouse BRCA1 showed consistently low (1.7-2.7 fold) induction (Fig. 2B). Dog sequences were consistently high in various human cells (Fig. 2D) but were not tested in canine cells. Cow sequences scored consistently low in all human cell lines (2.3-5.3 fold; Fig. 2C) and the cow BRCA1-CTD was inactive in bovine cells (1.7 fold; Fig. 1E).

The CTD from two disease-associated mutations of human BRCA1 (1853term (46) and 5382insC (change of frame at amino acid 1755(47)), failed to activate transcription (Fig. 2A). However, immunoblotting analysis of these proteins showed that they were less stable in mammalian cells when compared with proteins containing the native C-terminal ends (data not shown). These results support an alternative interpretation: that the activity of the human (and dog) BRCA1-CTD in one-hybrid assays reflects, to some extent, acidic content of the sequence, and may not elucidate a direct biological function of the BRCA1 protein in transcriptional activation (36, 45).
Full-length BRCA1 proteins lack one-hybrid transcriptional activation activity.

Fusion of the GAL4 DNA binding domain to full-length BRCA1 constructs from human, mouse and cow, or to the naturally-occurring mouse splice variant lacking exon 11 (D11), registered similarly to disease-associated BRCA1 mutant BRCA1-CTDs in all cell lines in which they were tested (Fig. 2A, B and C). The full-length fusion proteins were stable in mammalian cells and were detected at the predicted molecular weight in immunoblotting experiments with an antibody to the GAL4 DBD (data not shown). It is important to emphasize that assayed values for full-length BRCA1s compare precisely for all species, and that these in turn correlated with the values of mutant CTD domains assayed in this study (0.4-0.5 of control). Previous reports used similar human disease allele BRCA1-CTDs as standards of inactivity (14-16). Therefore, full-length BRCA1 proteins do not act as direct transcriptional activators, or inhibitors, in this assay.

Domain swapping between the human and mouse C-terminal domain (CTD) leads to chimeric proteins with reduced one-hybrid activity.

Since mouse and human BRCA1 proteins are interchangeable in a number of assays (11,12,48), we hypothesized that chimeric fusions of mouse and human BRCA1-CTD sequences should have modest effect on activities that are important for gene function. Using conserved restriction sites within the mouse and human CTD, we constructed chimeric proteins in which mouse sequences (referred to as regions A, B, and C in Fig. 2E) were fused in-frame with human sequences. Constructs that contained any mouse BRCA1 sequence proved to be less active in the GAL4 one-hybrid transcriptional activation assay when compared to the native human CTD,
even if this included substitution of the similarly charged region C (Fig. 2E). In T47D cells, the fusion protein using human region A and murine region B+C activated the reporter assay by 4.1 fold and the reverse fusion protein activated 12.9 fold. Replacing a longer region from human BRCA1 (amino acids 1380-1651, regions A+B) in the mouse BRCA1-CTD increased the one-hybrid transcriptional activation activity in 293T cells and MCF-7 cells, but not to the level of the continuous human BRCA1-CTD. All fusions were stable and expressed at equivalent levels as determined by immunoblot experiments (data not shown).

A comparison of charge clusters in human versus mouse CTDs was conducted based on the swapped segments (Fig. 2E). Regions A+B contained the most significant accumulations of net negative charge within the human CTD. Region A had a net negative charge contribution of -12 in human vs. -8 in mouse; region B had a net negative contribution of -13 in human vs. -3 in mouse. Region C, which corresponds to the tandem BRCT repeats, had a net negative contribution of -5 in human vs. -4 in mouse. This result would predict that fusing mouse regions A+B with the human region C would result in activity similar to the wild type mouse BRCA1-CTD, as is the case (Fig. 2E). The results of the swapping experiment showed that addition of any charged region from the human to the less charged mouse sequence increased the activity when compared to the mouse BRCA1-CTD alone. Conversely, reduction of negative charge in the human by addition of homologous mouse sequence decreased the activity in a stepwise manner. However, charge was not the only factor. Addition of human A+B with mouse C gave values that were significantly lower (3.9-6.8 fold) than present in the contiguous human CTD (12.3-20.8 fold), but higher than the mouse CTD (1.7-2.2 fold). Therefore, charge appears to be a strong contributing factor, but is likely not the only factor, in the one-hybrid response of various CTD domains.
Neither increased nuclear localization, nor alteration of the reporter construct, reveal measurable transcriptional activity in murine BRCA1-CTD fusions.

In addition to charge and phosphorylation state, we considered several additional potential reasons for a lack of activity by mouse CTD-GAL4 fusions. First, we tested several different GAL4 reporters that have been used in various studies of one-hybrid activity. The human BRCA1-CTD had the highest level of one-hybrid activation when combined with pFR-luc, a reporter consisting of five repeats of the GAL4-UAS fused to an adenovirus E1B promoter. The human BRCA1-CTD had four-fold lower transcription activation activity when assayed against reporters with the c-fos promoter (pWHGG). The mouse BRCA1-CTD had consistently minimal activity with all reporter constructs (these data are provided as supplemental Fig. #1). Next, we added nuclear localization signals (NLS) to the GAL4 constructs to assess the effect of improved nuclear localization on reporter activation. The nuclear localization of both human and mouse BRCA1-CTDs was significantly improved by addition of exogenous NLS sequences. NLS addition to the GAL4 vectors resulted in no increased activity in control GAL4 or mouse GAL4-BRCA1-CTD constructs, but resulted in significant enhancement of the activity of the human CTD (these data are provided as supplemental Fig. #2).

These data confirm that mouse BRCA1 has minimal activity in one-hybrid transcriptional activation assays. Furthermore, the activity of the human BRCA1-CTD construct is limited, requiring defined sequence composition and specific chimeric reporter design for efficient detection. From previous work with this assay (14-17,49,50), it is clear that one-hybrid type assays can be useful in characterizing disruptive mutations in the CTD of human BRCA1. The present study provides direct evidence that this strategy would be significantly less informative.
about the role of amino acid differences in other species and may not measure a conserved or native function of the BRCA1 protein.

BRCA1 interacts with hyper-phosphorylated RNA pol II complexes in preference to the more abundant hypo-phosphorylated pol II.

In normal cells, promoter-bound RNA pol II complexes contain a hypo-phosphorylated p220 subunit (form IIA) that is sequentially phosphorylated during initiation to generate the hyper-phosphorylated p220 (form IIO) which predominates in elongation complexes (51-53). Since human BRCA1 has been shown to associate with RNA pol II (13,27,28,32), we considered it important to test for this interaction with mouse and bovine BRCA1. We set out to determine whether the biology and timing of this interaction was consistent with BRCA1 acting in association with promoter-bound RNA pol II, as would be expected in the promoter and pre-promoter models discussed above. We immunoprecipitated the endogenous BRCA1 from undamaged human epithelial cells (HBL100 (Fig. 3B) and HeLa (data not shown)) and found that it associated specifically with heavily phosphorylated (IIO) p220. Similar results were obtained from bovine cells (MDBK cells, data not shown).

When compared to p220 catalytic subunit precipitated directly from human epithelial cells (N-20 antisera, Fig. 3B lane 1), it was clear that BRCA1 interacted preferentially with the slower migrating IIO form of p220. Interaction of BRCA1 with hyper-phosphorylated p220 was confirmed with antisera that recognize specific phosphorylation events within the heptad repeats of the p220 catalytic subunit. p220 co-purifying with BRCA1 showed evidence of phosphorylation at both the serine 5 (S5, Fig. 3B; lane 4) and serine 2 (S2, lane 6) positions of the heptad repeat, as well as showing a size shift consistent with maximal phosphorylation of the
When RNA pol II was directly precipitated from cells with anti-p220 antisera it was evident that a majority of RNA pol II was present in a minimally phosphorylated state (open arrow head, IIA; Fig. 3B lane 1). By binding the hyper-phosphorylated form of p220, BRCA1 shows considerable selectivity for the less abundant hyper-phosphorylated form (IIO) found in these cells (compare Fig. 3B, lanes 1 and 2). By comparison of RNA pol II immunoreactivity co-migrating with the minimally phosphorylated form (IIA) before or after phosphatase treatment, we estimated that only 5-10% of total p220 was present in BRCA1 complexes in these cells (see also Fig. 5C). In contrast, a large percentage of hyper-phosphorylated (IIO) p220 co-precipitated with BRCA1 (Fig. 3B).

To examine the specificity of BRCA1 interactions with RNA pol II, we also examined the BRCA1 that co-purified with different forms of p220 (Fig. 3C). Equivalent volumes of nuclear extract were immunoprecipitated with antisera to BRCA1, p220 or to two distinct forms of phospho-p220. Following immunoprecipitation, each fraction was split equally and half was treated with phosphatase (CIP). Subsequently, all fractions were immunoblotted with monoclonal antisera to BRCA1 (Ab-4, Oncogene Sciences). Two interesting results were evident from this experiment. First, similar amounts of BRCA1 (arrow) were precipitated with BRCA1 and p220 antisera. Second, the mobility of the BRCA1 bound by RNA pol II was not affected by phosphatase treatment (CIP treatment). These results provide evidence that a large fraction of BRCA1 associates with RNA pol II in undamaged epithelial cells and that the BRCA1 present is unphosphorylated, or minimally phosphorylated.

Following exposure of randomly cycling epithelial cells to DNA damaging agents known to cause phosphorylation of BRCA1, interaction with RNA pol II decreased (Fig. 3D). In this
experiment, cells were treated with the topoisomerase II inhibitor adriamycin (adr), the topoisomerase I inhibitor camptothecin (cpt), or the ribonucleotide reductase inhibitor hydroxyurea (HU) for one hour prior to cell lysis. It has been shown previously (54-56) that these agents induce double-strand DNA breaks and ATM/ATR-dependent phosphorylation of BRCA1 (not shown). Following treatments, there was a clear reduction of in phospho-p220 associated with the BRCA1 immune complexes. The residual interaction that was detected in damaged cells still targeted the hyper-phosphorylated (IIO) form of p220, and presumably represented residual unphosphorylated BRCA1 in these cells.

To further characterize the BRCA1-pol II complex we examined its susceptibility to phosphatases and to the DNA denaturant ethidium bromide (EtBr). In Figure 3, panel E, immunoprecipitated material was divided into two equal fractions, and both fractions were incubated at 37°C for 1 hour in the presence or absence of phosphatase (Fig. 3E). The cellular fraction of p220 that associated with BRCA1 (IIO form) was reduced to the lower molecular weight (IIA form) following phosphatase treatment (Fig. 3E, lane 2). Although some degradation of p220 was apparent following 37°C incubation (compare 3E lane 1 with 3B lane 2, or 3D lane 1) it is clear that phosphatase treatment effectively returns the BRCA1 bound p220 (IIO) to a form that co-migrates with unphosphorylated p220 (IIA). In panel F, immunoprecipitations were conducted in the presence of 50 μM EtBr, a DNA denaturant which is useful in distinguishing DNA-dependent and DNA-independent interactions (57). Immunoprecipitates were subsequently treated with CIP and immunoblotted for p220. From these experiments, it is clear that EtBr did not affect the ability of BRCA1 to bind hyper-phosphorylated RNA pol II (compare 3F, lanes 1 and 2).

In every preparation of BRCA1-RNA pol II complex we observed a very minor IIA-like
immunoreactive band (see the minor IIA-like band in Fig. 3B, lane 2). While the precise nature of the band is unknown, it may represent doubly phosphorylated (S5 + S2) that is not yet hyper-phosphorylated (note reactivity of S2 and S5 antisera to similarly migrating bands in lanes 3 and 5). Alternatively, the IIA-like band could represent the actions of endogenous phosphatases on the isolated IIO complex. We have documented the presence of FCP1 in BRCA1-RNA pol II immunoprecipitates (Fig. 3F, lower panel) using antisera provided by Dr. Michael E. Dahmus (UC Davis). FCP1 is an active p220 phosphatase required for maximum processivity and recycling of RNA pol II (58). Interestingly, FCP1 is not efficiently inactivated by the phosphatase inhibitors used in our extracts (NaF and Na$_3$VO$_4$). More complete inactivation of FCP1 requires EDTA and EGTA (59) which is absent from our buffers due to the requirement for divalent cations in some BRCA1 structures (60,61). As this IIA-like band represents a minor percentage of the total p220 present in BRCA1 complexes, we have not been able to fully document its origin, though this remains an interest.

*Unphosphorylated C-terminal domains from various mammalian BRCA1 orthologs interact preferentially with phosphorylated RNA pol II in human and other cells.*

Because of suitable antibody cross reactivity, we were able to show that both human and bovine BRCA1 proteins interacted with RNA pol II holoenzyme complexes that contained hyper-phosphorylated (IIO) p220 (above and data not shown). To demonstrate that this interaction was conserved more generally, and to study structural features of BRCA1 required for p220 association, we tested unphosphorylated BRCA1-CTDs (human, mouse, cow and dog) for their ability to bind RNA pol II complexes from various cells. To do this, GST fusions with the BRCA1-CTDs were purified from bacterial cells, and the purified proteins were incubated
with nuclear extracts. Specifically bound proteins were subjected to immunoblot analysis with antisera recognizing the 220 kDa catalytic subunit of RNA pol II (Fig. 4B). All four BRCA1-CTDs interacted with the hyper-phosphorylated RNA pol II (IIO). The presence of hyper-phosphorylated p220 in the complexes was confirmed by incubation of the blots with antibodies specific for phosphorylated forms of p220, including serine 2 (S2) and serine 5 (S5). In the same experiment, treatment of bound proteins with calf intestinal phosphatase resulted in a reduction in the molecular weight of the p220 subunit to that of the predicted size for the unphosphorylated p220 (IIA).

Importantly, unphosphorylated BRCA1-CTDs from all species tested behaved equivalently in binding the hyper-phosphorylated RNA pol II (Fig. 4B). In similar types of GST pull-down assays, the human and mouse BRCA1-CTDs bound specifically and preferentially with hyper-phosphorylated RNA pol II from mouse mammary epithelial cells (C57MG), and the human and cow BRCA1-CTDs interacted specifically and preferentially with hyper-phosphorylated RNA pol II from bovine MDBK (bovine kidney) cells (data not shown).

BRCA1-associated RNA pol II represents a large proportion of the catalytically active pol II activity in cycling cells.

To specifically demonstrate that the BRCA1-RNA pol II complex represented functional RNA pol II, we performed transcription runoff assays with proteins that co-precipitated with BRCA1 from human cells. These assays utilize a modification of the C-tail template assay known to require functional RNA pol II (43,44). The diagram (Fig. 5A) shows that a 45-nucleotide RNA transcript is generated when RNA pol II is added to a short double-stranded DNA template containing a 3’ poly-dC tail. As shown in Fig. 5B, the RNA pol II that associates
with BRCA1 is capable of transcribing full-length transcripts from this template.

Interestingly, BRCA1 immunoprecipitated fractions consistently demonstrated greater runoff transcription activity than was found in p220 precipitates from the same number of cells (compare Fig. 5B lane 3 with lane 6, and Fig. 5D, lanes 1 and 2). To test this further, an ELISA was developed to quantify the amount of p220 found in cells (Fig. 5C). Briefly, anti-BRCA1 or anti-p220 immune complexes were immobilized on immunoassay chambers and each complex was subsequently probed with a distinct p220 specific antisera (8WG16). These assays confirmed that BRCA1 immune complexes contained only 4-6% of cellular p220. When these values were used to normalize the runoff transcription assays (Fig. 5D), the RNA pol II found in BRCA1 precipitates was 6-fold more active than the total pool of p220 in the same volume of extract (compare Fig. 5D, lanes 3 and 4). These results indicate that BRCA1 interacted with a large percentage of the transcriptionally active RNA pol II in these cells.

**Discussion:**

From these studies, we propose that minimally phosphorylated BRCA1 interacts with post-initiation forms of RNA pol II and that this interaction diminishes following phosphorylation of BRCA1 as a result of genotoxic stress. In binding this specific form of the RNA pol II holoenzyme, BRCA1 is unlikely to play a direct or specific role in “transcriptional activation” (promoter activation) *per se*. However, interaction with processive RNA pol II places BRCA1 in a position where it can play a significant role in transcription, through roles in post-initiation events. In addition, association with the processive RNA pol II holoenzyme places BRCA1 in position to act as a sensor or regulator of multiple cellular functions, including chromatin remodeling, transcription-coupled repair, and genomic surveillance for DNA damage,
all of which are perturbed in BRCA1 deficient cells (62,63,69). Preferential interaction of unphosphorylated BRCA1 with the processive RNA pol II complex allows a close linkage of transcription and genomic repair processes in eukaryotic cells.

Several models of BRCA1 interaction with the transcriptional complex proposed that BRCA1 functioned in pre-initiation roles to regulate expression of specific genes, potentially including genes involved with DNA repair or the genomic stress response (20,64,65), and that the interactions affected promoter-associated complexes (19,23,25). A key element to this line of reasoning was the observation that CTD of human BRCA1 could act as a transcriptional activator in one-hybrid type assays (14-18). We present seven pieces of data that call aspects of the pre-initiation models into question: 1) full-length BRCA1 proteins (and D11 splice variants) isolated from several species, including human, lack one-hybrid transcriptional activation activity when tethered to a promoter element in GAL4-UAS one-hybrid assays; 2) one-hybrid transcriptional activation activity of BRCA1-CTDs is poorly conserved and correlates, in part, with negative charge; 3) closely related BRCA1-CTDs from mouse and cow lack significant one-hybrid transcriptional activation activity when tested in multiple cell types and under multiple conditions; 4) in-frame fusion of the mouse CTD onto portions of the human CTD sequence severely dissipates CTD-associated one-hybrid activity; 5) in vivo, BRCA1 proteins from all four species tested interact specifically with hyper-phosphorylated (IIO) RNA pol II, widely considered to represent post-promoter forms of the pol II complex; 6) in vivo, BRCA1 proteins fail to interact with hypo-phosphorylated (IIA) forms of RNA pol II that would be expected in pre-initiation stages of the transcriptional apparatus; 7) in vivo, BRCA1 interacts specifically with a very large percentage of the catalytically active RNA pol II while binding to a very small percentage of the total RNA pol II found in these cells, suggesting that BRCA1
interacts preferentially with post-promoter (late) forms of the transcriptional machinery.

Human BRCA1 rescues homologous recombination, viability, gametogenesis, and tumor-free survival in BRCA1-null mice and murine cell lines (11,12,48). If transcriptional activation were an intrinsic function of BRCA1, one would expect to see conservation of this activity in other species, and association of BRCA1 with promoter bound forms of RNA pol II. In the present study the interaction of BRCA1 proteins have been tested in a variety of assays aimed at assessing functional activity of the BRCA1-RNA pol II complex. Both full-length proteins and BRCA1-CTDs have been tested in protein-protein interaction studies, co-immunoprecipitation assays and two types of functional assays (one-hybrid transactivation studies, and runoff transcription assay). While CTD-associated transcriptional activation activity was poorly conserved, and correlated in part with charge in the four BRCA1 orthologs tested, all BRCA1 proteins interacted with the hyper-phosphorylated form of RNA pol II.

RNA pol II is sequentially phosphorylated during elongation of the mRNA transcript (66). As part of promoter initiation, serine 5 residues within the heptameric repeats of p220, are phosphorylated by cdk7, a subunit of TFIIH (67). Subsequently, cdk9/pTEFb phosphorylates serine 2 of the heptad to create highly processive post-promoter forms of RNA pol II holoenzyme (68). There are 52 repeats of this heptad sequence in mammalian p220 catalytic subunits, and phosphorylation of multiple heptads is required to generate the hyper-phosphorylated IIO form associated with elongation (51-53). In the present study, unphosphorylated full-length BRCA1 and BRCA1 derived CTDs were shown to interact with hyper-phosphorylated RNA pol II containing multiply-phosphorylated heptad repeats. Efficient detection of hyperphosphorylated IIO RNA pol II required rapid analysis of fresh nuclear extracts, which contained significant levels of endogenous RNA pol II phosphatases, including
Based on the present study, we conclude that unphosphorylated BRCA1 associates with processive RNA pol II and that the interaction is diminished following genomic stress. We suggest that effects observed on transcription, in cells engineered to constitutively over-express BRCA1, may result from complex regulation of transcription, as opposed to direct binding and recruitment of RNA pol II to specific promoters. It is clear that ultimate resolution of this issue will require analysis of BRCA1 interactions with chromatin from multiple regions within genes and across chromosomes. Interactions of BRCA1 with processive RNA pol II would likely position the protein to interact with chromatin at sites downstream of the promoter. While such interactions could contribute to post-initiation functions of RNA pol II (33-35), they would also position BRCA1 for other functions such as DNA-damage surveillance, which correlate well with other studies (5,54,69).

Acknowledgements:

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Li. We thank Amilcar Rizzo and Robert Lurvey for preparation of GST fusion proteins and the other members of the Lane lab for their many contributions to this project.

References:


Figure Legends:

Fig.1. Transcriptional activation by BRCA1 C-terminal domains (CTDs) is poorly conserved and correlates with net negative charge of the domain. (A) Structural features of BRCA1. Full-length BRCA1 proteins have a RING finger, tandem nuclear localization signals (NLS) and two BRCA1 C-terminal (BRCT) repeats. The CTD of BRCA1 is encoded by exons 12-24, including both BRCT repeats (exons 16-24) and an upstream region (exons 12-15) encoding ATM, ATR and other phosphorylation motifs. (B) To assess the stability of CTD domains from various species, cells were transfected with the GAL4 DNA binding domain (GAL4-DBD) alone (152 amino acids) (not shown in this image) or GAL4-DBD fused to cow (485 amino acids), dog (499 amino acids), human (483 amino acids) or mouse (479 amino acids) CTD. Cell lysates were analyzed by immunoblotting with antibody to the GAL4-DBD. (C) Human MCF-7 mammary epithelial cells were transfected with GAL4-DBD fusions to CTDs from human (h), dog (d), cow (c) or mouse (m) BRCA1 genes, or to the canonical activation domain of herpesvirus VP16. (C Inset) The total charge of each CTD was graphed versus its fold-activation, shown in part C. Transcriptional activation assays were also conducted in cells derived from various species, including: D) human 293T (black bars) or T47D cells (gray bars), (E) bovine aortic endothelial (BAE) cells, and (F) murine mammary epithelial cells (MNuMg, black bars) and murine 3T3 cells (gray bars). Transcriptional activation assays are plotted as the fold activation over control (GAL4 alone) after normalization to an internal Renilla luciferase standard, as described in the Experimental Procedures section. Error bars define ± one standard deviation (S.D.).
Fig. 2. **Transactivation activity is absent in all full-length BRCA1 proteins, but can be identified in constructs containing the most acidic C-terminal domains.** GAL4-UAS transcriptional activation activity of full-length BRCA1 proteins and various size fragments of BRCA1 proteins were compared in human 293T, T47D and MCF-7 cells. The following constructs were analyzed: (A) Full-length human BRCA1 (amino acids 1-1863), human BRCA1 CTD (amino acids 1380-1863), two tumor-associated truncating mutation (BRCA1-1853term (amino acids 1380-1852) and BRCA1-5382insC (amino acids 1380-1755)), and three additional fragments of the human BRCA1 CTD; (B) full-length mouse BRCA1 (amino acids 1-1813), the mouse CTD (amino acids 1334-1813), the naturally occurring Δ11 splice variant (amino acids Δ223-1328) and four additional fragments of mouse BRCA1; (C) full-length cow BRCA1 (amino acids 1-1849) and the cow CTD (amino acids 1364-1849); (D) The dog CTD (amino acids 1380-1879). (E) Fragments of human BRCA1 CTD (white bars) were fused in-frame to fragments of the mouse CTD (gray bars) and assayed for activity. (F) A scale model of the conserved RING finger, BRCT domains and CTD within full-length BRCA1. Fold activation was calculated as the mean of three transfections plus or minus one standard deviation (S.D.). ND, not determined. Gray boxes highlight the values for the CTDs.

Fig. 3. **Minimally phosphorylated BRCA1 proteins interact preferentially with hyper-phosphorylated RNA polymerase II (pol II).** (A) A schematic diagram of the catalytic subunit (p220) of RNA pol II showing epitopes identified by various antisera used in this study. N20 and 8WG16 are raised against minimally modified peptide fusions, whereas S2 and S5 were raised against phosphopeptides representing the heptad repeats of processive RNA pol II. (B) Nuclear lysates from human HBL100 epithelial cells were prepared in the presence of phosphatase inhibitors and then immediately immunoprecipitated (IP) with antisera ([]) to RNA
pol II (N20), or BRCA1 (BR). Immunoblotted material was then probed (WB) with β-pol II (N20) or β-phospho-pol II specific antisera (S5 or S2). (C) Minimally phosphorylated BRCA1 was found in complex with hyper-phosphorylated pp220. Nuclear lysates were mock immunoprecipitated (lane 1) or immunoprecipitated with antisera to BRCA1 (lanes 2 and 3), p220 (N20, lanes 4 and 5), phosphoserine 5 p220 (S5, lanes 6 and 7) or phosphoserine 2 p220 (S2, lanes 8 and 9). Precipitated material was split into two equal fractions and treated ± phosphatase (CIP). Immunoblotted material was then probed with BRCA1 antisera (Ab-4). (D) The interaction between RNA pol II and BRCA1 decreases after DNA damage. Cells were either untreated (c) or treated with adriamycin (adr), camptothecin (cmt), or hydroxyurea (HU), and lysed after 60 minutes. Proteins were immuno precipitated with BRCA1 specific sera (IP) and immunoblotted (WB) with β-pol II (8WG16). (E) The reduced mobility of BRCA1 associated p220 is due to phosphate. Nuclear fractions were immunoprecipitated with antisera to BRCA1 or RNA pol II (8WG16). The immunoprecipitated material was divided into two equal fractions and treated ± phosphatase (CIP), as indicated. Immunoprecipitated proteins were probed (WB) with β-pol II (8WG16). (F) The isolated BRCA1-RNA pol II complex is stable in the presence of DNA denaturants and contains FCP1, a marker of processive RNA pol II. Immune complexes were prepared ± 50 μM EtBr and subsequently treated with phosphatase prior to immunoblotting with β-pol II (8WG16) or β-FCP1. For all panels, immunoprecipitated complexes were run on a 5% SDS-PAGE gels prior to immunoblotting with the indicated sera. Hyper-phosphorylated (IIO) p220 is identified with solid arrowheads. Hypo-phosphorylated (IIA) p220 is indicated with open arrowheads in panels B, D and E.

Fig. 4. The unphosphorylated C-terminal domain (CTD) of BRCA1 proteins is sufficient to direct specific interaction with hyper-phosphorylated RNA pol II. (A) The map diagrams the
structure of the CTD constructs tested. (B) Bacterially expressed GST-CTD fusion proteins were incubated overnight at 4°C with nuclear extracts prepared from HBL100 cells in the presence of phosphatase inhibitors. Bound complexes were washed, and then treated ± phosphatase (CIP) for 60 minutes at 37°C. As a positive control, RNA polymerase II was precipitated with α-pol II antisera (8WG16, lanes 1-2). Proteins present in the pull-downs were analyzed on immunoblots and probed (WB) with the indicated antisera, followed by stripping and reprobing as indicated. IIO and IIA forms of RNA pol are indicated as in Figure 3. The membranes were then probed with antisera to GAL4 epitope tag (α-GST) to show the various fusion proteins present in lanes 5-12 (bottom panel).

Fig. 5. **BRCA1 interacts with a large proportion of functionally processive RNA polymerase II holoenzyme.** (A) Schematic representation of the modified RNA polymerase runoff transcription assay (C-tailed assay) used in this study. Double-stranded DNA templates were designed to generate a full-length (FL) RNA transcript of 45 nucleotides in the presence of active RNA pol II. (B) Runoff transcription assays were conducted with increasing amounts of immunoprecipitated pol II catalytic subunit (p220), or subunit that co-precipitated with BRCA1. Immunoprecipitations were carried out from equivalent amounts of HBL100 nuclear extract. Incorporation of [32P] NTPs into RNA was assessed by PAGE and dried gels were exposed to X-ray film. Product was quantified by Phosphoimager scans of the same gels and values were normalized to the highest BRCA1 fraction (100%). (C) To determine the relative catalytic activity of p220 precipitated by p220 and BRCA1 antisera respectively, a quantitative ELISA was developed. p220 catalytic subunit (8WG16) was quantified and normalized to the highest value. (D) A second RNA polymerase runoff assay was performed on complexes immunoprecipitated with α-BRCA1 or α-pol II antisera. Assays were run with either equivalent
amounts of nuclear extract (lanes 1 and 2) or equivalent amounts of immunoassayed p220 (lanes 3 and 4). BRCA1 precipitates isolate the majority of catalytically active p220 in these extracts.

Supplemental Figure #1. **Substitution of alternative GAL4-UAS reporter vectors does not enhance the activity of mouse GAL4-CTD fusions.** We compared the transcriptional activation of human and mouse GAL4-CTD fusions using four different well characterized eukaryotic UAS reporter vectors (pFR-luc, M1-luc, M2-luc and pWHGG). The number of GAL4-UAS repeats, the source of the minimal promoter, and the sequence of the TATA box are depicted for each vector. 293T cells were transfected with the indicated GAL4-CTD fusion, along with the indicated reporter vector, and a Renilla luciferase transfection control. Results represent the mean (± 1 S.D.) for triplicate transfections after normalization to the GAL4 epitope vector alone.

Supplemental Figure #2. **Increased nuclear localization of CTD constructs did not increase transcriptional activation of mouse BRCA1 CTD.** (A) Human and mouse CTDs, with or without an exogenous SV40 nuclear localization signal (NLS) were fused in-frame with green fluorescent protein (GFP) and transfected into 293T cells. GFP fluorescence demonstrated that an external NLS is required for effective nuclear import of CTD domains when expressed in isolation. (B) Human and mouse CTDs, with or without added nuclear localization signals, were then fused to the GAL4-DBD to compare UAS induction activity as described above. Data is presented as the mean of triplicate transfections (± 1 S.D.). (B inset) An immunoblot of similar
extracts was probed (WB) with antibodies against the GAL4 epitope tag (a-GAL4), to demonstrate equivalent expression of all constructs. Arrows identify the GAL4-CTD fusions. The GAL4 epitope alone (lane 1) runs as a monomer of approximately 28 kDa and was not included in this image.
Fig. 1

A. BRCA1 Structural Domains

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B. BRCA1 CTD fusions

WB: αGAL4

80 kDa

cow CTD
dog CTD
human CTD
mouse CTD

C. CTD Trx Activation Assay

Fold Activation vs. charge

MCF7
r² = 0.954

D. Other human cells

Fold Activation

GAL4 | h | d | c | m

T47D
293T

E. Bovine cells

Fold Activation

GAL4 | h | c

BAE

F. Mouse cells

Fold Activation

GAL4 | h | m

MNUmg
3T3
Fig. 2

A. Human

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D. Dog

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F. Conserved Domains of BRCA1
Fig. 3

**A.** RNA pol II, catalytic subunit

- **p220 (POLR2A)**
- **catalytic domain**
- **heptamer repeats (52 x 7mer)**

**B.** BRCA1 interacts with IIO form pp220

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**C.**

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**D.**

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**E.**

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<td><strong>WB:</strong></td>
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Fig. 5

A. Runoff template

45nt full-length transcript (FL)

→

5'

CC(n)

5'

poly C

45mer

65mer

B. Runoff template assay:

45nt

% activity: 5 18 75 10 30 100

IP: p220  

BRCA1

1 2 3 4

FL

C. p220 ELISA (normalized values)

IP: BRCA1 p220

D. Loading:

IP: BRCA1 p220

% activity:

100 71 100 16

equivalent extract  
equivalent p220
Supplementary Fig. 1
Supplementary Fig. 2

A. GFP fusion:

B. CTD Transactivation:

CTD Activity Assay: