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TITLE: Consequences of Cyclin D1/BRCA1 Interaction in Breast Cancer Progression

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<b>14. ABSTRACT</b>  The inheritance of one defective BRCA1 or BRCA2 allele predisposes an individual to developing breast, ovarian and T-cell cancers. In addition, in breast cancers where BRCA1 is not mutated, it is often functionally inactivated. Furthermore, cyclin D1 has been shown to be overexpressed in many cancers including breast cancer and its associates with BRCA1. Because of the crucial role of both of these proteins in cancer, it is reasonable to expect that this interaction has a significant role in tumor cells. The understanding of when this interaction occurs during cell cycle progression will help to determine the role of cyclin D1/BRCA1 binding in breast cancer cells. Therefore, I hypothesize that the direct interaction of cyclin D1 with BRCA1 results in the cell cycle dependent regulation of the activity of BRCA1. In this study, I wish to identify and confirm the cell cycle dependent cyclin D1/BRCA1 interaction in breast cancer cells, determine the biochemical consequence of cyclin D1/BRCA1 interaction in breast cancer cells, and determine the functional consequence of BRCA1 phosphorylation in breast cancer. BRCA1's phosphorylation by cyclin D1/cdk complexes may help to regulate BRCA1's localization to the nucleus, since BRCA1 has been shown to have a cytoplasmic expression pattern, but acts primarily in the nucleus. Phosphorylation may also be important in modulating BRCA1's ability to bind DNA, either as a transcription factor or as part of a DNA damage repair complex. Determining the consequences of the interaction of cyclin D1/BRCA1 could lead to a more complete understanding of how breast cancer occurs, thus leading to new treatment options.					
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## INTRODUCTION

The breast cancer susceptibility gene, BRCA1, is a major player in the progression to breast cancer. The inheritance of one defective BRCA1 or BRCA2 allele predisposes an individual to developing breast, ovarian, and T-cell cancers (1). Hereditary breast cancer constitutes only about 5-10% of all cases and 80% of these cases have mutations in either BRCA1 or BRCA2 (2,3). The remaining 90-95% of all breast cancer cases are considered sporadic breast cancer. While mutational inactivation of BRCA1 or BRCA2 genes in sporadic breast cancer is rarely observed (4), BRCA1 promoter is hyper-methylated in 10-15% of sporadic breast cancers (5-10). In addition, 30-40% of sporadic breast cancers exhibit reduced expression of BRCA1 mRNA and protein levels, especially in tumors with high nuclear grade (11-15).

The BRCA1 gene is composed of 24 exons and encodes a large protein of 1863 amino acids. At its N-terminus it contains a RING (Really Interesting New Gene) finger domain (amino acids 24-64), which has been shown to contain Ubiquitin E3 ligase activity (16). The C-terminus of BRCA1 contains an acidic transcriptional activation domain (TAD), which is transcriptionally active when fused to a GAL4 DNA binding domain (17,18). Within this TAD domain are two BRCT (BRCA1 C-terminus) motifs that are found in a large family of proteins important for DNA damage response, such as DNA ligase IV, p53BP1, and base excision response scaffold protein XRCC1 (19).

Functionally, overexpression of BRCA1 causes growth arrest in breast and ovarian cell lines (20), G2/M arrest (21), or apoptosis (22) depending on the report. BRCA1 expression can also inhibit growth of tumors formation in MCF-7 mice (20). One way BRCA1 exerts its growth arrest potential is through binding to Rb. BRCA1 can interact with Rb through both its C-terminus and the N-terminal region (23-25). This interaction is essential for BRCA1 induced cell cycle arrest as Rb-/- MEFs do not exhibit BRCA1 induced growth arrest (24). Other components of the cell cycle machinery also interact with BRCA1. Wang *et al.* demonstrated that the N-terminal of BRCA1 (aa 1-76) which contains the zinc finger domain, can interact with numerous cell cycle regulatory proteins including, cdc2, cdk2, cdk4, cyclin B, cyclin D, cyclin A, and E2F-4 (26). They were unable to detect interaction with cdk3, cdk5, cdk6, E2F-1, E2F-2, E2F-3, E2F-5, and cyclin E (26). Additionally, BRCA1 induces growth arrest through transcriptional activation of cell cycle checkpoint genes, such as p21/waf1, p27/kip1, and GADD45 $\alpha$ .

BRCA1 was first implicated in transcription when the C-terminus (aa 1560-1863) fused to Gal4 was able to activate transcription (18), with aa 1760-1863 being the minimal transactivation domain. Since that time numerous other findings have served to strengthen the connection between transcription and BRCA1. For instance, BRCA1 is part of the RNA polymerase II holoenzyme complex (27-29). One way BRCA1 may be recruited to this complex is through binding to RNA Helicase A, an enzyme that unwinds RNA and DNA molecules (29). Beyond the RNA polymerase II complex, BRCA1 also interacts with multiple cofactors and transcription factors. BRCA1 binds to the co-activators CBP/p300 in a phosphorylation independent manner, which resulted in the cooperative activation of the Rous sarcoma virus-long terminal repeat promoter (30). Conversely, another study found that BRCA1 downregulated p300 expression and that p300 expression rescued BRCA1 inhibition of ER mediated transcription (31). BRCA1 stimulates STAT1 in response to IFN $\gamma$  (32), NF- $\kappa$ B in response to tumor necrosis factor-alpha or interleukin-1beta (33), and p53 responsive promoters (34).

Among the genes found to be transactivated by BRCA1 are *mdm2*, *bax*, *p21/waf1*, *p27/Kip1*, and *GADD45 $\alpha$* , with *p21/waf1* and *GADD45 $\alpha$*  transactivation being independent of *p53* (22,34-38). Conversely, BRCA1 is able to inhibit transcription of estrogen responsive genes as well as telomerase reverse transcriptase gene expression. These studies also demonstrated BRCA1 on *pS2* (estrogen responsive gene) and *hTERT* promoters *in vivo* (39-41). Collectively, it appears that based on the promoter of interest, BRCA1 can have either activating or inhibitory effects.

BRCA1 is a phosphoprotein with its phosphorylated form predominating in S phase and subsequently becoming dephosphorylated after M phase (42,43). Cyclin A and D complexes have been shown to phosphorylate BRCA1 *in vitro* (42), whereas Cyclin A/cdk2 and Cyclin E/cdk2 complexes phosphorylated BRCA1 on Ser-1497 *in vivo* (44). Aurora-A binds and phosphorylates BRCA1 on Ser 308, which is important for the G2/M phase transition (45). In addition, multiple DNA damage protein kinases phosphorylate BRCA1 in response to DNA damage (46).

Cell cycle regulatory genes are often targeted in tumorigenesis mainly because this is one way of deregulating and increasing cell proliferation (47,48). The cyclin Ds in particular have been implicated in a variety of cancers such as breast and prostate cancers, and T-cell leukemia (49-51). Cyclin D1 is encoded by the *CCND1* gene localized to the chromosome band 11q13, which is amplified in 15-20% of breast cancer cases (52), while the protein levels of cyclin D1 are overexpressed in up to 35% of breast cancers (53). Cyclin D1 is classified as a weak oncogene because it is able to transform fibroblast in combination with activated H-Ras (54); making cyclin D1 the most commonly overexpressed oncogene in breast cancer (53,55-57). Cyclin D1 has also been shown to act as an oncogene through a study where the expression of cyclin D1 in mouse mammary glands led to hyperplasia and adenocarcinomas (58). Further studies have indicated that overexpression of cyclin D1 is associated with increased genomic instability (59). Finally cyclin D1 overexpression is correlated with tumor metastasis, possibly due to its contribution to cellular adhesion, migration, and invasion of endothelial cells and macrophages (60).

The most well know function of cyclin D1/cdk complexes is the phosphorylation of the retinoblastoma protein Rb. Rb is a tumor suppressor that functions as a gate keeper of the G1/S phase transition by binding to E2F transcription factor complexes and suppressing transcription. Progression through the G1/S checkpoint is accomplished mainly through the sequential phosphorylation of the retinoblastoma protein (Rb) by cyclin D/cdk4, 6 and cyclin E/cdk2 complexes. A recent model of how this occurs involves Rb and E2F complexes residing on E2F dependent promoters. Only after cyclin/cdk phosphorylation will Rb leave the promoter, thus allowing E2F to activate transcription of proliferative genes (61). Recently, it was demonstrated that cdk4 and cdk2 complexes phosphorylated Smad3 *in vitro* and *in vivo* resulting in inhibition of Smad3 transcriptional activity, specifically in the inhibition of anti-proliferative genes (62). Therefore, Smad3 phosphorylation by cyclin D1/cdk4 complexes adds another level by which cyclin D1 functions to stimulate cell cycle progression.

While various cyclin/cdk complexes, including cyclin D, have been shown to phosphorylate BRCA1, the consequence of cyclin D1/cdk phosphorylation on BRCA1 has not been determined. Based on the opposing roles of cyclin D1 and BRCA1 on cell cycle progression we hypothesize that cyclin D1/cdk phosphorylation of BRCA1 results in the inhibition of the tumor suppressor/cell cycle checkpoint role of BRCA1. Here I demonstrate through co-immunoprecipitation and *in vitro* binding assays, a direct interaction between cyclin D1 and BRCA1. Amino acids 1-500 and 504-802 of BRCA1 are important for the binding and

the phosphorylation by cyclin D1/cdk4 complexes. I have demonstrated that Ser 632 is an important site of phosphorylation for cyclin D1/cdk4 complexes. In addition, cyclin D1/cdk4 complexes phosphorylate BRCA1 *in vivo* as treatment with a cdk4 specific drug, Fascaplysin, resulted in decreased phosphorylation of BRCA1. Through chromatin immunoprecipitation assays (ChIP), I identified many novel BRCA1 bound promoters. Furthermore, the inhibition of cyclin D1/cdk4 phosphorylation of BRCA1 results in the increased presence of BRCA1 at SST and FHX promoters *in vivo*. These results suggest that cyclin D1/cdk4 phosphorylation of BRCA1 inhibits the ability of BRCA1 to be recruited to particular promoters *in vivo*.

## BODY

### Experimental Methods

Cell culture. MCF-7, and T47D are breast cancer epithelial cells derived from pleural effusions (36). MCF-10A is a normal breast epithelial cell line. T47D cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% streptomycin/penicillin. MCF-7 cells were grown in DMEM containing 10% (FBS), 1% L-glutamine, and 1% streptomycin/penicillin. MCF-10A cells were grown in Complete Mammary Epithelial Cell Medium plus 100 ng/ml of cholera toxin. All cells were grown in a 5% CO<sub>2</sub> incubator at 37°C. G<sub>0</sub> synchronization was performed as follows. Briefly, cells were brought to 95 % confluency, washed, and cultured in serum-free DMEM for 3 days. To stimulate cells DMEM plus 20% heat inactivated fetal calf serum (HIFCS) was added. MCF-7 cells were synchronized in S phase by treating with Hydroxyurea (2mM) for 48 hours at 60% confluency. Cells were released into DMEM plus 20% HIFCS and collected 4 hours later. G<sub>2</sub>/M phase cells were obtained by treating MCF-7 cells with 50 ng/ml of Nocodazole for 24 hours and collected the cells at 0hr.

Plasmids. pDC78 GST-BRCA1 (1-500), pDC79 GST-BRCA1 (452-1079), pDC80 GST-BRCA1 (1021-1552), pDC81 GST-BRCA1 (1501-1861), pDC99 GST-BRCA1 (504-802), pDC208 GST-BRCA1 (697-1276) were a kind gift from Dr. Tanya Paull at the University of Texas/ICMB. These constructs were original made in Dr. Elledge's lab at Vanderbilt University (37).

Transformation of GST-Plasmids. One µg of specified plasmid was incubated on ice for 2 hours with 200µl of the DH5α strain of *E. coli* competent cells. Cells were then heat shocked at 42°C for 90 seconds. Three hundred µl of LB broth (-Amp, +MgSO<sup>4</sup>, +dextrose) was added to the solution and the cells were incubated for 30 minutes at 37°C. Two hundred µl of solution was spread on Amp LB agar plates for selection. Plates were incubated overnight at 37°C. The next day, a single colony was selected and placed in 10ml LB broth (+Amp, +MgSO<sup>4</sup>, +dextrose), and shaken overnight at 37°C. Then, 5ml of *E. coli* was transferred into 150-200ml of LB broth (+Amp, +MgSO<sup>4</sup>, +dextrose) and shaken until the solution reached an OD of 0.5 to 0.6. The cells were then induced with 0.5 mM IPTG at 25°C overnight. After induction, *E.coli* was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C.

Purification of GST-Proteins. *E. coli* pellets were resuspended in 10 ml of PBS + 1% Triton X 100. *E.coli* was then lysed through sonication and freeze/thaw methods. After last thaw, the solutions were centrifuged at 10,000 rpm for 10 minutes. Supernatant was transferred to new tubes and pellet was discarded. Glutathione-Sepharose Beads were prepared by washing 3 times with PBS+1% Triton X-100, and a 30% slurry was created. Then, 100-200µl of a Glutathione-Sepharose Bead solution was added to 2-3 ml of lysed *E. coli* supernatant for binding overnight at 4°C. The next day, complexes were washed once in TNE300 + 0.1% NP-40 and once in PBS+1% Triton X-100. Complexes were resuspended in PBS + 1% Triton X-100. An aliquot of each GST-fusion protein was run on a 4-20% Tris-glycine gel by SDS-PAGE along with standard protein controls (BSA). Coomassie Blue staining was used to determine relative amounts of GST-protein as compared to standard protein concentrations.

TNT Binding Assays. <sup>35</sup>S-labeled HA-cyclin D1 was produced using the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's protocol. pcDNA3-HA cyclin D1 [a kind gift from Dr. Doris Germain (38)] was used to produce the <sup>35</sup>S-labeled protein. To confirm synthesis, proteins were separated by SDS-PAGE on a 4-20 % Tris-glycine polyacrylamide gel. The gels were dried and exposed to a PhosphorImager cassette. Binding assays included GST-BRCA1 constructs (1 µg), or GST (5 µg) (concentration determined by Coomassie Blue staining) and 5 µl of TNT reactions and were carried out at 4°C overnight. The next day, complexes were washed twice with TNE<sub>150</sub> + 1.0% NP-40 and once with TNE<sub>50</sub> + 0.1% NP-40. Complexes were separated on a 4-20% Tris-glycine gel by SDS-PAGE. The gel was dried and exposed to a PhosphorImager cassette.

Cell extract preparation. Cells were centrifuged at 4°C for 5 minutes at 2,000 rpm. Cell pellets were washed twice with D-PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>. Cell pellets were then resuspended in lysis buffer (50 mM Tris-Cl, pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (NP-40), 50 mM NaF, 1 mM DTT, 0.2 mM Na<sub>3</sub>VO<sub>4</sub> and one complete tablet of protease cocktail inhibitor/ 50 ml buffer) on ice and vortexed every 5 minutes for a total of 30 minutes. Cell lysates were then transferred to Eppendorf tubes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was then transferred to a new Eppendorf tube and the protein concentration taken using Bio-Rad protein assay.

Antibodies and Immunoblotting. Anti-cyclin D1 (M-20) and anti-BRCA1 (C-20) polyclonal antibodies were purchased from Santa Cruz. Total cellular protein was separated on 4-20% Tris-glycine polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) membranes (Immobilon-P transfer membranes; Millipore Corp.) overnight at 0.08 A. For the last thirty minutes of transfer, the amperage was increased to 200 A. Following the transfer, blots were blocked with 5% non-fat dry milk in 50 ml of TNE<sub>50</sub> (100 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) plus 0.1% NP-40. Membranes were probed with a 1:200 dilution of antibodies at 4°C overnight, followed by three washes with TNE<sub>50</sub> plus 0.1% NP-40. Next day blots were incubated with 10 ml of <sup>125</sup>I-protein G (Amersham, 50 µl/10ml solution) in TNE<sub>50</sub> plus 0.1% NP-40 for 2 hrs at 4°C. Finally, blots were washed three times in TNE<sub>50</sub> plus 0.1% NP-40 and placed on a PhosphorImager Cassette for further analysis.

Immunoprecipitation. One mg of cellular proteins and 5µg of appropriate antibody were used. Samples were rotated overnight at 4°C and the next day protein A and protein G agarose beads (Oncogene Research Products/Calbiochem catalog IP05) were added. This mixture was rotated for 90 minutes at 4°C. Samples were washed twice in the appropriate TNE buffer plus 0.1% NP-40 and once in TNE<sub>50</sub> plus 0.1% NP-40. Complexes were analyzed by reducing SDS-PAGE, on a 4-20% Tris-glycine gel.

Kinase assays. Cell extracts were immunoprecipitated (IP) overnight with the anti-cyclin D1 (M-20) rabbit polyclonal antibody. Protein G and protein A agarose beads were added to IPs and rotated for 2 hrs at 4°C. IPs were washed twice with the TNE<sub>300</sub> buffer, once with TNE<sub>50</sub> + 0.1% NP-40, and twice with kinase buffer (50 mM HEPES, 10 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 mM DTT, 50 mM NaF, 0.2 mM Na<sub>3</sub>VO<sub>4</sub> and one complete tablet of protease cocktail inhibitor/ 50 ml buffer). The appropriate substrate was added (GST-Rb or various GST-BRCA1 fusion proteins)



to each tube (1µg), with 1 µl of [ $\gamma^{32}$ P]-ATP (3000 Ci/mmol). Reactions were incubated at 30°C for 1 hour and stopped by the addition of 15 µl of 2X SDS sample buffer. The samples were separated by reducing SDS-PAGE on a 4-20% Tris-glycine gel. Gels were stained with Coomassie blue, destained, and then dried for 2 hr. Following drying, they were exposed to a PhosphorImager cassette and analyzed utilizing Molecular Dynamic's ImageQuant Software.

Chromatin Immunoprecipitation Analysis (ChIP). Chromatin immunoprecipitations were performed using a modification of previously published methods (36,37). Approximately  $5 \times 10^6$  cells were utilized per immunoprecipitation. Cells were cross-linked by addition of formaldehyde (1% final concentration). Cross-linking was allowed to proceed at 37° C for 10 min. Cells were washed with PBS three times. Next, cells were collected by trypsin and scraping. Cells were collected by centrifugation at 4000 rpm for 10 min, and the resulting pellet was lysis in 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 7.5) and incubated 10 min at room temperature. Lysates were sonicated on ice for 10 cycles to obtain an average DNA length of 500 to 1200 bp. Lysates were then cleared by centrifugation at 4000 rpm for 10 min and diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 7.5, 167 mM NaCl). Chromatin was pre-cleared with a mixture of protein A and protein G Sepharose (blocked previously with 1 mg/ml salmon sperm DNA and 1 mg/ml BSA) at 4°C for an hour. Pre-cleared chromatin was incubated with 5 µg of antibody at 4°C overnight. Next day, 60 µl of a 30% slurry of blocked protein A/G sepharose was added, and immune complexes were recovered. Immunoprecipitates were washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL, pH 7.5, 150 mM NaCl) twice with High salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL, pH 7.5, 600 mM NaCl), once with LiCl wash buffer (0.25 M LiCl, 1 mM EDTA, 1% NP-40, 1.0% deoxycholate, 10 mM Tris-HCl, pH 7.5), and once with TE buffer. IPs were eluted twice with elution buffer (1.0% SDS, 0.1 M NaHCO<sub>3</sub>) at room temperature for 15 minutes each. To the eluants and input, 20 µl of 5 M NaCl was added and cross-links were reversed by incubating samples at 65°C overnight. Next day, proteinase K (100 µg/ml) was added and samples incubated at 55°C for one hour. Samples were extracted with phenol:chloroform and ethanol precipitated. Pellets were resuspended in 50 µl of TE buffer and assayed by semi-quantitative PCR. Thirty-five cycles of PCR were performed in 50 µl with 10 µl of immunoprecipitated material, 10 pmole of primers and 1 units of *Taq* DNA polymerase. Finally, PCR products were electrophoresed on 1% agarose gels and visualized.

Immunofluorescent staining. MCF-7 cells were grown on coverslips and washed twice with D-PBS without Mg<sup>2+</sup> and Ca<sup>2+</sup>. Cells were fixed 20 minutes with 4% paraformaldehyde. Next, cells were permeabilized for 20 minutes with 0.2% Triton X-100 in D-PBS without Mg<sup>2+</sup> and Ca<sup>2+</sup>. Slides were then washed with D-PBS without Mg<sup>2+</sup> and Ca<sup>2+</sup> and blocked for 10 minutes with 10% bovine serum albumin (BSA). Coverslips were incubated for one hour at 37°C with the primary antibody, (1:200 dilution) in 10% BSA. Primary antibodies used were anti-cyclin D1 (M-20) rabbit polyclonal and anti-BRCA1 (Ab1) mouse monoclonal. Slides were then washed three times with D-PBS without Mg<sup>2+</sup> and Ca<sup>2+</sup>. Secondary antibodies (1:50), in 10% BSA, were added and the slides were again incubated for 1 hour, in the dark at 37°C. Secondary antibodies used were Fluor 488-goat anti-rabbit IgG and Texas Red (TR)-goat anti-mouse IgG from Molecular Probes. The previous washes were repeated. Slides were then incubated at room temperature for 20 minutes with 2 µM of TOTO-3, a dimeric cyanine nucleic acid stain (Molecular Probes), for nuclear staining. Slides were washed briefly with H<sub>2</sub>O and the excess

liquid was removed. Prolong anti-fade (Molecular Probes) was added to the slides to prevent photo-bleaching. After drying the coverslips were sealed.

Confocal laser scanning microscopy. Slides were viewed with a Bio-Rad MRC1024 confocal laser scanning microscope (Center for Microscopy and Image Analysis, George Washington University) using the 60 X objective. Optical sections were taken using z-dimensions of 1.0  $\mu\text{m}$ . Pictures were produced using Adobe Photoshop 5.0 and Bio-Rad plug-ins.

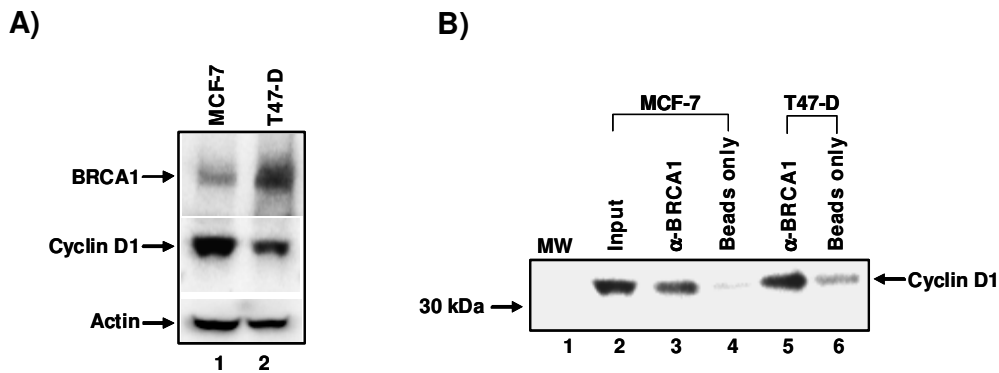
## **Results and Discussion**

### **Aim 1. Identify and confirm the cell cycle dependent cyclin D1 and BRCA1 interaction in breast cancer cells.**

**Task 1.** Initial experiments were designed to confirm that cyclin D1 and BRCA1 physically interact *in vivo*. Cyclin D1 has been shown to be overexpressed in many cancers, including breast cancer. Its association with BRCA1 has been demonstrated (31, 35). Because of the crucial role of both of these proteins in cancer it is reasonable to expect that this interaction has a significant role in the tumor cell. The understanding of when this interaction occurs during cell cycle progression will help to determine the role of cyclin D1/BRCA1 binding in breast cancer cells.

In order to initially address this aim, my first set of experiments examined the endogenous protein levels of BRCA1 and cyclin D1 in breast cancer cell lines. The breast cancer cell lines utilized were MCF-7 and T47D. MCF-7 and T47D are breast cancer epithelial cells derived from pleural effusions (36). As was proposed in Task 1 of Aim 1, asynchronous cultures were used to prepare protein extracts. The levels of cyclin D1 and BRCA1 were examined by immunoblotting utilizing anti-cyclin D1 and anti-BRCA1 rabbit polyclonal antibodies. Results from the western blot analysis indicate that the expression of cyclin D1 and BRCA1 is variable between cell lines. Please note that T47D cells expressed higher levels of BRCA1 compared to MCF-7 cells, whereas MCF-7 cells expressed higher levels of cyclin D1 as compared to T47D cells (Figure 1A). These results are interesting because the different expression levels of both cyclin D1 and BRCA1 endogenous proteins can be utilized to study effects of protein levels on their binding.

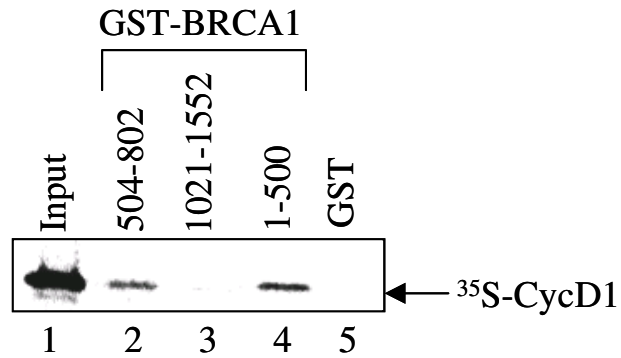
Next the ability of cyclin D1 to bind to BRCA1 in MCF-7 and T47D cells was examined through immunoprecipitation assays followed by western blotting. Anti-BRCA1 polyclonal antibodies were utilized in the immunoprecipitation assays, with Protein A and G agarose beads alone serving as the negative control. The immune complexes were examined by western blotting for cyclin D1. Results indicated that cyclin D1 was observed in immune complexes obtained by anti-BRCA1 antibodies (Figure 1B). In fact, this complex was resistant up to 1M NaCl, indicating that it is a very stable complex. *Therefore, in two different breast cancer cell lines, with variable cyclin D1 and BRCA1 protein expression levels, interaction between cyclin D1 and BRCA1 can be detected.*



**Figure 1: Cyclin D1 associates with BRCA1.** A) Seventy-five micrograms of total cellular protein was separated by SDS-PAGE on a 4-20% Tris-glycine gel, transferred, and blotted with anti-cyclin D1 and anti-BRCA1 rabbit polyclonal antibodies. The blot was stripped and reprobed with anti-actin goat polyclonal antibodies. The antigen-antibody complex was further detected with  $^{125}$ I-Protein G. B) MCF-7 and T47D cell extracts (1 mg) were immunoprecipitated with anti-BRCA1 rabbit polyclonal antibody or no antibody, overnight at 4°C. The complexes were precipitated with protein A+G agarose beads and washed with TNE<sub>600</sub> + 1.0% NP-40. Proteins were then separated by reducing SDS-PAGE on a 4-20 % Tris-glycine gel and transferred onto a PVDF membrane. A western blot was performed with anti-cyclin D1 rabbit polyclonal antibody.

To confirm these interactions, I performed *in vitro* binding assays with Glutathione S-transferase (GST)-BRCA1 fusion proteins and  $^{35}$ S labeled cyclin D1. Six GST-BRCA1 fragments spanning the BRCA1 protein were obtained from Dr. Tanya Paull (37). The GST-BRCA1 constructs were transformed into *Escherichia coli* and expressed as fusion proteins with glutathione S-transferase (GST). The fusion proteins were then purified on glutathione-Sepharose beads. For a negative control, GST alone was expressed and purified similarly.  $^{35}$ S labeled cyclin D1 was *in vitro* transcribed and translated using Promega's TNT kit.

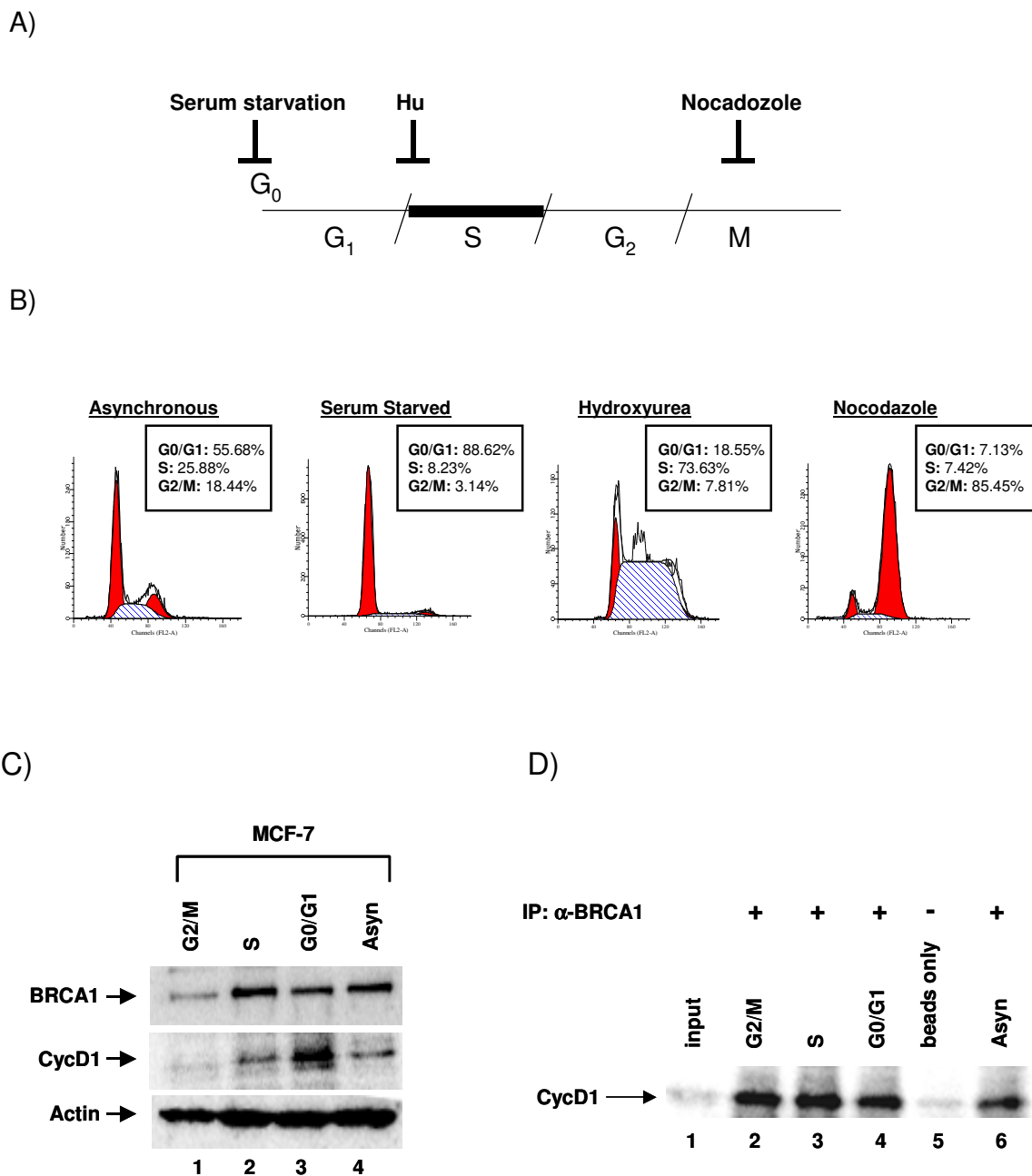
GST-BRCA1 proteins were incubated with  $^{35}$ S-cyclin D1 overnight. The next day protein complexes were washed and analyzed by SDS-PAGE. Results of a typical binding experiment are shown in Figure 2A. I observed cyclin D1 binding to multiple regions of BRCA1. There was strong binding observed on fragments 1-500, 504-802, and 452-1079. It is interesting to note that BRCA1 (504-802) is encompassed within the 452-1079 region indicating that this smaller fragment is the region that is necessary for binding. Also, it appears as though there is a separate binding site in the N-terminus of BRCA1 (1-500). Little or no binding was observed with fragment 1021-1552. Average binding of cyclin D1 to BRCA1 fragments is shown in Figure 2B. These results represent the average of five independent experiments. *Collectively, these results indicated that BRCA1 and cyclin D1 interact in vivo and that regions 1-500 and 504-802 of BRCA1 are particularly important for binding in vitro.*



**Figure 2. Cyclin D1 binds and phosphorylates BRCA1 *in vitro*.** A)  $^{35}\text{S}$ -labeled cyclin D1 ( $^{35}\text{S}$ -CycD1) was incubated with various GST-BRCA1 constructs (1.0 mg) or GST (2 mg). All GST tagged protein amounts were normalized to allow for quantitative comparison. Binding assays were carried out with GST-proteins purified from *E. coli* and 5  $\mu\text{l}$  of TNT reactions at  $4^\circ\text{C}$  overnight. The next day, complexes were washed (TNE150 + 0.1% NP-40), separated by SDS-PAGE on a 4-20 % Tris-glycine gel, dried, and exposed to a PhosphorImager cassette.

**Task 2.** In order to determine the cell cycle dependent interaction between cyclin D1/BRCA1, MCF-7 cells were analyzed at various points in the cell cycle. To obtain cells in different phases of the cell cycle, three different synchronization approaches were utilized (Figure 3A). G0/early G1 cells were obtained by serum starvation, S phase cells were collected by treated with HU, and a G2/M population was recovered through treatment with Nocodazole. Cells were stained with propidium iodine and analyzed by FACS analysis to ensure proper synchronization (Figure 3B). As can be seen in Figure 3B, an asynchronous MCF-7 population consists of 55.68% G0/G1 cells, 25.88% S phase cells, and 18.44% G2/M phase cells. Upon serum starvation for 3 days in 0% FBS, the G0/G1 population could be enriched to 88.62%. MCF-7 cells treated with 2mM Hydroxyurea, released into complete media and picked up 4 hours later, contained 73.63% of the cells in S phase. Finally, cells treated with Nocodazole for 24 hrs were 85.45% at G2/M phases.

Western Blotting was performed on the various cell cycle populations (Figure 3C). Both cyclin D1 and BRCA1 displayed a cell cycle dependent expression, with cyclin D1 being most highly expressed at G1 phase and BRCA1 at S phase (Figure 3C, lanes 3 and 2 respectively). Next, immunoprecipitation/western blot analysis was performed on the cell cycle time point samples (Figure 3D). Unexpectedly, cyclin D1 and BRCA1 were found to interact throughout the cell cycle. It is possible that this interaction only becomes functionally important at certain stages or in response to a stress signal. *In summary, I have observed that cyclin D1 and BRCA1 interact throughout all the stages of the cell cycle.*



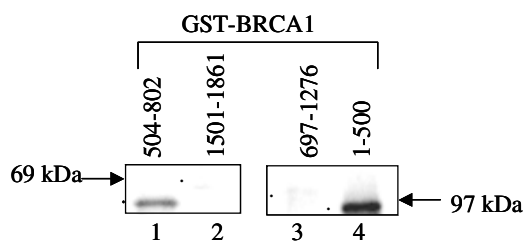
**Figure 3: BRCA1 and cyclin D1 interact throughout the cell cycle.** A) Diagram representing the three different approaches utilized to synchronize cell cultures. Serum starvation will result in a G0/early G1 cell population, Hydroxyurea (HU) will allow synchronization at the G1/S checkpoint, and Nocodazole arrests cells in M phase. B) Asynchronous cells, Serum Starved (0% FBS) cells picked up at 0hr, Hydroxyureas (2 mM) treated cells picked up at 4 hours post release, and Nocodazole (50 ng/ml) treated cells picked up at 0hr were analyzed by FACS using propidium iodine staining to measure DNA content. C) Seventy-five micrograms of protein extract from asynchronous and synchronized MCF-7 cells were separated by SDS-PAGE on a 4-20% Tris-glycine gel, transferred, and blotted with anti-cyclin D1 and anti-BRCA1 rabbit polyclonal antibodies. The blot was stripped and reprobed with anti-actin goat polyclonal antibodies. The antigen-antibody complex was further detected with <sup>125</sup>I-Protein G. D) MCF-7 cell extracts (1 mg) were Immunoprecipitated with anti-BRCA1 rabbit polyclonal antibody or no antibody overnight at 4°C. The complexes were precipitated with protein A+G agarose beads and washed with TNE600 + 1.0% NP-40. Proteins were then separated by reducing SDS-PAGE on a 4-20 % Tris-glycine gel and transferred onto a PVDF membrane. Western blot was performed with anti-cyclin D1 rabbit polyclonal antibody.

**Aim 2. Determine the biochemical consequence of cyclin D1/BRCA1 interaction in breast cancer cells.**

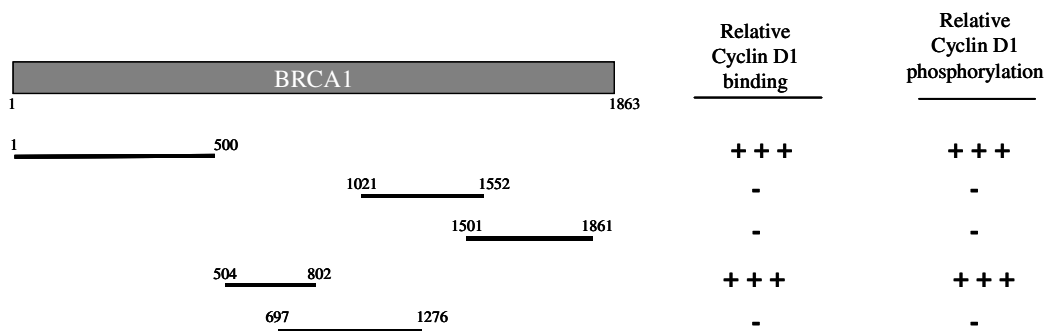
**Task 1.** One possible consequence of BRCA1/cyclin D1 interaction is the phosphorylation of BRCA1. This hypothesis is based on the facts that BRCA1 is a phosphoprotein and has been shown to have multiple cdk consensus phosphorylation sites along with nine RXL motifs, which are involved in the docking of cyclin/ckd complexes on their substrate (Chen et al., 1996; Ruffner and Verma, 1997; Ruffner and Verma, 1999). In addition, it has been previously shown that BRCA1 is phosphorylated by cyclin A/ckd2 (*in vivo*), cyclin E/ckd2 (*in vitro*), and cyclin D1/ckd4 (*in vitro*) (Chen et al., 1996; Wang et al., 1997; Ruffner et al., 1999). Therefore, re-confirmation and further detailed analysis about the phosphorylation of BRCA1 by cyclin D1/ckd4 complexes was initiated.

The functional activity of cyclin D/ckd complexes is often measured through *in vitro* kinase assays; therefore the ability of cyclin D/ckd complexes to phosphorylate BRCA1 was measured utilizing this assay. GST-BRCA1 (504-802) and (1-500) were both phosphorylated by cyclin D1/ckd complexes (Figure 4A, lanes 1 and 4), whereas GST-BRCA1 (1501-1861) and (697-1276) showed no phosphorylation (lanes 2 and 3). One representative kinase assay is shown in Figure 4A. A comparison between the *in vitro* binding results presented in Figure 2 and the *in vitro* kinase results presented here are shown in panel B. The kinase assay results and *in vitro* binding results correlate nicely, in that the same regions important for binding are also highly phosphorylated. It is the N-terminus (1-500) and the region of (504-802) of BRCA1 that appear to be important for the functional interaction between cyclin D1 and BRCA1. *In summary, the biochemical consequence of cyclin D1 interaction with BRCA1 is the phosphorylation of BRCA1, in particular the N-terminus aa 1-500.*

A)



B)

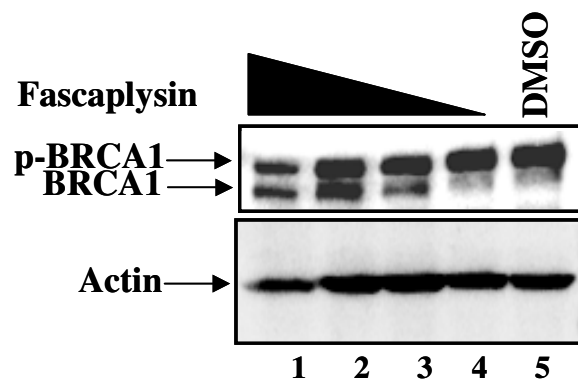


**Figure 4: Cyclin D1/cdk complexes phosphorylated BRCA1 *in vitro*.** A) MCF-7 cell extracts (1 mg) were immunoprecipitated with anti-cyclin D1 rabbit polyclonal antibodies overnight at 4°C. The complexes were precipitated with protein A+G agarose beads and washed twice with TNE300 + 0.1% NP-40, once with TNE50 + 0.1% NP-40, and twice with kinase buffer. Immune complexes were used for *in vitro* kinase assays using GST-Rb as a positive control, and various GST-BRCA1 fragments as substrates. Kinase reactions were separated on a 4-20% Tris-glycine gel, dried, and exposed to a PhosphorImager cassette. B) Summary of Cyclin D1 phosphorylating and binding to various BRCA1 fragments. Binding was demonstrated in Figure 2. These results are an average of five independent experiments. Relative binding and phosphorylation were calculated using ImageQuant Software. These phosphorylation results are representative of four independent experiments.



**Task 2.** The major goal of this task is to determine which cdk partner of cyclin D1 is responsible for phosphorylating BRCA1. In cell lines that overexpress cyclin D2, cyclin D2 has been shown to not only associate with cdk4 and cdk6, but also with cdk2 (14). In addition cdk2 complexes have been the major cyclin/ckd complexes shown to phosphorylate BRCA1 (34, 25). For these reasons it is important to determine which cdk is bound to the cyclin D1/BRCA1 complex in breast cancer cells.

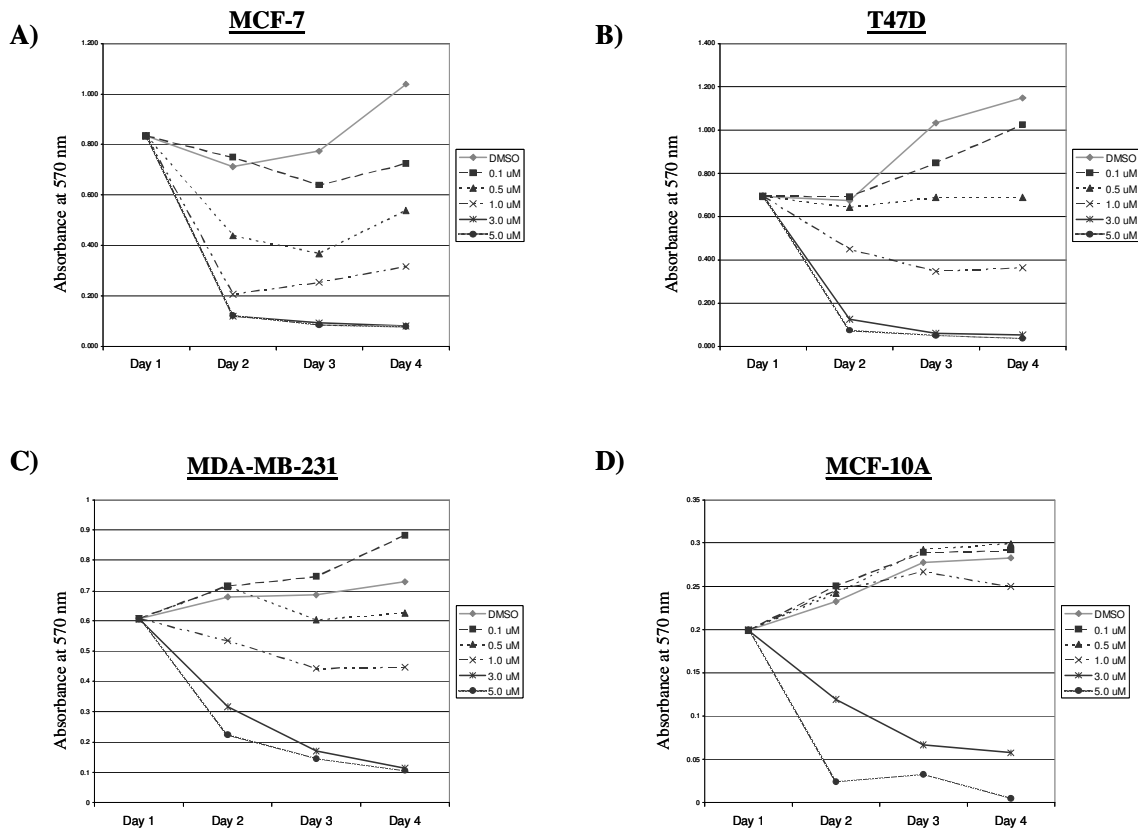
To differentiate between cdk2 and cdk4 kinase activity *in vivo*, a cdk4 specific drug, Fascaplysin (39) was obtained. Fascaplysin is a red pigment isolated from the marine sponge *Fascaplysinopsis sp.* It has been demonstrated to have an  $IC_{50}$  of 0.35  $\mu$ M against cyclin D1/ckd4, but a much higher  $IC_{50}$  of greater than 50  $\mu$ M against cdk2 complexes. Furthermore, Fascaplysin can inhibit cdk4 specific phosphorylation sites on the Rb *in vivo*. Therefore, Fascaplysin was utilized to verify that cyclin D1/ckd4 complexes phosphorylate BRCA1 *in vivo*. Asynchronous MCF-7 cells were treated for 24 hours with various concentrations of Fascaplysin (0.50, 0.75, 1.00, and 1.25  $\mu$ M). Significant inhibition of BRCA1 phosphorylation was observed beginning with 0.75  $\mu$ M of Fascaplysin (Figure 5A, lane 3). At 1.0  $\mu$ M, increased inhibition was observed, but at 1.25  $\mu$ M overall BRCA1 levels began to decrease possibly due to toxicity at this point. *Collectively, these results indicate that cdk4 is the cyclin D1 partner responsible for BRCA1 phosphorylation and that this phosphorylation occurs in vivo.*



**Figure 5: Cyclin D1/ckd4 phosphorylates BRCA1 *in vivo*.** MCF-7 cells were treated with DMSO or varying concentrations of the cdk4 specific inhibitor Fascaplysin (0.5, 0.75, 1.00, and 1.25 micromole) for 24 hours and processed for Western blotting. Fifty micrograms of total cellular protein was separated by SDS-PAGE on an 8% Tris-glycine gel, transferred, and blotted with anti-BRCA1 rabbit polyclonal antibodies. The blot was stripped and reprobbed with anti-actin goat polyclonal antibodies.

Due to the decreased protein levels observed at 1.25  $\mu$ M of Fascaplysin, experiments were designed to determine the *in vivo* effects of Fascaplysin on various breast cancer cells. An MTT assay was performed, which measures the ability of cells to metabolize MTT, a yellow tetrazolium salt. MTT metabolism results in the production of purple insoluble crystals, which are solubilized with a detergent, and quantitated by spectrophotometric means, allowing changes in proliferation to be quantitated. MCF-7, T47D, MDA-MB-231, and MCF-10A cells were treated with various concentrations of Fascaplysin and over a three day period analyzed by MTT assay. Day 1 represents cells before treatment and Days 2, 3, and 4 are following treatment. MCF-7 cells exhibited decreased proliferation (by about 50%) as early as Day 2 with as little as

0.5  $\mu\text{m}$  of Fascaplysin (Figure 6A), whereas T47D and MDA-MB-231 cells were virtually unaffected by 0.5  $\mu\text{m}$  of Fascaplysin at Day 2 (Figure 6B and C). T47D and MDA-MB-231 cells didn't demonstrated decreased proliferation in the presence of 0.5  $\mu\text{m}$  of Fascaplysin until Day 3. By Day 4, MCF-7 cells displayed decreased proliferation even in the presence of 0.1  $\mu\text{m}$  of Fascaplysin, being by far the most sensitive cell line tested. Interestingly, the non-tumorigenic breast epithelial cell line, MCF-10A, required 3.0  $\mu\text{m}$  to observe an effect on proliferation over the three day period (Figure 6D). The results of the MTT assays using Fascaplysin correlate nicely with the expression levels of cyclin D1 in the cell lines tested. That is that MCF-7 cells which express the highest levels of cyclin D1 were the most sensitive to Fascaplysin, whereas MCF-10A cells which express the lowest levels of cyclin D1 were the least sensitive.

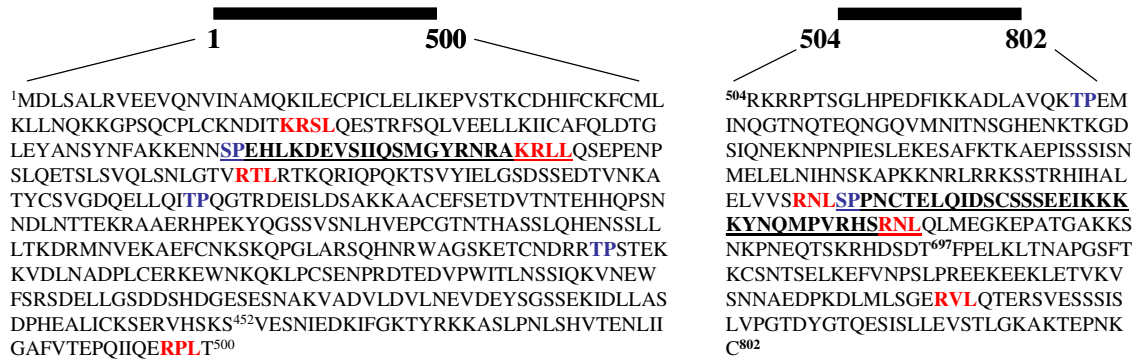


**Figure 6: Fascaplysin, a cdk4 inhibitor, inhibits proliferation of breast cancer cell lines.** Cells were plated at 5,000 cells/well in a 96 well format. Next day, Day 1 cells were analyzed by MTT assay and remaining cells were treated with either DMSO or various concentrations of Fascaplysin (0.1, 0.5, 1.0, 3.0, and 5.0  $\mu\text{m}$ ). MTT assays were then performed on Days 2, 3, and 4 following treatment. Cells analyzed were (A) MCF-7, B) T47D, C) MDA-MB-231, and D) MCF-10A. Results are an average of two independent experiments done in triplicate.

**Task 3:** The purpose of this task is to determine which site(s) are critical for cyclin D1/cdk4 binding/phosphorylation of BRCA1. Originally, it was proposed that a series of BRCA1 cDNA constructs mutated within the RXL motifs would be constructed and then used for *in vitro* binding and *in vitro* kinase assays. The methodology of this task has been modified in that instead of cDNA constructs, peptides spanning the potential sites of binding/phosphorylation are being constructed. This new methodology is a more feasible alternative to provide us with valuable information on the specific sites critical for binding/phosphorylation of BRCA1. The peptides to be utilized are shown in Figure 6. These peptides were picked based on a number of factors. First, the regions BRCA1 (1-500) and (504-802) were shown to be important for both binding to cyclin D1 and being phosphorylated by cyclin D1/cdk4 (shown previously in Figure 2 and 4). Secondly, cyclin/cdk complexes are known to phosphorylate their substrates through the use of a consensus cdk motif, Ser/Thr followed by Proline (indicated by Blue in Figure 7). This cdk consensus motif is often times precluded by a cyclin binding motif, RXL (indicated by Red in Figure 7) that is important for substrate docking and stability (40). Furthermore, the distance between the cdk consensus motif and the cyclin binding motif needs to be at least 12 amino acids apart in order to allow optimal phosphorylation to occur (41). It has been suggested that the RXL motif helps to specify which cdk phosphorylation site is targeted (41). In regions 1-500 and 504-802 of BRCA1, there are two spans of amino acids that fit the above criteria.

These peptides were utilized for *in vitro* kinase assays to determine if cyclin D1/cdk phosphorylates regions 112-141 or 630-669. As can be seen in Figure 7C, BRCA1 630-669 was efficiently phosphorylated by cyclin D1/cdk complexes *in vitro*, whereas BRCA1 112-141 showed minimal phosphorylation (compare lanes 1 and 2). Quantitation using ImageQuant Software indicated that BRCA1 112-141 had an intensity of 22,280 PhosphorImager Units, whereas BRCA1 630-669 was four times as intense at 84,542 PhosphorImager Units. Anti-HA antibody was used as a negative control (non-specific antibody) (lane 3). As an additional control to demonstrate that cyclin D1/cdk4 complexes phosphorylates Ser632, Ser632 was mutated to Ala632 and the resulting peptide utilized for *in vitro* kinase assays. Mutated BRCA1 630-669 was unable to be phosphorylated by cyclin D1/cdk4 complexes (Figure 7D, lane 2). In addition, BRCA1 630-669 was not phosphorylated when anti cyclin A and anti-cyclin G1 antibodies were utilized for the *in vitro* kinase assays (lanes 3 and 4). Finally, Fascaplysin inhibited the phosphorylation of BRCA1 630-669 *in vitro*. *Therefore, cyclin D1/cdk complexes phosphorylated BRCA1 630-669, specifically Ser 632, in vitro.*

A)



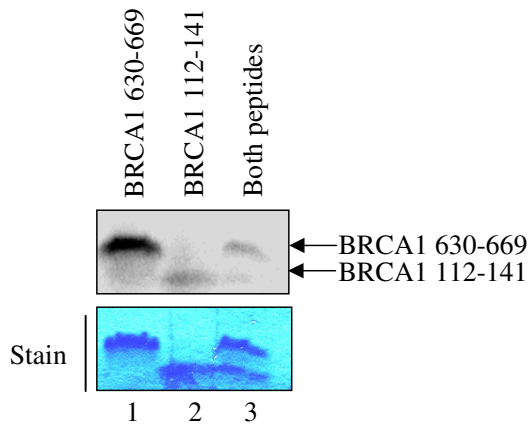
B)

**Peptides spanning possible  
binding/phosphorylation sites**

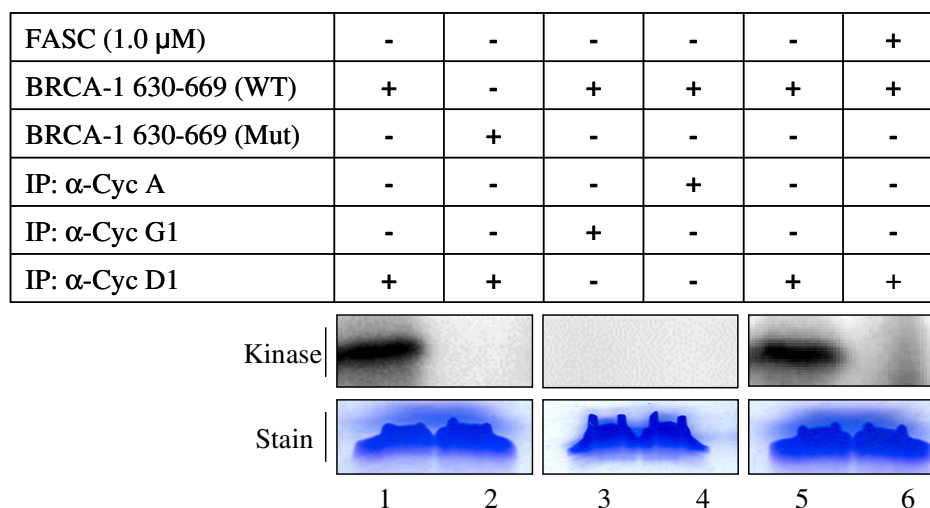
<sup>112</sup>NN**SPEHLKDEVSIHQSMGYRNRAKRL**LQSE<sup>141</sup>  
<sup>630</sup>NL**SPPNCTELOIDSCSSSEI**KKK**KYNQMPVRHSRNL**QLM<sup>669</sup>

C)

IP: α-Cyc D1	+	+	-
IP: α-NS Ab	-	-	+



D)



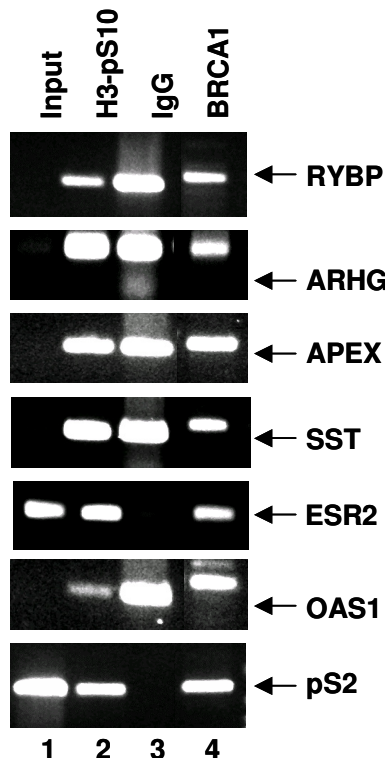
**Figure 7: Cyclin D1/cdk4 phosphorylates BRCA1 amino acids 630-669 peptide.** A) Potential cyclin D1/cdk4 binding and phosphorylation sites on BRCA1 based on bioinformatics and published research. Cyclin binding motifs (RXL) are indicated in Red and cdk phosphorylation sites (S/TP) are indicated in Blue. B) Peptides spanning these potential binding and phosphorylation sites C) MCF-7 cell extracts (1 mg) were immunoprecipitated with anti-cyclin D1 rabbit polyclonal or anti-HA mouse monoclonal (negative control) antibodies overnight at 4°C. The complexes were precipitated with protein A+G agarose beads and washed twice with TNE300 + 0.1% NP-40, once with TNE50 + 0.1% NP-40, and twice with kinase buffer. Immune complexes were used for *in vitro* kinase assays using peptides shown in panel A. Kinase reactions were separated on a 18 % Tris-glycine gel, dried, and exposed to a PhosphorImager cassette. D) Kinase assays were performed as described above with the additional of anti-cyclin A and anti-cyclin G1 antibodies as negative controls. BRCA1 630-669 (Mut) is mutated from Ser632 to Ala632.

### Aim 3. Determine the functional consequence of BRCA1 phosphorylation in breast cancer cells.

**Task 1:** At the time of my proposal, BRCA1 had been shown to bind preferentially to branched structures and long DNA stretches (42). This study implicated BRCA1 binding to DNA in a DNA damage response role such as homologous recombination. Therefore, I had proposed to determine the effect of phosphorylation of BRCA1 by cyclin D1 complexes through the utilization of electromobility shift assays (EMSA) with multiple branched DNA structures. However, over the past four years two important studies have provided further insight on the ability of BRCA1 to bind to DNA. Recently, Mark et al., narrowed the DNA binding region of BRCA1 to DB1 (498-663) and DB2 (936-1057) (66). This is significant because the area on BRCA1 that is highly phosphorylated by cyclin D1/cdk4 is aa 630-669, which overlaps with DB1. Finally, Cable et al. described the ability of BRCA1 to bind to a specific DNA sequence, TTC(G/T)GTTG (67). This finding suggests that BRCA1 could target genes for transcriptional activation or inhibition. In this study electromobility shift assays indicated that BRCA1 could bind to the consensus site *in vitro*. Furthermore, through transient transfection assays BRCA1

was able to transactivate the DNA consensus site, which was cloned into a pCAT reporter construct. Finally, the chromatin immunoprecipitation (ChIP) assay has become a widely used method to examine protein/DNA interaction *in vivo*. Thus, the methodology of this task has been changed in order to provide more valuable *in vivo* information about BRCA1 binding to specific DNA sequences.

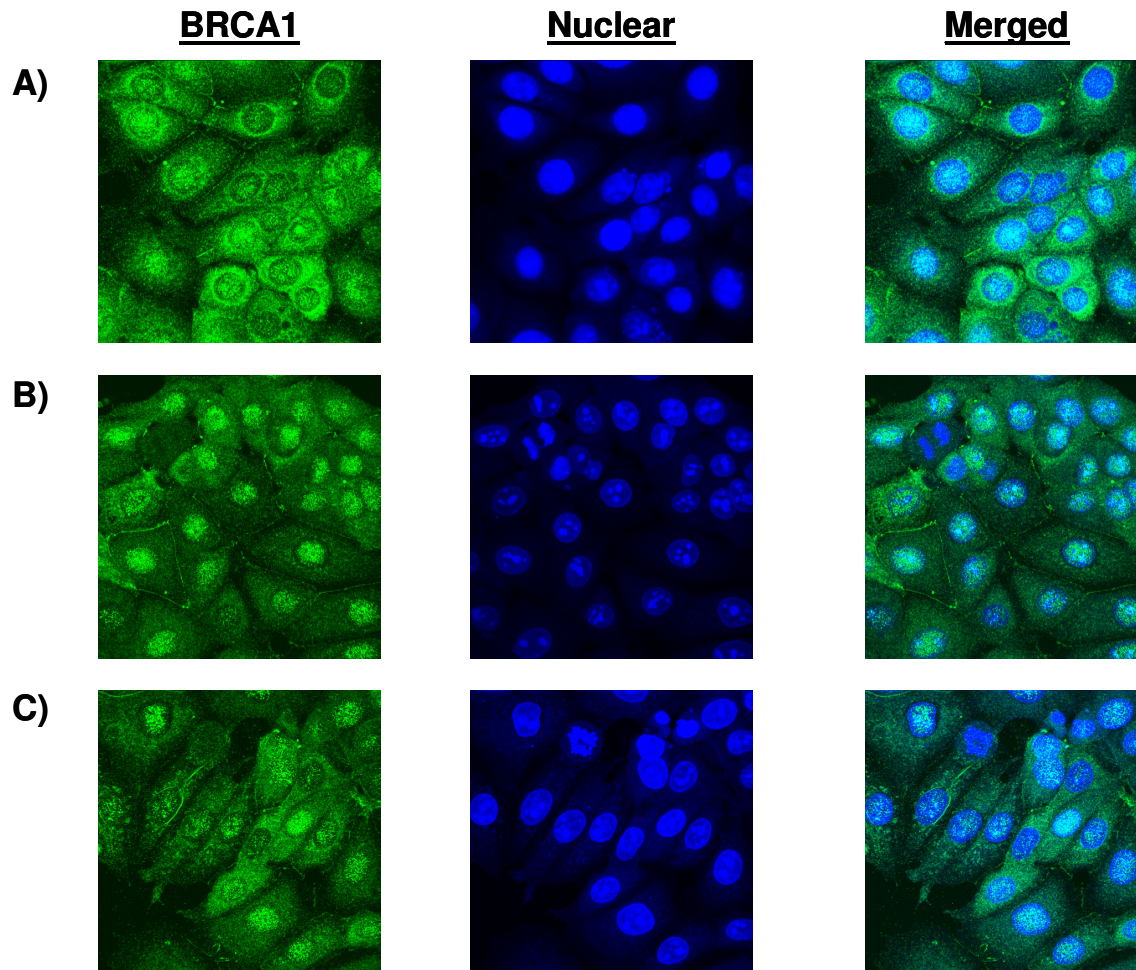
At the conclusion of the Cable *et al.* study, a total of 23 genes were identified through bioinformatics means that contained the BRCA1 DNA binding consensus sequence (67). However none of these genes had been experimentally tested *in vivo* to interact with BRCA1. Therefore, experiments were designed to determine if BRCA1 binds to these genes *in vivo*. The chromatin immunoprecipitation (ChIP) assay, which has become a widely used method to examine protein/DNA interaction *in vivo*, was utilized for this study. Upon design of the primers to be used for PCR, only 14 out of the 23 genes could be confirmed to contain the BRCA1 DNA consensus binding site. Therefore, only these 14 genes along with a positive control were further examined. The pS2 gene was utilized as a positive control, because BRCA1 has been shown through ChIP to bind to this particular promoter (39). For ChIP analysis, antibodies targeted against BRCA1, IgG as a negative control, and Histone H3-phosphorylated on Ser10 (H3-pS10) as a positive control were used. H3-pS10 was chosen as a positive control because this is a marker for activated transcription and has shown reproducible ChIP results in our hands. Out of the 15 genes tested for the presence of BRCA1, 7 were positive (Figure 8). These included RING1 and YY1 binding protein (RYBP), Ras homologue gene family member G, RhoG (ARHG), APEX nuclease (APEX), Somatostatin (SST), Estrogen receptor 2, ERbeta (ESR2), 2' 5' oligoadenylate synthetase 1 (OAS1), and Estrogen regulated gene (pS2). Importantly, none of these genes, except our positive control, pS2, have been shown previously to be bound by BRCA1 *in vivo*. Therefore, our results for the first time show that BRCA1 is associated with the RYBP, ARHG, APEX, SST, ESR2, and OAS1 promoters *in vivo*.



**Figure 8. BRCA1 binds to genes containing the BRCA1 DNA binding consensus sequence *in vivo*.** MCF-7 cells were processed for ChIP as described in Materials and Methods. Anti-bodies used were anti-BRCA1 (C-20) (10 ug), anti-IgG (10 ug), and anti-Histone H3-phosphorylated at S10 (H3-pS10) (5 ug). Results shown are representative of two independent experiments.

**Task 2:** BRCA1 is largely a nuclear protein, although it shuttles back and forth between the cytoplasm and the nucleus. However, in many breast cancer cells BRCA1 displays cytoplasmic staining (68), which may be one way to inactivate the activity of BRCA1 in absence of mutations. Furthermore, phosphorylation of BRCA1 has been shown to result in differential cellular localization. Using antibodies directed against phosphorylated BRCA1 at Ser 988 and Ser 1497, Coene *et al.* demonstrated that phosphorylated BRCA1 is mainly located in the nucleus and mitochondria, whereas unphosphorylated BRCA1 is located in the cytoplasm (69). Also, BRCA1 phosphorylated on Ser 988 was found on the inner chromosomal structure, centrosome, and cleavage furrow during prophase through telophase. In contrast, unphosphorylated BRCA1 was located on chromosomes from metaphase through telophase (70). Finally, cytoplasmic localization in response to DNA damage utilizes a CRM1-dependent pathway and is p53-dependent (71). Therefore, I examined the consequence of cyclin D1/cdk phosphorylation of BRCA1 with regards to its subcellular location.

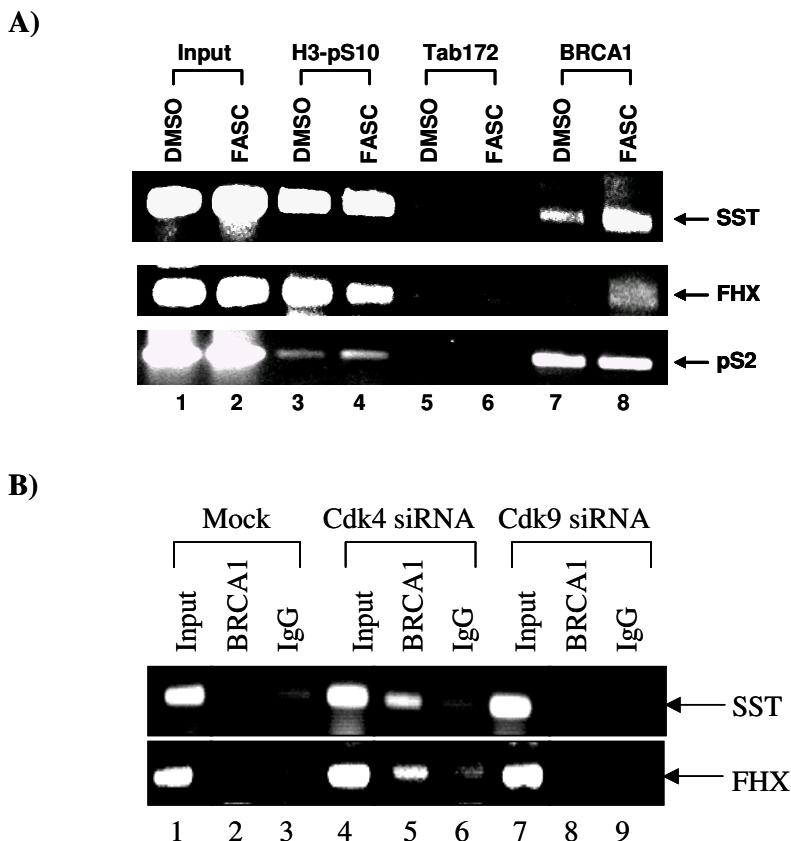
To determine if cyclin D1/cdk phosphorylation altered the subcellular localization of BRCA1, cyclin D1/cdk4 activity was inhibited using Fascaplysin. MCF-7 cells treated with 1.0  $\mu$ M Fascaplysin or DMSO were fixed and processed for confocal fluorescent microscopy. Cells were fluorescently labeled with anti-BRCA1 primary antibodies and Alexa Fluor 488 secondary antibodies as well as with the nuclear stain TOTO-3. As a positive control for increased cytoplasmic localization, MCF-7 cells were  $\gamma$ -irradiated and then processed 24 hours later (Figure 8A). This is because at extended periods after DNA damage BRCA1 has been shown to be exported from the nucleus and accumulate in the cytoplasm in MCF-7 cells (71). MCF-7 cells were also treated with either DMSO or Fascaplysin for 24 hours (Figure 8B and C respectively). Inhibiting cyclin D1/cdk4 kinase activity did not appear to have any effect on BRCA1 localization (compare panels B and C). *Therefore, these results suggest that cyclin D1/cdk4 phosphorylation of BRCA1 does not affect the subcellular localization of BRCA1.*



**Figure 9: Cyclin D1/cdk4 phosphorylation does not alter the subcellular localization of BRCA1.** As a positive control for increased cytoplasmic localization, MCF-7 cells were  $\gamma$ -irradiated and then processed for confocal imaging 24 hours later (panel A). MCF-7 cells were treated with either DMSO (Panel B) or 1  $\mu$ M Fascaplysin for 24 hours (Panel C). The green color indicates BRCA1 staining, which was detected by rabbit polyclonal anti-BRCA1 (C-20) primary antibody and Alexa Fluor 488 secondary antibody. The blue color indicates nuclear staining, which was detected utilizing TOTO-3, a dimeric cyanine nucleic acid stain. Confocal optical sections ( $z=1.0 \mu\text{m}$ ) after being projected and then merged are shown in all panels. Fascaplysin treatment data are representative of four independent experiments.  $\gamma$ -irradiation data were performed once.



**Task 3:** Experiments were next designed to examine the effects of cyclin D1/cdk4 phosphorylation on BRCA1 DNA binding. To this end, the cdk4 specific inhibitor, Fascaplysin was utilized. BRCA1 becomes highly phosphorylated at late G1 and cyclin D1 is most highly active during the G1 phase of the cell cycle. Therefore, cells at mid to late G1 phase would display the most pronounced cyclin D1/cdk4-dependent BRCA1 phosphorylation. MCF-7 cells were plated at low density and serum starved for 24 hours giving rise to an enriched G0/G1 population, 79.24% (data not shown). Cells were then stimulated with complete media for 8 hrs to accumulate cells in mid to late G1 phase and then treated with Fascaplysin for 24 hours to inhibit cyclin D1/cdk4 phosphorylation of BRCA1. To obtain the optimal inhibition of BRCA1 phosphorylation, Fascaplysin (1  $\mu$ M) was used. In parallel, cells were treated with DMSO as a negative control. After treatment, cells were processed for ChIP utilizing the antibodies described above. After treatment of MCF-7 cells with Fascaplysin, increased BRCA1 binding to SST was observed (Figure 10A). BRCA1 was also observed binding to FOXJ2 Foxhead transcription factor (FHX), which had not been observed in the absence of drug treatment (Figure 10A). Importantly, BRCA1 binding to pS2 promoter did not change. This serves as an additional negative control because it was recently described that BRCA1 binding to pS2 was decreased in the presence of excess cyclin D1 (72). However, this finding was cdk-independent. To confirm these results MCF-7 cells were treated with cdk4 siRNA. Cdk4 siRNA treatment resulted in increased BRCA1 association with SST and FHX genes (Figure 10B, lane 5). MCF-7 cells treated with cdk9 siRNA, as a negative control, did not display increased BRCA1 association with SST and FHX (lane 8). Collectively, these results suggest that cyclin D1/cdk4 phosphorylation of BRCA1 inhibits BRCA1 binding to SST and FHX promoters.



**Figure 10: Inhibition of cdk4 results in increased BRCA1 binding to genes containing the BRCA1 DNA binding consensus sequence.** A) MCF-7 cells were serum starved for 24 hours, released for 8 hours, and then treated for 24 hours with DMSO or 1.0  $\mu$ M Fascaplysin. Cells were then processed for ChIP using anti-BRCA1 (C-20) (10  $\mu$ g), anti-Tab172, and anti-H3-pS10 (5  $\mu$ g). B) MCF-7 cells were transfected with either cdk4 or cdk9 siRNA using metafectene. Cells were processed for ChIP as described in Materials and Methods. Antibodies used were anti-BRCA1 (C-20) (10  $\mu$ g) and anti-IgG (10  $\mu$ g).

## KEY RESEARCH ACCOMPLISHMENTS

1. Aim 1, Task 1 has been completed. I confirmed that BRCA1 and cyclin D1 interact in MCF-7 and T47D cells and showed that regions 1-500 and 504-802 of BRCA1 are particularly important for binding to cyclin D1 *in vitro*.
2. Aim 1, Task 2 has been completed. I have demonstrated that cyclin D1 and BRCA1 interact throughout all the stages of the cell cycle.
3. Aim 2, Task 1 has been completed. I have determined that the biochemical consequence of cyclin D1 interaction with BRCA1 is the phosphorylation of BRCA1, in particular regions 1-500 and 504-802.
4. Aim 2, Task 2 has been completed. I have established that cdk4 is the cyclin D1 partner responsible for BRCA1 phosphorylation and that this phosphorylation occurs *in vivo*.
5. Aim 2, Task 3 has been completed. I have narrowed down the specific sites that are important for the phosphorylation and binding of BRCA1 by cyclin D1/cdk4 complexes. Ser 632 is a major cdk phosphorylation site on BRCA1 that is targeted by cyclin D1/cdk4 complexes. In addition, amino acids 630-669 contains a RXL motif that is important for cyclin D1 binding to BRCA1.
6. Aim 3, Task 1 has been completed. I demonstrated that BRCA1 associates with RYBP, OAS1, ESR2, ARHG, SST, and APEX promoters *in vivo*.
7. Aim 3, Task 2 has been completed. My results indicated that cyclin D1/cdk4 phosphorylation of BRCA1 did not effect its subcellular localization.

8. Aim 3, Task3 has been completed. Through ChIP assays, I demonstrated that inhibition of cdk4 through siRNA or drug treatment resulted in increased association of BRCA1 with SST and FHX promoters *in vivo*.

## **REPORTABLE OUTCOMES**

**During the report time frame of March 2004-2005 the following reportable outcomes have been made:**

1. A manuscript pertaining to this work will be submitted to Oncogene by May 2006.
2. An R21 is being submitting to expand on the findings of this work for the June 2006 study section.
3. I am currently participating in a postdoctoral position at the Federal Bureau of Investigation through the Oak Ridge Institute for Science Education (ORISE). The DOD predoctoral fellowship has been instrumental in me obtaining this position.

## CONCLUSIONS

My final annual DOD report has indicated multiple findings and conclusions. First, I have confirmed that BRCA1 and cyclin D1 interact in MCF-7 and T47D cells. This complex is highly stable and was detected in asynchronous cell populations. This *in vivo* interaction could also be detected through confocal microscopy showing co-localization of cyclin D1 and BRCA1 in MCF-7 cells (data not shown). *In vitro* binding assays utilizing <sup>35</sup>S-cyclin D1 showed that regions 1-500 and 504-802 of BRCA1 are particularly important for binding to cyclin D1 *in vitro*. I have demonstrated that cyclin D1 and BRCA1 interact throughout all the stages of the cell cycle. This observation suggests that the interaction between cyclin D1 and BRCA1 is either important throughout the cell cycle, or that other circumstances, such as phosphorylation or protein binding partners, render this interaction functional.

Along these same lines, I have determined that the biochemical consequence of cyclin D1 interaction with BRCA1 is the phosphorylation of BRCA1. *In vitro* kinase assays indicated that the 1-500 and 504-802 regions of BRCA1 were consistently highly phosphorylated. This data suggests that there is more than one site of phosphorylation for cyclin D1/cdk complexes. Using a combination of bioinformatics and peptide kinase assays, Ser 632 was identified as a major site of cyclin D1/cdk4 phosphorylation. Cdk4 was established as the cyclin D1 partner responsible for BRCA1 phosphorylation through the use of the cdk4 specific inhibitor, Fascaplysin. Cyclin D1/cdk4 complexes were observed phosphorylating BRCA1 both *in vitro* and *in vivo*. This is the first time to my knowledge that cdk4 has been shown to phosphorylate BRCA1 *in vivo*.

Through chromatin immunoprecipitation assays (ChIP), I identified many novel BRCA1 bound promoters (SST, OAS1, ESR2, ARHG, APEX, FHX, and RYBP). Finally, the inhibition of cyclin D1/cdk4 phosphorylation of BRCA1 through cdk4 siRNA or drug treatment resulted in the increased presence of BRCA1 at SST and FHX promoters *in vivo*. These results indicate that cyclin D1/cdk4 phosphorylation of BRCA1 inhibits the ability of BRCA1 to be recruited to particular promoters *in vivo*.

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