Identification of BRCA1 Interacting Proteins

By

Sherry Lynn Winter

A thesis submitted in conformity with the requirements for the degree of M.Sc. Graduate Department of Molecular and Medical Genetics University of Toronto

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ABSTRACT

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Master of Science Thesis, 2000
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University of Toronto

The breast cancer susceptibility gene BRCA1 is mutated in many cases of familial breast and ovarian cancer. In order to increase our understanding of the function of this gene, a yeast two-hybrid study was performed to identify proteins that interact with the BRCA1 protein. The two-hybrid screen was performed using a human mammary gland cDNA library and a portion of the BRCA1 protein as bait. The screen yielded a total of 366 colonies, which grew on adenine media. The colonies were subsequently tested for lacZ reporter gene activation, autonomous activation of the reporter genes by the activating domain (AD) protein and for specific interaction by the AD-protein with BRCA1. A total of 16 putative positive interactions were identified, representing 12 distinct interacting proteins. These proteins included Protein Phosphatase 1β, the proteasome p27 subunit, 2 circadian clock proteins, importin α, and several other proteins. Future studies would include further analysis of the protein interactions to characterize the functional significance of the association of these proteins with BRCA1.
ACKNOWLEDGEMENTS

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ABBREVIATIONS

3-AT  3 amino-1,2,4-triazole
AD   activating domain
ade  adenine
ATM  ataxia-telangiectasia mutated
BAC  bacterial artificial chromosome
BAP1 BRCA1 associated protein 1
BARD BRCA1 associated RING domain
bHLH basic helix loop helix
BRAP2 BRCA1 associated protein 2
Brca1 Mouse homologue to human BRCA1 gene
Brca1 Mouse homologue to human BRCA1 protein
BRCA1/2 Human breast cancer susceptibility genes 1 and 2
BRCA1/2 Human breast cancer susceptibility proteins 1 and 2
BRCT BRCA1 c-terminus
cdk  cyclin dependent kinase
cfu  colony forming unit
CKII casein kinase II
CREB cAMP responsive element binding domain
CtBP carboxy terminal binding protein
CtIP carboxy terminal interacting protein
DARPP inhibitor of PP1, brain specific
DBD  DNA binding domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>DNA-PK</td>
<td>DNA protein kinase</td>
</tr>
<tr>
<td>EGF(R)</td>
<td>epidermal growth factor (receptor)</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen responsive element</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FLOT-2</td>
<td>flotillin-2</td>
</tr>
<tr>
<td>GADD45</td>
<td>growth arrest and DNA damage response gene</td>
</tr>
<tr>
<td>GEF-2</td>
<td>ganglioside expression factor - 2</td>
</tr>
<tr>
<td>HDAC1/2</td>
<td>histone deacetylase 1/2</td>
</tr>
<tr>
<td>his</td>
<td>histidine</td>
</tr>
<tr>
<td>JNK/SAPK</td>
<td>c-Jun N-terminal kinase/stress-activated protein kinase</td>
</tr>
<tr>
<td>leu</td>
<td>leucine</td>
</tr>
<tr>
<td>LIM</td>
<td>lin-11, isl-1, Mec-3 - zinc finger protein-protein interaction domain</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>NIPP</td>
<td>nuclear inhibitor of PP1</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAS</td>
<td>Period, Aryl hydrocarbon receptor nuclear transporter, Single minded - protein dimerization domain</td>
</tr>
<tr>
<td>PCNA*</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95, Dlg, ZO-1 - protein-protein interaction domain</td>
</tr>
<tr>
<td>Per2</td>
<td>period 2</td>
</tr>
<tr>
<td>PKA/C</td>
<td>protein kinase A/C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PNUTS</td>
<td>phosphatase I nuclear targeting subunit</td>
</tr>
<tr>
<td>PPI</td>
<td>protein phosphatase</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RbAp 46/48</td>
<td>Retinoblastoma associated protein 46/48</td>
</tr>
<tr>
<td>RHA</td>
<td>RNA helicase A</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting new gene</td>
</tr>
<tr>
<td>RNApolII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>SEKI</td>
<td>SAPK/ERK kinase-1 - upstream regulator of JNK/SAPK</td>
</tr>
<tr>
<td>STS</td>
<td>sequence tag site</td>
</tr>
<tr>
<td>TCR</td>
<td>transcription coupled repair</td>
</tr>
<tr>
<td>TFIIF, E, H</td>
<td>transcription factor II F, E, H</td>
</tr>
<tr>
<td>trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
</tbody>
</table>
INTRODUCTION

Breast cancer is a major concern among women today. One in nine women in the general population will develop breast cancer in her lifetime (1); however, the risk for women who inherit a mutated breast cancer susceptibility gene is greatly increased. The breast cancer susceptibility genes BRCA1 and BRCA2 have been identified thus far. The gene BRCA1 was cloned in 1994 by Miki et al. (2), and is mutated in many familial breast and ovarian cancers; however, it does not appear to be altered in most cases of sporadic cancer. Instead, decreased expression of BRCA1 may contribute to the development of sporadic cancer (3-6) and other genes that are part of the BRCA1 pathway may also be involved in these cancers. Furthermore, BRCA1 is expressed in all tissues examined; however, it is only implicated in breast and ovarian cancer. This may be due to unique protein interactions taking place in breast and ovarian cells due to hormonal signaling that occurs in these tissues. One method for elucidating the function of BRCA1 is to identify proteins that interact with the BRCA1 protein. The identification of such proteins will aid in our understanding of the role of BRCA1 and may identify how the interaction of protein(s) with BRCA1 affects its function.

BRCA1 and Cancer

Familial Cancer

BRCA1 is located at chromosome 17q21 and was initially cloned in 1994 using positional cloning methods (2). Mutations in BRCA1 are believed to account for approximately 50% of all familial breast cancer cases and 80% of cases of familial breast and ovarian cancer (7). Familial breast cancer itself is responsible for roughly 5-10% of
all cases of breast cancer (7). A single inherited copy of a mutant allele of BRCA1 causes predisposition to breast cancer and a loss or inactivation of the wild type allele is required for malignancy. The finding that a lack of BRCA1 prevents breast cancer suggests that it is a tumour suppressor gene.

**Involvement of BRCA1 in Sporadic Cancer**

Although mutations in BRCA1 have been observed in familial breast and ovarian cancers, the gene does not appear to be mutated in sporadic breast cancer and mutations have been identified in only a few ovarian cancers (8; 9). As an alternative mechanism to mutations in BRCA1 that result in a loss of function of the protein, it is possible that sporadic cancer cells may have decreased expression of the BRCA1 gene. Several studies have been performed examining the expression levels of BRCA1 in both breast cancer cell lines and primary breast tumours and a significant decrease in gene expression has been observed in cancer tissue compared to wild type controls (3-6).

Methylation of the promoter region of BRCA1 is one method the cell may utilize to decrease BRCA1 expression. The BRCA1 promoter contains a CpG island, a region that is often located in the promoter of a gene containing unmethylated CpG motifs. Methylation of promoter CpG bases often represses the transcription of a gene. Although no CpG motifs located in the promoter of BRCA1 were methylated in samples from normal tissue, methylation has been observed in the BRCA1 promoter of some sporadic tumours (5; 10). In one sample, methylation occurred at the putative site of binding for the CREB (cAMP responsive element binding) transcription factor. Gel shift assays indicate that when these bases are methylated, CREB cannot bind to the BRCA1 promoter.
A direct correlation between the methylation of a \textit{BRCA1} promoter and the expression of \textit{BRCA1} from that specific promoter has not yet been reported. Recently, a study was performed to identify the amount of methylation present in DNA isolated from sporadic breast and ovarian cancers, and only 11% of sporadic breast (of 96 studied) and 5% of sporadic ovarian (of 43 studied) cancers had abnormal methylation at their promoter (11). The authors did find, however, that there was a significant correlation between methylation and estrogen and progesterone receptor status in breast cancer cases. Estrogen receptor status is an important indicator of the responsiveness of the tumour to treatment with tamoxifen (12). None of the \textit{BRCA1} promoters in ER\textsuperscript{+}/PR\textsuperscript{+} tumours were methylated in the analyzed region, while 10 out of 11 methylated \textit{BRCA1} promoters were from ER\textsuperscript{-}/PR\textsuperscript{-} tumours (11). The remaining one methylated \textit{BRCA1} promoter was from an ER\textsuperscript{+}/PR\textsuperscript{-} tumour (11). These results suggest that methylation is not the sole mechanism for the reduction of \textit{BRCA1} in sporadic tumours, but may be important for the development of a subset of tumours.

\textbf{\textit{BRCA1} Expression}

\textbf{mRNA Expression}

\textit{BRCA1} expression in human cells changes throughout the cell cycle, increasing in S phase and remaining elevated through to M phase (13). Expression of murine \textit{Brcal} transcripts are observed in all tissues studied including the breast and ovaries, and are present at highest levels in proliferating and differentiating cells (14). The RNA \textit{in situ} hybridization analysis of murine \textit{Brcal} mRNA in mouse tissues correlates with the localization of the proliferation cell nuclear antigen PCNA\textsuperscript{+}, indicating that \textit{Brcal} is
expressed in growing and dividing cells (15). In the adult mouse, Brca1 expression is observed in most proliferating tissues. However, in the mouse testis, Brca1 transcripts are expressed for more stages of meiosis during spermatocyte development than the PCNA+ protein, suggesting that Brca1 functions beyond DNA replication. The highest level of expression of Brca1 in the mouse testis is in cells undergoing meiosis, whereas pre-meiotic germ cells express little or no Brca1 (15; 16).

The expression patterns of Brca1 and Brca2 also parallel that of the mouse Rad51 protein (a protein involved in DNA repair and recombination) in spermatogonia and spermatocytes (15). Along with colocalization data indicating that human BRCA1 and BRCA2 interact with Rad51 (17; 18), the expression patterns of all three mouse genes suggests a role for BRCA1 and BRCA2 in DNA repair and recombination.

**Alternative Splicing of BRCA1**

Several distinct BRCA1 mRNA species have been identified that are the result of alternative splicing of the primary BRCA1 transcripts (Figure 1). Dual promoters and alternative splicing generates two forms of BRCA1 exon1 mRNA that are referred to as either exon 1a or 1b and are detected in all tissues studied (19). Of the two transcripts, the major transcript expressed in mammary glands is exon 1a. However, exon 1b is the major transcript expressed in the placenta, indicating a possible tissue specific expression pattern. Using 5' RACE, it was determined that exon 1a contains 99 bases of the published exon1 sequence (Genbank Accession #U14680 (2)) plus an additional 22 bases at the 5' end; and the 5' end of exon 1b contains a region between the published exon 1
Alternative splicing of BRCA1. Dual promoters generate two alternatively spliced BRCA1 mRNA that encode the wild type protein. All or part of exon11 may also be spliced out of the mRNA, generating alternative proteins. In addition to these, other alternatively spliced mRNA and protein have been identified. Black represents the published BRCA1 sequence, light grey represents the additional 22 bp found at the start of exon1a, the hatched box represents novel exon1b sequence and white represents BRCA1 protein. The BRCA1 coding sequence commences with exon2.
and exon 2 (19) sequence. There is no overlapping sequence between exon 1a and b, and the genomic 5' region of both exon 1a and exon 1b contains an initiator element as well as several putative binding sites for transcription factors, suggesting that the two exons may have different promoters. Since the major open reading frame of both transcripts starts at the beginning of exon 2, the transcripts do not produce different proteins. The presence of two different promoters and 5' UTRs (untranslated region) for the BRCA1 gene may result in tissue specific expression of BRCA1.

Exon 11 of BRCA1, which contains a nuclear localization signal (NLS) (20), is often spliced out of the primary transcripts, suggesting that differential splicing may affect the subcellular localization of the protein, as a protein lacking an NLS might not be transported into the nucleus. However, it is possible that the BRCA1 protein is transported into the nucleus along with another nuclear protein (20). A second splice variant, designated Δ11b, removes much of exon 11, with the exception of a region at the amino terminus of the exon (20). This splice variant does not encode a nuclear localization signal and is reduced or absent in some breast and ovarian tumour lines relative to full length BRCA1 (20), implying a potential role for the Δ11b splice variant in normal cell function. Both the full length and Δ11 variants are expressed as proteins. Several other tissue-specific splice variants have been observed and Western analysis of BRCA1 proteins in mouse embryonic fibroblasts indicates the presence of multiple BRCA1 polypeptides, suggesting that the other splice variants are expressed (21). However, it is possible that these polypeptides are degradation products of the full-length BRCA1 protein. Alternative splicing may be important in the functional regulation and/or cellular localization of BRCA1.
BRCA1 Protein Analysis

Protein Localization

BRCA1 encodes a 220-kDa protein that is localized in the cell nucleus and has cycle-specific cell expression patterns. BRCA1 appears to be homogeneously distributed throughout the cell in G1, and is punctate at the onset of S phase (17; 18; 22). During meiosis, BRCA1 is located at the axial elements of unsynapsed human synaptonemal complexes (17); however during mitosis a hypophosphorylated form of BRCA1 can be detected at the centrosome (23).

Controversial localization results have indicated potential cytoplasmic staining, or even secretion of BRCA1. The presence of a putative granin domain in exon 11 of BRCA1 originally suggested that BRCA1 might behave as a secreted protein (24). Granin proteins have a high percentage of acidic amino acids, are post translationally modified by glycosylation, phosphorylation or sulfation, and may undergo proteolytic cleavage leading to biologically active peptides. BRCA1 has an acidic amino acid content and 21 predicted glycosylation sites, 20 of which are within the granin domain (24). Based on immunofluorescence analysis, one group of investigators have suggested that BRCA1 is secreted and have identified BRCA1 as a 190-kDa protein (24); however, these results may have been due to cross reactivity of the BRCA1 carboxy terminus antibody with the human epidermal growth factor receptor protein (25). Additional studies have indicated that BRCA1 may be localized to the cytoplasm in breast cancer cell lines and the nucleus in non-cancer cell lines (26). Other studies have suggested BRCA1 is nuclear at all times (27). Problems with antibodies, or potential splice variants
of BRCA1 that lack the nuclear localization signal, may be confounding these localization experiments.

**BRCA1 Protein Domains**

The *BRCA1* gene encodes a protein with several domains that have an effect on the function, localization and protein-protein interactions of BRCA1. The amino terminus of BRCA1 contains a zinc finger known as the RING (Really Interesting New Gene) domain, which is implicated in protein-protein interactions (28). Two nuclear localization signals (NLS) are located in exon11 of BRCA1 and control the movement of BRCA1 into the nucleus (29). The carboxy terminus of the protein contains two tandem BRCT (BRCA1 carboxy terminal) domains that are also found in a variety of proteins, including those that have a role in DNA repair (30).

**BRCA1 Protein Expression and Phosphorylation**

The phosphorylation status and expression levels of BRCA1 fluctuate throughout the cell cycle. As cells enter S phase, BRCA1 protein expression rapidly increases to a maximum and remains elevated until the end of M phase (31), similar to the increase in mRNA expression observed at S phase. In addition, a phosphorylated form of BRCA1 becomes evident at mid to late G1, rising to a maximum at S phase, and phosphorylation levels remain elevated throughout M phase (31; 32). The identities of the kinases responsible for BRCA1 phosphorylation are unknown. However, BRCA1 coimmunoprecipitates the kinase cdk2, which has been shown to phosphorylate BRCA1
at serine 1497, and may phosphorylate the protein at additional sites (33). In addition, cyclins A and D also co-immunoprecipitated BRCA1 and those immunoprecipitates contained a kinase that phosphorylated the BRCA1 protein (31). This kinase may include but is not limited to cdk2.

BRCA1 binds to and is also phosphorylated in vitro by casein kinase 2 (CK2) at serine 1572. These results suggest that many kinases may be responsible for the phosphorylation of BRCA1 at multiple sites (34). Phosphoamino acid analysis has indicated that BRCA1 is predominantly phosphorylated on serine residues, with additional phosphorylation on threonine and tyrosine residues (32; 33). Two dimensional tryptic peptide analysis has also been utilized to identify 13 tryptic peptides that are phosphorylated on serine residues (32).

A fragment of the BRCA1 protein, including amino acids 329 to 435, copurifies with a kinase that is capable of phosphorylating BRCA1 in vitro (35). Deletion and tryptic peptide analysis was utilized to further delineate the minimal region of BRCA1 that is phosphorylated and copurifies with the kinase, identifying a region of BRCA1 from amino acids 379 to 408 (35) that is encoded by exon11. Even without the presence of another kinase, this BRCA1 peptide is phosphorylated in vitro after the addition of γ-32P-ATP, indicating that the phosphorylating activity is associated with BRCA1. Autophosphorylation by BRCA1 has been ruled out due as the kinase activity can be separated from BRCA1 by incubation with 0.1% SDS, after which phosphorylation is restored following incubation with a human cell lysate. In addition, BRCA1 that has been purified on an SDS-PAGE gel and renatured in situ is unable to autophosphorylate after incubation with γ-32P-ATP (35). Substitution of individual serine residues with
alanine did not affect the levels of phosphorylation of the BRCA1 peptide, suggesting that more than one serine is phosphorylated. It is also possible that there may be a preferred site for phosphorylation in vivo; however, other secondary sites may be substrates for phosphorylation in vitro or in the absence of the preferred site (35). Deletion of the region in exon11 that is phosphorylated results in a loss of the growth suppressive activity of BRCA1 that occurs when the full-length gene is transfected into cells (35). Phosphorylation of the region of BRCA1 from amino acids 379 to 408 by the kinases PKA (protein kinase A), PKC (protein kinase C) or CKII (casein kinase II) did not occur in vitro, indicating that these kinases are not responsible for phosphorylation of this region (35).

BRCA1 is also phosphorylated on at least one tyrosine residue (36; 37). An inverse correlation has been noted between the tyrosine phosphorylation levels of BRCA1 and proliferation mediated by members of the erbB family of receptor tyrosine kinases p185 neu and the epidermal growth factor receptor (EGFR) (37). A tyrosine hyperphosphorylated form of BRCA1 is present in quiescent cells and in cells arrested at the G2/M checkpoint, suggesting that tyrosine hyperphosphorylation of BRCA1 may inhibit cell proliferation (37). Reduced levels of tyrosine-phosphorylated BRCA1 are observed in cell lines that have been transformed with oncogenic p185 neu, correlating with unrestricted cell cycle progression and proliferation. There are three putative tyrosine phosphorylation sites encoded in the BRCA1 sequence, one of which is located at the carboxy terminus within the region that may be involved in the transactivation of genes. A mutation in BRCA1 that has been observed in breast cancer tumours
(T1561→I) disrupts this phosphotyrosine motif (37), suggesting that tyrosine phosphorylation at this site may be important for BRCA1 function.

**Growth Suppression Mediated by BRCA1**

Several methods have been used to study the growth suppressive effect of BRCA1 on some types of cells. Overexpression of BRCA1 in breast cancer cell lines that do not express a high amount of BRCA1 (MCF-7, MDA-MB157) as well as in ovarian cancer cells (Caov-4, Es-2, PA-1) results in decreased growth of the cells. By contrast, increased expression of BRCA1 in lung (FK111) and colon (OK3) cancer cell lines and fibroblasts (W138) has little effect on cell growth (38). In addition, retroviral transfer of wild type BRCA1 results in the suppression of cell growth, whereas mutant BRCA1 does not. Interestingly, mutant BRCA1 does not suppress ovarian cell growth in cases where the BRCA1 mutation was in the 5' portion of the gene (38), but otherwise suppresses growth similarly to WT BRCA1. This suggests that the site of mutation on BRCA1 may determine the affect of the mutation in ovarian tumours. A growth suppressive role for BRCA1 is also suggested by evidence that growth of tumours caused by implantation of MCF-7 cells into nude mice is suppressed by transfection with wild type but not mutant BRCA1 (38).

Inhibition of BRCA1 expression using anti-sense mRNA was also used to study the role of BRCA1 in cell growth. Mouse embryonic fibroblasts expressing *BRCA1* antisense mRNA had a decreased level of BRCA1 protein and also had an accelerated growth rate, anchorage independence and tumorigenicity in nude mice (21). In addition, cells expressing antisense mRNA grew in the absence of serum, unlike control cells
expressing sense mRNA or wild type NIH 3T3 cells (21). BRCA1 antisense mRNA added to the cultures of primary mammary epithelial cells and MCF-7 breast cancer cells also resulted in a 70-90% reduction of mRNA levels and an increase in the proliferative rate of the cells, providing increasing support for a growth suppressive role of BRCA1 (6).

Microarrays were utilized to analyze the genes affected by the induction of BRCA1. Induction of BRCA1 using a tetracycline-inducible system causes apoptosis of cells within 24 hours of BRCA1 expression (39). The DNA damage response gene GADD45 was activated by BRCA1 expression and is believed to stimulate the stress activated protein kinase JNK/SAPK, implying that GADD45 may be mediating the affect of BRCA1-induced apoptosis on cells (39). The induction of GADD45 occurred in cells lacking p53, indicating that BRCA1 is capable of causing apoptosis independently of a p53 induced apoptotic pathway. Disruption of the JNK/SAPK signaling pathway by dominant negative SEK1, an upstream regulator of JNK/SAPK family members, prevented apoptosis caused by the induction of BRCA1, but had no affect on the increase in expression of GADD45.

**BRCA1 Mouse Studies**

Mouse knock-out experiments are an important tool to study the effects of the loss of a gene on the development of the mouse and also the effect of the loss of the gene on a specific tissue. These experiments have indicated that the mouse Brca1 gene is essential for development. The mouse and human proteins are 51% identical overall and are 80% identical within the RING finger and carboxy terminal regions (40; 41).
*Brca1* knockouts have been constructed in mice by deleting exons 5 and 6, leading to a truncated protein. These mice die before day 7.5 of embryogenesis and are poorly developed with no evidence of mesoderm formation (42). Mice with a deletion in exon 2 of *Brca1*, resulting in a truncated protein, also die at this stage (43). Embryos without functional *Brca1* show reduced cell proliferation and decreased expression of the cell cycle protein cyclin E. They also have decreased expression of mdm2, an inhibitor of the p53 protein, resulting in a p53 dependent increase in the expression of the cyclin dependent kinase inhibitor p21^{WAF1/CIP1} (42). The period of resorption of the mutant embryos indicates that *Brca1* is essential during E5.5-E6.5, at which time mouse embryos undergo gastrulation resulting in a dramatic increase in size (42). The growth deficit of the *Brca1* mutant embryos is not due to an increase in apoptosis, but is the result of a decrease in cell growth and proliferation. Unlike human females heterozygous for a *BRCA1* mutation who have in increased susceptibility to breast and ovarian cancer, mice heterozygous for the *Brca1*^{Δ5-6} mutant allele are phenotypically normal and fertile beyond 11 months of age (42).

The expression of *mdm2* was examined in mice, due to the phenotypic similarity between *Brca1* and *mdm2* null mice (42). Mdm2 inhibits p53 and also targets it for degradation. Since the level of mdm2 was decreased in Brca1 null mice but the p53 protein level was unchanged, a net increase in p53 activity resulted. p21^{WAF1/CIP1}, a downstream target of p53, showed elevated levels suggesting that p53 activity is higher in *Brca1*^{Δ5-6/-} cells and may impair growth in *Brca1* mutants, although other regulators in addition to p53 may also have an effect on the level of p21^{WAF1/CIP1} protein.
Mice mutant for both Brca1 Δ5-6-/- and p53 +/- or Brca1 Δ5-6-/- and p21^WAF1/CIP1-/- were generated to test the hypothesis that if the primary cause of cell death in Brca1 Δ5-6-/- mice was increased p53 expression, then deletion of p53 or p21^WAF1/CIP1 may rescue the Brca1 Δ5-6-/- phenotype (44). Results from the double knockout p53 +/-/Brca1 Δ5-6-/- and Brca1 Δ5-6-/-/p21^WAF1/CIP1-/- mice indicated that mutations in both p53 and p21^WAF1/CIP1 prolonged the survival of Brca1 mutant animals, although none survived to birth. p53 +/-/Brca1 Δ5-6-/- mice survived until day 9.5 of gestation; however, the Brca1/p53 double null mutants were smaller and less developmentally advanced than wild type animals. This result has been confirmed in Brca1^exon2-/-/ p53 +/- double knock-out mice where more advanced development than that detected in mice null for Brca1^exon2-/- was observed (43). Brca1 null mice that were heterozygous for p53 expression did not have increased survival of the embryo past the stage that is observed in Brca1 Δ5-6-/- mice (or Brca1^exon2-/- mice) (43; 44).

Brca1 Δ5-6-/-/p21^WAF1/CIP1-/- double mutants survived to E11.5 before resorption, unlike Brca1 Δ5-6-/-/p21^WAF1/CIP1+/+ mice, which did not survive past E7.5, similar to Brca1 Δ5-6-/- single mutants (44). All double mutant embryos were much more developed than Brca1 Δ5-6-/- mutants. It is possible that the absence of Brca1 affects genome stability, resulting in the up-regulation of p21^WAF1/CIP1 by p53 and subsequent cell death. The partial rescue observed, resulting from a lack of p53 and p21^WAF1/CIP1 on the Brca1 Δ5-6-/- phenotype, indicates that these genes are involved in early embryonic lethality caused by a Brca1 deletion.

Mouse cells homozygous for an allele of Brca1 with a deletion in exon11, resulting in a stop codon and truncated protein, exhibit hypersensitivity to γ-irradiation
and have chromosomal abnormalities (45). Deletion of exon11 results in a less detrimental phenotype than a Brca1Δ5-6/− deletion, presumably due to the alternative splicing of exon11 of Brca1 that normally occurs in cells (45; 46). Embryos with a deletion in exon11 survive longer than Brca1Δ5-6/- mice, and Brca1 exon11/- embryonic cells are viable. After receiving γ- irradiation, the number of trophoblast cells of the control blastocysts were only slightly reduced, while the trophoblast cells of the Brca1 exon11/- null blastocysts were reduced from 20-30 cells per embryo to 5-10 cells per embryo.

E7.5 Brca1 exon11/- embryos were karyotyped and 70% retained a normal chromosome number compared to 97% in the control. p53+/− mice were crossed with the Brca1 exon11/- mice and survived on average two days longer; however, 72% of the double knockouts displayed chromosomal abnormalities. Translocations and dicentric chromosomes were observed in the double knock-out mice, indicating that accumulated DNA damage may lead to embryonic cell death in the double null animals. The p53 mediated cell cycle checkpoint in Brca1 exon11/- mice, which is activated due to a lack of Brca1-dependent DNA repair, is abolished in Brca1 exon11+/−p53+/− mice, allowing the accumulation of massive chromosomal changes (45).

The Effect of DNA Damage on BRCA1

In response to DNA damage mediated by hydroxyurea, UV light, mitomycin C or γ-irradiation treatment (agents which cause DNA damage but do not necessarily arrest DNA synthesis), BRCA1 phosphorylation and localization patterns change. After exposure to hydroxyurea and UV-light, BRCA1 relocates from nuclear dots to PCNA+ replication structures that are the site of DNA replication and repair (22). The DNA
damage response protein Rad51 and BARD1 (BRCA1 associated RING domain), a novel protein that interacts with BRCA1, are associated with BRCA1 in S phase nuclear dots, and also undergo changes in subcellular location to DNA damage, moving with BRCA1 to these PCNA\(^{+}\) structures (17; 22). In cells that have not been subjected to DNA damaging agents, PCNA\(^{+}\) staining in late S phase becomes focal and throughout S phase BrdU incorporation into replication forks overlays PCNA\(^{+}\) staining. In these cells, the BRCA1 punctate dots do not overlap with PCNA\(^{+}\) foci (22).

Recently, BRCA1 was shown to interact with Rad50 and colocalize with the Rad50/Mre11/p95 complex in nuclear dots following ionizing radiation (47). Rad50 is involved in end processing, and Rad51 is involved with DNA strand exchange in a subsequent step of DNA repair. It is possible that BRCA1 facilitates the coupling of different stages of DNA repair (47). Cells show either Rad50/BRCA1 or Rad51/BRCA1 colocalization; they do not contain both at the same time.

Three forms of BRCA1 are observed after treatment with hydroxyurea. Phosphatase treatment of the protein alters gel mobility and collapses the three bands into the single band that is observed in G1, when BRCA1 is not phosphorylated. Therefore, the three bands are thought to be the unphosphorylated form, the phosphorylated form identified during S phase and a hyperphosphorylated form of BRCA1 observed after the addition of DNA damaging agents. The increase in phosphorylation above the amount normally observed in S phase can also occur in cells that are lacking two DNA damage-sensing protein kinases: DNA-PK and ATM, indicating that these kinases are not required for hypophosphorylation of BRCA1 after DNA damage (22). Significant alteration in gel mobility is observed 20 to 30 minutes after exposure of cells to UV light.
and significant dispersal of nuclear foci is seen 25 minutes after treatment, suggesting that location of BRCA1 may be affected by the phosphorylation status of the protein (22).

The Involvement of BRCA1 in Transcription Coupled Repair

Recent studies indicate that BRCA1 is involved in transcription-coupled repair (TCR) (48; 49). TCR occurs more rapidly on the strand of DNA being transcribed than on the genome as a whole. Several types of DNA damage, including damage caused by UV treatment, ionizing irradiation and oxidizing agents, are repaired by TCR. Embryonic stem cells undergo transcription coupled repair in cells heterozygous for a wild type and mutant allele of Brca1 with a deletion in exon11 (Brca1^exon11-/-), but not in the cells homozygous for the Brca1^exon11-/- allele (48). The Brca1 null cells do, however, undergo repair of oxidized bases that is not coupled to transcription, indicating that Brca1 is required for TCR of oxidative or UV induced DNA damage, but is not required for the general removal of oxidized bases. These results were confirmed by Abbott et al. (45), who determined that HCC1937 cells, which express only a truncated form of BRCA1, are hypersensitive to ionizing radiation and do not undergo TCR. Addition of BRCA1 results in the restoration of TCR. Several cell lines were tested with ionizing radiation, and cells with two copies of BRCA1 were less sensitive than those containing one copy of BRCA1, which were in turn less sensitive that the BRCA1-null cell line HCC1937. HCC1937 cells were capable of performing double strand break repair at levels comparable to cells with WT BRCA1, but were defective for TCR, suggesting that the BRCT domain of BRCA1 is required for TCR but not double strand break repair (49).
Although Brca1<sup>exon11</sup> cells do not repair transcriptionally active DNA preferentially after DNA damage (48), there is an increase in the sensitivity of Brca1<sup>exon11</sup> cells to DNA damage induced by UV-light or the oxidizing agent H<sub>2</sub>O<sub>2</sub>. Therefore, BRCA1 may be involved in another aspect of repairing the damage caused by UV or H<sub>2</sub>O<sub>2</sub> other than TCR. It is not known whether BRCA1 is directly involved in TCR or if it is responsible for the transactivation of genes involved in this repair mechanism.

Several agents, including adriamycin, camptothecin and UV light, lead to decreases in BRCA1 and BRCA2 mRNA levels at doses that lead to little or no cytotoxicity or apoptosis in MCF-7 breast cancer cells (50). Cells transfected with a dominant negative p53 protein require 10-fold higher doses of adriamycin to down-regulate BRCA1 and BRCA2 transcript levels indicating there may be a p53-sensitive component to the down-regulation of BRCA1 and BRCA2 (50). A decrease in BRCA1 and BRCA2 protein levels was also observed. Although the subcellular redistribution of BRCA1 and Rad51 occurs rapidly, down-regulation of BRCA1 and BRCA2 occurs 6 to 12 hours after treatment in mammary cells (50). Therefore, down-regulation of BRCA1 and BRCA2 may be an indirect effect that occurs downstream of the initial DNA damaging events. Another study using ovarian cells indicates that although down-regulation of BRCA1 and BRCA2 occurs 8 to 16 hours after treatment with adriamycin and UV light, it does not occur after treatment with several other DNA damaging agents including camptothecin, nitrogen mustard, taxol, vincristine and etoposide (51). Interestingly, breast and ovarian cells are appear to react differently to damage caused by camptothecin; however, the overall trend of down-regulation of BRCA1 and BRCA2 after
DNA damage is similar for the two cell types. The authors speculate that down-regulation of *BRCA1* and *BRCA2* allows cells to repair their DNA and re-enter the cell cycle, rather than undergo BRCA1-mediated apoptosis (51).

BRCA1 may be involved in several different types of repair. Adriamycin is an intercalator and topoisomerase II inhibitor that causes single and double stranded DNA breaks (52). UV irradiation causes the formation of thymidine dimers that are repaired by the nucleotide excision repair pathway (53). At present, evidence suggesting the specific type of lesion or repair pathway that requires BRCA1 is inconclusive.

**The Effect of BRCA1 on the Transactivation of Genes**

The acidic nature of the carboxy terminus of the BRCA1 protein suggests that it may be involved in the transactivation of genes. The region of BRCA1 encompassing amino acids 1760 to 1863, when fused to a Gal4 DNA binding domain, was determined to be the minimal region required for the transactivation of Gal4 responsive reporter genes in a yeast two hybrid system (54; 55). Additional sequences 5’ of the minimal transactivating domain are required for maximal transactivation activity. Moreover, mutations in the carboxy terminus of BRCA1 that are associated with predisposition to breast cancer abolish the ability of BRCA1 to transactivate the Gal4 responsive reporter genes (54). The carboxy terminus of BRCA1 also interacts with RNA helicase A (56), as well as other components of the RNA pol II holoenzyme (57), CtIP (58; 59), a protein potentially involved in transcriptional repression and the CREB binding protein transcription factor (CBP) (60). These interactions further suggest an involvement of BRCA1 in the transactivation of genes (56).
Cotransformation of BRCA1 with a luciferase reporter gene under the control of either the p21\textsuperscript{WAF1/CIP1} or \textit{mdm2} promoter indicates that BRCA1 is capable of activating those promoters in a p53-\textit{independent} manner (61-63). Furthermore, BRCA1 transfected into p21\textsuperscript{WAF1/CIP1\textsuperscript{+/+}} cells inhibits S phase progression as well as DNA synthesis by 50%, while transfection of BRCA1 into p21\textsuperscript{WAF1/CIP1\textsuperscript{+-}} cells does not (61). Missense mutants of BRCA1 that are deficient in the transactivation of the p21 promoter are also deficient in cell cycle inhibition. p21 signals growth arrest both dependently and independently of p53 induction. It is, therefore, possible that BRCA1 is the signal for growth arrest of cells in the absence of p53.

BRCA1 can also increase p53-\textit{dependent} gene expression (60; 62; 63). BRCA1 stimulates the p53 responsive promoters p21\textsuperscript{WAF1/CIP1}, \textit{bax} and \textit{mdm2} to higher levels in the presence of p53 (62; 63). BRCA1 is therefore able to transactivate p21\textsuperscript{WAF1/CIP1} and \textit{mdm2} in a p53-dependent and p53-independent manner, however \textit{bax} requires both proteins for activation. p53 and BRCA1 physically interact in the cell, suggesting that the two proteins may work together to effect growth arrest and apoptosis (60; 62; 63).

Contrary to the above results, a recent study using oligonucleotide arrays to analyze the effect of BRCA1 induction on cellular gene expression indicates that p21\textsuperscript{WAF1/CIP1} expression is not changed, nor is the expression of the p53-dependent genes \textit{bax} and \textit{mdm2} or the p21\textsuperscript{WAF1/CIP1} and p53 protein levels in the cell (39). It is possible that differences observed between the two studies are a result of the promoter-reporter assays not accurately depicting the activation status of genes in cells or the differences may be a result of the variant cell types that were used in the studies. The cell line used to perform the oligonucleotide array contained inducible tetracycline-regulated
expression of BRCA1 and constitutively expressed bcl-2 to prevent BRCA1 mediated apoptosis. It is possible that expression of bcl-2 prevented normal activation of p21WAF1/CIP1 or mdm2 by BRCA1 (39).

BRCA1 also has an affect on c-Myc-mediated transactivation and interacts with the c-Myc protein (64). c-Myc is a weak transactivator of genes including cdc25A, which plays a role in the G1-S phase transition by activating cyclin-dependent kinases and the translation initiation factors eIF-2a and eIF-4E. BRCA1 represses the transactivation of a leucine reporter gene under the control of a minimal E1b promoter containing c-Myc binding sequences. A BRCA1 protein lacking the amino terminus (the protein contained residues 773 to 1863) that could not interact with c-Myc had little affect on the transactivation by c-Myc of the reporter genes. BRCA1 was also able to repress c-Myc activity on the cdc25A promoter itself.

BRCA1 has also been shown to repress estrogen receptor signaling (65). Once activated by estradiol, the estrogen receptor enters the nucleus, dimerizes and activates genes containing estrogen responsive elements (ERE). A reporter gene containing an ERE was repressed after cotransfection of estradiol and BRCA1, compared to transfection with estradiol alone. It is possible that BRCA1 represses estrogen receptor mediated cell growth, and loss of that repression may contribute to the development of cancer.
Protein-Protein Interactions

Colocalization, co-immunoprecipitation and yeast two hybrid studies indicate that many proteins are capable of interacting with the BRCA1 protein (Figure 2, Table 1), potentially affecting the function of BRCA1.

BARD1

BARD1 (BRCA1 associated RING domain) was identified as a BRCA1 RING finger binding protein using a yeast two hybrid assay. This observation was confirmed using mammalian two hybrid and co-immunoprecipitation assays (66) and BRCA1 and BARD1 were also found to colocalize during S phase in nuclear dots (41). BARD1 itself contains a RING finger domain and three tandem ankyrin repeats, which may also be involved in protein-protein interactions. In addition, the carboxy terminus of BARD1 has a BRCT domain homologous to the BRCT domains located at the carboxy terminus of BRCA1. BRCA1 missense mutations associated with breast cancer susceptibility disrupt the interaction of BRCA1 and BARD1.

The BARD1 coding sequence in 58 ovarian tumours, 50 breast tumours and 60 uterine tumours was examined for mutations (67). Two somatic missense mutations were identified in the BRCT domain of BARD1, one in a breast tumour and the other in an endometrial carcinoma. In addition, the germline alteration Gln564His was detected in a clear cell ovarian tumour that also had LOH of the wild type BARD1 allele. Although the genomic location of BARD1, 2q34-q35, is not frequently altered in breast or ovarian cancer, the identification of mutations coupled with LOH suggests that BARD1 may encode a tumour suppressor protein (67).
Proteins that interact with BRCA1. Exon11 of BRCA1 was used in this study and has been found to interact with p53, BRAP2, importin α, Rad50 and Rad51 and an undetermined kinase. The location that γ-tubulin, a component of the centrosome, and the location that the cyclin dependent kinase cdk2 interact with BRCA1 has not been identified.
Table 1: Summary of Identified BRCA1 Interacting Proteins

<table>
<thead>
<tr>
<th>Identity of Interacting Protein</th>
<th>BRCA1 Interaction Domain</th>
<th>Method for Identification of Interaction</th>
<th>Function of Interacting Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP1 (BRCA1 assoc. protein 1)</td>
<td>RING finger</td>
<td>yeast two-hybrid assay, GST pull down, colocal</td>
<td>ubiquitin carboxy terminal hydrolase</td>
</tr>
<tr>
<td>BARD1 (BRCA1 assoc. RING domain)</td>
<td>RING finger</td>
<td>yeast &amp; mammalian two-hybrid, GST pull down</td>
<td>unknown, mutated in cancers</td>
</tr>
<tr>
<td>E2 Ubiquitin conjugating enzyme</td>
<td>RING finger</td>
<td>yeast two-hybrid, GST pull down</td>
<td>ubiquitination</td>
</tr>
<tr>
<td>c-Myc</td>
<td>exons 8 - 11(aa 175-303, 433-511)</td>
<td>yeast two-hybrid, GST pull down, co-immuno.</td>
<td>proto-oncogene</td>
</tr>
<tr>
<td>p53</td>
<td>exon11 and carboxy terminus</td>
<td>GST pull down, co-immunoprecipitation</td>
<td>tumour suppressor</td>
</tr>
<tr>
<td>BRAP2 (BRCA1 assoc. protein 2)</td>
<td>nuclear localization signal (exon11)</td>
<td>yeast two-hybrid assay, GST pull down</td>
<td>cytoplasmic retention</td>
</tr>
<tr>
<td>importin α</td>
<td>nuclear localization signal (exon11)</td>
<td>yeast two-hybrid assay, GST pull down</td>
<td>nuclear localization</td>
</tr>
<tr>
<td>Rad50</td>
<td>exon11 (aa 341 to 758)</td>
<td>yeast two-hybrid, GST pull down, co-localization, co-immunoprecipitation</td>
<td>DNA repair, recombination, non-homolog, end joining</td>
</tr>
<tr>
<td>Rad51</td>
<td>exon11 (aa 758 to 1064)</td>
<td>co-immunoprecip., colocal, Sos recruitment</td>
<td>DNA repair</td>
</tr>
<tr>
<td>BRCA2</td>
<td>1314 to 1756</td>
<td>co-immunoprecip., colocalization</td>
<td>tumour suppressor</td>
</tr>
<tr>
<td>RNA PolII, RHA, CBP</td>
<td>c-terminus (aa 1650 to 1800)</td>
<td>co-purification, GST pull down,</td>
<td>transactivation of genes</td>
</tr>
<tr>
<td>CtIP</td>
<td>c-terminus (aa 1602 to 1863 )</td>
<td>yeast two-hybrid assay, GST pull down</td>
<td>transcriptional repression</td>
</tr>
<tr>
<td>casein kinase II (CKII)</td>
<td>c-terminus (aa 1642 to 1863)</td>
<td>yeast two-hybrid, GST pull down</td>
<td>phosphorylates BRCA1 at Ser 1572</td>
</tr>
</tbody>
</table>

Summary of Known Interacting Proteins. At the commencement of this project, BARD1, Rad51, importin α and RNA Pol II were known to interact with BRCA1. Subsequently, several more interactions were identified. γ-tubulin and the cyclin dependent kinase, CK2 also interact with BRCA1 at an undetermined location.
The use of anti-sense \textit{Brd1} to repress expression in mouse cells results in several changes, including altered cell morphology (68), an increase in cell size, aberrant cell cycle repression, multinucleated cells and an ability to overcome contact inhibition. Together, these results suggest that repression of \textit{Brd1} in cells results in a premalignant phenotype, further supporting its role as a tumour suppressor.

\textbf{BAP1}

The BRCA1 associated protein BAP1 is a second protein identified by a yeast two-hybrid assay that interacts with the RING domain of BRCA1 (69). BAP1 has homology to ubiquitin C-terminal hydrolases and, therefore, may be involved in cleaving ubiquitin moieties from substrates. \textit{In vitro} studies show that BAP1 is capable of hydrolyzing the glycine 76 ethyl ester of ubiquitin. Proteins encoded by alleles of \textit{BRCA1} mutated in the RING finger domain have been observed in breast cancer families and do not bind to BAP1. Both BRCA1 and BAP1 are present in punctate nuclear domains in the cell, several of which overlap, although colocalization is not complete. The function of this protein may be to stabilize BRCA1, to stabilize proteins bound to BRCA1, or to affect the cellular localization of BRCA1 by cleaving ubiquitin from BRCA1 or other proteins, preventing ubiquitin-mediated proteolysis (69).

\textbf{The Ubiquitin Conjugating Enzyme E2}

The discovery of another RING-finger containing protein (A07) that interacted with the ubiquitin conjugating enzymes E2 led to the discovery that several other RING
fingers, including the one found in BRCA1, also bound to E2 and facilitated E2-dependent ubiquitination (70). The ubiquitination was diminished when a chelator of divalent cations was added indicating that coordination of the RING finger with a Zn$^{2+}$ ion is required for the interaction. The authors speculate that the RING finger and surrounding regions associate with E2 and provide a favorable environment for the transfer of ubiquitin from E2 to a lysine residue on the protein. Binding of BRCA1 to E2 suggests that BRCA1 is itself targeted for ubiquitin mediated protein degradation.

**Importin α and BRAP2**

There are two nuclear localization signals (NLS) in exon 11 of BRCA1. Mutation of these NLS resulted in a loss of movement of the BRCA1 protein to the nucleus (71). Both of the BRCA1 NLS bind importin α and BRAP2 (BRCA1 associated protein 2) in yeast two-hybrid screens (71). Mutations in the NLS that prevented BRCA1 protein transport to the nucleus abrogated protein binding to importin α. BRAP2, a protein that is mainly localized to the cytoplasm, was also identified as a BRCA1 binding protein in the same two-hybrid screen. BRAP2 binds to the NLS motif encoded by the wild type BRCA1 gene but not one encoded by a mutant. It may function as a cytoplasmic retention protein that is involved in the regulation of BRCA1 localization and transport (29). In yeast two hybrid and GST pull-down assays, BRCA1 and BRAP2 interact with 5 fold higher affinity than BRCA1 and importin α, suggesting that BRAP2 may retain newly synthesized BRCA1 in the cytoplasm until further signaling dissociates BRCA1 from BRAP2, allowing binding to importin α and transport into the nucleus.
**Rad51**

Cell localization studies and co-immunoprecipitation assays indicate an interaction between a domain within exon11 of BRCA1 and Rad51 (17). Rad51 is a member of a protein family that mediates DNA strand exchange functions leading to normal recombination and double strand break repair. It is present in nuclear dots that show significant, but not complete, overlap with BRCA1 foci and there is some cell to cell variability in the degree of overlap. In addition, during meiosis Rad51 and BRCA1 are partially colocalized on the unsynapsed axial elements of human synaptonemal complexes, suggesting a role for BRCA1 in meiotic recombination. Incomplete co-immunoprecipitation is observed between BRCA1 and Rad51. This may be due to an excess of Rad51 over BRCA1, an indirect association between Rad51 and BRCA1, or possibly because only a fraction of Rad51 binds to BRCA1. Co-immunoprecipitation assays are not able to discriminate between these possibilities. Deletion analysis was performed to determine the region required for co-immunoprecipitation of BRCA1 and Rad51, and the minimal region of BRCA1 required for binding includes amino acids 758 to 1064 of BRCA1. Furthermore, mutations in this region have been observed in breast cancer suggesting that the interaction of BRCA1 and Rad51 may be required for normal cell function. The interaction of Rad51 and BRCA1 supports a role for BRCA1 in the maintenance of genome integrity.

**Rad50**

The amino-terminal portion of Rad50 also interacts with BRCA1 at amino acids 341 to 748, indicated by both a GST pull down assay and yeast two-hybrid system (47).
Rad50 forms a complex with Mre11 and p95/nibrin that functions in homologous recombination, non-homologous end joining, meiotic recombination, DNA damage response and telomere maintenance. The colocalization of Rad50/Mre11/p95 with BRCA1 after cells are subjected to ionizing radiation suggests that BRCA1 and Rad50 also play a role in DNA damage repair.

**BRCA2**

An interaction has been identified between the two breast cancer susceptibility gene products, BRCA1 and BRCA2 (18). Colocalization experiments indicate that BRCA2 is located in the nuclear punctate dots that contain BRCA1 and Rad51, and BRCA2 also interacts with Rad51. BRCA2 also undergoes a shift in its staining pattern after DNA damage and relocates to areas overlapping PCNA+ staining along with BRCA1 and Rad51. Staining for BRCA2 in meiotic cells indicates that there is also colocalization between BRCA1, BRCA2 and Rad51 on the unsynapsed axial elements of chromosomes. These proteins may all work in a complex to ensure proper meiotic recombination.

To identify the region of BRCA1 that is required for an interaction with BRCA2, co-immunoprecipitation studies were performed in HCC1937 cells that contain one truncated 210kDa allele of BRCA1 and no wild type copy (18). An antibody to the amino terminus of BRCA1 was able to co-immunoprecipitate BRCA2, indicating that the extreme carboxy terminal amino acids of BRCA1 are not required for an interaction with BRCA2. The carboxy terminal fragment of BRCA1 including amino acids 1314 to 1756 was identified as the domain required for this interaction through the use of fragments of
BRCA1 that overlapped the entire reading frame. Intact BRCT domains in BRCA1 are not required for this interaction, as truncated BRCA1 mutants with part of their second BRCT domain deleted are still able to bind to BRCA2. The domain encoded by exon 11 of BRCA1 that is needed for the interaction with Rad51 was not necessary, indicating that Rad51 is not required to mediate the interaction between BRCA2 and BRCA1.

c-Myc

BRCA1 also interacts with the proto-oncogene c-Myc, as indicated by coimmunofluorescence and yeast two-hybrid experiments (64). Two regions of BRCA1 are involved in c-Myc binding – amino acids 175 to 303 and 433 to 511. These two regions of the BRCA1 protein are encoded by exons 8, 9, 10 and the amino portion of exon 11. The interaction between the BRCA1 and c-Myc proteins requires the helix loop helix motif of c-Myc, which is known to mediate protein dimerization with Max; however, BRCA1 does not interact with Max itself (Wang et al., 1998).

The affect of BRCA1 on c-Myc mediated transformation was examined by transforming rat embryonic fibroblast cells with c-Myc and h-Ras and then transfecting the cells with BRCA1 (64). Transfection with BRCA1 reduced the number of transformed foci by approximately 90%. BRCA1 does not, however, lower the number of transformed foci when cells were transformed by SV40, suggesting that the effect of BRCA1 on cell transformation is specific for c-Myc transformed cells. Since Myc-Max dimers are required for Myc function, and both BRCA1 and Max bind to the basic helix-loop-helix motif of Myc, it is possible that BRCA1 down-regulates Myc activity by preventing the formation of Myc-Max dimers. This has not yet been confirmed by direct
studies of the effect of BRCA1 on Myc-Max dimerization. BRCA1 may also repress transcriptional activation of genes by Myc that are required for cellular transformation.

p53

Both p53 and BRCA1 are altered in response to DNA damage by stabilization or hyperphosphorylation, respectively, and are implicated in the regulation of gene transcription, control of cell growth, and response to DNA damage. Exon11 of BRCA1 including amino acids 224 to 500 was shown to interact with p53 through the use of a GST-p53 fusion protein to precipitate either in vitro translated or cellular BRCA1 protein (63). Co-immunoprecipitation experiments using endogenous p53 and BRCA1 proteins were also performed to confirm the interaction and the carboxy terminal domain of p53, including amino acids 300-393, was identified as the region required for the association (63).

An additional region of BRCA1 has been shown to interact with the DNA-binding domain of p53. GST pull down and co-immunoprecipitation experiments using a BRCA1 protein lacking all or most of exon11, including the previously identified p53 binding domain, indicates that the second BRCT domain of BRCA1 from amino acids 1760 to 1863 is also sufficient for BRCA1 and p53 interaction (60). The central region of p53 from amino acids 79 to 307, which is necessary for sequence-specific DNA binding, is required for the interaction of p53 with BRCA1 (72). Interestingly, BRCA1 proteins lacking exon11 are not able to stimulate p53-independent transactivation of the p21 promoter upstream of a luciferase reporter gene, suggesting that region(s) in exon11 are required for p53-independent transactivation of the p21 promoter (60). Ouchi et al.
(62), also identified binding of BRCA1 to the central domain of p53 from amino acids 271-393, but did not identify the region of BRCA1 required for the interaction.

RNA pol II holoenzyme

The carboxy terminus of BRCA1 has been implicated in the transactivation of genes, and also interacts with several proteins involved in transactivation, including hSRB7, a component of the RNA polII holoenzyme (57). Antibodies to BRCA1 co-immunoprecipitate a polymerase activity as well as the basal transcription factors TFIIF, TFIIE, and TFIIH (57). A truncated form of BRCA1, with a deletion of the carboxy terminal 111 amino acids, did not associate with the hSRB7-containing complex indicating that those residues of BRCA1 are required hSRB7 binding. However, since BRCA1 also interacts with the polymerase complex through RNA helicase A (RHA), a lack of hSRB7 binding does not preclude binding of BRCA1 to the RNA pol II holoenzyme (73).

RNA helicase A

Yeast two-hybrid and GST fusion protein assays were used to show that RNA helicase A also appears to link BRCA1 to the RNA polII holoenzyme complex (56). Both BRCT domains of BRCA1 from amino acids 1650 to 1800 are necessary for binding to RHA. The interacting region is comprised of at least three distinct RHA binding domains from residues 1650 to 1700, 1701 to 1750 and 1751 to 1800. The regions necessary for RHA binding were also necessary to interact with the RNA pol II holoenzyme, suggesting that RHA is mediating the interaction. Furthermore, a missense
BRCA1 mutation, M1775E, encoding a protein that is defective for transactivation, also binds with less stability to RHA. A yeast two-hybrid assay determined that amino acids 230 to 625 of RHA were necessary for the interaction with BRCA1 (56). Co-transfection of BRCA1 fused to a Gal4 DNA binding domain and of a truncated RHA molecule that lacked most of its polymerase binding domain but retained its BRCA1 binding domain, resulted in a 2.5-fold reduction in the previously observed transcriptional activation of the Gal4 responsive reporter gene by the BRCA1 carboxy terminus fused to the Gal4 DNA binding domain (56). The mutant RHA may have acted as a dominant negative and out-competed wild type RHA from interacting with BRCA1 and transactivating the reporter gene promoter, resulting in decreased activation of the luciferase reporter gene. This implies that the carboxy terminus of BRCA1 interacts with RHA, and therefore RNA pol II to activate the luciferase reporter gene.

**CREB binding protein**

In addition to interacting with p53, the second BRCT domain interacts with the CREB binding protein (CBP) transcription factor, a member of the RNA polymerase II holoenzyme complex that binds to the CREB binding site on DNA and also interacts with RHA (60). This result further suggests that the interaction of the BRCT domain with components of the RNA pol II holoenzyme is required to mediate the transactivation of genes.
CtIP

The carboxy terminus of BRCA1 interacts with another protein involved in transcriptional activation. CtIP (Carboxy terminal Interacting Protein) interacts with amino acids 1602 to 1863 of BRCA1, but does not interact with a truncated form of BRCA1 that lacks transactivation activity (58; 59). CtIP associates with the transcriptional repressor CtBP (Carboxy terminal Binding Protein), indicating a possible role for CtIP in transcription. CtIP cDNA from 89 tumour cell lines derived from several different tissue types was sequenced and 5 missense variants were observed (59). A possible requirement for CtIP in the normal function of the cell is indicated by a tumour cell line that contains a variant form of CtIP encoding a lysine to glutamic acid change. This cell line has no expression of the wild type CtIP allele, suggesting that either loss of heterozygosity or decreased expression has occurred. Binding of the variant CtIP to BRCA1 was not affected, however, and it has not yet been determined if CtIP is mutated in human tumours.

Components of the Histone Deacetylase Complex (Rb, RbAp46/48, HDAC1/2)

The retinoblastoma (Rb) associated protein RbAp46 was identified as an interacting protein of the BRCT domain of BRCA1 using Far Western analysis. Also, the homology between RbAp46 and another Rb-associated protein, RbAp48, led to the identification of RbAp48 as another BRCT-associated protein (74). Because RbAp46 and RbAp48 interact with Rb, the direct association of BRCA1 with Rb was analyzed. This analysis demonstrated that Rb could interact with the BRCT domain of BRCA1 in the presence or absence of RbAp46 (74). Colocalization experiments indicate that
RbAp48, RbAp46 and Rb colocalize in punctate nuclear dots with BRCA1, although colocalization is not complete (74).

Rb acts through histone deacetylases to repress transcription, and RbAp46 and RbAp48 are members of the histone deacetylase complex involved in chromatin remodeling. Interestingly the BRCT domain of BRCA1 can interact with the catalytic subunits of the histone deacetylase complex, HDAC1 and HDAC2 (74). This association of BRCA1 with the histone deacetylase complex is of interest given the evidence that BRCA1 may be involved in DNA repair and recombination, both of which require chromatin remodeling. These data also raise the possibility that BRCA1 can repress transcription, as histone deacetylase complexes are involved in nucleosomal condensation and transcriptional repression.

BRCA1 Association with Protein Kinases

Immunoprecipitation with antibodies to BRCA1 has led to the identification of several protein kinases, including the cyclin dependent kinase CDK2, that associate with BRCA1 (31). CDK2 was determined to phosphorylate serine 1497 of BRCA1, however the region of interaction between BRCA1 and CDK2 has not been identified.

The interacting region between the β subunit of casein kinase 2(CKII) and the carboxy terminus of BRCA1, including amino acids 1712-1863 (34) has been ascertained. CKII, a heterotrimeric protein that controls G0 to G1 and G1 to G2 transitions in the cell cycle, was subsequently shown to be required for the phosphorylation of serine 1572 of BRCA1. It remains a possibility that CDK2 and CKII, as well as other kinases, may phosphorylate BRCA1 at other locations.
Centrosomal Association with BRCA1

The centrosome of cells is responsible for organizing microtubule directed events during mitosis and controls the organization of animal cell structures in interphase. Immunofluorescence studies indicate that BRCA1 is associated with the centrosome during mitosis (23). γ-tubulin, a centrosomal component required for the nucleation of microtubules, co-immunoprecipitates a hypophosphorylated form of BRCA1, which co-migrates with an in vitro translated protein and is not believed to be a deletion product, since antibodies to the amino and carboxy terminus recognize the protein.

Identification of the proteins that interact with BRCA1 is necessary to increase our understanding the function of the protein. Evidence of BRCA1 interactions with several proteins suggests that BRCA1 is involved in the transactivation of genes. Differential expression and phosphorylation of BRCA1 argues that the protein may be transactivating genes in a cell cycle-specific manner. In addition, BRCA1 may be required to activate genes that are needed for a p53-independent apoptotic pathway. Colocalization experiments, the response of the BRCA1 protein and BRCA1 null cells to DNA damaging agents and the interaction with DNA repair genes also suggest that BRCA1 plays an important role in repairing DNA damage, although the type of DNA damage and the mechanism of repair is not understood.
Methods for Identifying Protein Interactions

A Yeast Two Hybrid System for Identifying Protein-Protein Interactions

This study utilized a yeast two-hybrid system (Figure 3) to identify interactions between the BRCA1 protein and proteins expressed from a human mammary gland cDNA library, and has identified several putative proteins that have not previously been reported to interact with BRCA1. The identification of proteins that interact with BRCA1 is important for understanding the function of BRCA1 and to elucidate the molecular pathways in which BRCA1 is involved. At the time I began my project, relatively few BRCA1-interacting proteins had been discovered - the novel protein BARD1 as well as importin α, Rad51 and the RNA polII were believed to interact with BRCA1. An interaction with these proteins suggests that BRCA1 has an effect on the transcription of genes as well as DNA repair. However, the function of BRCA1 has not been determined and it was thought that more proteins might interact with the BRCA1 protein. In addition, non-mammary gland libraries had been utilized to identify interacting proteins. Because BRCA1 is expressed in the majority of tissues studied, but is only affected in breast and ovarian cancer, the tissue type for performing a yeast two hybrid screen may be important. This yeast two-hybrid experiment as well as others have subsequently identified several BRCA1 interacting proteins.

Fields and Song first introduced the yeast two-hybrid system in 1989 as a method to identify protein-protein interactions (75). The gene encoding the protein of interest is fused to a DNA binding domain (DBD) and a cDNA expression library is fused to an activating domain (AD). Interaction between the protein of interest and a protein expressed from the cDNA library results in the AD being brought proximal to the
Yeast two hybrid system: BRCA1 is fused in frame to the Gal4 DNA binding domain (DBD), which interacts with the Gal4 upstream activating sequence (UAS) found in the promoter of three reporter genes Gal2-ade2, Gal1-his3 and Gal7-lacZ. Proteins expressed from a human mammary gland cDNA library are fused in frame to the Gal4 activating domain (AD), which is capable of activating the transcription of genes. Interaction between BRCA1 and a protein encoded by the cDNA library brings the AD proximal to the promoter region, resulting in the activation of transcription of the reporter genes. This allows yeast to grow on plates that do not contain adenine or histidine and to have a positive result in a lacZ assay.
promoter of a reporter gene, activating the gene. The system used for this study makes use of the Gal4 transactivator, which can be divided into two distinct domains: the DBD that binds to the upstream activation sequence (UAS) of a Gal4 responsive promoter, and the AD that is capable of transactivating the promoter of reporter genes. The gene of interest, in this case BRCA1, is ligated in frame to the DNA binding domain coding sequence in a yeast expression vector. A fusion protein is created, capable of binding to a Gal4 UAS in promoters. Yeast strains have been constructed that contain reporter genes under the control of Gal4 responsive promoters, such as the ADE2, HIS3 and lacZ genes. This allows for detection by the production of colour, in the case of the lacZ gene (due to the hydrolysis of a substrate by β-galactosidase, resulting in the production of a colour), or for growth on media lacking the required amino acid, in the case of the ADE2 or HIS3 reporter genes. The yeast strains are also deleted for the Gal80 gene; Gal80 is an inhibitor of the Gal4 protein in the absence of galactose and would consequently interfere with the screen when yeast cells are grown on glucose (76). An interaction between the DBD-BRCA1 fusion protein and a protein expressed from a human mammary gland cDNA library clone that is fused to the Gal4 activating domain brings the AD proximal to the promoter and activates the reporter genes.

Although exogenous mammalian proteins were used in this screen, yeast is the host that has been utilized. Yeast has the advantages of easy transformation and isolation of colonies as well as convenient retrieval of plasmids. In addition, many nutritional markers are available that can be utilized as reporter genes, and endogenous yeast proteins are less likely to bind a mammalian target protein than the proteins found in mammalian cells. Many proteins not normally expressed in the nucleus can be targeted
to the yeast nucleus as fusion proteins due to the NLS in the DBD or AD. Interactions have been identified with the yeast two-hybrid system using nuclear, cytoplasmic, membrane associated and extracellular proteins. In addition, many protein modifications occur in yeast that also occur in mammalian cells, including serine and threonine phosphorylation. The affinity of one protein to the other, concentration of proteins, extent of nuclear import, accessibility of the interacting domains to each other and accessibility of the AD to the transcription machinery affect the success of the screen (77).

The two-hybrid system is capable of detecting protein interactions undetectable by other methods. For example, the interaction between the guanine-nucleotide-binding protein Ras and the serine/threonine kinase Raf was initially detected by the yeast two-hybrid system (78; 79) but was not detectable by coimmunoprecipitation of endogenous proteins (79). The yeast two hybrid system amplifies the signal when transient transactivations result in the transcription of the reporter gene to produce a stable mRNA that is repeatedly translated into the reporter protein (78). An interaction that takes place in the yeast two hybrid system may be further strengthened by interactions between the Gal4 AD and factors at the promoter, which encourage the AD-protein fusion to remain at the promoter. The reporter gene promoters used in the yeast two-hybrid assay also have multiple copies of upstream activating sequences that increase the sensitivity of the assay (77).

Yeast two hybrid systems can be used to test the interaction of known proteins and to delineate the domains involved in the interaction between proteins. By creating deletions in the genes to be studied, the region of the gene necessary for an interaction
can be determined. It can also be used to define amino acids that are critical to the interaction.

A necessary requirement for the screen is that the protein being tested does not have the ability to activate the transcription of reporter genes without the interaction of a protein fused to the AD, resulting in the production of false positives. Different promoters upstream of two or more reporter genes also reduce the number of false positives because the promoter regions of the reporter genes are only similar at the upstream activating sequences (80). Therefore, any library proteins that bind to sequences on one promoter and activate the reporter gene may not bind to sequences on the second promoter. Also, by maintaining plasmids under conditions requiring the activation of the reporter gene, e.g. growing in media without histidine or adenine, any library plasmid that might have cotransformed with a plasmid encoding an interacting protein, but which does not itself encode an interacting protein, may be eliminated.

False positives may occur if the AD fusion protein binds to the promoter of the reporter gene itself or through another endogenous protein at the promoter. It is also possible that the AD-fusion protein is interacting non-specifically with the DBD-fusion protein; hence, it must be tested with other non-related DBD-fusion proteins to ensure that binding is specific. It is also possible that the AD-fusion protein requires the presence of the Gal4 DBD hybrid to function but does not itself interact with that protein. This may be because the Gal4 DBD hybrid is altering the chromatin structure around the promoter of the reporter gene, allowing the activator with a weak DNA binding domain of its own to bind to the promoter and activate transcription (81). False positives may also occur if the interacting proteins are not normally expressed within the same region in
the cell; however, they may interact when transported to the nucleus because of a common protein dimerization region such as a leucine zipper or helix-loop-helix motif (77).

It is possible that some protein interactions are missed using this system. A lack of sequences surrounding the protein fragment or the exposure of charged or hydrophobic basis may also affect protein conformation. Proteins may also not be transported to the nucleus properly if there are localization signals other than the NLS (present within the DNA binding domain or activating domain) in the protein (77). Alternatively, the correct protein modifications may not occur in yeast, including glycosylation, phosphorylation or the creation of disulfide bonds. Although yeast is capable of glycosylating and phosphorylating proteins, the specific residues that are modified may not be altered in yeast (77). It is also possible that the AD or DBD blocks accessibility to the interacting region of the protein. In addition, an interaction that occurs between two mammalian proteins that are overexpressed in yeast may not occur in mammalian cells between the endogenous proteins located in the cell.

For all of the reasons given above, it is necessary to confirm protein interactions using another method, such as co-immunoprecipitation, that makes use of endogenous proteins that are not overexpressed, and that are localized in the cell at their normal intracellular sites.

**Alternative Methods for Identifying Protein Interactions**

Several methods are available for detecting protein interactions, other than the yeast two-hybrid method. These methods are useful when the yeast two hybrid system
cannot be utilized, due to autonomous activation of reporter genes by the protein of interest, or if the protein is not correctly produced or modified in yeast.

A technique known as phage display is used to identify protein-protein interactions (82). A library of proteins is fused in frame to a gene encoding one of the phage coat proteins, resulting in its expression at the surface of the phage, and a protein of interest is radioactively labeled and used to probe the λ library. Subsequent washes ensure specificity of interaction and the phage is isolated for re-infection of host bacteria to enrich for the phage retaining the interacting protein, which is subsequently analyzed. While this system is advantageous in that the interacting gene is available for identification, bacteria may not post translationally modify or fold the protein in the manner required for the protein interaction to occur.

A similar method to phage display is the Far Western, where the cDNA library is expressed in phage, which is then used to infect bacterial cells (74). Protein production by the phage is induced and the cells are incubated with filters, which are then washed, blocked and probed with a histidine-tagged protein in an effort to identify interactions with that protein. The filters are then probed with an anti-histidine antibody, followed by incubation with the appropriate horseradish peroxidase conjugated secondary antibody and chemiluminescence is used to visualize plaques containing putative interacting proteins. This method has the same advantages and shortcomings as the phage display method, with the additional advantage of using immunological methods rather than radioactive methods to identify interacting proteins.

Immunoprecipitation or protein purification assays can also be performed in order to determine the protein(s) that co-immunoprecipitate or co-purify with the protein of
interest. It is difficult, however, to identify or obtain the clone of the interacting protein, as the protein bands on a gel must be purified and sequenced in order to identify the protein. A yeast two-hybrid system automatically provides the cDNA encoding the interacting protein once an interaction has been identified and the plasmid has been isolated from the yeast. An advantage of a co-immunoprecipitation assay is the production of protein in mammalian cells, allowing the proper post-translational modifications to take place. In addition, interactions between endogenous proteins can be tested, which is a necessary step to determine if the protein interactions occur naturally in cells and are not the result of overexpression of one or both of the proteins.

Exon11 of BRCA1 was utilized in this yeast two-hybrid study to identify proteins encoded by a human mammary gland cDNA library that are capable of interacting with the BRCA1 protein. A total of 366 colonies showed activation of the adenine reporter gene and were analyzed further. Twelve putative interacting proteins have been identified including protein phosphatase 1β, the proteasome subunit p27, two circadian clock proteins and GEF-2, a protein involved in the expression of gangliosides. Additional studies are now required to characterize these interactions.
MATERIALS AND METHODS

Construction of Vectors

A clone containing the entire coding region of BRCA1 plus a 3' untranslated region (UTR) in the bluescript vector, pBS, was provided by Myriad Genetics. The 3' UTR was later removed by Dr. M. Sauer and the resulting vector pBS-BRCA1ΔUTR was used for subsequent cloning steps. Fragments of BRCA1 were ligated in frame into the DNA binding domain (DBD) yeast expression vectors pAS1 (GAL4 (1-147) DNA-BD, TRP1, amp') and pAS2 (Clontech [GAL4 (1-147) DNA-BD, TRP1, amp', CYH2]) (Figure 4). pAS2 is identical to pAS1, except it contains the cycloheximide sensitivity gene. This facilitates the removal of the DBD plasmid in subsequent steps in the yeast strains Y190 and CG1945. Both vectors encode a TRP1 gene, resulting in the production of tryptophan, to facilitate the identification of yeast that has been transformed by the vector. A vector containing sequences from 1 to 4000 base pairs (bp) (pAS Bam) was constructed, effectively removing the activating domain from BRCA1. A DNA binding domain vector containing primarily exon 11 was also created (DBD-BRCA1exon11), including sequences from 900bp to 4000bp, as well as a pAS Eco-Xba vector that contained sequences from 900bp to 4600bp, including exon 11 and sequences proximal to the activating domain (Figure 5). BRCA1 sequences were ligated in frame with the DBD to produce a DBD-BRCA1 fusion protein that is expressed in yeast. The plasmids were then sequenced across the junction to ensure that no cloning errors disrupted the reading frame of the vector.
Figure 4: Construction of DBD-BRCA1 vector

pAS2 (8414 bp)

pBS-BRCA1 Δ3'UTR (8534 bp)
Figure 5: BRCA1 Domains Ligated into pAS Vectors

Construction of DNA binding domain vectors. Four vectors containing different fragments of BRCA1 were ligated into the vectors pAS1 and pAS2. pAS-BRCA1 contains the full length BRCA1 cDNA; pAS-Bam removes the carboxy terminal activating domain; pAS-BRCA1exon11 was utilized in the screen and encompasses exon11 of BRCA1; pAS-Eco-Xba contains exon11 of BRCA1 plus some carboxy-terminal residues.
Restriction Digests

Restriction digests were performed according to Sambrook et al. (83). If required, bands were purified using a Qiagen Gel Extraction Kit according to manufacturer's instructions. Enzymes were purchased from Boehringher Mannheim, New England Biolabs, Gibco BRL, and Pharmacia.

Ligation Reactions

Ligation reactions were performed according to Sambrook et al. (83). DH5α (supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) (83) bacterial cells were used for transformations after ligation.

Transformation of Bacteria

Bacteria were made chemically competent and were transformed using the methods outlined in Sambrook et al. (83).

Plasmid DNA Mini-Preps from bacteria

DNA was extracted according to Sambrook et al. (83). 5µL of the miniprep DNA was digested with the appropriate restriction enzymes to determine if the ligation was successful. Large scale DNA preps were performed using the Qiagen Plasmid Maxi Kit.

Yeast Strain Genotypes

The yeast strains Y190 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trpl-901, leu2-3, 112, gal4Δ, gal80Δ, cyh2, LYS2::GAL1_UAS-HIS3_TATA-HIS3, URA3::GAL1_UAS-
GAL1_{TATA-lacZ} (84), CG1945 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, cyh2, LYS2::GAL1_{UAS-GAL1_{TATA-HIS3}}, URA3::GAL4_{17-met(a3)} -CyC1_{TATA-lacZ} (85), pJ69-4A (MATa trp1-901, leu2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::gal1-HIS3, GAL2-ADE2, met2::GAL7-lacZ) (80) and Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met, gal80Δ, URA3::GAL1_{UAS-GAL1_{TATA-lacZ}}) (86) were utilized in this study. The yeast strains used contain mutations in the ADE2 gene, resulting in a pink colony colour. A loss of the pink colony colour occurs with the activation of the ADE2 reporter gene in pJ69-4A, however the other yeast strains do not contain an ADE2 reporter gene and should remain pink.

Transformation of Yeast

Yeast were transformed according to the LiAc transformation method (87). 100μL of each transformation was plated onto selective SD agar plates (6.7g/L yeast nitrogen base without amino acids, 20g/L agar, 50mL/L of 40% glucose stock) and was incubated at 30°C until colony formation occurred.

Testing for Autonomous Activation by the DBD Vector

After transformation plasmids were plated onto SD agar plates after transformation containing a 10X drop-out (D.O.) solution lacking tryptophan (85). Resulting colonies were streaked onto multiple trp/his' SD Agar plates containing 3-amino-1,2,4-triazole (1 to 3mM for pJ69-4A, 1 to 10mM for CG1945 and 10 to 50mM for Y190) to test for autonomous activation of the histidine reporter gene. Colonies were
also tested for autonomous lacZ reporter gene activation by using a β-galactosidase colony filter assay (Y190, CG1945) or a liquid β-galactosidase assay (pJ69-4A). pJ69-4A was also tested for adenine reporter gene activation by streaking colonies onto trp⁺/ade⁻ SD minimal media plates.

**Colony Filter β-galactosidase Assay (used with Y190 and CG1945)**

A 7.5 cm VWR filter paper (grade 413) was placed onto a plate of growing yeast and the colonies were lifted onto the filter. The filter was frozen in liquid N₂ for 10 seconds. 1.5mL of Z-buffer (16.1g/L Na₂HPO₄•7H₂O, 5.5 g/L NaH₂PO₄•H₂O, 0.75 g/L KCl, 0.246 g/L MgSO₄•7H₂O)/X-gal [5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside in N,N-dimethylformamide at 20mg/mL] solution was added to a 100 mm petri dish and a clean filter paper was added to the plate to soak up the liquid. The filter with the colony lifts was placed on the presoaked filter colony side up and the filters were incubated at 30°C until blue colonies appeared. The colonies that were blue on the filter were matched to the colonies on the plate to identify the positives.

**Liquid β-galactosidase Assay (used with pJ69-4A)**

Yeast colonies were inoculated into 3mL of trp⁺ SD medium and grown to saturation. 2 mL of saturated inoculum was added to 8 mL of YPD and grown to mid log-phase (O.D₆₀₀ of 0.6 to 0.8). 1.5 mL of cells were pelleted in a 2mL microtube, washed in Z-buffer containing 2.7mL/L β-mercaptoethanol and pelleted again. 150μL of Z buffer/β-mercaptoethanol, 50μL of chloroform and 20μL of 0.1%SDS was added to the pellet and the pellet was vortexed for 45 seconds. Microtubes were placed in a 30°C
water bath, and 700µL of ONPG(1.2 mg/mL o-nitrophenyl β-D-galactopyranoside in Z buffer) solution was added, noting the time of addition. 0.5 mL of 1M Na₂CO₃ stop solution was added when the sample turned yellow and the stop time was recorded. The absorbance at 420nm was measured. β-galactosidase activity was measured in miller units using the following calculation: (OD₄₂₀ x 1000)/(OD₆₀₀ x time x mL) of cells.

Results of the liquid β-galactosidase assay performed on each colony were normalized to the background levels of lacZ expression obtained in yeast transformed with the DBD-BRCA1exon11 plasmid alone in order to account for variations in lacZ expression levels from experiment to experiment. Due to the number of samples the liquid β-galactosidase experiment was only performed once per colony. This was done in order to obtain a positive or negative result, rather than a quantitative result for the number of Miller Units of lacZ expression for each colony.

**Protein Extraction from Yeast and Western Analysis of Protein Products**

Western assays were performed to determine if the BRCA1 protein was being produced in yeast. 100mL of cells grown to saturation were pelleted and resuspended in ice cold water. Cells were pelleted again and transferred into a 2 mL microtube and pelleted again. The remaining water was aspirated. Two pellet volumes of acid washed glass beads were added to the pellet. An equal pellet volume of lysis buffer (50mM Tris-Cl pH 7.5, 250 mM NaCl, 50 mM NaF, 5mM EDTA, 0.1% NP-40 [Nonidet P-40], 1mM PMSF [Phenylmethysulfonyl Fluoride], 0.6 mM Dimethylaminopurine, 1µg/mL Leupeptin, 1µg/mL Pepstatin, 10µg/mL TPCK [Tosyl Phenylalanine Chloromethyl Ketone], 10µg/mL Soybean Trypsin Inhibitor) was added and the tube was vortexed 2 x
45 seconds at 4°C. Beads and cells were pelleted, and the supernatant was removed and centrifuged for 10 minutes. The supernatant was removed into a clean tube. Protein concentration was measured using a Bradford Assay (88). An equal volume of 2X protein loading buffer (0.01% Bromophenol Blue, 100 mM Tris, pH 7.4, 4% SDS, 20% glycerol, 2% β-mercaptoethanol) was added, and the sample was boiled for 1 to 2 minutes and transferred to ice. The sample was loaded on a 6% acrylamide gel (Separating gel: 1.5 mL 40% [29:1] acrylamide, 2.5 mL 4X separating buffer [38 g/L tris base, 4 g/L SDS, dH₂O to 1L, pH 8.8], 50 µL 10% APS [ammonium persulfate], 5µL TEMED [N, N', N', N'-tetra methyl ethylenediamine], 5.95 mL dH₂O; Stacking gel: 0.65 mL 50% acrylamide [29:1], 1.25 mL 4X stacking buffer [60.5 g/L Tris, 4 g/L SDS, dH₂O to 1L, pH 6.8], 25µL 10% APS, 2.5 µL TEMED, 3.05 mL dH₂O) and electrophoresed along with high molecular weight protein markers (Gibco BRL) at 200V until the dye reached the bottom of the gel (Running buffer: 3 g/L Tris, 14.4 g/L glycine, 1g/L SDS, dH₂O to 1L). The proteins were transferred to nitrocellulose (transfer buffer: 14.4g/L glycine, 3g/L Tris, 200 mL MeOH, dH₂O to 1 L) using 100V for 1 hour, then the membranes were blocked for 1 hour to overnight in 5% skim milk-TBST (4.5 g/L NaCl, 1.32 g/L Tris-Cl, 0.195 g/L Tris base, dH₂O to 1L, add 0.1% Tween 20). The nitrocellulose was washed with TBS-T, then probed for 1 hour with an antibody directed against either the amino terminus (Ab-1, Oncogene) or exon 11 (BRCA1-17F8 clone 3A2 GeneTex) of BRCA1. Filters were washed again with TBS-T, probed with a horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research Laboratories, Inc.) against the heavy chain of the primary antibody, then washed again with TBS-T. BRCA1 protein bands were visualized using Enhanced Chemiluminescence (ECL
Western blotting detection reagents kit [Amersham Pharmacia Biotech]). The blots were wrapped in plastic wrap and exposed for various times to Kodak X-Omat™ Blue XB-1 film.

**Titre of Library:**

1μL of library was added to 1 mL of LB broth and was mixed to create Dilution A. 1μL of Dilution A was added to 1mL of LB broth creating Dilution B. 1μL from Dilution A was added to 50μL of LB broth, and the entire volume was spread onto an LB Agar plate. 50μL and 100μL aliquots from Dilution B were spread onto LB Agar plates as well. Plates were incubated at 30°C overnight, and the number of colonies on each plate was counted. The titre was calculated using the following formulas: (# colonies on Dilution A) \( \times 10^3 \times 10^3 = \text{cfu/mL} \); or (# colonies on Dilution B/plating volume) \( \times 10^3 \times 10^3 \times 10^3 = \text{cfu/mL} \).

**Amplification of Library:**

The Human Mammary Gland cDNA library (Clontech) was amplified according to manufacturer's instructions: 50,000 colonies were plated onto 200 LB Amp plates (50μg/mL Amp) to obtain approximately 3x the number of independent clones in the library. The plates were grown for 2 days at 30°C. Colonies were scraped into 2L of LB Amp medium, and were shaken at 30°C for 4 hours. A Qiagen Plasmid Giga Kit was performed to obtain the plasmid DNA.
Autonomous Activation by the Activating Domain Protein

To isolate the AD-plasmid from the DBD-BRCA1exon11 plasmid, colonies were grown to saturation in minimal medium without leucine and plated in a grid onto leu'/trp' medium and leu' medium. Those colonies that grew on leu' medium, but not leu'/trp' medium had lost the DBD-BRCA1exon11 plasmid. The resulting colonies were then plated onto leu'/ade' medium to identify those AD plasmids that could activate the adenine reporter gene in the absence of the DBD-BRCA1exon11 plasmid.

Plasmid Isolation From Yeast

To isolate the Activating Domain plasmid from yeast, 5mL of SD leu' media was inoculated with a single colony and was incubated at 30°C or room temperature until the culture reached saturation (24 to 36 hours). Cells were pelleted by centrifugation and the supernatant was decanted. Cells were resuspended in residual liquid and transferred to a 1.5mL microtube. 0.2mL lysis solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris [pH 8.0], 1.0 mM EDTA), 0.2mL phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3g of acid washed beads were added. Tubes were vortexed for 2 minutes and then centrifuged for 5 minutes at room temperature. The supernatant was transferred to a clean tube, then a 1/10 volume of 3M NaOAc, pH5.2 and 2.5 volumes of ethanol were added. The DNA was precipitated by centrifugation and the ethanol was aspirated. The pellet was washed with 70% ethanol, and the supernatant was aspirated. The pellet was then dried under vacuum and resuspended in 20μL of 1X TE buffer.
Electroporation of bacteria

Electrocompetent Epicurian coli XL10-Gold bacteria (TetR Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI93ΔM15 Tn10 (TetR) Amy CamR]) were thawed on ice. 30μL of bacteria and 2μL of DNA were added to an electroporation chamber (0.2 cm gap, BIORAD). 1.7V (25 μF capacitance, 200 ohms resistance) was used to electroporate the bacteria immediately after which 1 mL of LB was added and the cells were transferred to a 1.5 mL microtube. The tubes were incubated at 37°C with shaking for 45 minutes and centrifuged at room temperature for 1 minute at 14000 rpm. The supernatant was decanted, and cells were resuspended in the residual liquid. This was plated onto 100mm LB Agar plates containing 50μg/mL of Ampicillin.

Yeast mating

pJ69-4A yeast (α mating type) containing an AD vector was mated with Y187 yeast (α mating type) containing a DBD vector (Figure 6). Yeast were streaked together onto a mating patch on YPD plates (YPD + 20 g/L agar) and incubated overnight. Yeast from the mating patch was streaked onto leu'/trp' SD agar plates and incubated at 30°C until colonies were visible. A single colony from each mating was streaked onto a leu'/trp'/ade' plate (5/plate), and plates were incubated at 30°C until colony growth was visible.

Sequencing

Sequencing of the DBD-BRCA1 vectors was done using Thermosequenase (Amersham Pharmacia Biotech) with the Gal4 Binding Domain Sequencing primer
Confirmation of the specificity of the interaction between exon11 of BRCA1 and the AD-protein: pJ69-4A expressing the AD-protein were mated to Y187 yeast containing one of four unrelated DBD vectors (including the pAS-BRCA1 exon11 used in the screen). The yeast were plated onto media lacking leucine, tryptophan and adenine after mating to determine if the ADE2 reporter gene was activated. 139 colonies were mated: 19 AD-proteins activated the reporter gene after mating with any DBD-plasmid, 104 no longer interacted with the pASBRCA1 exon11 protein; and 16 interacted specifically with exon 11 of BRCA1.
(Clontech cat#6474-1). Sequencing from the pACT-cDNA plasmid was done using the Matchmaker AD LD-Insert Screening Amplimer Set (Clontech) with either Thermosequenase (Amersham Pharmacia Biotech) or automated sequencing from the Samuel Lunenfeld Sequencing Facility.

**Identification of cDNA**

The sequence for each cDNA encoding a putative interacting protein was entered into the BLAST program (NCBI) to determine the identity of the protein.
RESULTS

Transformation into Yeast Strains Y190 and CG1945

The yeast strains Y190 and CG1945 were transformed with one of four DBD-BRCA1 vectors that contained fragments of the BRCA1 coding sequence ligated into the pAS1 and pAS2 DBD vectors. Y190 is a commonly used yeast strain for yeast two hybrid studies (86); it contains both HIS3 and lacZ reporter genes under the control of two different promoters, HIS3 and Gal1 respectively, both of which include the Gal1 Upstream Activating Sequence (UAS). Y190 is leaky for HIS3 reporter gene expression, resulting in the production of histidine in the absence of reporter gene activation, and therefore requires high levels of the competitive inhibitor 3-amino-1,2,4-triazole (3-AT) to suppress background colony growth (85).

The yeast strain CG1945 is derived from the strain HF7c (89). It also contains HIS3 and lacZ reporter genes under the control of Gal1 and CyC1 promoters, respectively. These promoters contain the Gal1 UAS or 3 tandem copies of a Gal4 17-mer consensus sequence that makes up the UAS. The HIS3 gene of CG1945 is not as leaky as the HIS3 gene of Y190 due to the use of the Gal1 promoter in CG1945. A lower background level of HIS3 allows a smaller amount of 3-AT to be used, potentially permitting weaker interactions to be identified; however, lacZ gene expression from CG1945 is not as strong as that from Y190 (85), possibly resulting in some false negative results in the screen.

Plasmids were transformed into Y190 and CG1945 yeast cells with an average transformation efficiency of $1 \times 10^4$ cfu/μg. The resulting transformed yeast were then tested for autonomous activation of the HIS3 and lacZ reporter genes by the DBD-
BRCA1 proteins. If activated, the reporter genes produce the enzymes imidazole acetol phosphate transaminase and β-galactosidase, resulting in yeast growth on minimal medium without tryptophan and histidine, or in a positive β-galactosidase test. Yeast that had been transformed with the DBD-BRCA1 plasmids were, therefore, plated onto medium lacking tryptophan and histidine (trp'/his'), that contained either 0-5 mM 3-AT when testing autonomous activation in the yeast strain CG1945 or 25-55mM 3-AT for Y190. Although growth of the transformed Y190 yeast on histidine could be reduced using 35-55mM 3-AT (Table 2), this level also suppressed the anticipated activation of the HIS3 reporter gene by the full length BRCA1 protein. The HIS3 reporter gene in CG1945, under the control of a Gal1 promoter, was activated solely by the full length BRCA1 protein and not by proteins encoded by the other DBD-BRCA1 plasmids (Table 2).

Colony lift filter assays were performed in order to assess the activation of the lacZ reporter gene by the DBD-BRCA1 proteins and these indicated that the lacZ reporter gene was activated by all constructs in both yeast strains. In the strain Y190, yeast transformed with DBD-BRCA1 turned blue after 2 to 3 hours, and the positive control yeast that were transformed with pAS1-Cln2/pACT-Pho85, became blue after 5 to 6 hours. All colonies containing the other DBD-BRCA1 constructs became blue in fewer than 8 hours; however, the negative control, pAS1-Cln2, remained white. The CG1945 colonies transformed with the DBD-BRCA1 plasmids became blue upon testing with the colony lift filter assay more rapidly than the Y190 strain did; however, the end results were identical to those observed with Y190. Therefore, in the strains Y190 and CG1945, all DBD-BRCA1 plasmids were autonomously activating the lacZ reporter
Table 2: Autonomous Activation of Reporter Genes in Y190 and CG1945

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Activation of HIS3 in Y190 (0-35mM 3AT)</th>
<th>Activation of HIS3 in CG1945 (0-5mM 3AT)</th>
<th>Activation of lacZ in Y190</th>
<th>Activation of lacZ in CG1945</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS-BRCA1</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>pAS-Bam</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BRCA1exon11</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pAS-Eco/Xba</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pAS-Cln2/ pACT-pho85 (pos)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pAS-Cln2 (neg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Autonomous activation of the HIS3 and lacZ reporter genes in Y190 and CG1945. All plasmids, with the exception of the negative control, activated both reporter genes in Y190. In the yeast strain CG1945, the lacZ reporter gene was activated by all constructs; however, the HIS3 reporter gene was only activated by the full length BRCA1 protein.
gene making these strains unusable for the yeast two-hybrid analysis. The G1 cyclin Cln2 and the cyclin dependent kinase (cdk) Pho85 were the positive controls used for this experiment, and have previously been identified as interacting proteins (90).

**Transformation into pJ69-4A**

Autonomous activation of the reporter genes in the yeast strains Y190 and CG1945 by the DBD-BRCA1 proteins required another yeast strain to be tested for use in the yeast two hybrid screen. The pAS1 DBD-BRCA1 plasmids were used to transform the strain pJ69-4A, with an average transformation efficiency $5 \times 10^4$ cfu/μg. Only pAS1 plasmids were used in this experiment, as the pJ69-4A strain of yeast does not contain a gene encoding cycloheximide resistance, and consequently the cycloheximide sensitivity gene located on pAS2 was not required. The yeast were tested for autonomous reporter gene activation by plating onto minimal medium lacking tryptophan and adenine (trp⁻/ade⁻) or tryptophan and histidine (trp⁻/his⁻) that contained 1, 2 or 3 mM 3-AT. The histidine reporter gene in pJ69-4A is relatively stringent compared to Y190 and should require only 1 to 3 mM 3-AT to inhibit background expression. After the yeast were transformed and plated onto medium lacking tryptophan and adenine, no colonies grew for any of the constructs except for the DBD-BRCA1 full length construct (Table 3). Twenty to forty colonies grew after plating yeast onto medium lacking tryptophan and histidine (plus 1 to 3 mM 3-AT) regardless of the amount of 3-AT present (Table 3). Due to the relatively small size of the colonies that grew on the trp⁻/his⁻ plates, it was thought that they might be distinguishable from true positives that arose during the screen that was subsequently performed.
Table 3: Testing for Autonomous Reporter Gene Activation in pJ69-4A

<table>
<thead>
<tr>
<th>plasmid</th>
<th>growth on trp⁺ plate</th>
<th>activation of adenine gene</th>
<th>activation of histidine gene(1-3mM 3AT)</th>
<th>β-gal (mil.units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS-BRCA1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4.7</td>
</tr>
<tr>
<td>pAS-Bam</td>
<td>+</td>
<td>-</td>
<td>40 colonies</td>
<td>2.2</td>
</tr>
<tr>
<td>pAS-BRCA1exon11</td>
<td>+</td>
<td>-</td>
<td>20 colonies</td>
<td>2.3</td>
</tr>
<tr>
<td>pAS-Eco/Xba</td>
<td>+</td>
<td>-</td>
<td>30 colonies</td>
<td>1.8</td>
</tr>
<tr>
<td>pAS-Cln2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>pAS-Cln2/pACT-Pho85</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9.6</td>
</tr>
<tr>
<td>pAS1 (neg)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Testing for autonomous activation by the BRCA1-DBD plasmids in the yeast strain pJ69-4A. Growth on plates lacking tryptophan indicates the presence of the DBD plasmid in the yeast. Growth on histidine or adenine, as well as an increase in β-galactosidase activity over the negative control indicates autonomous activation by the plasmid of the reporter genes. The pAS-BRCA1 plasmid encoding full length BRCA1 protein activated all reporter genes. The other plasmids only activated the HIS3 reporter gene.
To test for autonomous activation of the lacZ reporter gene by the DBD-BRCA1 plasmids, liquid β-galactosidase assays were performed using chemical means to lyse the cells since the use of liquid nitrogen for lysis activates the Gal7 promoter (80) of the lacZ reporter gene. The DBD-BRCA1 plasmid encoding full-length BRCA1 protein activated the Gal7 promoter resulting in 4.7 Miller Units of β-galactosidase activity. Transformation with the other DBD-BRCA1 constructs resulted in lacZ levels that were comparable to the 2.3 Miller Units obtained after testing the pAS1 vector alone, whereas transformation and lacZ testing of the positive control pAS1Cln2/pACT1Pho85 resulted in 9.6 Miller Units of β-galactosidase activity (Table 3, Figure 7). The above results indicate that only the full length BRCA1 construct activated all reporter genes. Although the histidine reporter gene appeared to be minimally activated by all of the constructs, the adenine and lacZ reporter genes could still be utilized to screen the library with one of the DBD-BRCA1 constructs, other than the full length DBD-BRCA1 plasmid.

**Western Assay for BRCA1 Protein Production in pJ69-4A**

To ensure that the BRCA1 protein encoded by the DBD-BRCA1 plasmids was being produced in pJ69-4A, a Western blot was performed using an antibody to exon 11 of BRCA1 (17F8) (Figure 8). Results indicated that a BRCA1 protein of approximately the correct size was produced in yeast; however, significant degradation occurred. The full length BRCA1 protein appeared almost totally degraded. Although the pAS1-Bam protein appears less degraded, Ponceau S staining indicated that less protein had been loaded onto the lane compared to the other samples, suggesting that degradation of the pAS-Bam protein was likely comparable to the other BRCA1 proteins. A lack of protein
**Figure 7: β-Galactosidase Assay Testing for Autonomous Reporter Gene Activation**

Identification of autonomous activation of the lacZ reporter gene by the DBD-BRCA1 plasmids. Full length BRCA1 activates the lacZ gene compared to the control vector (pAS1) alone. The positive control pAS1-Cln2/pACT-pho85 activates the lacZ reporter gene relative to the level observed in yeast containing pAS1-Cln2 alone. The vector used in the study, pAS1-BRCA1exon11 did not appear to activate the lacZ reporter gene above the level observed for the vector alone.
Protein expression in yeast using a monoclonal antibody directed at exon 11 (17F8). Protein degradation is observed in all lanes due to the large size of the protein being expressed in yeast, although lower amounts of protein loaded into the pAS-Bam lane makes it appear to be less degraded. Due to the amount of degradation, pAS-BRCA1exon11 was used for the study, as it was the smallest fragment of BRCA1 that had been constructed. Antibody binding is specific to BRCA1, as no bands are observed in the lane with the untransformed negative control yeast.
bands in the negative lane indicates that the extra bands observed on the gel were due to degradation and not to non-specific binding by the antibody, although Ponceau S staining indicated that the amount of protein loaded onto the gel for the negative lane was comparable to other lanes (data not shown). Due to the degradation observed of the BRCA1 proteins encoded by all constructs, the DBD-BRCA1exon1 plasmid that encoded the smallest fragment of BRCA1 was used to screen the library, as the smaller size of the protein may have lessened the degree of degradation, unless sequences within exon11 were being specifically degraded by yeast proteases.

**Transformation with library**

Initially, 50μg of a human mammary gland cDNA library (Clontech) was used to transform yeast containing the DBD-BRCA1exon1 plasmid. A transformation efficiency of 1.2 x 10^3 cfu/μg was achieved, and 6.5x10^4 independent clones were screened with this transformation, resulting in the growth of 36 colonies on medium that did not contain adenine, leucine or tryptophan (trp'/leu'/ade). In order to screen a larger number of clones, we repeated the transformation using more library. 500μg of library cDNA was used in the second transformation and 330 colonies grew on leu'/trp'/ade' plates over a period of two weeks. The library was transformed with roughly the same efficiency as the previous transformation, i.e. 1x10^3 cfu/μg, resulting in 5x10^5 independent clones. The 330 colonies, plus the 36 colonies from the original transformation, were used in the subsequent steps to determine true positive protein interactions. The following calculations were used to determine the number of clones screened:
Calculation of Transformation Efficiency:

\[
\text{A1-36: } \frac{60\text{cfu} \times 1000\mu\text{L}}{100\mu\text{L} \times 1/10 \times 50\mu\text{g}} = 1.2 \times 10^3 \text{ cfu/µL}
\]

A transformation efficiency of \(1 \times 10^3\) cfu/µL was obtained for the B1-330 transformation.

Number of Clones Screened:

First transformation: \(1.3 \times 10^3\) cfu/µg x 50µg = \(6.5 \times 10^4\) clones

Second transformation: \(1.0 \times 10^3\) cfu/µg x 500 µg = \(5 \times 10^5\) clones

Total number of clones screened = \(5.65 \times 10^5\) clones

Testing for Activation of the HIS3 Reporter Gene

To identify activation of the histidine reporter gene, colonies were plated onto leu' /trp'/his' plates containing 3mM 3-AT. The original test for autonomous activation of the HIS3 gene by the DBD-BRCA1 vectors indicated there was some activation of this reporter gene; however, the resulting colony size was small. It was, therefore, possible that increased growth of colonies on trp'/leu'/his' plates due to an interaction of the AD-protein with the protein encoded by DBD-BRCA1 exon11 would be discernible from background growth. After plating the transformants on the trp'/leu'/his' plates, however, all colonies grew and true positives were indistinguishable from background growth. Colonies were re-plated onto trp'/leu'/ade' plates to maintain selection for the interacting
AD plasmid, and liquid β-galactosidase assays were performed. 40 colonies did not grow after replating, resulting in 326 colonies for further testing.

**Liquid β-galactosidase Assay**

To determine if the interaction of the AD-protein with exon11 of BRCA1 resulted in activation of the lacZ reporter gene, liquid β-galactosidase assays were performed. Colonies were grown to saturation the cell pellets were disrupted using SDS and chloroform. The β-galactosidase activity of the DBD-BRCA1exon11 plasmid alone was used as a negative control and to normalize values. Any colony containing an AD-protein and a DBD-BRCA1exon11 protein that activated the lacZ reporter gene above the level observed for the DBD-BRCA1exon11 plasmid was considered positive (see Figure 9 for the lacZ results for putative interacting clones). Of the 326 colonies that were analyzed, 68 were negative for lacZ activation, leaving 258 colonies to be analyzed further for false positive interactions.

**Testing for Autonomous Activation by the AD Plasmid**

The AD-plasmid was isolated from the DBD-BRCA1exon11 plasmid in order to test for autonomous activation of the reporter genes by the AD-protein. Of the 243 colonies analyzed, 17 activated the adenine reporter gene autonomously and were therefore not tested further, leaving two hundred and twenty six colonies to analyze.

After growing the colonies to saturation and plating them onto leu- medium to isolate AD plasmids, it became evident that, in some cases, there was a difference in colony colour or size, suggesting that two yeast species were present. This may have
Figure 9: β-galactosidase Results for Putative Positives, Relative to DBD-BRCA1 exon11
been due to contamination; however, it also may have been due to the presence of two AD plasmids in the same yeast colony that separated out into different colonies. For this reason, 22 additional colonies were added to the screen, resulting in 248 colonies that were analyzed further for specific interaction with the DBD-BRCA1 exon11 protein.

**Isolation of DBD-BRCA1 exon11 Plasmids to Test for Autonomous Activation**

It was possible that the DBD-BRCA1 exon11 plasmid had mutated during the screen, enabling it to autonomously activate reporter genes, resulting in false positives (91). To test for this possibility, the DBD-BRCA1 exon11 plasmid was isolated from a subset of colonies that were positive in the two-hybrid assay, and was examined for its ability to transactivate the adenine reporter gene. Of 105 isolates tested, 24 were now able to activate the adenine reporter gene. In contrast, freshly transformed yeast containing the DBD-BRCA1 exon11 plasmid from the original DNA preparation gave no transactivation. Those colonies containing the DBD-BRCA1 exon11 plasmid continued to be utilized in the study since the AD plasmid was later extracted, transformed into fresh yeast and tested for interaction with several unrelated DBD plasmids. Of the 7 colonies containing activating DBD-BRCA1 exon11 plasmids later tested in the mating assay, none contained an AD-protein that truly interacted with the DBD-BRCA1 exon11 protein.

**Testing for Non-Specific Protein Interactions**

To test for non-specific protein interactions of the AD-protein with exon11 of BRCA1, pJ69-4A colonies containing the AD plasmid were mated to yeast of the
opposite mating type (Y187) that had been transformed with the DBD-BRCA1 exon11 plasmid or plasmids encoding proteins unrelated to BRCA1 i.e. pAS1-Snf1, pAS1-Cln2 or pAS1-lectin. Plasmids from a total of 137 colonies were tested for non-specific interaction of the AD-protein with the other DBD-proteins and a positive interaction with the DBD-BRCA1 exon11 protein was not reproduced in a number of cases. 16 of the 140 colonies that have been tested were positive for a specific interaction with the DBD-BRCA1 exon11 protein. Non-specific interactions with the other proteins tested was observed in 19 of the 137 clones, and 104 AD-proteins did not interact with proteins encoded by pAS1-lectin, -Cln2, -SNF1 or DBD-BRCA1 exon11. Figure 10 contains a breakdown of the number of colonies analyzed at each step of the yeast two-hybrid system.

**Identification of the Interacting Proteins**

The sequence of the AD plasmids encoding putative BRCA1 exon11 interacting proteins was determined and a BLAST ([www.ncbi.nlm.gov/BLAST](http://www.ncbi.nlm.gov/BLAST)) search was performed on the sequence. Eleven distinct proteins were identified as putative BRCA1 interacting proteins (Table 4), some of which were identified more than once in the screen. Of the proteins identified, the proteasome p27 subunit was identified four times in the screen, with three clones being identical. In addition, two different overlapping clones encoding Per2, a homologue of the *Drosophila* Per2 circadian clock protein (also known as KIAA0347) interacted with exon 11 of BRCA1. The cDNA inserted into the B4 AD plasmid was approximately 2kb and included the 1kb of Per2 cDNA inserted into the B56 AD-plasmid. Per2 has six regions of homology with RIGUI, another human
Figure 10: Number of Colonies Analyzed at Each Step of the Two-Hybrid System

Transformation of pJ69-4A containing DBD-BRCA1exon11 with the cDNA library
First Transformation: 36 colonies grew on leu-/trp-/ade- media
Second Transformation: 330 colonies grew on leu-/trp-/ade- media
Total number of colonies for next step: 366

Analysis of Histidine Reporter Gene Activation
All colonies grew
Total number of colonies for next step: 366

Replating onto Leu-/Trp/Ade- Plates
40 colonies did not grow
Total number of colonies for next step: 326

Analysis of lacZ Reporter Gene Activation
68 were negative for lacZ reporter gene expression
Total number of colonies for next step: 258

Determination of Autonomous Activation by the AD-protein
243 colonies were tested, 17 autonomously activated the adenine reporter gene
Total number of colonies for next step: 226

Determination of Autonomous Activation by the DBD-BRCA1exon11 protein
105 tested, 25 autonomously activated the adenine reporter gene
Those colonies were still utilized in subsequent steps
Total number of colonies for next step: 226

Testing for Specificity of Interaction between the DBD-BRCA1exon11 and AD-proteins
140 colonies tested
105 did not interact 19 were non-specific interactions, 16 were specific interactions with exon11 of BRCA1
Total number of colonies for next step: 16

Sequence of cDNA for Putative Interacting Proteins
12 distinct proteins were identified
Table 4: Summary of Putative Interacting Clones

<table>
<thead>
<tr>
<th>Identity of Interacting Clone</th>
<th>Number of Times Isolated</th>
<th>Number of Clones</th>
<th>% homology/bp sequenced</th>
<th>Function of Putative Interacting Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Phosphatase 1β</td>
<td>1</td>
<td>1</td>
<td>99%/604bp</td>
<td>Serine/Threonine Phosphatase</td>
</tr>
<tr>
<td>Proteasome p27 subunit</td>
<td>4</td>
<td>2</td>
<td>A2-97%/569bp, A13-94%/140bp, A34-97%/564bp, B53-98%/547bp</td>
<td>stimulates association of 26S with PA700</td>
</tr>
<tr>
<td>KIAA0346</td>
<td>1</td>
<td>1</td>
<td>92%/426bp</td>
<td>unknown</td>
</tr>
<tr>
<td>H. sapiens Period 2 (Per2) (KIAA0347)</td>
<td>2</td>
<td>2</td>
<td>B4-100%/138bp, B56-95%/226bp</td>
<td>circadian clock protein</td>
</tr>
<tr>
<td>RIGUI</td>
<td>1</td>
<td>1</td>
<td>100%/547bp</td>
<td>circadian clock protein</td>
</tr>
<tr>
<td>GEF-2</td>
<td>2</td>
<td>1</td>
<td>B25 - 93%/494bp, B155 - 97%/617 bp</td>
<td>ganglioside expression factor</td>
</tr>
<tr>
<td>Importin α</td>
<td>1</td>
<td>1</td>
<td>94%/124bp</td>
<td>nuclear import</td>
</tr>
<tr>
<td>FLOT2 mRNA</td>
<td>1</td>
<td>1</td>
<td>91%/484 bp</td>
<td>not homologous with coding sequence of mRNA</td>
</tr>
<tr>
<td>chromosome 17 BAC</td>
<td>1</td>
<td>1</td>
<td>98%/590 bp</td>
<td>unknown</td>
</tr>
<tr>
<td>B87 novel</td>
<td>1</td>
<td>1</td>
<td>non-homologous</td>
<td>unknown</td>
</tr>
<tr>
<td>LOMP</td>
<td>1</td>
<td>1</td>
<td>98%/476bp</td>
<td>LIM/PDZ domain protein</td>
</tr>
</tbody>
</table>

Summary of putative interacting proteins that were identified in the screen. The proteasome p27 subunit was identified four times in the screen, with two different AD plasmids encoding the protein; and the Per2 protein was identified two times and was encoded by two different interacting clones. GEF-2 was encoded by two different clones and the remaining putative interacting proteins were encoded by a single clone.
circadian clock protein that was also identified as a BRCA1 interacting protein in this screen.

Importin-α was identified as a BRCA1 binding protein. This protein has previously been shown by others to interact with the nuclear localization signal of BRCA1 (71), located within exon11, and is one of five previously identified interacting proteins that could potentially have been identified in this screen (BRAP2, p53, Rad51 and Rad 50 also interact with exon11 of BRCA1 (17; 29; 47; 63)).

Protein phosphatase 1β (PP1β), a serine/threonine phosphatase appeared to interact with BRCA1, and a putative consensus binding sequence for PPI binding ((K/R)(V/I)XF) (92) is located in exon 11 of BRCA1. The entire 1kb PP1β coding sequence is located in the AD plasmid.

Clone A17 encodes a recently identified protein referred to as LOMP that contains a cysteine rich protein binding domain known as a LIM domain (lin-11, isl-1, mec-3)(93) and a PDZ domain (PSD-95 [Prosynaptic Density Protein–95], Dlg [Drosophila lethal discs-large tumour suppresser gene], and the mammalian tight junction protein ZO-1)(94). Both PDZ and LIM domains are involved in protein-protein interactions. The LIM domain may be mediating the interaction between LOMP and BRCA1, as the fragment of LOMP mRNA encoded by the pACT plasmid includes the LIM domain, but does not include the PDZ domain.

A ganglioside expression factor (GEF-2) bound the BRCA1 exon11 protein in the screen. The protein encoded by GEF-2 is a short protein of approximately 100 amino acids that contains two protein kinase C phosphorylation motifs and one potential tyrosine phosphorylation site (identified by the NCBI MOTIF – Searching Protein &
Nucleic Acid Sequence Motifs program [www.ncbi.nlm.nih.gov/htbin-post/0min/dispmim?600185]).

Three novel proteins of unknown function also bound to BRCA1 in the screen. One of these is encoded by a sequence homologous to a bacterial artificial clone (BAC) containing a chromosome 17 sequence (clone hRPK.180_P_8, accession AC005972). A blast search identifying Sequence Tag Sites (STS) that were derived from the chromosome 17 clone determined that it maps to a region in 17q21. Sequence analysis of the cDNA fused to the activating domain identifies a very short peptide that is in the correct reading frame with the activating domain (ORF finder program, NCBI [www.ncbi.nlm.nih.gov/orf/orf.html]). The peptide is 19 amino acids long and may represent the carboxy terminus of a true protein, or may not represent a protein if it derived from an untranslated region of mRNA.

Another of the novel BRCA1 interacting proteins is 92% homologous (over 426 base pairs) to a protein encoded by the EST clone KIAA0346. This protein was identified during a screen of brain cDNAs with large open reading frames that might encode proteins (95). However, the function of KIAA0346 is unknown and its sequence has no homology with any other proteins or nucleotide sequences. The third novel sequence, B87, does not share homology with any other nucleotide sequence or protein and is also not homologous to any known ESTs. A peptide of 36 amino acids is fused to the activating domain and appears to be interacting with BRCA1.

One of the AD plasmids contained a sequence that was homologous to Flotillin-2 (FLOT2) mRNA, but not protein (96). The sequence of the AD plasmid insert commences after the coding sequence of FLOT2 ends and encodes a very short peptide of
11 amino acids fused in frame behind the activating domain. Therefore, it is likely that the plasmid contained the 3' untranslated region of the FLOT2 mRNA and consequently this peptide would not normally be made in mammalian cells.

The identification of interacting proteins in a yeast two hybrid system is the initial step in the isolation and characterization of proteins that are implicated in the BRCA1 pathway. Further characterization of each of the interactions by co-immunoprecipitation is necessary to confirm the interaction, followed by subsequent analysis of the interacting domain, colocalization studies of the proteins in cells and determination of the functional significance of the interaction.
DISCUSSION

A yeast two-hybrid system was utilized to identify potential BRCA1-interacting proteins. Several different constructs were created that contained either full length BRCA1 or fragments of the gene, in the DNA binding domain vectors pAS1 and pAS2. Since the carboxy terminus of BRCA1 had previously been determined to activate Gal4 responsive reporter genes when fused to the Gal4 DNA-binding domain (DBD) (55), we reasoned that the full length BRCA1 protein, which includes the activating carboxy terminus, would also activate Gal4 responsive reporter genes and consequently would not be useful in the screen. For that reason, plasmids were constructed containing various fragments of BRCA1 that removed either the carboxy terminus alone or both the amino terminus including the RING finger and the carboxy terminus. After testing for autonomous activation by the DBD-BRCA1 proteins and observing the production of the BRCA1 protein in yeast, exon11 of BRCA1 was utilized in the screen.

Rational for Performing the Yeast Two-Hybrid Screen

At the time this screen was initiated only Rad51 and importin α had been identified as putative BRCA1 exon 11 binding proteins (17; 71). Yeast two-hybrid analysis had primarily been performed on the RING-finger domain of BRCA1, as RING domains are believed to mediate protein-protein interactions. However, exon11 encodes 60% of the BRCA1 protein and many interactions and important functions may be occurring within this region.

To further underscore the importance of exon11 for BRCA1 function, transgenic mice that have a neomycin cassette within exon 11 and produce a truncated form of
BRCA1 are not viable even though a splice variant that removes exon11 (and, therefore, the neomycin cassette) may be present (46). In addition, mouse embryonic fibroblasts with the disrupted exon11 of BRCA1 have a defective G2/M checkpoint and contain multiple centrosomes leading to unequal chromosome segregation and abnormal division (97). Together, these studies suggest a role for exon 11 of BRCA1 in controlling chromosomal organization during mitotic division, potentially by binding to the centrosomes to effect function.

A human mammary gland cDNA library obtained from Clontech was utilized in the yeast two-hybrid screen described in this thesis. Other two-hybrid screens for BRCA1 interacting proteins used libraries that are not mammary gland or ovarian in origin. The source of the tissue used to create the library may influence the results of the screen since BRCA1 is expressed in all tissues examined, but is only implicated in breast and ovarian cancer. This implies a potential tissue-specific role for BRCA1, suggesting that protein interactions that take place in the breast or ovary may not occur in other cell types.

**Testing the Yeast Strains Y190 and CG1945**

The DBD-BRCA1 plasmids were originally tested for autonomous reporter gene activation in two yeast strains: Y190 and CG145 and all of the constructs activated the lacZ reporter gene in both strains. The lacZ gene in Y190 contains a Gal1 TATA box and CG1945 contains a CYC1 (cytochrome C) TATA initiation site. Promoters for the reporter genes contain either the Gal1Upstream Activating Sequence (UAS) or three tandem copies of a 17-mer sequence that is homologous to the UAS consensus sequence
in 15 of the 17 nucleotides. The degree of homology of the 17-mer is greater than the true UAS of the Gal promoters themselves and a single copy of this 17-mer sequence is enough to activate transcription of the Gal genes to almost wild type levels. It is possible that BRCA1 autonomously activates the Gal1 promoter, and that the synthetic promoter, given its similarity to the Gal1 promoter and the fact that there was three copies present in the promoter resulting in a stronger signal, was similarly activated.

When testing for autonomous HIS3 reporter gene activation by the DBD-BRCA1 proteins, the HIS3 reporter gene in the yeast strain CG1945 was only activated by the full-length BRCA1 construct but in the strain Y190, all transformants activated the gene. Both the CG1945 and Y190 HIS3 reporter genes are under the control of a Gal1 upstream activating sequence; however, the initiation site for the CG1945 HIS3 reporter gene is a Gal1 TATA box, while Y190 has a HIS3 TATA box as the initiation site. Since the lacZ gene for Y190 was activated by the BRCA1 proteins and is also under the control of a Gal1 UAS and Gal1 TATA box, it is surprising that the HIS3 reporter gene of CG1945 was not activated. It is possible that sequences surrounding the Gal1 UAS and TATA box were different for both genes or those factors that the BRCA1 proteins interact with were not present at the CG1945 HIS3 promoter.

**Use of the Yeast Strain pJ69-4A for the Screen**

The yeast strain, pJ69-4A was utilized for the yeast two-hybrid screen due to the activation of one or both of the reporter genes in the other strains of yeast. Although a study has been published recently which did make use of the activating carboxy terminus of BRCA1 in a yeast two hybrid study by comparing the degrees of activation of the lacZ
gene to identify the interacting protein CtIP (59), background activation may have compromised the ability to identify weak interactions. Therefore, it was necessary to utilize a yeast strain with reporter genes that were not activated by exon11 of BRCA1.

pJ69-4A encodes the reporter genes HIS3, adenine and lacZ under the control of three different Gal4 responsive promoters - Gal1, 2 and 7 respectively. The use of three different promoters reduces the autonomous activation of reporter genes by proteins that activate the genes in a promoter specific manner. The sequence of the UAS is different for each promoter, potentially reducing the number of false positives caused by proteins fused to the Gal4 activating domain that are able to interact with the specific UAS and, therefore, activate the reporter genes autonomously. Also, the use of three different promoters reduces the possibility that the DBD-protein is interacting with a transactivating protein located specifically at that promoter. A DBD-construct that activates one of the reporter genes can still be utilized, as there are two other reporter genes in pJ69-4A that can be used in the screen to identify interacting proteins and reduce the number of false positives observed.

Identifying False Positive Interacting Proteins

Autonomous Activation of the Reporter Genes by the DBD-BRCA1exon11 Plasmid

Several of the DBD-BRCA1exon11 plasmids from the transformed colonies were also tested for autonomous activation of the ADE2 reporter gene after isolation away from the AD-plasmid, and it was observed that in 25 of the 105 colonies tested the DBD-BRCA1exon11 plasmid was now able to activate the ADE2 reporter gene. This result is in direct contrast to the result obtained when the DBD-BRCA1exon11 vector was
originally tested for autonomous ADE2 reporter gene activation. Autonomous activation by the DBD-BRCA1 exon11 plasmid may be due to a mutation in the plasmid that occurred after transformation into the yeast. It is possible that some of the protein interactions that were occurring between exon11 of BRCA1 and the AD-protein were slightly toxic to the yeast, or that expression of exon11 of BRCA1 was itself toxic to the yeast. Deletions or mutations in BRCA1 could have taken place that alleviated such toxicity. These alterations may have been activating mutations, allowing the DBD-BRCA1 exon11 protein to activate the reporter genes.

In agreement with these results, another study has been reported in which the calcyphosine gene, ligated to the DBD, had deletions in 7 of the 9 cases that appeared positive in the screen, resulting in autonomous activation of the reporter gene (91). Different deletions were observed that didn't alter the reading frame, but all resulted in activation of the reporter gene. The authors speculate that the deletions uncovered an acidic domain that could act as an activator, or resulted in loss of the protein structure that exposed acidic regions of the protein.

Loss of Interaction Between exon11 of BRCA1 and the Putative Interacting Proteins

A number of AD-proteins that originally seemed to interact with the DBD-BRCA1 exon11 protein in the screen appeared not to interact with the DBD-BRCA1 exon11 in the mating assay. The DBD-BRCA1 exon11 plasmid and the other non-related DBD control plasmids were used to transform the Y187 strain of yeast just prior to the mating assay. The use of the mating assay may have eliminated many of the false positives since activating mutations that could have occurred in the DBD-BRCA1
gene fusion, resulting in the activation of the reporter genes, may not have occurred in the mating assay. In addition, although pJ69-4A had been re-streaked, the yeast was originally transformed with the library plasmids several months prior to the mating assay. It is possible that the AD-protein was no longer being produced at high enough levels in the yeast for a detectable interaction to occur. For that reason, the AD-plasmids were extracted from the yeast, purified by transformation and DNA prepped in bacteria, and the plasmids were then re-transformed into fresh pJ69-4A. Therefore, any specific interaction that was actually taking place between the DBD-BRCA1exon11 protein and the AD-protein should have been identified in the mating assay. It is possible, though, that if more than one AD-plasmid was initially transformed into the yeast cell a non-interacting plasmid may have been utilized in subsequent steps. It would therefore appear that the colony didn’t contain an interacting protein, although in the original screen there may have been an interaction taking place.

**Putative Positive Interacting Proteins Identified in the Yeast Two-Hybrid Screen**

Several proteins were identified as potential BRCA1 interacting proteins in this screen. So far, twelve putative BRCA1 interacting proteins have been identified; some of which were isolated more than once in the screen. These proteins include a novel protein designated KIAA0346; protein phosphatase 1β (PP1β); the proteasome p27 subunit; the nuclear import protein importin α that has previously been identified as a BRCA1 binding protein; two circadian clock proteins Per2 and RIGUI; a ganglioside expression factor; and several novel proteins. Below, I discuss each of these putative BRCA1 binding proteins.
Circadian Clock Proteins Per2 and RIGUI

KIAA0347 is a protein that was first identified in a screen by Nagase et al (95), in which they identified 100 brain-derived cDNA clones that potentially encoded large proteins. It has since been designated as Period 2 (Per2), a human homologue of the Drosophila Per 2 circadian clock protein (96). Circadian clock proteins respond to signals of light and dark and their protein levels vary according to the time of day (98). The master regulatory centre for these proteins is the suprachiasmatic nucleus, a region of the hypothalamus that is located behind the eyes. Circadian clock proteins are found oscillating in many tissues outside of the brain, and it is thought that signals sent from the suprachiasmatic nucleus to other tissues regulate the circadian rhythm of peripheral tissues (98). Although the presence of circadian clock proteins has not been tested for in the breast, rat Per2 has been identified in most rat tissues examined, suggesting that circadian proteins may also be present in the majority of human tissues (96). Furthermore, rat fibroblast cells in culture express their clock proteins in a circadian fashion in response to serum, which contains many growth and signaling factors (99). It is possible that the circadian cycle in the suprachiasmatic nucleus responds directly to light and other signals to set the clock and signals are sent out to other regions of the body to set the peripheral circadian rhythms. For example, melatonin is released from the pineal gland at high levels at night, and is believed to be responsible for such behavior as hibernation, mating cycles, and other seasonal activity (98). Other hormones also follow a circadian rhythm and may affect when the potential clock proteins in breast cells are expressed (100).
Per2 has 6 regions of homology to another circadian clock protein, RIGUI, which was also identified in the screen as a potential BRCA1 interacting protein. Both proteins contain a PAS (Per ARNT Sim) domain that is commonly found in circadian proteins and mediates protein-protein interactions. The RIGUI protein also contains a basic helix loop helix (bHLH) motif at the amino terminus that may facilitate an interaction with the promoter of genes, resulting in circadian expression of the genes (101), however, the bHLH motif is not a region that is homologous between Per2 and RIGUI. Therefore, it is unlikely that the motif is responsible for a false positive interaction, assuming that a region in common between Per2 and RIGUI is facilitating the interaction of BRCA1 with the proteins; alternatively a distinct region in RIGUI may be responsible for a false positive interaction. In addition, the region of Per2 cDNA that is present on the AD-plasmid does not appear to encode the PAS domain of Per2, indicating that the PAS domain is also not mediating the protein-protein interaction in this case.

The functional significance of the association of BRCA1 and circadian clock proteins is unknown. Circadian clock proteins have not yet been implicated in cancer, nor does BRCA1 appear to be expressed in a circadian fashion. That does not imply that BRCA1 is not involved in the circadian rhythm in cells, as proteins such as Drosophila's clock protein are expressed continuously and have an effect on the expression of the two clock proteins Per (a Per2 homologue) and Timeless (Tim) (98); therefore, it is possible that BRCA1 has an effect on the circadian expression of proteins. Phosphorylation plays a role in the function of the Per protein in Drosophila. Both human Per2 and RIGUI contain multiple sites that are potentially phosphorylated and, as will be discussed later, protein phosphatase 1β has also been identified in the screen as a BRCA1 interacting
protein. Not only is it possible that BRCA1 is itself being dephosphorylated by PP1β, but also it may be directing the actions of PP1β to other proteins that interact with BRCA1, such as RIGUI or Per2 to affect their function. Further study to determine if breast cells express the clock proteins Per2 and RIGUI in a circadian pattern, and the identification of the effect of BRCA1 on the expression or function of the proteins must be performed to determine if the association is real, and to identify its importance.

**Ganglioside Expression Factor - 2**

A clone expressing a ganglioside expression factor (GEF-2) was isolated twice in this screen as a BRCA1 exon11 binding protein. Gangliosides are carbohydrate-rich sphingolipids with acidic sugars that are at their highest concentration in the nervous system (102). Several types of tumours including melanoma, breast, lung, lymphomas and neuroblastomas have been found to contain significantly higher amounts of gangliosides, or to produce abnormal types of gangliosides, compared to normal tissue (103). Gangliosides mediate cell to cell or cell to matrix interactions and also modulate functional membrane proteins (104). For example, the ganglioside GM3 inhibits tyrosine autophosphorylation of the epidermal growth factor receptor (EGFR) that occurs with the addition of the epidermal growth factor (EGF) thereby inhibiting cell growth (105; 106). However, another ganglioside, De-N-acetyl-GM3 (which does not contain the N-acetyl group of sialic acid) enhances serine phosphorylation of the EGFR and stimulates cell growth (105-107).

A study by Thomas et al. (108) has shown that tumours with high ganglioside levels exhibit increased radioresistance, but decreased metastatic capabilities. Tumour
cells expressing either high or low levels of gangliosides that are injected into immunosuppressed mice acquire high levels of gangliosides at the site of injection, but have significantly lower levels at the site of lung metastasis, suggesting that changes in ganglioside levels are required for metastases to occur. Furthermore, cells expressing varying levels of gangliosides have different degrees of spreading and adhesion to plates coated with glycosphingolipids (109). Cells expressing a higher degree of $G_{M3}$ exhibited an increased rate of cell adhesion, cell spreading and cell motility, suggesting that those functions can be controlled by glycosphingolipid-glycosphingolipid interactions (109). However, increased cell adhesion may decrease the level of metastasis and a lower amount of ganglioside expression would, therefore, be required for the tumour to metastasize.

Large amounts of gangliosides are shed by malignant cells, creating a microenvironment around the tumour with a high concentration of tumour derived gangliosides. This may inhibit the immune response of the host by inhibiting the function of leukocytes that infiltrate the tumours, allowing the tumour cell to escape the host immune response (104). The gangliosides that are shed from tumour cells are significantly more immunosuppressive than gangliosides from normal cells. The length of the ceramide structure of the ganglioside has a large influence on the ability of the ganglioside to suppress the immune response. Ganglioside species with ceramides that have shorter fatty acyl chains are more potent at inhibiting the immune response, and are also the same species that are preferentially shed from the tumour cell. This suggests that the tumour cell optimizes the gangliosides that will be lost from the cell membrane for better evasion from the host immune response (104).
The importance of gangliosides in cancer progression is still under study; however, their affect on growth factor receptors and on the host immune system implies that they may play an important role in the development of cancer. An association of BRCA1 with a factor that has an affect on the production of gangliosides may, therefore, have an affect on the progression of cancer. It is unknown whether GEF-2 has an enhancing or repressing affect on the production of gangliosides, or whether BRCA1 would have a positive or negative influence on GEF-2. Further study on the relationship between the expression of GEF-2 and gangliosides as well as the affect of BRCA1 on the expression of gangliosides is required to understand the functional significance of this interaction.

**Novel Protein Sequences KIAA0346, B87, A16 and A25**

The protein KIAA0346 also interacts with exon11 of BRCA1 in the yeast two hybrid system. KIAA0346 was identified by the same group that identified Per2 (KIAA0347)(95); however, there is no sequence similarity between the two proteins. KIAA0346 bears no homology to any other proteins or sequences found in Genbank. The coding sequence contains potential tyrosine phosphorylation sites as well as other sites commonly found in proteins; however, there are no sequences that indicate a potential function for this protein.

The pACT plasmid p87 encodes another novel protein that has no homology to any nucleotide or protein sequence. There is also no homology of the inserted sequence to any expressed sequence tags, indicating that either this insert may not normally be made into a protein, or that an EST homologous for this sequence has not yet been
discovered. The pACT insert includes an ORF of 198 base pairs, encoding the activating domain plus 36 amino acids. The B87 protein may represent part of the non-coding region of mRNA ligated in frame behind the activating domain, or it may be a small fragment of a novel protein.

The clone A16 is a novel protein that has 98% nucleotide homology (over a sequence of 590 base pairs) to a Bacterial Artificial Clone (BAC) containing sequences from chromosome 17. A blast search identifying Sequence Tag Sites that were derived from the chromosome 17 clone determined that it maps to a region in 17q21. Although loss of heterozygosity (LOH) in this region may be solely due to BRCA1, LOH at 17q21 has been identified in several other types of cancers (including colon, gastric and prostate) and may be due to another gene in the region (110-112). Sequence analysis of the cDNA fused to the activating domain identifies a short peptide that is in the correct reading frame with the activating domain. The peptide is 19 amino acids long and may not represent a true protein. It is possible that a non-coding region of mRNA was ligated behind the activating domain to make a small protein that is able to weakly interact with BRCA1. A BLAST search does not reveal any homology with other proteins, suggesting that this sequence is not encoding the carboxy terminus of a protein that interacts with BRCA1, unless the interacting protein is novel. It is also possible that, if this sequence is part of a larger protein, it would not normally interact with BRCA1 due to the protein conformation of the larger protein that may obscure the interacting domain of the peptide.

The clone A25 has 91% homology over 484 base pairs to the 3’ non-coding region of Flotillin 2 (FLOT2), a calveolar associated integral membrane protein (113). Further analysis of the AD plasmid revealed that the AD is fused to a very short peptide
of 11 amino acids, with a stop codon 36 base pairs after the activating domain. This peptide is apparently binding to BRCA1; however, under physiological conditions this peptide would not be made as it is not part of the coding sequence of the FLOT2 mRNA, and therefore has no significance to BRCA1 function. As there is not perfect homology with the FLOT-2 mRNA, it is also possible that this represents a FLOT-2 homologue that is not entered into Genbank.

LIM-PDZ Domain Protein LOMP

Another potential BRCA1-interacting protein is a novel zinc-finger domain containing protein, referred to as LOMP. The protein contains a cysteine rich domain involved in protein-protein interactions, known as a LIM domain, as well as a PDZ domain that is capable of mediating protein-protein interactions, primarily by binding at the carboxy terminus of proteins (114). Proteins with PDZ domains are generally localized at the plasma membrane and are involved in synaptic functions or focal adhesion (94). A class of PDZ proteins referred to as MAGUKS (Membrane Associated Guanylate Kinase) are often found at the synapses in the brain and may be involved in targeting proteins to the submembranous compartment of the cell (94). There is another class of proteins that contain LIM and PDZ domains as well as a kinase domain. These proteins are located in the cytoplasm and nucleus and are involved in the polymerization of actin (115). The LOMP protein does not appear to contain a kinase domain; however, it is possible that BRCA1 could interact with other LIM-domain containing proteins through its association with LOMP. Alternatively, if BRCA1 interacts directly with the LIM domain of LOMP, it may interact with other proteins that contain this domain. The
PDZ domain of LOMP is located in the amino half of the protein, and the LIM domain is more carboxy-terminal. The fragment of LOMP mRNA encoded by the pACT plasmid encompasses the LIM domain suggesting that this may be the domain mediating the protein-protein interaction. To date, no function or cellular location has been defined for the LOMP protein.

The protein encoded by A26 (LOMP) also interacted weakly with the DBD-Cln2 protein. Although this may represent a non-specific protein interaction, it is possible that this protein genuinely interacts with cyclins. Further study is required to confirm that the interaction between LOMP and BRCA1 occurs naturally in mammalian cells.

**Proteasome p27 Subunit**

The proteasome p27 subunit was identified on four separate occasions in this screen as a BRCA1 exon11 binding protein. The p27 subunit is part of a modulator complex consisting of three proteins: p27, p24 and p50 (TBP1) (116). The trimer complex stimulates the association of PA700 (the regulatory subunit of the 26S proteasome), and 20S (the catalytic subunit of the 26S proteasome), by 3 to 8 fold. The structures and properties of p27 are unknown; however, disruption of the *S. cerevisiae* homologue NAS2 that has 39% identity with the p27 subunit, has no effect on cell viability. This result is in contrast to other essential proteasome subunits, where disruption of genes crucial to the ubiquitin family results in cell death, suggesting that the p27 subunit is not a necessary component of the ubiquitin pathway.

Proteasome subunits are often identified in yeast two-hybrid screens. A list of false positives identified in yeast two hybrid screens, including proteasome subunits, has
been provided at the site www.fcnc.edu/research/labs/golemis/Table.html. However, the exact identity of the false positive proteasome subunits has not been disclosed. Although it is possible that the interaction of the BRCA1 exon11 protein with the proteasome p27 subunit is a false positive, BRCA1 has previously been linked to the ubiquitination pathway by its association with BAP1, a ubiquitin decarboxyl hydrolase (69). BAP1 is a member of a family of proteins that cleaves ubiquitin chains from substrates. It is possible that BRCA1 controls the ubiquitination of some proteins by promoting the cleavage of ubiquitin moieties from substrates through its association with BAP1 and promoting ubiquitination through its association with the p27 subunit, depending on the stage of the cell cycle or extracellular signals that are received. The effect of the association of BRCA1 and p27 on the association of PA700 and 20S should be analyzed to determine the mechanism of action of the two proteins.

The Serine/Threonine Phosphatase PP1β

Protein Phosphatase 1β was also identified in the screen as a putative BRCA1 interacting protein. Protein phosphorylation is important for the function of many proteins, both in signaling and cell cycle pathways. Protein phosphatase 1 (PP1) has three subunits - α, β and γ that, with the exception of the amino and carboxy termini, are extremely conserved. The cellular location of the three subunits differs, with the β subunit being detected at the chromosomes during both interphase and mitosis (117). This may correlate with BRCA1 location, since BRCA1 is located in nuclear dots that are potentially located along the chromatin. BRCA1 has also been localized to the centrosomes during mitosis (23) similarly to the PP1α subunit, which is localized at the
centrosomes during both interphase and mitosis (117). The association of BRCA1 and PP1 has not been reported, however, and it is unknown what the degree of colocalization of the two proteins is at the centrosome. It is possible, due to the degree of homology between the PP1 subunits that BRCA1 is capable of interacting with all three. However an interaction of BRCA1 with either the amino or carboxyl terminus of the PP1 subunits may confer specificity of binding to PP1β alone.

There is evidence that PP1 is involved in cell cycle regulation. Disruption of the PP1 homologue in S. cerevisiae, Aspergillus and Drosophila indicates that PP1 is essential for the completion of mitosis, and cells without functional PP1 homologues are unable to separate their chromosomes properly in anaphase (72; 118; 119). Similarly, injection of a neutralizing PP1 antibody into mammalian cells blocks cells in metaphase before chromosome separation occurs and injection of PP1 into one of the anaphase poles accelerates anaphase in that pole (120). A deletion of exon11 of BRCA1 in mouse embryonic fibroblasts results in the loss of the G2/M cell cycle checkpoint and the creation of multiple centrosomes, leading to abnormal nuclear division and aneuploidy (97). In addition, a hypophosphorylated form of BRCA1 associates with γ-tubulin, a component of the centrosome that is required for nucleation of microtubules during mitosis (23). Together, this evidence suggests that BRCA1 and PP1 may work together at the centrosome during mitosis to ensure the correct separation of chromosomes. PP1 may be required to dephosphorylate BRCA1 for its association with the centrosomes, or PP1 may be transported to the centrosomes by BRCA1 where it then dephosphorylates other components that are required to allow chromatin segregation to occur.
PP1 activity and localization are altered by an interaction with several modulator proteins. In addition to regulatory subunits involved in glycogen metabolism, several proteins that inhibit PP1 function have been identified: Inhibitor 1, a cAMP regulated phosphoprotein and its neuronal homologue DARPP-32 (121); Inhibitor 2 and NIPP-1 (122) have been shown to interact directly with PP1 (123). Other proteins such as PNUTS (phosphatase 1 nuclear targeting subunit) (124) also interact with PP1 and modulate the localization or activity of the phosphatase. It is possible that BRCA1 is acting as a regulatory subunit and is directing PP1 activity to another protein rather than being directly dephosphorylated by PP1 itself.

A potential PP1 binding motif with the sequence (K/R)/(V/I)/XF has been identified in PP1 interacting proteins; however, searching for this protein sequence in Genbank identifies the motif in approximately 10 per cent of all proteins, indicating that additional sequences may be required for PP1 binding (125). Alternatively, the domain may be unavailable for binding due to protein folding in some cases, or it is possible that not all amino acids are acceptable in the "X" location. It is also possible that the motif may be found on extracellular proteins, or in the extracellular domain of some proteins preventing them from interacting with PP1 (125). The motif KVTF is located in exon 11 of BRCA1 and may be the region that is mediating the binding of BRCA1 to PP1. Further delineation of the precise region of interaction is required, as well as the determination of whether BRCA1 is itself being dephosphorylated by PP1β, or if it is directing PP1β to dephosphorylate another protein. Since the phosphorylation state of proteins is often very important to their function, understanding the role of PP1β in
BRCA1 function may be an important step for understanding the role of BRCA1 in the cell.

The identification of proteins that interact with BRCA1 is an essential first step to elucidate the function of this important tumour suppressor gene. Further characterization of the interactions is necessary to confirm that they are occurring, as well as to determine the functional significance of the interactions. Coimmunoprecipitation assays as well as colocalization analyses in breast cells are required to study the interacting proteins in an environment more typical of the one actually occurring in breast and ovarian cells. By identifying proteins that interact with BRCA1, some understanding of the function of BRCA1 may be gained. Interacting proteins may affect or be affected by the action of BRCA1 and could themselves be important in the development of breast cancer.
Several putative BRCA1 interacting proteins were identified in my yeast-two hybrid study; however, the interaction between BRCA1 and the serine/threonine phosphatase Protein Phosphatase 1β (PP1β) could have the greatest impact on our understanding of the role of BRCA1 in cancer. Analysis of the association between these two proteins may help to elucidate how BRCA1 function is affected by phosphorylation, which has been shown to be very important in the function of several other proteins involved in cancer including p53, pRb and signaling proteins (126-128). It may also suggest ways in which the dephosphorylating action of PP1β is regulated.

Background

The phosphorylation status of BRCA1 changes in a cell cycle-dependent manner. BRCA1 is hypophosphorylated in G1, and becomes phosphorylated with the onset of S phase. The protein remains phosphorylated until M phase, and a hypophosphorylated form of BRCA1 has been identified that interacts with the centrosomes during mitosis (31). In addition, during meiosis BRCA1 and Rad51 colocalize at the unsynapsed elements of the synaptonemal complex (17). BRCA1 also becomes hyperphosphorylated with the addition of DNA damaging agents and the localization pattern of the protein changes (22). These experiments suggest that BRCA1 may perform many roles at different locations in the cell depending on the cell cycle and that the phosphorylation status may have an effect on the function of the BRCA1 protein.

A change in the phosphorylation status of a protein often has important consequences for its function. The phosphatases responsible for removing the phosphate
groups from BRCA1 have not been identified and may be equally important in affecting the function of BRCA1. Other proteins that are crucial for cell function are controlled by their phosphorylation status. For example, the Retinoblastoma protein, pRb has a phosphorylation pattern similar to BRCA1, with a phosphorylated form of the protein appearing from S to M phase. Hypophosphorylated pRb is believed to sequester transcription factors that are necessary for the cell cycle to progress. PP1 has been identified as the phosphatase responsible for dephosphorylating pRb, and therefore stopping the cell cycle, at the end of M phase (129; 130). The functions of other proteins such as p53 and signal transduction factors are also dependent on the phosphorylation status of the protein (126; 128). To date, at least two kinases have been identified that phosphorylate BRCA1 on specific serine residues, although BRCA1 is phosphorylated on other sites as well, and may also be phosphorylated on threonine and tyrosine residues (33; 34). Although many kinases have been observed that are responsible for the phosphorylation of proteins, very few phosphatases have been identified, suggesting that the specificity of phosphatases may be affected by other regulatory proteins.

Protein phosphatase I (PP1) has three isoforms encoded by three separate genes (131). PP1α, PP1β (also referred to as PP1δ) and PP1γ are conserved across the catalytic domain, but are divergent primarily across the carboxy terminus, with some amino acid changes at the amino terminus of the protein. The three isoforms are localized to different areas in the cell and it is possible that they are being directed to specific sites by regulatory proteins that complex with the PP1 enzyme.

Microinjection of a neutralizing PP1 antibody into mammalian cells prior to mitosis results in a metaphase block. However, microinjection of the antibody into one
of the two anaphase poles speeds up cytokinesis (132). Similarly, disruption of PP1 homologues in yeast, *Drosophila* and *Aspergillus nidulans* results in an inability to complete anaphase separation (72; 133). The nuclei of *Aspergillus* cells that contain a mutation in the PP1 homologue bimG cycle between condensed and uncondensed states; however, anaphase is not competed successfully. Furthermore, higher amounts of nuclear phosphoproteins are observed in the bimG mutant (72). A large number of nuclear proteins require phosphorylation for mitosis to occur, and it is possible that dephosphorylation of these proteins is required to complete mitosis successfully (72). In many species multiple isoforms of PP1 exist and it is necessary to delete all PP1 isoforms in order to observe a phenotype, suggesting there are overlapping functions for the different PP1 enzymes. Not all PP1 functions overlap, however, as male mice bearing a mutation in the PP1γ gene are infertile unlike homozygous females (134). Impairment of spermiogenesis and polyploid spermatids was observed, suggesting that although some of the functions of the PP1 isoforms may overlap, isoform specific functions also exist within the cell and PP1γ is required for meiosis to occur in male mice.

Since the level and activity of the PP1 protein is constant throughout the cell cycle, the activity of PP1 to specific proteins is believed to be a function of regulatory proteins that direct the actions of PP1. Several proteins that modify PP1 function contain a potential interacting domain ([K/R]/[V/I]XF) (125). This domain is present in exon11 of BRCA1 suggesting that BRCA1 may also regulate PP1 function.

It is possible that BRCA1 directs PP1 to function at a specific site within the cell, or that PP1 dephosphorylates BRCA1, thereby modifying the function of BRCA1 in an as yet undetermined way. BRCA1 could interact with PP1β at the chromosomes, or it
could interact with PP1α at the centrosome during mitosis (Figure 11, Figure 12). Further analysis of the location and time during the cell cycle that the interaction occurs may suggest the functional significance of the interaction and will help determine if the actions of PP1 are modified by BRCA1, or if BRCA1 activity is affected by PP1.

Specific Studies

Several studies could be performed to analyze the interaction of BRCA1 and PP1β as described below. These studies would include:

1. **Characterization of the Interaction of BRCA1 and PP1β.** Co-immunoprecipitation and colocalization would be utilized to confirm the interaction of BRCA1 with the PP1 protein and to identify the area in the cell where the interaction occurs. In addition, fragments of the BRCA1 or PP1 proteins would be used to identify the interacting domains of the two proteins.

2. **Identification of the Effect of Mutations on the Interaction of BRCA1 and PP1 and Analysis of the Status of PP1 in breast tumours.** Mutations in the interacting region of the BRCA1 or the PP1 protein would be utilized to determine if the interaction of the two proteins is disrupted. In addition, mutations in the PP1 coding sequence could be investigated in breast tumours using Single Stranded Conformation Analysis and Sequencing.
Figure 11: Model of the Interaction of PP1β with BRCA1 at the chromosomes during G1 and M phase

Potential Interaction of PP1β with BRCA1 at the chromosomes during G1 and Mitosis. During G1 a hypophosphorylated form of BRCA1 is present with diffuse nuclear staining. PP1β also has diffuse nuclear staining and is associated with the chromatin. It is hypothesized that PP1β (black) interacts with and dephosphorylates BRCA1 (dephosphorylated BRCA1 is hatched) during G1. Rad51 (light grey) associates with BRCA1 (phosphorylated BRCA1 is dark grey) during S and G2 phase, forming punctate nuclear dots. During M phase both PP1β and the BRCA1/Rad51 complex associate with the chromosomes, at which time PP1β successfully competes with Rad51 to associate with and dephosphorylate BRCA1. It is possible that the phosphorylation of BRCA1 allows the cell cycle to proceed, and the phosphorylated form of BRCA1 is involved in DNA damage repair along with Rad51, or that PP1β is released by BRCA1 during S-G2 to dephosphorylate other proteins involved in the initiation of the cell cycle.
**Figure 12: Model for the Interaction of PP1α and BRCA1 at the Centrosomes during Mitosis**

**Early mitosis**

**Late mitosis**

BRCA1 and PP1α could interact at the centrosomes during mitosis to allow chromosome segregation. Both PP1α and a hypophosphorylated form of BRCA1 have been localized to the centrosomes (white) during mitosis, where BRCA1 has been shown to interact with γ-tubulin. It is possible that BRCA1 (hatched) interacts with the γ-tubulin at the centrosomes, at which time PP1α (black) interacts with BRCA1 and is brought proximal to the microtubules. PP1α then dephosphorylates BRCA1 as well as the microtubules, causing the microtubules to shorten and draw the chromosomes towards the spindle poles.
3. Analysis of BRCA1 Phosphorylation and the Effect on BRCA1 Phosphorylation by PPI. The ability of PPI to dephosphorylate BRCA1 would be analyzed. In addition, mutational analysis of potentially phosphorylated residues could be used to determine the identity of phosphorylated amino acids in BRCA1.

4. Identification of the Effect of the Association of BRCA1 and PPI on other proteins. The association of BRCA1 with Rad51 may interfere with the BRCA1/PPI interaction. The ability of BRCA1 and Rad51 to associate in the presence of PPI, as well as their colocalization could be analyzed.

BRCA1 also associates with the Retinoblastoma protein, pRb, which has been shown to be dephosphorylated by PPI (135). The association of BRCA1 and pRb in the presence and absence of PPI, as well as the colocalization of the three proteins could be studied.

Co-Immunoprecipitation and Colocalization of PPI and BRCA1

In order to confirm the interaction identified in the yeast two-hybrid study between PPIβ and BRCA1 it is necessary to perform co-immunoprecipitation and colocalization experiments using the endogenous proteins found in breast epithelial cells. It is possible that the interaction observed does not occur under normal physiological conditions, either due to the proteins being overexpressed in yeast or incorrect post-translational modification of the proteins. It is also conceivable that the proteins may not normally be expressed in the same location or at the same point in the cell cycle for an interaction to occur. Therefore, it is important to show the interaction under natural
conditions by co-immunoprecipitation. Because other cell types may not express BRCA1 or PP1β in the correct form or location, nuclear extracts from normal and cancer breast cell lines will be used for the experiment.

There are three PP1 isoforms, which differ only in their NH2 and COOH termini. Due to the high degree of homology across the central catalytic domain, it is possible that BRCA1 interacts with all three isoforms. Isoform specific antibodies (Santa Cruz) will be utilized to examine the interaction of BRCA1 with all three PP1 isoforms.

The three PP1 isoforms are found in different locations at various points of the cell cycle (117). PP1α is located to the nuclear matrix and centrosomes during interphase, and the centrosomes during mitosis. PP1γ is located to the nucleoli during interphase and the mitotic spindle during mitosis, and PP1β is located on the chromosomes during both interphase and mitosis, although there is some conflicting data regarding the localization of the isoforms (136). Since BRCA1 is reported to have diffuse nuclear staining during G1, consistent with PP1β staining during interphase, it is possible that the two proteins interact at this point in the cell cycle. BRCA1 is also reported to localize to the chromosomes during mitosis, similar to PP1β. In addition, a hypophosphorylated form of BRCA1 has also been localized to the centrosome during mitosis, which coincides with the PP1α location (23). Both BRCA1 and PP1α have been implicated in chromosome separation, suggesting that colocalization of the proteins at the centrosome may be necessary for the correct division of chromosomes.

Co-immunoprecipitation and colocalization of proteins from extracts of synchronized cells at G1, S, G2 and M will identify if there is a cell cycle specific association of BRCA1 with PP1, or if the association with distinct PP1 isoforms is
specific for a time in the cell cycle and will confirm the localization of PP1. The cells will be synchronized using nocodazole, an agent that arrests cells in G2 by disrupting kinetochore microtubules, as serum starvation is not effective for some cancer cell lines due to their lack of dependence on serum growth factors.

Colocalization experiments will be performed on synchronized breast cells using BRCA1 and an antibody directed against PP1 (in the event that BRCA1 interacts with all PP1 isoforms), or an antibody specific for the isoform of PP1β, which BRCA1 interacted with in the yeast two hybrid study. A fluorescein tagged anti-mouse secondary antibody will be utilized against the primary BRCA1 antibody, and an anti-rabbit rhodamine tagged fluorescein antibody will be utilized against the PP1 antibody. Fluorescein emits green light after excitation with UV light, and rhodamine emits red light, allowing the two antibodies to be distinguished. A third antibody will be utilized to determine if the two proteins are interacting at a specific sight within the cell, or if they interact with other proteins known to interact with BRCA1. For example, if the proteins interact during M phase an anti-γ-tubulin primary antibody and a secondary antibody coupled with aminomethylcoumarin acetate (AMCA) can be utilized to identify if BRCA1 and PP1 are interacting at the centrosome. Antibodies against BRCA2 and Rad51 will also be utilized to identify colocalization with both BRCA1 and PP1. The secondary antibodies are tagged with three fluorochromes that emit light in different spectra, producing different colours upon excitation with UV light, allowing the proteins to be distinguished in the cell.
Identification of Regions of Interaction between BRCA1 and PP1

A putative PP1 interacting domain is present within exon11 of BRCA1 (Figure 13). To determine if this is in fact the only region that is required for an interaction of BRCA1 and PP1 co-immunoprecipitation experiments followed by western immunoblots will be performed to identify the region of exon11 of BRCA1 that is required for interaction with PP1. Fragments including the entire EcoRI/BamHI fragment will be constructed using PCR to generate the EcoRI and BamHI restriction sites at the end of the PCR fragments to facilitate in-frame cloning in to a FLAG epitope from a pFLAG-CMV2 vector. A smaller fragment surrounding the putative binding sequence will also be constructed. In the event that an interaction occurs with this fragment, the PP1 binding sequence within exon11 of BRCA1 will be mutated using in vitro PCR mutagenesis (see later section) to determine if the interaction is due to that sequence. The same experiment will be performed using fragments of PP1β that have PCR-generated HindIII and BamHI restriction sites at the end of the fragments fused to the FLAG epitope. HindIII and BamHI will be utilized, as they are not present within the coding sequence of PP1β. In the event that BRCA1 interacts with all PP1 isoforms, it is likely that the putative binding domain on BRCA1 will interact with the central catalytic domain of PP1 that is common in all three isoforms. If BRCA1 only interacts with PP1β, fragments of the entire PP1β protein will be used to identify the interacting region.
Determination of binding region between BRCA1 and PP1. Initially, four fragments will be utilized to identify the interacting region between BRCA1 and PP1. More fragments will be constructed, once the initial binding region is determined. A small protein around the putative PP1 binding site will be utilized, as well as a larger one in the event that sequences surrounding the binding domain are important. In addition, fragments including the NH2 or COOH region of the Eco-Bam fragment that was utilized to make the BRCA1exon11 vector will be used to identify a possible region of interaction. Primers will be designed that contain EcoRI or BamHI sites to ligate the fragment in frame to the pFLAG-CMV2 expression vector, thereby producing a protein that is fused to the FLAG epitope to facilitate immunological detection.
The Effect of Mutations on the Interaction of PP1 and BRCA1

Missense mutations in BRCA1 that segregate with breast cancer have been constructed by Dr. M. Sauer, a research associate in Dr. Andrulis' laboratory, using in vitro PCR-mutagenesis in BRCA1 cDNA that is fused to the FLAG epitope (Figure 14). Co-immunoprecipitation experiments to determine if the interaction of PP1 and BRCA1 is disrupted by these mutations will be performed using antibodies against the FLAG epitope and against PP1. The mutations that will be utilized will be in the region of BRCA1 determined to interact with PP1. Additional mutations will be constructed in both BRCA1 and PP1 that are within the region of interaction, if necessary. Previous studies have shown that the interaction of PP1 and a second protein is disrupted by mutating the (arginine/lysine) – (valine/isoleucine) – X – phenylalanine motif by substituting the valine or phenylalanine residues with an alanine residue (125), therefore this binding site will be mutated if it is the site of interaction. A disruption of the interaction of the two proteins with a tumour-associated mutation in BRCA1 would suggest that this interaction is disrupted in breast cancer and that the interaction of the two proteins may be required for the proper function of the cell. The closest missense mutation that has been reported in BIC (Breast Cancer Information Core Database [http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/bic/Member/index.html]) in a BRCA1 tumour is a Gly890-Val mutation (the interacting domain is from amino acids 898-902). Although this mutation is not directly within the putative binding domain, it is possible that disruption of the sequences around the domain have an effect on the interaction. If a mutation does disrupt the interaction of the two proteins, the effect of the mutation on the colocalization of the proteins will also be studied. The endogenous PP1
Missense Mutations Previously Constructed in the pFLAG-CMV2 Expression Vector. Several missense mutations within the BRCA1 coding sequence, resulting in a BRCA1 protein containing a missense mutation fused to a FLAG epitope tag. Several of the missense mutations are within the part of the protein encoded by exon11 and may, therefore, disrupt the interaction between BRCA1 and PP1. More mutations may be constructed, depending on the location of the interaction between BRCA1 and PP1 to identify if the mutation disrupts the interaction of the two proteins.
protein and a BRCA1 mutation fused to the FLAG epitope will be employed, as a mutation may affect the localization of the protein thereby preventing the interaction from taking place.

Analysis of the Status of BRCA1 in Breast Tumours

Due to the association of PP1β and BRCA1, it is possible that PP1β is itself involved in the development of breast cancer. Single Stranded Conformational Polymorphism (SSCP) analysis will be performed on the interacting subunit of PP1, or all three subunits if required, to identify any sequence alterations. The samples used for SSCP will be performed on tumour specimens from hereditary cancer cases that are part of the familial cancer registry.

The sequence alteration may change the function of the protein by changing the amino acid sequence. In order to examine if the amino acid change is in fact a mutation as opposed to a benign polymorphism, functional studies will be performed. Co-immunoprecipitation analysis will be performed to test the interaction between the mutated PP1 protein fused to a FLAG epitope and BRCA1. In addition, the mutation may affect the catalytic function of PP1, and its ability to dephosphorylate BRCA1 will also be analyzed if, in fact, BRCA1 is dephosphorylated by PP1. The mutation might also affect the localization of PP1, preventing an association with BRCA1. The protein localization will therefore be studied.

It is unknown whether the association with and possible dephosphorylation by PP1 activates or disrupts BRCA1 function. PP1 could, therefore, be acting as either a tumour suppressor gene or an oncogene. A study by Sogawa et al. (137) identified
overexpression of PP1γ in invasive ductal carcinoma using immunohistochemistry. Since amplification is a common method for oncogenic activation of a gene, the amplification of PP1 will be analyzed using quantitative PCR on PP1 DNA.

The Effect of PP1 on the Phosphorylation status of BRCA1

BRCA1 may associate with and be dephosphorylated by PP1, similar to the Rb protein, or it may function as a regulator of PP1 either by directing PP1 to a specific location in the cell, or by modulating the function of PP1. Although all of the proteins that have thus far been identified to have the binding motif are regulatory proteins, it is possible that BRCA1 is dephosphorylated by PP1. Therefore, the dephosphorylation of BRCA1 by PP1 will be analyzed. The individual subunits will be analyzed for their ability to dephosphorylate BRCA1 in an effort to elucidate which PP1 subunit functions physiologically to modify BRCA1. BRCA1 will be labeled by incubating cells with $^{32}$P-ATP, and will then be mixed with cell extract, or immunoprecipitations from all three PP1 isoforms or the PP1 α, β or γ isoform. In addition okadaic acid, an inhibitor of PP1, will be utilized to determine if the dephosphorylation is occurring due to PP1. Phosphorylase α will be used as a positive control for the dephosphorylation activity of PP1. This experiment will show if BRCA1 is dephosphorylated by PP1 and will also identify which isoform has the greatest activity toward BRCA1. In addition, if any mutations are identified within PP1 the effect of the mutations on the catalytic ability of PP1 will be studied by performing the above experiment with the mutated PP1 that will be isolated by immunoprecipitating the FLAG-PP1 fusion protein.
Identification of the Residues Dephosphorylated by PP1

Tryptic peptide analysis has shown that BRCA1 is phosphorylated mainly on serine residues, with some threonine and tyrosine phosphorylation. At least 13 tryptic BRCA1 peptides that are phosphorylated have been identified (32). In the event that BRCA1 is shown to be dephosphorylated by PP1, isolation of labeled BRCA1 from cells, incubation with PP1 and subsequent tryptic digestion followed by two dimensional gel electrophoresis will identify the fragments that are dephosphorylated, by comparing the resulting tryptic phosphopeptide pattern to that obtained without incubation of BRCA1 with PP1. Site-directed mutational analysis will be performed on the potential phosphorylation sites in the peptide to determine the effect of phosphorylation on the BRCA1 protein. The residues that are potentially phosphorylated will be altered to alanine, to mimic a dephosphorylated status, or aspartate to mimic a constitutively phosphorylated status. Analysis of the localization of BRCA1 in the cell, as well as analysis of any altered cell phenotype will be performed to determine an effect of the mutated BRCA1. In the event that PP1 dephosphorylates many tryptic peptides, phosphorylation in exon11 will be analyzed as a hypophosphorylated form of BRCA1 has been shown to localize to the centrosome (23), and BRCA1 mutants with a deletion of exon11 are deficient in chromosome separation (97). This suggests that exon11 may be involved in chromosome separation and would be further supported by an association of BRCA1 with PP1α at the centrosome.
The Effect of the Association of BRCA1 and PP1 on Other Proteins

Exon11 of BRCA1 also interacts with Rad51, and the putative PP1 binding sequence on BRCA1 is within the Rad51 binding site. If BRCA1 does interact with PP1 at this sequence, it is possible that BRCA1 cannot interact with both at the same time, or that PP1 is mediating the interaction between BRCA1 and Rad51, as only weak co-immunoprecipitation and colocalization data has been utilized to indicate the interaction between BRCA1 and Rad51. The ability of BRCA1 to coimmunoprecipitate Rad51 in the presence and absence of PP1 will be evaluated. Since BRCA1 is hyperphosphorylated after DNA damaging events occur, it is possible that Rad51 displaces PP1 in the event of DNA damage, allowing BRCA1 to become phosphorylated.

Additionally, PP1 is believed to dephosphorylate pRb, and BRCA1 has recently been shown to interact with a hypophosphorylated form of pRb (138). BRCA1 mediated growth suppression occurs only in cells that contain a functional pRb protein, and pRB<sup>−/−</sup> cells that express high levels of BRCA1 are undergo G1 arrest (138). It is possible that BRCA1 is a necessary regulator protein that brings PP1 proximal to pRb, dephosphorylating pRb to allow growth suppression to occur. The interaction of BRCA1 and pRb in the presence and absence of PP1 will be studied, as will the interaction of PP1 and pRb in the presence and absence of BRCA1 to determine if the proteins have an effect on the strength of the interaction. Studies will be performed as described previously to determine if the three proteins colocalize, and where and when in the cell cycle colocalization occurs.

The study of the functional significance of the interaction between BRCA1 and Protein Phosphatase 1 is a necessary step to increase our understanding of the role of
BRCA1 in breast cancer. Phosphorylation plays an important role in cell signaling and in modulating the effect of several proteins including oncogenes and tumour suppressor genes such as pRb, and the dephosphorylation of these proteins may be equally significant. The studies outlined will serve as a first step for characterizing the interaction of these proteins and will suggest a way in which the function of the BRCA1 protein is regulated, or will suggest an involvement of BRCA1 in the dephosphorylation and function of other proteins.
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