INVESTIGATION OF A NOVEL +ACA *BRCA1* PROMOTER POLYMORPHISM AND ITS IMPACT ON THE BREAST CANCER SUSCEPTIBILITY PHENOTYPE

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ABSTRACT

KRISTEN KING WHITE: Investigation of a Novel +ACA BRCA1 Promoter Polymorphism and Its Impact on the Breast Cancer Susceptibility Phenotype
(Under the direction of William B. Coleman, Ph.D.)

We identified a +ACA *BRCA1* promoter polymorphism located -600bp from the *BRCA1* exon1a transcriptional start site. The +ACA insertion creates a consensus FAC1 transcriptional repressor binding site (AACAACAC). We determined the frequency of the +ACA allele in 1760 DNA samples from the general population and breast disease patients. We observed a significantly higher allelic frequency of the +ACA *BRCA1* promoter in African-American cases (27%) compared to African-American controls (17%, P=0.0005), while no significant difference among Caucasian cases and controls were observed (34% versus 37%, P=0.50). Furthermore, we observed statistically significant reduction in functional activity in the +ACA polymorphic promoter in both the absence and presence of exogeneous FAC1 compared to the wild-type *BRCA1* promoter. The results of the study enabled expansion of the two-hit model of breast cancer susceptibility to include a FAC1-mediated *BRCA1* silencing in patients that carry the *BRCA1* promoter polymorphism.

For my husband, John Anthony and son, Matthew Austin
and
In Loving Memory of my Mom,
Elizabeth Ladd Loy King
April 16 1949 – October 24 1988

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LIST OF ABBREVIATIONS

AA	African American
+ACA	3 base pair BRCA1 promoter polymorphism
Adeno	Adenocarcinoma
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiestasia and RAD3 related
bp	base pair
BPP	*BRCA1* promoter primer
BRCA1	breast cancer susceptibility gene 1
BRCA2	breast cancer susceptibility gene 2
CBCS	Carolina Breast Cancer Study
cDNA	complementary deoxyribonucleic acid
Chk2	checkpoint homolog 2
CK 5/6	cytokeratin 5/6
CK8/18	cytokeratin 8/18
CREB	cAMP-responsive element binding
DCIS	ductal carcinoma *in situ*
DMEM-H	Dulbecco's modified eagle media-high glucose
DNA	deoxyribonucleic acid
DMNT3b	DNA (cytosine-5-)-methyltransferase 3 beta
DTMA	Dartmouth-Hitchcock Tissue Microarray
EMEM	minimal essential media
ER1	estrogen receptor alpha
FAC1	fetal alzheimers-50 clone1

FBS	fetal bovine serum
Her1	human epidermal growth factor receptor 1
Her2	human epidermal growth factor receptor 2
I	Native American
LOH	loss of heterozygosity
mRNA	messenger ribonucleic acid
NaPyr	sodium pyruvate
ND	not done
NEAA	non-essential amino acids
OR	odds ratio
p21	cyclin-dependent kinase inhibitor 1A
p53	Tumor protein 53
Pag	Paget's disease
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PR	progesterone receptor
PSF	pencillin, antibiotic
RB1	retinoblastoma gene
RNA	ribonucleic acid
RPMI	RPMI 1640 media
SEM	standard error of the mean
SNP	single nucleotide polymorphism
STK11	serine/threonine kinase 11

Tm	Temperature
U	Unknown
W	Caucasian
WT	wild-type

INTRODUCTION

Statistics, Epidemiology and Risk Factors for Breast Cancer

Second to skin cancer, breast cancer is the most frequently occurring cancer in women in the United States. In 2008, approximately 182,000 new breast cancer cases will be diagnosed in the United States (American Cancer Society, 2008a). The *American Cancer Society* estimates that the average lifetime risk for developing breast cancer in women is 1:8 (American Cancer Society, 2008a). In 2008, 40,500 breast cancer-related deaths are expected to occur in the United States. Mortality associated with breast cancer is the second leading cause of cancer-related deaths, second only to lung cancer (American Cancer Society, 2008a).

Factors that contribute to breast cancer risk include gender, age, genetic predispositions, family history, previous history of breast cancer, race, breast density, early age of menses (<12 yrs), late menopause (≥55 yrs), radiation exposure at a young age, and diethylstilbestrol (DES) drug exposure *in utero* (American Cancer Society, 2008b). In addition, certain life-style factors are associated with increased breast cancer risk, including late or no parity, use of birth control (exposure to exogenous hormones), hormone replacement therapy (exposure to exogenous hormones), not breastfeeding, alcohol consumption (≥1 drink/day), obesity, and lack of exercise (American Cancer Society, 2008b). Beyond these accepted risk factors, more research is required to investigate

uncertain or controversial associations between breast carcinogenesis and high fat diets, environmental pollutants, second-hand tobacco smoke, and night work (circadian rhythm changes) (American Cancer Society, 2008b). Other factors including use of antiperspirants, wearing underwire bras, abortions/miscarriages, active tobacco use, and breast implants have not been found to be directly causally-related to breast carcinogenesis. However, some of these factors are closely associated with known risk factors (for instance, alcohol consumption and tobacco smoke exposure), complicating the discernment of causative factors. Additionally, some potentially contributing factors to breast carcinogenesis (such as breast implants) may not have been in common use long enough to assess long-term exposure risk (American Cancer Society, 2008b).

Risk of breast cancer increases with age in a manner similar to other malignancies. The median age of breast cancer diagnosis is 61 years-old and 50% of all breast cancers occur in women this age or older (American Cancer Society, 2007). The clinical behavior of breast cancer varies considerably between younger and older patients. Five-year survival rates are lower for women who are diagnosed with breast cancer before the age of 40 (American Cancer Society, 2007). This observation suggests that younger women develop more aggressive forms of breast cancer, resulting in a poorer prognosis. Race-associated discrepancies in breast cancer incidence have been observed between Caucasian and African-American women. Caucasian women are more likely to develop breast cancer after the age of forty. However, African-American women have a higher breast cancer incidence before 40 years of age and a higher breast cancer mortality rate at all ages

(American Cancer Society, 2007). For the years 2001-2005, breast cancer related mortality rates in the United States for Caucasian and African American women younger than 50 years were 24 and 34 per 100,000 women, respectively (Rieset al, 2007). Furthermore, African-American women diagnosed with breast cancer have a lower five year survival rate (77% surviving at 5 years-post diagnosis) compared to Caucasian women diagnosed with breast cancer (90% surviving at 5 years-post diagnosis) (American Cancer Society, 2007; Rieset al, 2007). Historically, this race-related difference in survival was largely attributed to a higher prevalence of more aggressive forms of breast cancer (basal subtype) among younger African-American women (Carey et al, 2006).

Mechanisms of Breast Carcinogenesis

Cancer development is a multi-step process through which cells acquire increasingly abnormal proliferative and invasive behaviors. Cancer also represents a unique form of genetic disease, characterized by the accumulation of multiple somatic mutations in a population of cells undergoing neoplastic transformation (Bishop, 1991; Lengaueret al, 1998). Genetic lesions represent an integral part of the processes of neoplastic transformation, tumorigenesis, and tumor progression, and as such represent potentially valuable markers for cancer detection and staging (Mao and Sidransky, 1994; Sidransky, 1995). Several forms of molecular alteration have been described in human cancers, including gene amplifications, deletions, insertions, rearrangements, and point mutations (Lengauer et al, 1998). In many cases specific genetic lesions have been identified that are associated with

neoplastic transformation and/or tumor progression in a particular tissue or cell type (Bishop, 1991). Statistical analyses of age-specific mortality rates for different forms of human cancer predict that multiple mutations in specific target genes are required for the genesis and outgrowth of most clinically diagnosable tumors (Renan, 1993). In accordance with this prediction, it has been suggested that tumors grow through a process of clonal expansion driven by mutation (Loeb and Loeb, 2000), where the first mutation leads to limited expansion of progeny of a single cell, and each subsequent mutation gives rise to a new clonal outgrowth with greater proliferative potential. The idea that carcinogenesis is a multi-step process is supported by morphologic observations of the transitions between premalignant (benign) cell growth and malignant tumors. In colorectal cancer (and some other tumor systems), the transition from benign lesion to malignant neoplasm can be easily documented and occurs in discernible stages, including benign adenoma, carcinoma *in situ*, invasive carcinoma, and eventually local and distant metastasis (Cohen et al, 1997). Moreover, specific genetic alterations have been shown to correlate with each of these well defined histopathologic stages of tumor development and progression (Kinzler and Vogelstein, 2001). However, it is important to recognize that it is the accumulation of multiple genetic alterations in affected cells, and not necessarily the order in which these changes accumulate, that determines tumor formation and progression.

Genetic Mechanisms of Breast Carcinogenesis

Hereditary Breast Cancer Hereditary breast cancers account for approximately 10% of all breast cancers (American Cancer Society, 2007; American Cancer Society, 2008b; Couch and Weber, 1998). Several breast cancer susceptibility genes have been identified, including *BRCA1, BRCA2, p53, and ATM,* that are mutated in many, but not all, hereditary breast cancer (Chapentier, 2002; Hedenfalk et al, 2001). Among these breast cancer susceptibility genes, genetic alterations affecting *BRCA1* and *BRCA2* are most frequently associated with the early-onset hereditary breast cancer syndromes (Couch and Weber, 1998). Annually, *BRCA1* and *BRCA2* mutations account for approximately 7280 to 9100 (40-50%) and 5460 to 7280 (30-40%) of the new cases of hereditary breast cancer, respectively (Couch and Weber, 1998). This results in 1820-5460 (10-30%) remaining cases of familial breast cancer that are not associated with *BRCA1* and *BRCA2* mutations. Rarely, some of these familial breast cancer cases can be attributed to syndromes such as Li-Fraumeni, Ataxia telangiectasia, Peutz-Jehgers and Cowden that are associated with mutations in *p53*, *ATM*, *STK11/LKB1*, or *PTEN,* respectively (Lacroix, and Leclercq, 2005). Given that these cancer syndromes occur rarely and do not always involve breast cancer, it is difficult to determine the contributions of these syndromes to familial breast cancer incidence. However, the family history and clinical presentation of breast cancers related to these syndromes differ significantly from those of families with suspected *BRCA1* mutations.

In the mid-1990's, *BRCA1* was the first tumor suppressor gene to be associated with a significant increase in risk for developing familial breast cancer (Friedman et al, 1994a; Friedman et al, 1994b; Miki et al, 1994). *BRCA1* was discovered to be

mutated among families with multiple members that developed early-onset breast and ovarian cancer (Friedman et al, 1994a; Friedman et al, 1994b; Miki et al, 1994). *BRCA1* mutations are autosomal dominant with high penetrance. The risk for women affected with a *BRCA1* mutation results in an 80% chance of developing breast cancer before the age of 70, compared to a 12% lifetime risk in women who do not have a *BRCA1* mutation (American Cancer Society, 2007; American Cancer Society, 2008b). Null mutations of *BRCA1* have been shown to be embryonic lethal in mice suggesting that this gene plays a significant role during development (Hakem et al, 1998). At least 909 *BRCA1* mutations, polymorphisms, and variations have been reported (Catteau and Morris, 2002). The majority of these are frameshift, missense, and nonsense mutations that result in the production of a truncated protein (Catteau and Morris, 2002).

While mutations in breast cancer susceptibility genes *BRCA1* and *BRCA2* account for the majority of hereditary breast cancers, it is now recognized that non-BRCA1/2 hereditary breast cancers exist (Hedenfalk et al, 2001; Hedenfalk et al, 2003; Lacroix and Leclercq, 2005). This group of patients have been designated BRCAx and display all of the characteristics expected of a familial cancer (early age of onset, bilaterality, family history), but lack detectable mutations in *BRCA1* or *BRCA2* (Hedenfalk et al, 2001; Hedenfalk et al, 2003; Lacroix and Leclercq 2005). Despite a lack of detectable mutation, some BRCAx patients demonstrate loss of BRCA1 expression. While it is possible that an undetected *BRCA1* mutation may account for a portion of the BRCAx patients that have loss of BRCA1 expression, it is plausible that alternative mechanisms may govern the loss of BRCA1 expression

in some BRCAx patients. This suggests that the BRCAx breast cancers that also demonstrate loss of *BRCA1* expression may have an alternative molecular mechanism of *BRCA1* loss.

Sporadic Breast Cancer Sporadic breast cancer accounts for approximately 90% of all breast cancer cases (American Cancer Society, 2007; Couch and Weber, 1998). In contrast to hereditary breast cancers, *BRCA1* mutations are extremely rare in sporadic breast cancer (Catteau and Morris, 2002; Futreal et al, 1994; Khoo et al, 1999; Merajver et al, 1995; Uhrhammer et al, 2008; van der Looij et al, 2000). However, *BRCA1* expression is down-regulated or lost in approximately 30% of all sporadic breast tumors (Thompson et al, 1995; Wilson et al, 1999; Yoshikawa et al, 1999). The molecular mechanisms that account for loss of BRCA1 in sporadic breast cancer have not been fully elucidated, but may include an epigenetic mechanism in some cases. Frequently, loss of heterozygosity (LOH) affecting 17q21 occurs in sporadic breast cancer, which accounts for the first hit of *BRCA1* loss of function (Catteau and Morris, 2002; Cropp et al, 1993; Ford et al, 1994).

Polymorphisms, Polygenes, and Breast Cancer Polymorphisms occur frequently throughout the sequence of human DNA. Some polymorphisms result in an altered protein product which in some cases display altered protein function. Polymorphisms have been identified in various genes in breast cancer as well as in a range of other pathological processes with an associated risk of disease. Polymorphisms that occur in coding regions of genes often produce changes in

amino acid sequence that could potentially alter protein function. Twenty-one *BRCA1* variants from 50 Japanese breast cancer families were described by Kawahara *et al.* (Kawahara et al, 2004). Four of the *BRCA1* single nucleotide polymorphisms (SNP's) were associated with protein-truncating mutations, not previously described (Kawahara et al, 2004). Of the remaining 17 *BRCA1* SNP's, 13 were observed in healthy volunteers and discounted, perhaps erroneously, from association with increased breast cancer risk (Kawahara et al, 2004). Of the remaining 4 *BRCA1* SNP's that were present only in affected Japanese breast cancer families, 2 *BRCA1* SNP's (*BRCA1* intron 14, IVS14+14A>G and intron 22, IVS22+33A>T) were located at the boundaries between introns and exons. However, splicing alterations were not detected in the corresponding *BRCA1* mRNA (Kawahara et al, 2004). Lastly, 2 *BRCA1* SNP's [*BRCA1* exon 11, G275D (824G>A) and exon 3, H41R (122A>G)] were substitutions that resulted in an amino acid changes. Functional assays were not perform in order to evaluate if these amino acid changes generated proteins that were variable compared to wild-type protein, due to testing limitations (Kawahara et al, 2004). This study indicates that polymorphisms occur frequently in the coding region of *BRCA1,* which are potentially altering protein function and contributing to tumorigenesis in breast cancer. It has been suggested that breast cancer syndromes may actually be heritable through multiple gene combinations where each allele carries a low to moderate breast cancer risk individually, but in combination, risk of breast carcinogensis may become more pronounced. This concept has been termed the polygenic model for cancer susceptibility (Cebrian et al, 2006; Dragani et al, 1996; Pharoah et al, 2004; Pharoah

et al, 2008). Certain polymorphisms, in various genes represent an important component of this model of multi-gene breast cancer susceptibility.

Epigenetic Mechanisms of Breast Carcinogenesis

Hereditary Breast Cancer In addition to genetic mutation, it is now thought that DNA methylation-dependant silencing of the *BRCA1* promoter may also contribute to a portion of familial breast cancer that is associated with loss of BRCA1 expression. Tapia *et al.* observed that the *BRCA1* promoter was hypermethylated in 50% of the tumors they sampled (these tumors had been determined to lack *BRCA1* and *BRCA2* mutations) (Tapia et al, 2008). However, there was a subset of tumors with loss of *BRCA1* expression that did not demonstrate *BRCA1* promoter hypermethylation (Tapia et al, 2008). While a portion of these patients may harbor an undetected mutated BRCA1 gene, it is possible that a subset of familial breast cancer syndrome patients may be associated with loss of *BRCA1* through some nonmutational mechanism.

Sporadic Breast Cancer Several studies have shown that loss of *BRCA1* expression in sporadic breast cancers are frequently associated with DNA methylation-dependant epigenetic silencing of the *BRCA1* promoter (Biancoet al, 2000; Catteau et al, 1999; Catteau and Morris, 2002; Dobrovic and Simpfendorfer, 1997; Esteller et al, 2000; Magdinier et al, 2000; Mancini et al, 1998; Niwa et al, 2000; Rice et al, 2000). In these studies, the *BRCA1* promoter was found to be hypermethylated in 11-33% of the sporadic breast cancers analyzed (Catteau and

Morris, 2002). Like the familial breast cancer syndromes, methylation silencing of the *BRCA1* promoter appears to contribute to a subset of sporadic breast cancers that are characterized by loss of BRCA1 expression. As with hereditary breast cancer syndromes, there remains a subset of sporadic breast cancer patients that we do not understand the mechanism of *BRCA1* function loss.

Polymorphism, Polygenes, and Breast Cancer Polymorphisms that occur in transcriptional control regions (such as gene promoters or enhancers) could result in the alteration of transcriptional activator binding sites, introduce aberrant transcriptional repressor binding sites, or inhibit *cis*-acting enhancer elements. A study of promoter polymorphisms in genes expressed in the brain found that the presence of specific polymorphisms was associated with altered transcriptional activity (Buckland et al, 2004). These investigators characterized polymorphisms in 8 genes that were associated with significant increases in functional activity (Buckland et al, 2004). This study illustrates that promoter polymorphisms can affect the regulation of transcription. Another study characterized a *DNMT3b* promoter polymorphism (C46359T), which represents a T to C nucleotide change at -149 bp from the transcriptional start site (Montgomery et al, 2004). This polymorphism was found to be associated with a marginal increase in breast cancer risk (OR=1.5) among subjects of a British cohort (Montgomery et al, 2004). The Montgomery *et al.* observation of an association between the *DNMT3b* C allele and breast cancer risk conflicts with the results of two additional studies. Shen *et al*. observed an association of the T allele of *DNMT3b* with an increased risk of lung cancer (all T

allele OR=2) and Wang *et al.* observed a 30% increase in *DNMT3b* promoter functional activity compared to the C allele *in vitro* (Shen et al, 2002; Wang et al, 2004). The likely consequence of aberrantly increased *DNMT3b* activity is DNA hypermethylation, which is associated with epigenetic gene-silencing. However, there are several possible explanations for the discrepancy between these studies, including variable roles of *DNMT3b* in different cell types or linkage disequilibrium. Nevertheless, these studies illustrate that promoter polymorphisms can functionally alter promoter activity that confers risk of multiple diseases. Polymorphic variants that dysregulate methylation machinery genes, such as *DNMT3b* could affect methylation status of the *BRCA1* promoter or *BRCA1* promoter transcriptional regulators, resulting in the epigenetic silencing of *BRCA1* (Cebrian et al, 2006; Roll et al, 2008). Several *BRCA1* promoter polymorphisms have been identified and characterized in breast cancer (Catteau et al, 1999; Chan et al, 2008). Catteau *et al.* reported a C/G *BRCA1* promoter polymorphism that was associated by linkage disequilibrium with another *BRCA1* exon 11 polymorphism where neither polymorphism contributed to increased breast cancer risk (Catteau et al, 1999). However, they concluded, that the strength of linkage disequilibrium between the C/G and exon 11 polymorphisms allowed for the potential use as a screening tool to detect LOH (Catteau et al, 1999). Four *BRCA1* promoter polymorphisms were discussed by Chan *et al.* Notably, they focused on a c.-2265 C/T polymorphism that they suggested was associated with a decreased breast cancer risk in Chinese women (Chan et al, 2008). At present, no *BRCA1* promoter polymorphisms have been reported to be associated with increased breast cancer risk.

Molecular Classification of Breast Cancer

In this era of specialized medicine, classification of breast cancer has been evolving rapidly towards better prediction of patient outcome and improved guidance for treatment. The basal breast cancer subtype was first identified using immunohistochemistry for cytokeratin proteins that are typically associated with the basal cells of the breast, which are located in the cell layer closest to the basement membrane (Wetzels et al, 1989) More recently, microarray-based gene expression studies uncovered a gene expression signature that is associated with basal breast cancer (Perou et al, 2000). These gene expression studies also identified several other molecular subtypes characterized by the distinction of estrogen receptor (ER) expression status and human epidermal growth factor receptor 2 (Her2) expression status (Perou et al, 1999; Perou et al, 2000; Sorlie et al, 2001) ER-positive expressing neoplasms are classified as a luminal subtype and can be further subdivided into luminal A and luminal B based on Her2 expression status (negative and positive, respectively). Neoplasms that are negative for ER expression are divided into two categories, based on Her2 expression status, basal tumors are Her2 negative (triple negative) and Her2 expressing tumors are aptly designated the Her2+ subtype. Luminal-type tumors express cytokeratins (CK8/18) that are associated with upper layers of more differentiated breast epithelia while basal tumors express CK5/6 (Hu et al, 2006; Sorlie et al, 2006). Based on numbers from the Carolina Breast Cancer Study, the distribution of breast cancer subtypes are estimated to be: 67% luminal, 20% basal, and 13% Her2 positive (Brenton et al,

2005). Luminal A tumors are associated with the best prognosis for breast cancer and represent the most prevalent breast cancer subtype. The basal tumor subtype is associated with poor breast cancer prognosis. It is interesting to note that a large portion of breast cancers from patients with *BRCA1* mutations are of the basal subtype (Lacroix and Leclercq, 2005; Millikan et al, 2007). Furthermore, basal breast cancer incidence is highest among pre-menopausal African-American women. These women tend to have several basal breast cancer risk factors (multi-parity, parity prior to <26 years, never having breastfed, and a waist to hip ratio of >0.77) (Millikan et al, 2007).

Functions of the BRCA1 Protein Product

BRCA1 is a relatively large gene located at 17q21. *BRCA1* is composed of 24 exons that span over 100 kb genomic DNA resulting in a 1863 amino acid protein that normally localizes to the nucleus (Scully et al, 1997a; Yang and Lippman, 1999). The *BRCA1* gene encodes for a multifunctional protein that interacts with numerous other proteins in the cell and plays primary roles in cell cycle regulation and DNA repair (Figure 1) (Venkitaraman, 2001). Increasing evidence suggests that BRCA1 is involved in all phases of the cell cycle and interacts with over 50 molecules (Deng and Brodie, 2000; Deng, 2006). Consistent with the expectation that BRCA1 is involved in many cell-cycle processes, cells that have loss of BRCA1 function exhibit slowing of growth, increased apoptotic activity, inefficient repair of DNA damage, defective cell-cycle checkpoints, and chromosomal abnormalities (Brodie, and Deng, 2001; Deng, 2002; Venkitaraman, 2002).

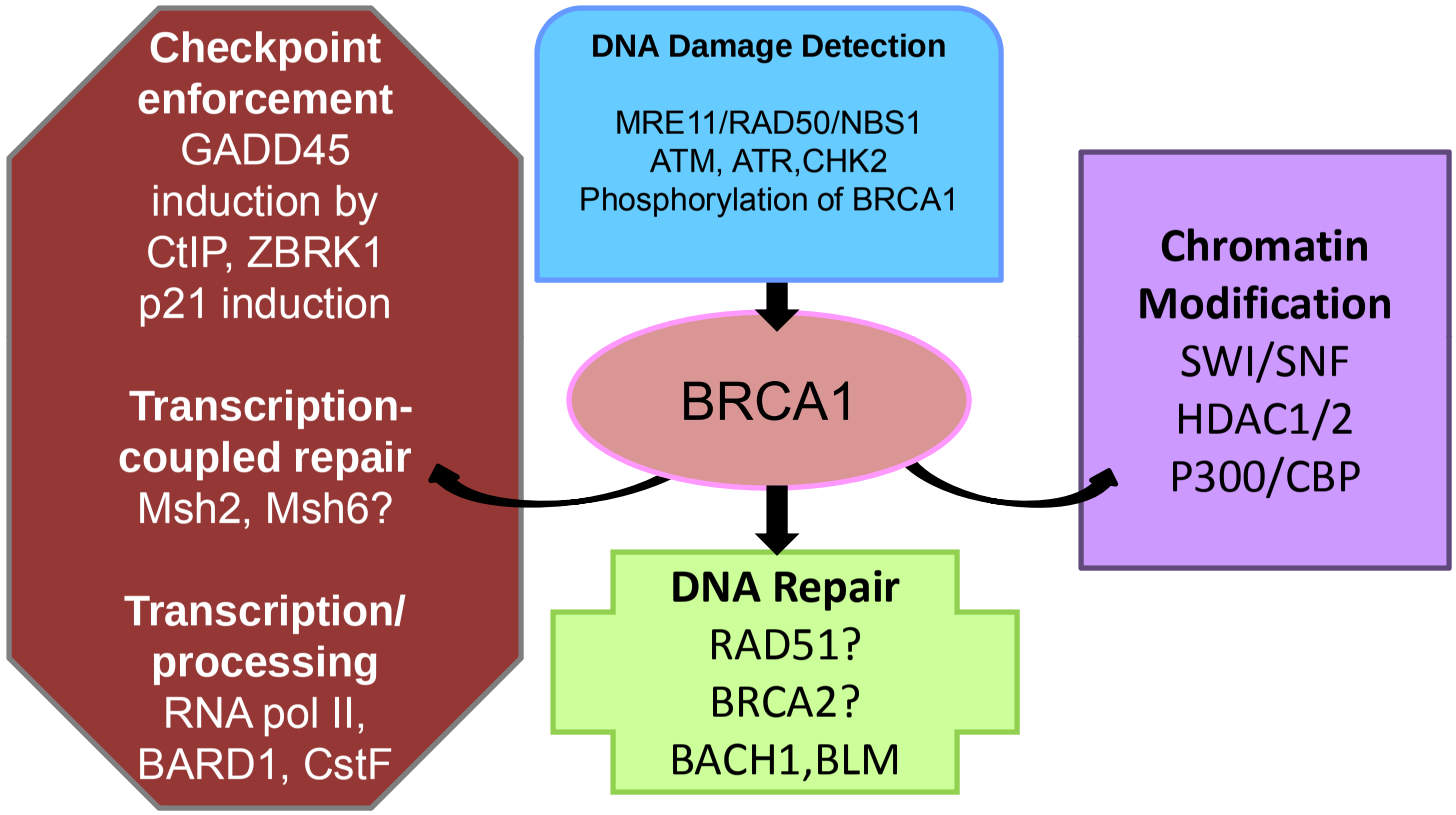

Figure 1. BRCA1 DNA Damage Response Functions and Protein Interactions. Schematic illustrates several BRCA1 primary functions and the protein interactions associated with each. The list represented here is not all-inclusive, but highlights some of the functions discussed in the text. Adapted from Venkitaraman (2001).

Structural features of BRCA1 include a N-terminus RING finger and an acidic residue-rich C-terminus which are domains that are associated with transcription factors and trans-activators (Figure 2) (Kerr and Ashworth, 2001; Starita and Parvin, 2003; Welcsh et al, 2000). Additionally, the BRCA1 RING finger domain has been shown to bind to BRCA1-associated protein-1 (BAP1), a de-ubiquitinating enzyme (Jensen et al, 1998). The presence of these structures suggests that transcriptional regulation and ubiquitination are functions of *BRCA1* (Chapman and Verma, 1996; Lovering et al, 1993; Miki et al, 1994; Yang and Lippman, 1999). Additionally, BRCA1 has been shown to act as a p53 co-activator and complex with RNA polymerase II holoenzyme, which further supports *BRCA1* involvement in transcription regulation (Ouchi et al, 1998; Scully et al, 1997a). Therefore, loss of BRCA1 function could result in aberrant reduction in transcription of genes that are involved DNA damage repair, cell-cycle checkpoints, and apoptosis.

BRCA1 interacts with BRCA1 C-terminal repeats (BRCT), which are motifs found in other proteins, such as BRCA2 and p53 (Figure 2) (Kerr and Ashworth, 2001). Furthermore, p53 is involved in DNA repair and metabolism, suggesting that BRCA1 may associate with p53 during DNA repair (Callebaut and Mornon, 1997). DNA repair pathway functional involvement is also supported by biochemical interaction between BRCA1 with BRCA2, RAD51, and RAD50, all of which are required in the DNA damage repair process (Chen et al, 1998; Scully et al, 1997c; Zhong et al, 1999). Additionally, *BRCA1* becomes phosphorylated via *ATM* in response to DNA damage, which allows *BRCA1* to complex with other proteins involved in the DNA damage repair pathways (Cortez et al, 1999; Scully et al, 1997b). However, a

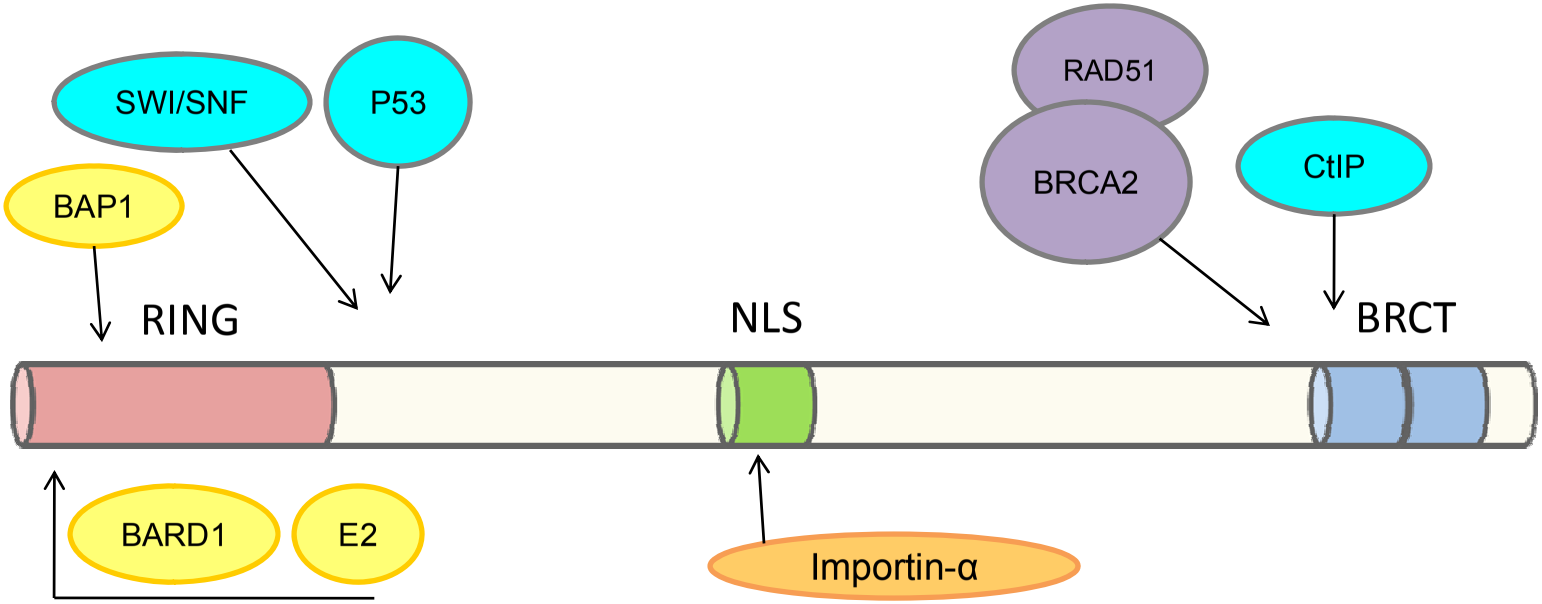

Figure 2. Functional domains of BRCA1. The RING finger domain creates a protein interaction site that plays a role in ubiquitination. BRCA1 contains a nuclear localization sequence (NLS) region. The BRCA1 C-terminal repeat sequence (BRCT) is a protein interaction site that is commonly found in proteins involved in DNA repair and metabolism. Adapted from Kerr and Ashworth (2001), Welcsh *et al*. (2000), and Startita and Parvin (2003).

common error that occurs in studies examining *BRCA1* function is the incapacity to promote double stranded break repair (Scully et al, 1999). Many genes have been associated with BRCA1 in the G1/S, S, G2/M, and the spindle checkpoints, including RB, p21, Chk1, ATM, ATR, and Chk2 (Deng, 2006). If BRCA1 is lost, DNA replication cell-cycle checkpoints that occur to correct replication DNA errors will not function appropriately possibly resulting in genetic errors or general genetic instability. With BRCA1 involved in several different levels of cell cycle checkpoint, DNA repair, and chromosomal stability, tumorigenesis occurs as a result of errors in multiple pathways.

Goals

In the study described in this thesis we aimed to identify *BRCA1* promoter sequence variations that potentially impact on the transcription of the *BRCA1* gene and that might contribute to alternative mechanisms for loss of *BRCA1* transcription in breast cancer. This investigation led to the discovery of a +ACA insertional polymorphism in the *BRCA1* promoter that creates a binding site for the FAC1 transcriptional repressor protein. We determined the frequency of the +ACA *BRCA1* promoter polymorphism in both the general population and in patients with breast disease. Furthermore, we examined functional differences between the wild-type *BRCA1* promoter and the +ACA polymorphic promoter. The results of the study enabled expansion of the two-hit model of breast cancer susceptibility to include a FAC1-mediated *BRCA1* silencing in patients that carry the *BRCA1* promoter polymorphism.

EXPERIMENTAL PROCEDURES

Experimental Subjects

Genomic DNA samples were collected from subjects representing patients with breast cancer (sporadic and hereditary), patients with ductal carcinoma *in situ* (DCIS), and various control subjects that represented the general population and were not known to be at increased risk for breast carcinogenesis. Protection of patient privacy and handling of tissue specimens followed strict policies of the Institutional Review Board of the University of North Carolina School of Medicine.

Patients with Malignant Breast Cancer

Patients with Sporadic Breast Cancer Four cohorts of patients with sporadic breast cancer were utilized. These cohorts include (i) patients from the UNC archives, (ii) patients from the UNC 9830 study, (iii) patients from the Carolina Breast Cancer Study (CBCS), and (iv) patients from the tissue archives of the Dartmouth-Hitchcock Medical Center (Lebanon, NH). Characteristics of sporadic breast cancer patients are described in Table 1.

UNC Archived Tumor Samples. Forty-one cases of sporadic breast cancer were selected from UNC tumor archive and provided with the assistance of Dr. Ruth Lininger and Dr. William Funkhouser (Department of Pathology and Laboratory Medicine). The patients in this cohort ranged in age from 43-94, with 64 and 65 years being the median and the mean, respectively. 39/41 (95%) of sporadic breast

Table 1. Characteristics of Sporadic Breast Cancer Patients

Study Set	Number in Study	Age Range	Sample Type	Tests performed	Age Matched Controls
UNC tumor archival	41	43-94 years	DNA from micro dissected tumor sections	IHC, Genotyping	No
UNC 9830 Study	46	28-82 years	DNA isolated from immortalized lymphocyctic cell lines established from whole blood	Genotyping only	Yes
DTMA[1]	175	32-85 years	No DNA available	For IHC only, known molecular subtype	No
CBCS[2]	620	20-74 years	DNA isolated from whole blood	Genotyping only Subset has known molecular subtype	Yes

[1] DTMA = Dartmouth-Hitchcock Tissue Microarray
[2] CBCS = Carolina Breast Cancer Study

cancer subjects were women and 2 were men. Most of the patients with sporadic breast cancer were Caucasian (28/41), with the other 13 patients comprised of one Native American, eight African-Americans, and four subjects of unknown race. All of the cases were classified as invasive ductal carcinomas.

UNC 9830 Study Subjects. A forty-six patient subset of the UNC 9830 study sporadic breast cancer cohort were derived from patients from UNC Hospitals that were newly diagnosed with invasive primary breast cancer cases. The patients in this cohort ranged in age from 28-82, with 54 years being both the median and the mean, respectively. The majority of these women (40/46) were Caucasian, with the remainder including one Hispanic, one Asian, and four African-American females. The majority of the cases (44/46) were invasive ductal carcinomas and the other cases were classified as tubular carcinoma or carcinoma not otherwise specified. ER, PR, Her2, Her1, and CK5/6 tumor marker status was analyzed and the patients were distributed into the following categories: luminal A (ER+, PR+, Her2-), luminal B (ER+, PR+, Her2+), Her2 positve PR-, Her2+), basal (ER-, PR-, Her2-, with either CK5/6+, or Her1+), and unclassified (samples that did not match the previous combinations). The distribution of molecular subtype categories among these patients was 21 luminal A (46%), 6 luminal B (13%), 8 basal (17%), 6 Her2+ (13%), and 5 unclassified cases (11%).

Carolina Breast Cancer Study Subjects. The Carolina Breast Cancer Study (CBCS) cohort of experimental subjects consisted of six hundred and twenty cases from Phase 1 of the study (Carey et al, 2006). Access to these patients was provided by Dr. Robert Millikan (Department of Epidemiology and UNC Lineberger

Comprehensive Cancer Center). The CBCS samples were obtained after informed consent from patients ranging in age from 20-74 years. These patients had been diagnosed with invasive breast carcinoma between 1993-1996 (Millikan et al, 2007). Patient recruitment intentionally oversampled for African-American cases so that both Caucasian (372/620, 60%) and African-American (248/620, 40%) women were equally represented (Millikan et al, 2007). Additionally, the patient recruitment intentionally oversampled for younger women in order to equally represent pre-menopausal (276/555, 50%) and post-menopausal women (279/555, 50%). The molecular subtypes of 350 of the CBCS cases were determined based upon immunohistological analysis of ER, PR, Her2, Her1, and CK5/6. The distribution of molecular subtypes among these 350 women, consisted of 179 luminal A (51%), 53 luminal B (15%), 24 Her2+ (7%), and 74 basal tumors (21%). Twenty cases with immunohistological data could not be classified (6%).

Dartmouth Tissue Microarray. Two tissue microarrays were commissioned from the Dartmouth-Hitchcock Medical Center through Dr. Gregory J. Tsongalis and Dr. Wendy A. Wells (Department of Pathology and Laboratory Medicine, Dartmouth-Hitchcock Medical Center, Lebanon, NH). These two microarrays (DTMA1 and DTMA2) contain 92 and 83 invasive primary human breast tumors, respectively. Tumors were categorized based on the results of the immunohistological molecular subtype markers (ER, PR, Her2, Her1, and CK-5/6 status). These microarrays are composed of 122 luminal A, 17 Her2+, and 30 basal tumors, as well as 15 unclassified cases. Breast cancers of the luminal B subtype were not represented in this cohort.

Patients with BRCA1 Mutation Fifteen female and three male patients with documented *BRCA1* mutations were identified. The *BRCA1* mutations affecting these patients include point mutations, small insertions, and small deletions (described in Table 2). Access to these patients was provided by Dr. Jessica Booker (McClendon Clinical Laboratory, Department of Pathology and Laboratory Medicine). The majority of these mutations were detected by protein truncation assays at UNC Hospitals. The race for these *BRCA1* mutant patients was not available.

BRCAx Patients Twenty-four breast cancer subjects with characteristics of hereditary cancer (based on age of onset, bilaterality, and/or family history), but lacking documented *BRCA1* mutations were included in our analysis of the *BRCA1* promoter sequence (Table 3). One additional unaffected BRCAx patient was included (Table 3). Access to these patients was provided by Dr. Jessica Booker (McClendon Clinical Laboratory, Department of Pathology and Laboratory Medicine). Among the twenty-four patients that were diagnosed with breast cancer, age of onset was between the ages of 29-65 years old (average = 43 years old). Seven patients (29%) had bilateral breast cancer (concurrent in three patients; metachronous in four patients, with 1, 7, 8, and 17 year intervals). Two patients (8%) were diagnosed with ovarian cancer prior to the onset of breast cancer. 15 patients (63%) had primary breast cancer affecting one breast. BRCAPRO scores were calculated to predict the likelihood of a patients risk for a *BRCA1* mutation (Berry et al, 2002). A BRCAPRO score is derived from a statistical model and

Table 2. Characteristics of Subjects with a *BRCA1* Mutation.

Subject	Sex	History/Age at Diagnosis	BRCAPRO Score	Mutation Detected
BRCA1mut 1	F	No information	Not given	IVS5-11 T>G
BRCA1mut 2	M	Unaffected at 57	Not given	188 deletion 11[1]
BRCA1mut 3	F	Unaffected at 23	40	5296 deletion 4
BRCA1mut 4	F	BrCa at 66	Not given	2508 deletion AG
BRCA1mut 5	F	Bilateral BrCa at 37	Not given	4603 G>T(R1459M) splicing error
BRCA1mut 6	M	No information	Not given	3430 NT deletion loss exon 21&22
BRCA1mut 7	F	No information	Not given	5404 insertion G
BRCA1mut 8	M	No information	Not given	5661 C>T
BRCA1mut 9	F	BrCa at 27	Not given	3450 deletion 14
BRCA1mut 10	F	BrCa at 47	Not given	3490 deletion TC
BRCA1mut 11	F	Unaffected at 22	Not given	4603 G>T[1]
BRCA1mut 12	F	Bilateral BrCa at 31,32	70	2800 deletion AA
BRCA1mut 13	F	Unaffected at 38	Not given	E908X [1]
BRCA1mut14	F	Unaffected at 29	Not given	185 deletion AG[1]
BRCA1mut 15	F	Unaffected at 43	Not given	Exon 21/22 deletion [1]
BRCA1mut 16	F	Unaffected at 54	Not given	5193 G>C[1]
BRCA1mut 17	F	BrCa at unknown age	Not given	IVS5-11 T>G [1]
BRCA1mut 18	F	Unaffected at 29	Not given	5191 C>T[1]

[1] Specific diagnostic testing for known familial mutation, as opposed to full *BRCA1* mutation screening.

Table 3. Characteristics of BRCAx Subjects.

Subject	History/Age at Diagnosis	BRCAPRO Score	Mutation Diagnostic Facility
BRCAx 1	Breast Cancer at 42	64	UNC Hospitals
BRCAx 2	Breast Cancer at 44	57	UNC Hospitals
BRCAx 3	Bilateral Breast Cancer at 43,43	90	Myriad
BRCAx 4	Ovarian Cancer at 49, Breast Cancer at 50	56	Myriad
BRCAx 5	BrCa at 33	74	UNC Hospitals
BRCAx 6	BrCa at 32	95	UNC Hospitals
BRCAx 7	Bilateral BrCa at 29,46	56	UNC Hospitals
BRCAx 8	OvCa at 33,BrCa at 54	Not given	Mryiad
BRCAx 9	BrCa at 39	89	UNC Hospitals
BRCAx 10	Bilateral BrCa at 46	Not given	UNC Hospitals
BRCAx 11	Bilateral BrCa at 34,41	89	UNC Hospitals
BRCAx 12	BrCa at 40	78	UNC Hospitals
BRCAx 13	BrCa at 42	90	UNC Hospitals
BRCAx 14	BrCa at 36	64	UNC Hospitals
BRCAx 15	BrCa at 38	50	UNC Hospitals
BRCAx 16	Bilateral BrCa at 61,62	20	UNC Hospitals
BRCAx 17	3 synchronous BrCa 61	10	UNC Hospitals
BRCAx 18	BrCa at 44	17	UNC Hospitals
BRCAx 19	BrCa at 57	10	UNC Hospitals
BRCAx 20	Bilateral BrCa at 65.73	20	UNC Hospitals
BRCAx 21	BrCa at 29	10	UNC Hospitals
BRCAx 22	Unaffected at 31	25	UNC Hospitals
BRCAx 23	BrCa at 48	9	UNC Hospitals
BRCAx 24	BrCa 31	10	UNC Hospitals
BRCAx 25	BrCa at 65	32	UNC Hospitals

software that takes into account family history and population genetics (Berry et al, 2002). The average BRCAPRO score was 50, with 54% of these patients having BRCAPRO scores 50 or higher. All BRCAx patients were evaluated for *BRCA1* mutation using a protein truncation assay (n=22, UNC Hospitals) or DNA sequencing (n=3, Myriad Genetics). No *BRCA1* mutations were detected in any of the BRCAx patients (0/25, 0%). The racial distribution for the BRCAx patients was not disclosed.

Patients with Ductal Carcinoma *In Situ* (DCIS)

Fourteen female patients with ductal carcinoma *in situ* (DCIS) were identified. Access to DCIS patients was provided by Dr. Gregory Tsongalis and Dorothy Belloni (Department of Pathology and Laboratory Medicine, Dartmouth-Hitchcock Medical Center). No additional patient information was obtained.

Control Subjects

Four different cohorts totaling nine-hundred and ninety-seven unaffected individuals, identified among patients having standard well care testing or attending the genetics clinic for screening for other conditions. These patients do not have breast cancer, have no history of breast cancer, and have no known elevated risk for development of breast cancer.

Mayo Clinic Controls. Two hundred and eighty-five patients representing unaffected individuals were from a cohort at the Mayo Clinic (Rochester, Minnesota). Access to these control subjects was provided with the assistance of Dr. W. Edward Highsmith. The Mayo study was comprised of Caucasian control subjects that had

samples submitted to the Mayo Clinic Molecular Genetics Laboratory for cystic fibrosis carrier screening. After completion of the ordered service, samples annotated from patients with Caucasian or Northern European ethnicity were selected and strictly anonymized. Although no information other than the ethnicity of the individuals corresponding to these samples was retained, this group of individuals was composed primarily of females of childbearing age.

UNC Hospital Connexin 26 Study Controls. Sixty-four individuals who were previously evaluated for Connexin 26 mutation at UNC Hospitals (which causes a non-syndromic hearing loss) were identified and access to these patients was provided by Dr. Jessica Booker. The age, sex, and racial distributions for the UNC Hospital control subjects were not disclosed.

UNC 9830 Study Controls. Forty-six unaffected individuals were indentified through the UNC 9830 study as age and race matched subjects corresponding to the sporadic breast cancer patients in the UNC 9830 study. This cohort of control subjects ranged in age from 27-86, with 54 and 53 years being the median and the mean, respectively. The race of the UNC 9830 control subjects matched that of the cases: forty Caucasian, one Hispanic, one Asian, and four African American females.

Carolina Breast Cancer Study Controls. Six hundred and two CBCS unaffected individuals (age, race, and pre/post-menopause matched) were included in our analysis of the *BRCA1* promoter sequence. These CBCS study control subjects were recruited using lists from the NC Drivers' License and medicare beneficiary (Millikan et al, 2007).

Cell Lines and General Culture Conditions

Nineteen human breast cancer cell lines were obtained from the UNC Lineberger Comprehensive Cancer Center Tissue Culture Facility: BT20 (ATCC # HTB 19), BT549 (ATCC # HTB 122), Hs578T (ATCC # HTB 126), MCF7 (ATCC # HTB 22), MDA-MB-134 (ATCC # HTB 23) MDA-MB-175C VII (ATCC # HTB 25), MDA-MB-231 (ATCC # HTB 26), MDA-MB-415 (ATCC # HTB 128), MDA-MB-435S (ATCC # HTB 129), MDA-MB-436 (ATCC # HTB 130), MDA-MB-453 (ATCC # HTB 131), MDA-MB-468 (ATCC # HTB 132), SKBR3 (ATCC # HTB 30), UACC812 (ATCC # CRL 1897), and ZR751 (ATCC # CRL 1500). Human breast cancer cell lines HCC1937 and SUM149 were provided as a kind gift from the laboratory of Dr. William K. Kaufmann (Department of Pathology and Laboratory Medicine). Likewise, human breast cancer cell lines SUM102 and SUM185 were a kind gift from the laboratory of Dr. Carolyn I. Sartor (Department of Radiation Oncology). Additionally, two cultures established from normal breast epithelium were purchased from the American Type Culture Collection (ATCC) for control purposes: MCF10-2a (ATCC # CRL 10781), MCF12a (ATCC # CRL 10782). Cell line culture conditions, tissue origin, patient race, and tumor classification are included in Table 4. Cells were grown in 5% carbon dioxide at 37°C. When the cell cultures grew to 80-100% confluency, the cells were passaged.

A tissue array was constructed in the UNC Anatomical Pathology Translational Core Lab by Courtney Boyd (Department of Pathology and Laboratory Medicine) containing a subset of 16 breast cancer cell lines. This subset included BT20, BT549, Hs578t, MCF7, MDA-MB-134, MDA-MB-231, MDA-MB-415, MDA-MB-435s,

Table 4. Breast Cancer Cell Lines, Race, Origin, Tumor Type, and Culture Conditions.

Breast Cancer Cell Line	Race[1]	Origin	Tumor Type	Medium[2]
BT-20	C	Breast	Ductal Carcinoma	EMEM, NEAA, NaPyr, insulin
BT-549	C	Breast	Ductal Carcinoma	RPMI
Hs578T	C	Breast	Carcinosarcoma	EMEM, NEAA, NaPyr, insulin,
MCF7	C	Pleural Effusion	Adenocarcinoma	EMEM, NEAA, NaPyr, insulin
MDA-MB-134	C	Pleural Effusion	Ductal Carcinoma	EMEM, NEAA, NaPyr, insulin
MDA-MB-175C	AA	Pleural Effusion	Ductal Carcinoma	DMEM-H, insulin
MDA-MB-231	C	Pleural Effusion	Adenocarcinoma	EMEM, NEAA, NaPyr, insulin
MDA-MB-415	C	Pleural Effusion	Adenocarcinoma	DMEM-H, insulin, 2mM glutathione
MDA-MB-435S	C	Pleural Effusion	Ductal Carcinoma	DMEM-H, insulin
MDA-MB-436	C	Pleural Effusion	Adenocarcinoma	DMEM-H, insulin, 15% FBS*
MDA-MB-453	C	Pericardial Effusion	Adenocarcinoma	DMEM-H, insulin
MDA-MB-468	AA	Pleural Effusion	Adenocarcinoma	Leibovitz's L-15
SKBR3	C	Pleural Effusion	Ductal Carcinoma	McCoy's 5A
UACC812	NI	Breast	Ductal Carcinoma	DMEM-H, insulin
ZR751	C	Ascites	Ductal Carcinoma	RPMI
SUM102	NI	NI	NI	DMEM/F12, *5% Horse Serum
SUM149	NI	NI	NI	DMEM/F12, *5% Horse Serum
SUM185	NI	NI	NI	DMEM/F12, *5% Horse Serum
HCC1937	NI	Breast	Ductal Carcinoma	RPMI
MCF12a[3] (Normal)	C	Breast	Reduction Mammoplasy	DMEM/F12, *5% Horse Serum

[1]C = Caucasian, AA = African American, NI = not indicated

[2] Each medium (Gibco/Invitrogen Life Technologies, Carlsbad, CA) contains 10% fetal bovine serum (Hyclone, Logan, UT) and 1% Antibiotic-Antimycotic (PSF = penicillin, streptomycin, and fungicide) (Gibco/Invitrogen) unless otherwise noted (*). Other additives concentrations are: 10 µg/ml insulin (GIBCO/Invitrogen), 1% NEAA = Non essential amino acids (Gibco/Invitrogen), 1% NaPry = Sodium pyruvate (Gibco/Invitrogen).

[3] Normal breast epithelial cells

MDA-MB-436, MDA-MB-453, MDA-MB-468, SKBR3, UACC812, ZR751, SUM102, SUM149, SUM185, and HCC 1937. Additionally, MCF12a, a normal breast epithelial cell line was included. Confluent cultures of MCF12a and breast cancer cell lines were harvested by trypsinization and pelleted by centrifugation. Cell pellets were clotted by sequential addition of 200 µl of Ci-trol reagent (Dade Behring/Siemens, Deerfield, IL) and 200 µl of Thrombin reagent (Dade Behring/Siemens). After 2 minutes at room temperature, the clotted cell pellets were transferred to a nylon biopsy bag, placed in a labeled cassette, and fixed in 10% buffered formalin. Fixed cell pellets were transferred to a molding tray filled with paraffin and appropriately oriented. Once the paraffin had solidified, the embedded cell pellet paraffin block was cored and assembled in the cell line array block. 5 µm sections of the cell array were prepared and used for immunohistological analysis.

DNA Preparation and Purification from Blood Samples, Tumor Samples and Breast Cell Lines

Constitutional DNA samples from whole blood of the BRCAx subjects, BRCA1 mutant subjects, CBCS sporadic cases, CBCS controls, UNC controls, and Mayo Clinic controls were prepared using QIAamp DNA Blood Mini kit for whole blood (Buffers: AL, AW1, AW2, AE; and RNase A; Qiagen/Gentra Inc., Valencia, CA). Briefly, 20 µl of proteinase K was added to the bottom of a microcentrifuge tube, followed by the addition of 200 µl of whole blood. 4 µl of RNase A solution (100 mg/ml) to the sample and was mixed. 200 µl of Buffer AL was added to the sample and vortexed for 15 seconds, incubated for 10 minutes at 56°C, and pulse

centrifuged to remove any sample from the lid. 200 µl of 100% ethanol was added, mixed for 15 seconds, and pulse centrifuged. The sample was transferred to a QIAamp spin column/ collection tube and centrifuged at 6,000 rcf for 1 minute. After centrifugation, the spin column was transferred to a new collection tube, 500 µl of Buffer AW1 was added, and was centrifuged at 6000 rcf for 1 minute. 500 µl of Buffer AW2 was added to the spin column and was centrifuged at 20,000 rcf for 3 minutes. The spin column was transferred to a new collection tube and centrifuged for an additional minute at 20,000 rcf. To elute the DNA, the spin column was transferred to a 1.5 ml microcentrifuge tube, 200 µl of Buffer AE was added, incubated at room temperature for 5 minutes and centrifuge at 6,000 rcf for 1 minute. DNA was kept at -20°C for long term storage.

Lymphocytic cell lines corresponding to patients and controls from the UNC 9830 study were established by Dr. Steve Oglesbee of the Lineberger Comprehensive Cancer Center's tissue culture facility. Immortalization of these cell lines was achieved using Epstein-Barr virus transformation (Ryan et al, 2006). Dr. Patricia Basta isolated DNA from Epstein-Barr virus immortalized lymphocytic cell lines by processing the cell lysate DNA on the Autopure, automated DNA isolation system (Qiagen/GentraInc., Valencia, CA). Briefly, samples are loaded in an automated rack, the appropriate program for the concentration of cells is selected, cultured cells are lysed and the protein is precipitated. The DNA containing supernatant is separated, and the DNA is precipitated, washed and hydrated.

In order to isolate DNA from paraffin-embedded DCIS tissue and UNC tumor sections, tissue was micro-dissected from the slide, collected in a microcentrifuge

tube, and processed using the QiaAmp® DNA Mini Kit (Buffers: ATL, AL, AW1, AW2, and AE; Qiagen/Gentra Inc.) following the manufacturer's tissue protocol. To the tissue, 180 µl of room temperature ATL buffer (Qiagen/Gentra Inc.) and 20 µl of proteinase K was added, pulse vortexed for 15 seconds and held at 56°C overnight. Following the overnight incubation, 200 µl of AL buffer (Qiagen/Gentra Inc.) was added, mixed by pulse vortexing for 15 seconds, 200 µl of 100% ethanol was added, mixed thoroughly and held at 25°C for 5 minutes. The DNA lysate was transferred to a QiaAmp MinElute column/collection tube and centrifuged at 6,000 rcf for 1 minute and transferred to a new collection tube. 500 µl of AW1 buffer (Qiagen/Gentra Inc) was added, centrifuged at 6,000 rcf for 1 minute and transferred to a new collection tube. AW2 buffer (500 µl) (Qiagen/Gentra Inc.) was added, the sample was centrifuged at 6,000 rcf for 1 minute, transferred to a new collection tube and was followed by a 3 minute 20,000 rcf centrifugation to completely remove all ethanol. The MinElute column was transferred to a collection tube, 35 µl of room temperature AE buffer was added to the column, incubated at 25°C for 5 minutes and centrifuged for 1 minute at 20,000 rcf and stored at -20°C.

DNA was isolated from breast cell lines using a PureGene DNA Isolation Kit for cell and tissues (Qiagen/Gentra Inc.). Briefly, cells were washed using Hank's Balanced Salt Solution (Cellgro, Lawrence, KS), and released from the plate using 0.05% trypsin-EDTA (Sigma, St. Louis, MO). The cells in 0.05% trypsin-EDTA was neutralized with the addition of standard growth medium. Subsequently, the medium containing cells was transferred to a centrifuge tube. The cells were centrifuged at 1,000 rcf for 5 minutes, the medium was removed from the cell pellet, and the cells

were resuspended 300 µl of Cell Lysis Solution by inversion mixing (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis, MN). 3 µl of a 20 mg/ml proteinase K was added to the lysate, inverted 25 times and incubated at 55°C overnight. RNA was removed from the cell lysate using 1.5 µl of a 4 mg/ml RNase A solution (Puregene DNA Isolation Kit), inverting 25 times, and incubated at 37°C for 1 hour. Protein Precipitation Solution was added to the lysate and incubated for 5 minutes on ice. Subsequently, the cell lysates were centrifuged at 20,000 rcf for 5 minutes. DNA was precipitated by adding 300 µl 100% isopropanol, followed by a 300 µl 70% ethanol wash. DNA was resuspended in nuclease free water, held at 65°C for 30 minutes and stored at -20°C.

For all methods of DNA isolation, DNA concentrations were determined using a Beckman DU-600 (Beckman Coulter Inc., Fullerton, CA) UV/Vis spectrophotometer.

Preparation of RNA from Cell Lines

RNA was prepared using either a standard Trizol isolation procedure (Gibco/Invitrogen Life Technologies, Carlsbad, CA) or the Zymo Mini RNA Isolation II Kit (Zymo Research Corp., Orange, CA). For RNA isolation using the Trizol protocol, Dulbecco's phosphate buffered saline (PBS) without calcium chloride or magnesium chloride (1x concentration Gibco/Invitrogen Life Technologies,) was added to a 100 mm plate of adherent cells and removed. 5 ml of Trizol was added to each plate, agitated by pipette mixing, and incubated for 5 minutes at room temperature. The cell extract was transferred to a 50 ml collection tube and 1 ml of chloroform was added. The tube was shaken vigorously for 15 seconds to mix the

chloroform and Trizol and incubated at room temperature for 3 minutes. Tubes were centrifuged at 4ºC for 20 minutes at 2,850 rcf. The aqueous phase (was transferred to a new 15 ml tube, 2.5 ml of 100% isopropanol was added, incubated for 10 minutes at room temperature, and centrifuged at 4ºC for 40 minutes at 2,850 rcf. The supernatant was removed and the RNA was resupsended in Diethyl Pyrocarbonate (DEPC)-treated water for immediate quantitation with a Beckman DU-600 (Beckman Coulter Inc.) spectrophotometer. RNA was either used immediately for conversion to cDNA or stored at -80°C for future use.

For isolation of RNA using the Zymo Mini RNA Isolation II Kit all centrifugation steps were at 12,000 rcf (Zymo Research Corp.). 600 µl of ZR RNA buffer was added to a washed cell culture plate. Cell lysates were scraped from the plate, transferred to a Zymo-Spin Column/Collection Tube, centrifuged for 1 minute and flow-through was discarded. 350 µl of RNA Wash Buffer was added, the sample was centrifuged for 1 minute, and the flow-through was discarded. A DNase cocktail master mix was prepared by mixing (per sample) 6 µl of RNase-free DNase I, 5 µl of 10X Reaction Buffer, and 39 µl of RNA Wash Buffer. To each sample, 50 µl of the DNase I cocktail was added directly to the column and incubated for 15 minutes at room temperature. Following the DNase I cocktail treatment incubation, 100 µl of RNA Binding Buffer was added to the column, centrifuged for 1 minute, followed by the addition of 350 µl RNA Wash Buffer, and centrifuged for 1 minute. The wash step was repeated and the column was transferred to a new 1.5 ml centrifuge tube. 50 µl of Elution Buffer was added to the column, incubated at room temperature for 2 minutes, and centrifuged for 1 minute. RNA was quantified by

NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was either used immediately for conversion to cDNA or stored at -80°C for future use.

Experimental Procedures Related to Genotyping of Experimental Subjects

BRCA1 Promoter PCR and Primers

Primers were designed based upon the known DNA sequence U37574 (Genbank, www.ncbi.nih.gov) to analyze 1526 bp of the 1581bp *BRCA1* promoter in three segments (BPP-1, BPP-2, BPP-3) (Table 5 and Figure 3) (Xuet al, 1995). These primers were synthesized by the UNC Oligodeoxynucleotide Synthesis Core Facility (Chapel Hill, NC). *BRCA1* promoter segments were scanned for variants through PCR amplification, cloning, and DNA sequencing. The BPP-1 primer set amplified the 5' end of the *BRCA1* promoter and spanned 515 bps. The BPP-2 primer set amplified the middle segment of the *BRCA1* promoter and spanned 699 bps. The BPP-3 primer set amplified the 3' end of the *BRCA1* promoter and spanned 349 bps. The amplicons generated using these primer sets overlapped for sequencing of the *BRCA1* promoter.

A 3 bp *BRCA1* promoter polymorphism was detected at -600 bp from the exon 1a transcription start site and BPP-99 bp, BPP-132 bp, and BPP-250 bp primer sets were designed for specific and rapid screening of this insertion (Table 5 and Figure 3). The BPP-99 bp, BPP-132 bp, and BPP-250 bp primers (± 5' Hex) were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 _www.cgi) (Rozen, and Skaletsky, 2000) and were synthesized by MWG-Biotech AG (High Point, NC). PCR reactions were performed in a 50 µl total volume of buffer

Table 5. PCR Primers and PCR Conditions.

Name[1]	Sense primer	Antisense Primer	Tm[2]	Cycles	PCR Product
BPP-1	5'-TGCGGGGCCCGCTGAGCCCG-'3	5'-CGGTCTTTGCATTGCCGCCT-'3	60°	35	515 bp
BPP-2	5'-AGGCGGCAATGCAAAGACCG-'3	5'-CCCCCACTCTTTCCGCCCTAATGG-'3	60°	35	699 bp
BPP-3	5'-TAGGGCGGAAAGAGTGGGGG-'3	5'-TAATTCCCGCGCTTTTCCGT-'3	60°	35	349 bp
BPP-99	5'-AAGGCCTAGTTTCTGCTTTCAA-'3	5'-TCCAGTGGATAGATTGGAGACC-'3	62°	35	99/101 bp
BPP-132	Paired with 5' Hex Labelled BPP-250 Sense Primer	5'- CTTGTCAACAGTTATGGACTCG -'3	60°	35	129/132 bp
BPP-250 5' Hex BPP-250	5'-CTACTTGCCCCAGACTCCTG-'3 Hex 5'-CTACTTGCCCCAGACTCCTG-'3	5'-TCGCCACAGTGTTCCTTAGA-'3	63°	35	247/250 bp
BPP-850 [3]	5'-GATCA [A▼GATC▲T]CAAGACTACTTGCCCCAGACTC-'3	5'- GATCA [A▼AGCT▲T]TCCCGGGACTCTACTACCTTT-'3	62°	40	847/850 bp
Actin[3] (DNA)	5'-AGAAAATCTGGCACCACACC-'3	5'-CCACTCACCTGGGTCATCATCTT-'3	59°	35	124 bp

[1] BPP = *BRCA1* Promoter Primer

[2] Tm = Annealing Temperature, held for 1minute 30 seconds

[3] Forward primers contains a Bgl II restriction site and the reverse primer contains a Hind III restriction cut site for cloning

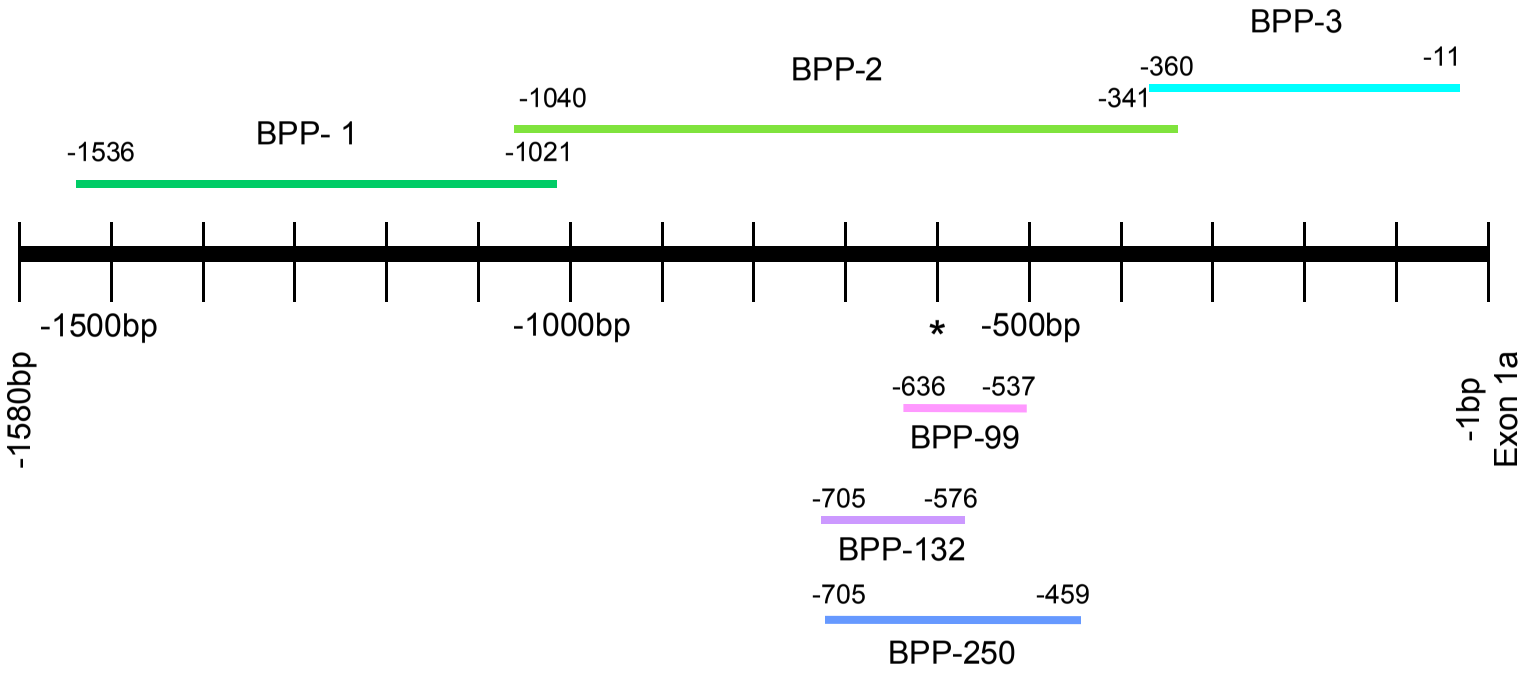

Figure 3. Location of *BRCA1* Promoter Primers. Three primer sets were designed to span 1526 bps of the 1581 bp *BRCA1* promoter (BPP-1, BPP-2, and BPP-3). To investigate the frequency of the +ACA (*) *BRCA1* promoter polymorphism located at -600 bp, three additional primer sets were designed (BPP-99, BPP-132, and BPP-250). Nucleotide numbering is based on GenBank accesion number U37574 (Xu et al. 1995).

containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM of each dNTP (EasyStart Micro 50 PCR mix in a tube, Molecular BioProducts, San Diego, CA), 0.4 µM of each primer, and 1.25 units GoTaq® Flexi DNA Polymerase (Promega, Madison, WI), and 50 ng of sample. Amplifications were carried out using a 9700 Perkin-Elmer step-cycle program consisting of multiple cycles of 94°C for denaturing (1 minute), variable (59°C to 63°C range) annealing temperature (1 minute 30 seconds), and 72°C for extension (2 minutes). Specific cycles and annealing temperatures are given in Table 5. Two final extension cycles, a 72°C (10 minutes) and 60°C (60 minutes), followed the 40 step cycle. A portion of each PCR product was analyzed on a 2% agarose gel containing 40 mM Tris-acetate/1.0 mM EDTA (pH 8.0) and visualized by ethidium bromide staining. The remaining PCR sample was used for cloning and sequencing, and/or capillary electrophoresis.

High Resolution Analytical Agarose Gel Electrophoresis

PCR products resulting from amplifications using +ACA polymorphism 99 bp and 250 bp primers were analyzed on 3% Super Fine Resolution (SFR) agarose gel (Amresco, Solon, OH) containing 40 mM Tris-acetate/1.0 mM EDTA (pH 8.0) and visualized by ethidium bromide staining. SFR gel analysis facilitated the resolution of *BRCA1* PCR product size polymorphisms (99 bp versus 102 bp, and 247 bp versus 250 bp, respectively), allowing for the visual detection of the presence of the amplicon representing the +ACA polymorphic allele. Select patient samples were cloned and sequenced to verify the results of SFR gel electrophoresis.

Cloning and DNA Sequencing

PCR products were ligated into pGEM-T Easy plasmid vector following the manufacturer's protocol (Promega). Recombinant plasmids were transformed into competent JM109 cells (Promega). At least four clones were selected through blue-white colony screening for each patient-amplicon. Plasmid DNA was purified from selected clones using the Wizard DNA purification system (Promega, Madison, WI). Verification of cloned inserts was accomplished through restriction enzyme digestion in 20 µl total volume consisting of 2 µl NEB Buffer 2, 10 U NcoI, 20 U NdeI restriction enzyme (New England Biolabs, Beverly, MA), and 1 µg of purified clone. Restriction enzyme digests were resolved on a 0.8% agarose gel containing 40 mM Tris-acetate/1.0 mM EDTA (pH 8.0) and visualized by ethidium bromide staining. Cloned PCR products were sequenced using the MI3R3 primer and automated DNA sequencing performed by the UNC Genomic Analysis Facility.

Sequences generated from each clone were compared to the known DNA sequence for the *BRCA1* promoter region using Genbank submission U37574 as the standard (Xu et al., 1995).

Capillary Electrophoresis

A 5' Hex-labeled BPP-250 sense primer was synthesized and used in conjunction with the unlabeled BPP-99 bp, BPP-132 bp, or BPP-250 bp anti-sense primer (177/180 bp, 129/132 bp, and 247/250 bp amplicons, respectively) for analysis on the ABI 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) (Table 5).

1.5 µl of each PCR amplicon was mixed with 12 µl of molecular biology grade formamide and 0.75 µl of ROX-HD internal standard (Applied Biosystems Inc.), denatured at 95°C for 3 minutes, cooled to 4°C for 2 minutes and loaded on the instrument. Size of the PCR amplicons were determined by capillary electorphoresis with a 16 capillary (36 cm length) ABI PRISM 3130 Genetic Analyzer using POP6 and buffer supplied by manufacturer at 60°C. Electrophoresis conditions using dye set D were 10 second injection time, 1.2 kV injection voltage, 15.0 kV electrophoresis voltage, 6500 steps of polymer fill volume, 180 second preinjection electrophoresis, and 20 minute collection time for each sample. Electronic images were analyzed by Gene Mapper analysis software ver.3.7 (Applied Biosystems Inc.). Select patient samples were cloned and sequenced to confirm the results of the ABI 3130 genetic analyzer. A representative result of capillary electrophoretic analysis of the *BRCA1* promoter PCR products is shown in Figure 4.

Electrophorectic Mobility Shift Assay

Protein extracts were prepared by detergent lysis on ice in a buffer containing 0.1% Nonidet P-40, 10 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 15 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A, and 1 µg/ml leupeptin (Jordan-Sciutto et al, 1999b). After centrifugation at 800 rcf for 5 minutes, the nuclei were collected and the supernatant was saved as the cytosolic extract. The pellet containing the nuclei was further extracted using a high salt buffer containing 0.42 M NaCl, 20 mM HEPES (pH 7.9), 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A, and 1 µg/ml leupeptin and incubating on ice for 10 minutes. To

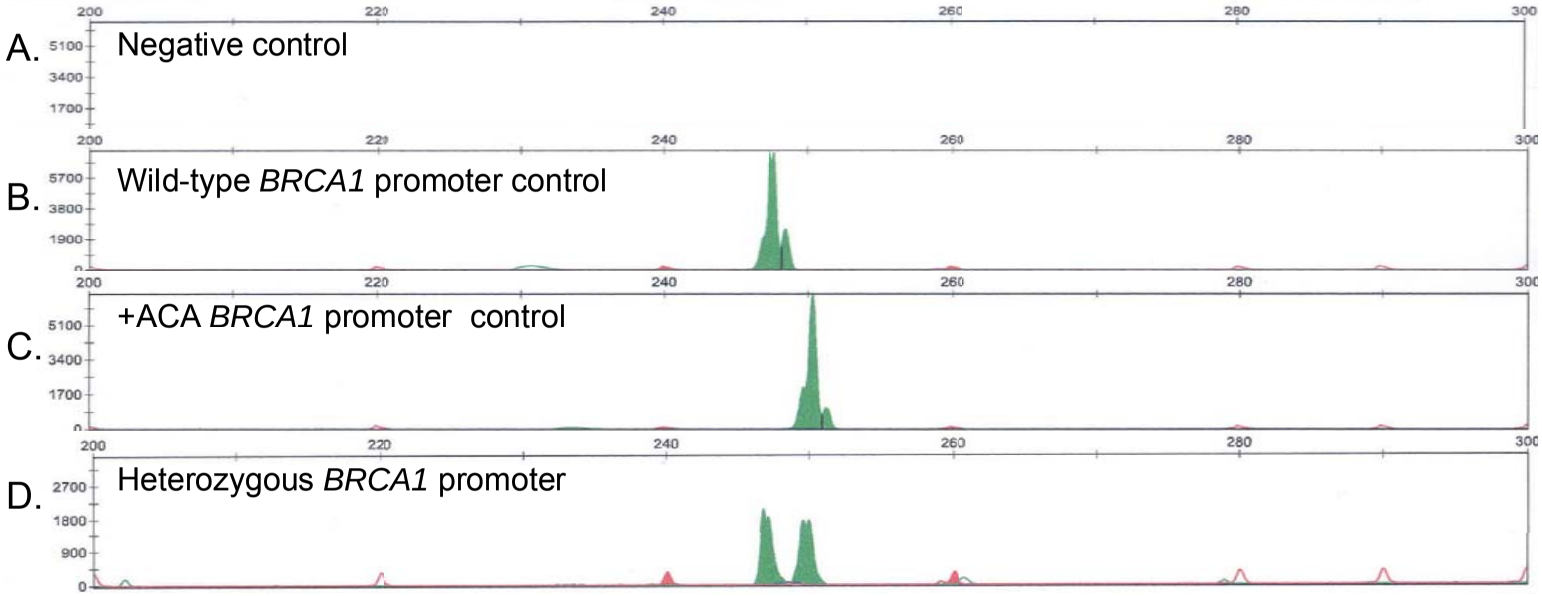

Figure 4. Representative Genotypic Analysis of *BRCA1* Promoter Region by Capillary Electrophoresis. Red peaks correspond to ROX HD internal standard and green peaks correspond to PCR amplicons. A. No DNA template PCR control. B. Wild-type *BRCA1* promoter DNA template control (previously verified by sequencing). C. +ACA *BRCA1* promoter DNA template control (previously verified by sequencing). D. Representative heterozygous patient.

remove residual insoluble material, centrifugation at 14,000 rcf was carried out for 5 minutes. The supernatant fraction was collected as the nuclear extract. Protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

For purified fusion protein, ~50 ng of protein and 0.5 ng of ^{32}P-end-labeled double-stranded oligonucleotides was incubated in 20 µl of EMSA buffer containing 200 mM KCl, 20 mM Hepes (pH 7.9), 20% glycerol, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol for 20 minutes at room temperature (Jordan-Sciutto et al, 1999b). Prior to addition of labeled probe to reduce nonspecific DNA-protein interactions, 20-30 µg of protein was incubated with salmon sperm DNA as a nonspecific competitor (1 µg of competitor/10 µg of protein) in EMSA buffer. For competition reactions, unlabeled competitive molecules were preincubated with the protein for 5 minutes on ice before adding labeled probe. The reaction mixture was run on 4% nondenaturing polyacrylamide gel and electrophoresed at 100 V. After 1.5 hours, the polyacrylamide gel was removed from the apparatus, dried, and exposed to autoradiography film.

Gene Expression Analysis of BRCA1 and FAC1

RT-PCR

RNA was converted using SuperScript™ First-Strand Synthesis System for RT-PCR (Gibco/Invitrogen Life Technologies, Carlsbad, CA). 2 µg of total RNA was resuspended with DEPC-treated water to give a final volume of 4.0 µl. 1 µl of a 1:40 dilution of 1 µM Oligio cDNA Synthesis primer was added to each RNA sample,

incubated at 70ºC for 3 minutes and then cooled on ice. Following cooling, 2 µl of 5X First Strand Buffer, 2 µl of 5 mM dNTP mix, and 1 µl Superscript II reverse transcriptase was added to each RNA sample and incubated for 42ºC for 1 hour. The reaction was stopped by heating to 75ºC for 10 minutes, and cooled on ice. Subsequently, 90 µl of DEPC-treated water was added to the 2 µg/10 µl cDNA sample to result in a 20 ng/µl working concentration. Gene-specific oligonucleotide primers were designed using Primer3 software and were synthesized by MWG-Biotech (High Point, NC) based upon the known cDNA sequences (Genbank, www.ncbi.nih.gov) for mRNAs of interest (Rozen and Skaletsky, 2000). The RT-PCR primer sequences for gene-specific primers are given in Table 6. Verification of equal cDNA template concentrations between samples was accomplished using either mouse GAPDH or human *β-actin* primers Table 6. PCR reactions were performed in a 50 µl total volume of buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM of each dNTP (EasyStart Micro 50 PCR-mix-in-a-tube, Molecular BioProducts, San Diego, CA), 0.4 µM of each primer, and 1.25 units GoTaq® Flexi DNA Polymerase (Promega). Reactions were carried out in an 9700 Perkin-Elmer (Perkin Elmer/Cetus, Foster City, CA) step-cycle program as follows: 30-40 cycles at 94°C for denaturing (1 minute), 58-62°C for annealing (1 minute 30 seconds), and 72°C for extension (2 minutes) (Table 6). PCR products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA and visualized by ethidium bromide staining.

Quantitative Real-time PCR

Table 6. Reverse Transcriptase-PCR (RT-PCR) Primers and PCR Conditions.

Name [1]	Sense primer	Antisense Primer	Tm[4]	Cycles	PCR Product
RT-PCR mGAPDH	5'-CCATGGAGAAGGCTGGGG-'3	5'-CAAAGTTGTCATGGATGACC-'3	58°	30	194 bp
RT-PCR hACTIN[2]	5'-AGAGATGGCCACGGCTGCTT-'3	5'-ATTTGCGGTGGACGATGGAG-'3	58°	30	446 bp
RT-PCR hFAC1	5'-GGCGACAACACAACAAATGC-'3	5'-TTCCAGATTAGGCTGGATGG-'3	60°	40	213 bp
RT-PCR hBRCA1 (3') [3]	5'-CAGAGGACAATGGCTTCCATG-'3	5'-CTACACTGTCCAACACCCACTCTC-'3	62°	35	101 bp

[1] m = mouse, h = human

[2] hActin primer designed by UniSTS

[3] hBRCA1 primer designed by Vissac, C. *et al* 2002

[4] Tm = Annealing temperature, held for 1 minute and 30 seconds

20 µg of RNA, isolated from human breast cancer cells lines, was treated with RQ1 RNase-free DNase and RNasin Plus RNase inhibitor (Promega, Madison, WI) to remove any residual DNA. Briefly, in a final total volume of 50 µl, 20 µg of RNA was mixed with 5 µl 10x RQ1 buffer, 1 µl RQ1 DNase, and 1 µl RNasin Plus RNase Inhibitor and held at 37ºC for 30 minutes. The sample volumes were adjusted to 100 µl by adding 50 µl of nuclease-free water (Promega) and the RNA samples were processed with the RNeasy Mini RNA Cleanup kit (Qiagen Inc., Valencia, CA) according to standard protocol. Briefly, 350 µl of freshly prepared buffer RLT/beta-mercaptoethanol (β-ME) mix was added to the 100 µl RNA samples (1% β-ME into buffer RLT) followed by the addition of 250 µl 100% ethanol. RNA samples were transferred to a mini column/collection tube and centrifuged at 8,000 rcf for 15 seconds and flow through was discarded. 500 µl of buffer RPE was added to the column, transferred to a second collection tube, and centrifuged for 2 minutes at 8,000 rcf. The column was transferred to a third 1.5 ml collection tube and centrifuged for 1 minute at 8,000 rcf to remove residual buffer RPE. RNA was eluted by transferring the spin column to a 1.5 ml microcentrifuge tube, adding 20 µl of nuclease-free water, and centrifuging for 8,000 rcf for 1 minute for a final concentration of 1 µg/µl. The DNA-free, purified RNA was converted to cDNA using a High-Capacity cDNA reverse transcriptase kit from Applied Biosystems. Briefly, 10 µg of RNA (10 µl) was mixed with 10 µl 10X RT buffer, 4 µl of 25X dNTP (100 mM), 10 µl of 10X random primer, 5 µl of Reverse Transcriptase and 61 µl of nuclease-free water, and processed at 25ºC for 10 minutes, 37ºC for 120 minutes, 85ºC for 5 seconds, and cooled to 4ºC. A volume of 200 µl of nuclease-free water was added

and cDNA stored at -20ºC. For real-time analysis of cDNA, 5 µl of either *BRCA1* (Hs00173233_m1) or *FAC1* (Hs00189461_m1) TaqMan® Gene Expression Assay primer was mixed with 12.5 µl TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems Inc.), and 7.5 µl of cDNA. Each sample was run in triplicate on the Applied Biosystems 7500 Real-time PCR system for a 10 minute 95°C enzyme activation and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Immunohistochemcial Analysis of BRCA1 and FAC1 Expression and Localization

5 µm paraffin sections of normal and tumor tissues were prepared and analyzed by immunohistochemistry for CK18, BRCA1, and FAC1 protein. Antibody host, clonality, staining region, controls, working dilutions, and antibody manufacturer are provided in Table 7. Tissue sections were deparaffinized by incubating slides at 60°C for 30 minutes followed by two 5 minute xylene baths. Tissue sections were rehydrated in a step down series of ethanol washes (100%, 95%, and 70%) for 10 rapid submersions, followed by 3 minutes at each level. Following the 70% ethanol rehydration, sections were placed for 10 minutes in a final concentration of 0.3% hydrogen peroxidase in methanol, to block endogenous peroxidase activity. Slides were rinsed in distilled water followed by a hydration for 5 minutes in Dulbecco's PBS. Antigen retrieval citrate buffer (pH 6.1) was warmed for 50 seconds in the microwave at high power, slides were placed in the warmed citrate buffer, steamed for thirty minutes, and allowed to cool for thirty minutes (Dako North America,

Table 7. Primary Antibody Characteristics and Working Dilution.

Antibody	Host	Target Protein Molecular Weight	Antibody Type	Staining Regions	Positive Controls	Working Dilution	Manufacturer
CK-18 (DC-10)	Mouse	Not Done	Monoclonal	Nuclear	Normal or Cancerous Breast Tissue	IHC 1:1000	Santa Cruz
Pan cytokeratin (B311.1)	Mouse	Not Done	Monoclonal	Cytoplasmic	Normal or Cancerous Breast Tissue	IHC 1:25	AbCam
BRCA1 [8F7]	Mouse	Not Done	Monoclonal	Nuclear	Normal Breast	IHC 1:500	GeneTex
BRCA1 (MS110)	Mouse	Not Done	Monoclonal	Nuclear	Normal Breast and MCF7 cells	IHC 1:250	Calbiochem
GAPDH (ab9385)	Rabbit	38 kDa	Polyclonal	Cytoplasmic	Housekeeping gene	WB 1:400	Abcam
FAC1 (non-commerical)	Rabbit	97.5 kDa	Polyclonal	Cytoplasmic +/or Nuclear	Human Hippocampus or Hs578t	IHC 1:1500	Generously provided by Dr. Robert Bowser

Carpinteria, CA). Sections were removed from the antigen retrieval citrate buffer, placed in a humid box with 4 drops of serum-free protein block (Dako) for 10 minutes, and rinsed in PBS. 300 µl primary antibody was applied at a dilution shown in Table 7 using Dako antibody diluent and incubated for 2 hours at room temperature. Excess unbound primary antibody was removed with a PBS wash, followed by a 10 minute application of LSAB-2 biotinylated link IgG secondary antibody (Dako) to the sections. Sections were rinsed in PBS, covered for 10 minutes with LSAB-2 Streptavidin Horseradish Peroxidase (HRP) enzyme, rinsed in PBS, covered for 2 minutes with DAB (3,3'-diaminobenzidine) chromogen substrate, then rinsed in distilled water, and counterstained with Mayer's hematoxylin for 2 minutes. Following counterstaining, slides were rinsed in distilled water, dipped in PBS for 15 seconds, and returned to distilled water. The sections were then dehydrated in 3 consecutive ethanol washes (70%, 95%, and 100%) for 3 minutes each, followed by two xylene washes (2 minutes and 5 minutes), and coverslips were mounted. Digital images were captured using light microscopy and viewed on the computer for side by side scoring comparison by a single evaluation. Tissue quality control (CK18/pan-cytokeratin) antibodies were scored negative or positive, BRCA1 and FAC1 scoring was graded from 0-4+: 0 = no staining, 1+ = light staining (<40%), 2+ = medium staining (40-60%), 3+ = moderate staining (61-80%), and 4+ = heavily stained (80-100%). Additionally, FAC1 antibody localization was documented (mostly nuclear, nuclear and cytoplasmic, or mostly cytoplasmic). Tumors were classified as negative/reduced for BRCA1 and FAC1 with staining of 1+ or less, whereas 2+ or greater BRCA1 and FAC1 staining was considered

positive. Cores were excluded from grading if the tissue quality control antibodies (CK18/pan-cytokeratin) sample was missing, negative, or greatly reduced, or if one or both of the BRCA1 or FAC1 antibody stained core was missing. Normal adjacent breast tissue served as internal positive controls, and slides of normal breast tissue from reduction mammoplasty were included as external BRCA1 positive controls and generally scored ≥2+. Histologic sections of human hippocampus were included as a positive control for FAC1 and stained in conjunction with the subject samples. Sections of hippocampus were provided by Dr. Kinuko Sukuzi and Courtney Boyd (Department of Pathology and Laboratory Medicine).

Functional Analysis of Wild-type and +ACA BRCA1 Promoters

BRCA1 Promoter Luciferase Constructs

PCR products from two UNC Hospital control subjects that were homozygous for either the wild-type or +ACA *BRCA1* promoter allele were ligated independently into pGL4.17 luciferase reporter vector following the manufacturer's protocol (Promega, Madison, WI). Briefly, the BPP-850 sense primer was designed to include a 5' Bgl II restriction enzyme cut site while the anti-sense primer was designed with a 5' Hind III restriction enzyme cut site that amplified an 847 base pair sequence containing the putative minimal *BRCA1* promoter (Thakur and Croce, 1999) and extended 137 bases into exon 1a, to result in a forward directional vector (Figure 5. The BPP-850 sense primer was designed by Visaac *et al.,* the BPP-850 anti-sense primer was designed using Primer3 software, and both sense and antisense primer were synthesized by MWG-Biotech (High Point, NC) (Table 5) (Rozen and Skaletsky,

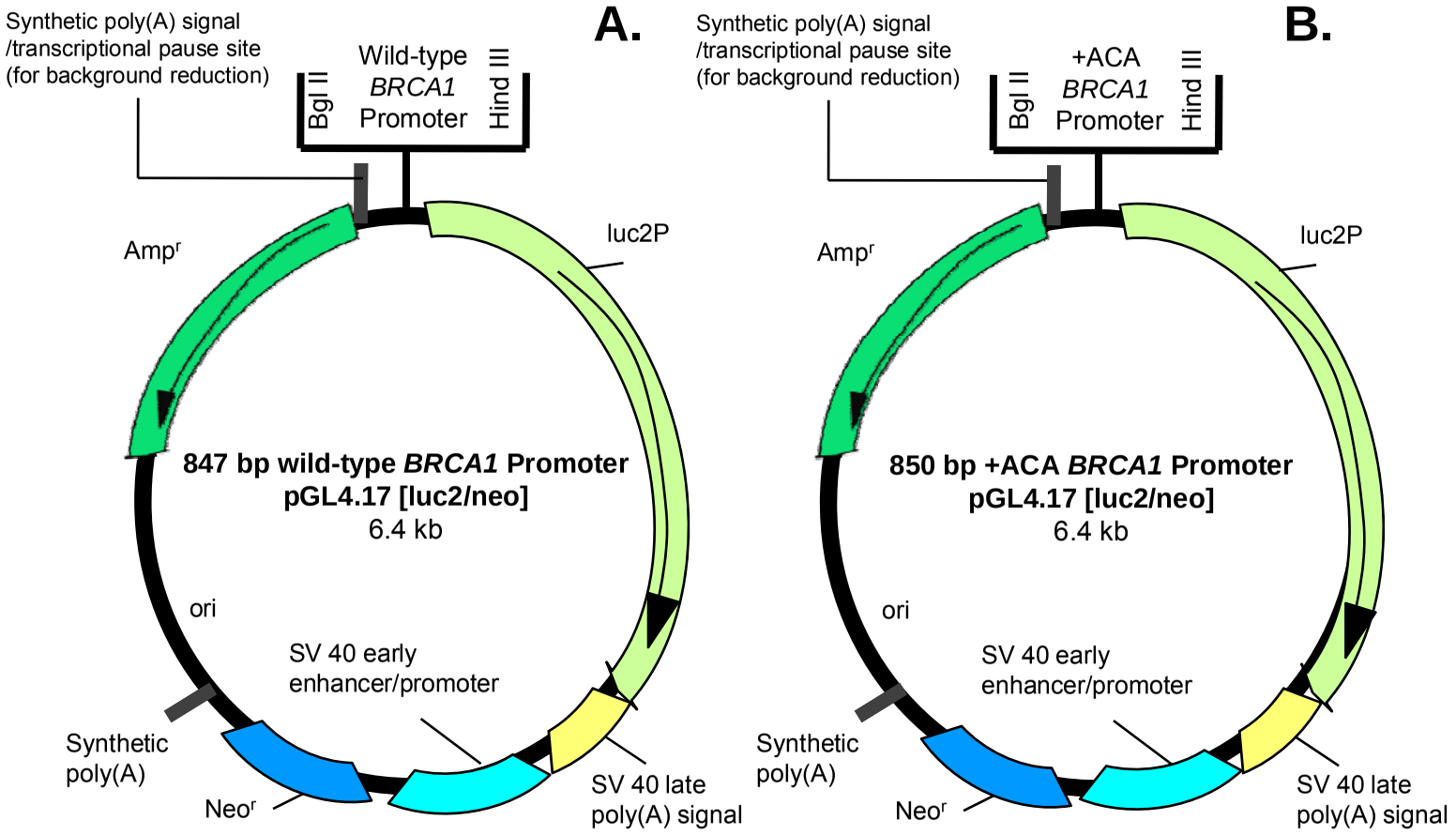
A.
Synthetic poly(A) signal
/transcriptional pause site
(for background reduction)
Bgl II
Wild-type
BRCA1
Promoter
Hind III
Ampr
luc2P
847 bp wild-type BRCA1 Promoter
pGL4.17 [luc2/neo]
6.4 kb
ori
SV 40 early
enhancer/promoter
Synthetic
poly(A)
Neor
SV 40 late
poly(A) signal
B.
Synthetic poly(A) signal
/transcriptional pause site
(for background reduction)
Bgl II
+ACA
BRCA1
Promoter
Hind III
Ampr
luc2P
850 bp +ACA BRCA1 Promoter
pGL4.17 [luc2/neo]
6.4 kb
ori
SV 40 early
enhancer/promoter
Synthetic
poly(A)
Neor
SV 40 late
poly(A) signal

Figure 5. Wild-type and +ACA *BRCA1* Promoter-Driven Luciferase Reporter Constructs. A. The 847 base pair *BRCA1* promoter segment (wild-type) inserted into a pGL4.17 luciferase reporter construct. B. The 850 base pair *BRCA1* promoter segment (+ACA) inserted into the pGL4.17 luciferase reporter construct. The pGL4.17 vector contains a Neomycin cassette for tissue culture selection purposes. The Bgl II and Hind III restriction enzyme cut sites were utilized for ligation of the *BRCA1* segments. pGL4.17 diagrams adapted from Promega (Madison, WI).

2000; Vissac et al, 2002). Recombinant pGL4.17 luciferase reporter vector constructs were transformed into JM109 competent cells (Promega), and successful transfectants were selected and purified. Verification of cloned inserts was accomplished through DNA sequencing, capillary electrophoresis comparison to known wild-type and +ACA *BRCA1* promoter controls, and restriction enzyme digestion. Restriction enzyme digestion was performed in 15 µl total reaction volume of buffer containing with 10 U Bgl II, 20 U Hind III restriction enzymes, and 1 µg of each purified clone. Samples were resolved on a 0.8% low melting temperature agarose gel containing 40 mM Tris-acetate/1.0 mM EDTA (pH 8.0) and visualized by ethidium bromide staining for either the 847 and 850 bp amplicon corresponding to the wild-type or +ACA *BRCA1* promoter allele, respectively. The wild-type or +ACA *BRCA1* promoter pGL4.17 luciferase constructs were linearized using Bsu 361 restriction enzyme digestion, prior to transfection (1 µg/transfection condition) of NIH-3T3 cells.

FAC1 Expression Construct

A Zeo-resistant *FAC1* pcDNA3.1(+) expression construct was generated, by subcloning an 810 amino acid coding region of *FAC1* from a Neo-resistant pcDNA3.1(+) reporter construct (a generous gift from Dr. Robert Bowser's laboratory, Department of Pathology and Laboratory Medicine, University of Pittsburgh, Pittsburgh, PA) into the pcDNA3.1(+)/Zeo vector (Figure 6). The FAC1 excision was performed utilizing the Not I and Hind III restriction enzyme digestion sites in the Neo-resistant pcDNA3.1(+) reporter construct. In parallel 2 µg of

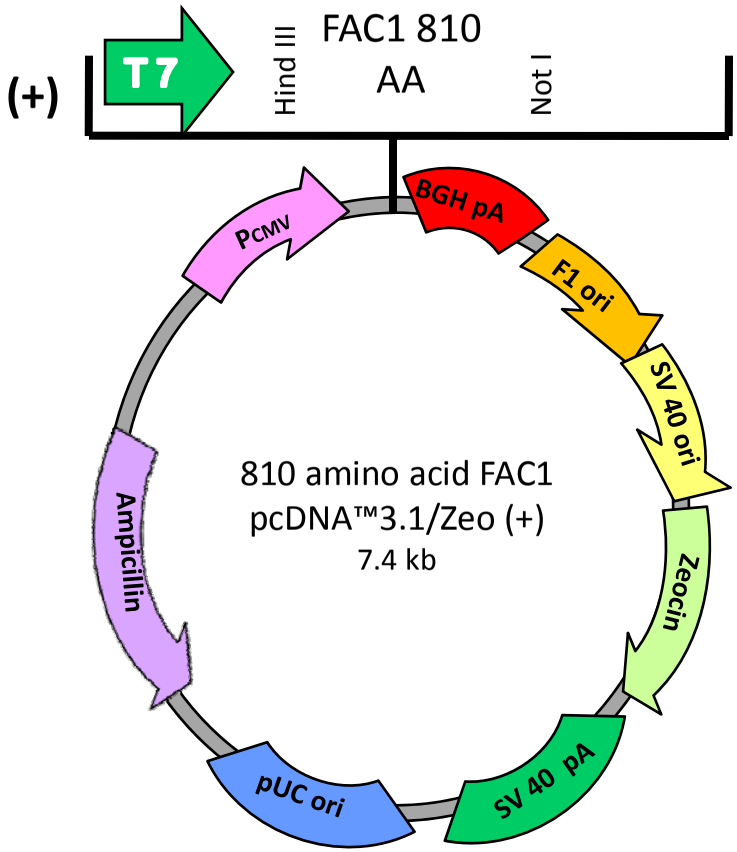

Figure 6. FAC1 Expression Construct. An 810 amino acid segment of FAC1 was ligated into a pcDNA3.1/Zeo(+) that contains a Zeocin cassette for selection of cells containing the FAC1 construct. The Hind III and Not I restriction enzyme cut sites were utilized for the ligation of the FAC1 segment. pcDNA 3.1/Zeo (+) diagram adapted from Gibco/Invitrogen Life Technologies (Carlsbad, CA).

pcDNA3.1(+)/Zeo vector was digested to create matching Not I/Hind III sticky end in preparation for the ligation of the 2.4 kb *FAC1* insert. Restriction enzyme digestion was completed in 30 µl total reaction volume of 10 U Not I, 20 U Hind III restriction enzymes, 3 µl Buffer 2, 3 µl 10X BSA, and 17 or 18 µl of deionized water, respectively. The *FAC1* insert was verified by DNA sequencing using a universal T7 sequencing primer that was synthesized by MWG-Biotech (High Point, NC). Subcloning was accomplished with the assistance of Dean Staus (Department of Pathology and Laboratory Medicine).

Stable Transfection of *BRCA1* Promoter Luciferase Reporter Constructs into NIH-3T3 Cell Lines

NIH-3T3 mouse fibroblasts were utilized in these studies because they do not express FAC1. Non-transfected cells were maintained in DMEM-H growth medium prepared as described in Table 4 (Gibco/Invitrogen Life Technologies). An empty vector control was transfected with a promoter-less luciferase/Neomycin resistant pGL4.17 vector. Additionally, the NIH-3T3 fibroblasts were transfected with a luciferase/Neomycin resistant pGL4.17 vector containing either an 847 bp wild-type or 850 bp +ACA section of the *BRCA1* promoter. Transfections were performed when cells reached 50-70% confluency. Briefly, for each transfection, 250 µl of Opti-MEM medium (Gibco/Invitrogen Life Technologies) was mixed with 7.5 µl of TransIT-3T3 transfection reagent (Mirus, Madision, WI) and incubated at 25°C for 15 minutes. Transfection complex formation was completed by adding 1 µg of plasmid/DNA constructs to the Opti-MEM/TransIT-3T3 mixture for 20 minutes at

25°C. Liposome-coated transfection complexes were added dropwise to the appropriate cells and were incubated at 37°C overnight. 24 hours post-transfection, the medium was removed from the transfected cells and non-transfected control cells, and was replaced with medium containing 600 µg/ml Neomycin [Geneticin (G-418), Gibco/Invitrogen Life Technologies]. Cells were observed and selection medium was refreshed every 3-4 days, until the non-transfected Neomycin treated NIH-3T3 cells were no longer viable.

Stable Transfection of *FAC1* Expression Construct into NIH-3T3 Cells Contianing Wild-type and +ACA *BRCA1* Luciferase Reporter Constructs

Zeo-resistant FAC1 pcDNA3.1(+) expression construct was transfected as described above into both NIH-3T3 cells containing the wild-type or +ACA *BRCA1* promoter-driven luciferase constructs. 24 hours post-transfection, the medium was removed from the transfected cells and non-transfected control cells, and medium containing 50 µg/ml Zeocin was added to the cells (Gibco/Invitrogen Life Technologies). Cells were observed and selection medium was refreshed every 3-4 days, until the non-transfected Zeocin treated NIH-3T3 cells were no longer viable.

Luciferase Assay

Transfected cells were plated in quadruplicate at an equal dilution density in twenty-four well plates and grown to 75-90% confluency. Luciferase activity was measured in triplicate and averages were calculated. Medium was removed from the wells, sterile Dulbecco's PBS was applied to rinse residual medium and removed. To each

test well, 300 µl of Glo lysis buffer was added and lysates were incubated at room temperature with mixing for 12 minutes. Steady Glo substrate assay buffer was prepared during this incubation as follows: Steady Glo substrate (10 mg/ml final concentration) was added to 300 µl/well Steady Glo assay buffer. Following the room temperature incubation, 300 µl of the 10 mg/ml Steady Glo substrate assay buffer was added to each well. In triplicate, 200 µl of the protein lysate/substrate buffer mix was transferred to a translucent microplate, covered to protect from light, and incubated at room temperature for 7 minutes with mixing. Immediately following this incubation, the microplate was placed on a Berthold Detection System reader and sample luminescence was measured using the Simplicity 2.1 program (Berthold Detection Systems, Oak Ridge, TN). Luciferase activities corresponding to each construct were determined for each transfected cell line.

MTT Cell Viability Assay

Transfected cells were plated in duplicate as described above for MTT cell viability assay (used to normalize luciferase activity among repeats and conditions). Medium was removed from the transfected cells and 1 ml of MTT (500 µg/ml final concentration) in culture medium was added and incubated in the dark at 37°C for 4 hours. After the incubation, 1 ml of solubilizing solution was added to each well and mixed until no precipitate remained. From each MTT assay well, 200 µl of sample was transferred in triplicate to a 96 well flat bottom transparent microplate and read on a spectrophotometer at 570 nm wavelength. Results for each condition were averaged. Averages were divided into 1.0 to establish a multiplication correction

factor for the luciferase activity in order to normalize the results among transfected cell lines.

Statistical Analysis

Statistical descriptive tests included simple ranges, means, medians, and standard error of the mean (SEM). Error bars depicted in graphs represent the standard error of the mean (SEM). Unpaired two tailed t-test ($p < 0.05$) were performed using KaleidaGraph® (Synergy Software, Reading, PA). Statistical analyses for the Carolina Breast Cancer Study including Fisher's Exact test, odds ratio, p-values were performed by Dr. Robert Millikan (Department of Epidemiology) using version 8.2 SAS software (SAS Institute Inc., Cary, NC). Chi-square and Fisher's exact test, for samples outside of the CBCS, were performed using GraphPad (GraphPad Software, La Jolla, CA).

RESULTS

Discovery of the +ACA BRCA1 Promoter Polymorphism

PCR amplification, cloning, and DNA sequence analysis was performed to identify *BRCA1* promoter sequence alterations in constitutional DNA from BRCAx breast cancer patients. We sequenced 1526 bp of the 1581 bp *BRCA1* promoter from 7 BRCAx patients and a commercial pooled normal human control (Promega, Madision,WI). Sequencing of the *BRCA1* promoter was accomplished secondary to PCR amplification of multiple replicate clones for each of three *BRCA1* promoter segments and patients (ranging from 1-4 and averaged 3 per subject). In total, 72 *BRCA1* promoter sequences were analyzed (28 from the BPP-1 segment, 29 from the BPP-2 segment, and 15 from the BPP-3 segment). All patient and control samples were analyzed for the BPP-1 and BPP-2 segments (8/8, 100%), while analysis of the entire promoter sequence was completed in 5 of the BRCAx subjects (BRCAx 2, 3, 8, 13, and 15) and the normal human control sample. Cloned DNA sequences were analyzed and the GenBank sequence for the *BRCA1* promoter (U37574) was used for comparison (Xu et al, 1995). A comparative sequence analysis is shown in Figure 7. In many of the *BRCA1* promoter segment clones, slight sequence variations were observed including deletions, insertions, and substitutions compared to the prototype sequence. The majority of these variations were neither consistent between subjects nor clones from an individual, and may reflect an expected level of sequencing infidelity. However, we identified a novel

Figure 7. Sequence Analysis of the *BRCA1* Promoter. Examples of sequence analysis results from PCR amplicons generated with BPP-132 primer set. The sequence shown in black is based on and numbered according to GenBank accession number U37574 (Xu et al. 1995). The sequence shown in blue corresponds to the wild-type allele and matches the GenBank sequence. The sequence shown in green corresponds to the +ACA insertion polymorphic allele.

+ACA insertion in 3/7 (43%) of the patients' samples (BRCAx 2, 13, 15) and in the normal human control DNA. This +ACA insertion is located in the BPP-2 segment at -600 nucleotides from the exon 1a transcription start site of the *BRCA1* promoter (Figures 3 and 8) (White et al, 2006; White et al, 2005; Xu et al, 1995). We observed the +ACA insertion in 2/4 clones corresponding to pooled normal human control DNA, suggesting the +ACA allele is carried by some portion of unaffected individuals. The +ACA insertion was present in all of the clones from BRCAx subjects 2 and 13 suggesting that these patients are homozygous for the +ACA *BRCA1* promoter sequence. In contrast, 3/4 clones corresponding to BRCAx subject 15 contained the +ACA insert, suggesting that this patient is heterozygous for the +ACA *BRCA1* promoter sequence. Additionally, the +ACA insertion was absent in all clones sequenced from BRCAx subjects 3 and 8, suggesting that these patients are homozygous for the wild-type *BRCA1* promoter sequence. Overall, the +ACA insertion was present in 11/29 (38%) of all BPP-2 segment clones analyzed.

To verify that this three base pair insertional polymorphism was a real sequence variation rather than a result of PCR infidelity, we employed three additional primer sets (BPP-99, BPP-132, and BPP-250, Figure 7) to specifically and rapidly screen subjects by PCR for the +ACA insertion. We confirmed all genotypes that were generated by DNA sequencing, using PCR amplification followed by either capillary electrophoresis and/or high resolution agarose. Representative results of these techniques are shown in Figures 4 and 9, respectively.

BRCA1 Promoter Genotyping Analysis

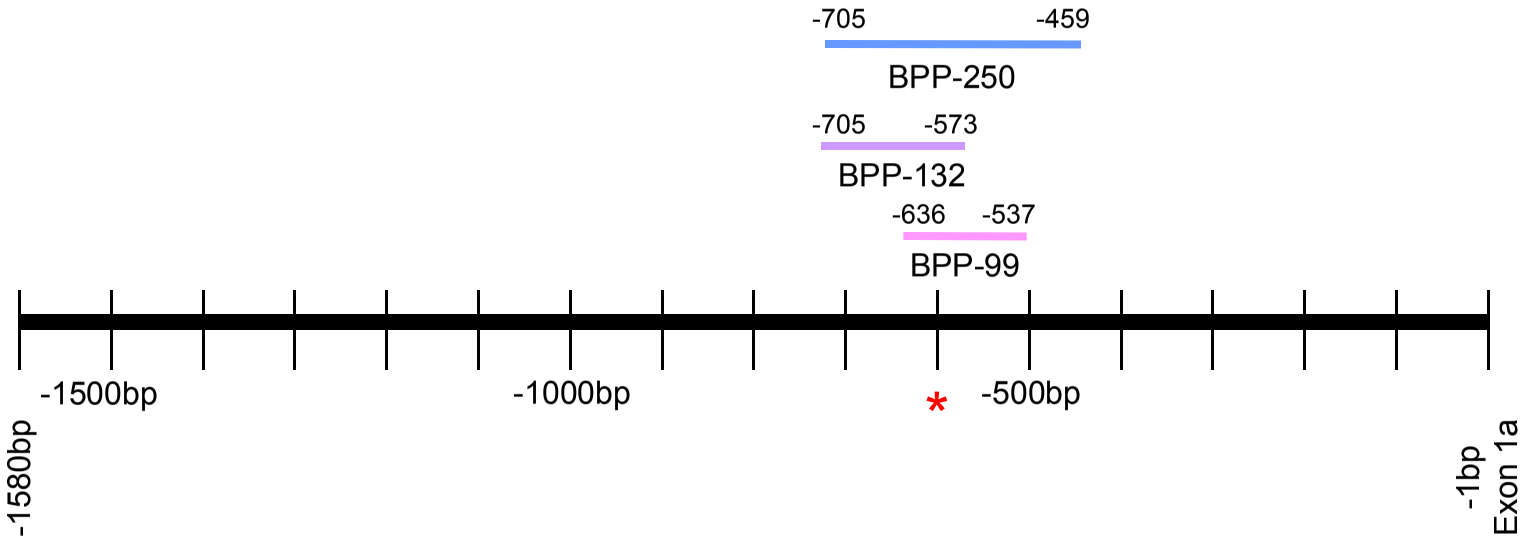

Figure 8. Location of the +ACA *BRCA1* Promoter Polymorphism. The +ACA *BRCA1* promoter polymorphism is located at -600 bps* from the exon 1a transcriptional start site. Three sets of *BRCA1* promoter primers (BPP) were designed for high throughput and specific screening of subjects for +ACA genotype analysis (BPP-99, BPP-132, and BPP-250). Nucleotide numbering is based on GenBank accession number U37574 (Xu et al. 1995).

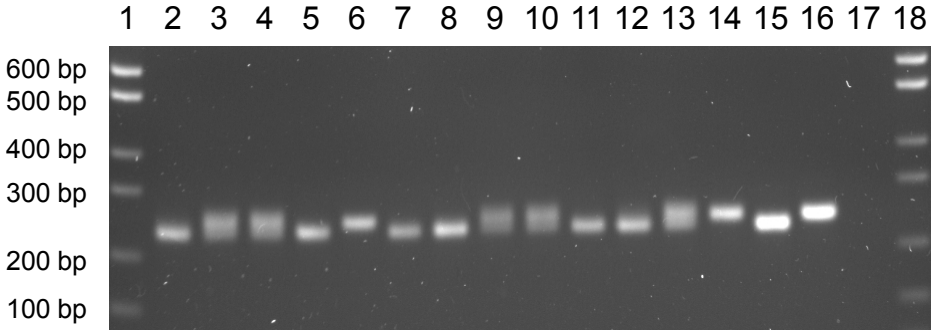

Figure 9. Detection of the +ACA *BRCA1* Promoter Polymorphism in Patients with Ductal Carcinoma *in situ* (DCIS). Representative 3% SFR agarose gel of PCR products from patients with DCIS. Expected PCR amplicon size is 247 bp (wild-type homozygotes), 247 and 250 bp (heterozygotes), and 250 bp (ACA homozygotes). Samples: Lanes 1 and 18, DNA molecular size ladder; Lane 2 – Lane 11, Genotype analysis for 10 individual DCIS patients; Lane 12, known wt/wt genotype template; Lane 13, known WT/ACA genotype template; Lane 14, known ACA/ACA genotype template; Lane 15, cloned wt *BRCA1* promoter template; Lane 16, cloned ACA *BRCA1* promoter template; Lane 17, no DNA template control. Samples were resolved at 125 volts for 1 hour and 30 minutes. This analysis was provided by Dorothy Belloni and Greg Tsongalis, Dartmouth-Hitchcock Medical Center, Lebanon, NH.

Frequency of the +ACA *BRCA1* Promoter Polymorphism in Unaffected Individuals

We analyzed the genotypic distribution of the *BRCA1* promoter polymorphism in unaffected individuals from four separate cohorts (Mayo Clinic Study, Minneapolis, MN; UNC Hospital Connexin 26, UNC 9830 Study, and the Carolina Breast Cancer Study, Chapel Hill, NC) to estimate of the frequency of the +ACA allele in the general population. The overall genotypic distribution in the 997 unaffected individuals was 481/997 (48%) WT/WT, 432/997 (43%) WT/ACA, and 91/997 (9%) ACA/ACA (Figure 10). This distribution was similar among the individual cohorts of unaffected individuals: Mayo Clinic Study [119/285 (42%) WT/WT, 133/285 (47%) WT/ACA, and 33/285 (11%) ACA/ACA]; UNC Hospital Connexin 26 Study [38/64 (59%) WT/WT, 24/64 (38%) WT/ACA, and 2/64 (3%) ACA/ACA]; UNC 9830 Study [23/46 (50%) WT/WT, 17/46 (37%) WT/ACA, and 6/46 (13%) ACA/ACA]; and the Carolina Breast Cancer Study [301/602 (50%) WT/WT, 258/602 (43%) WT/ACA, and 43/602 (7%) ACA/ACA]. Genotypic frequencies for the *BRCA1* promoter +ACA polymorphism corresponding to individual cohorts are represented in Figure 10.

Hardy-Weinberg Equilibrium values were calculated for the expected genotypic distribution and compared to the observed genotypic distribution (Table 8). No deviations from the Hardy-Weinberg equilibrium were found between the observed and expected values for the unaffected individuals (p=0.7).

Frequency of the +ACA *BRCA1* Promoter Polymorphism in Breast Disease

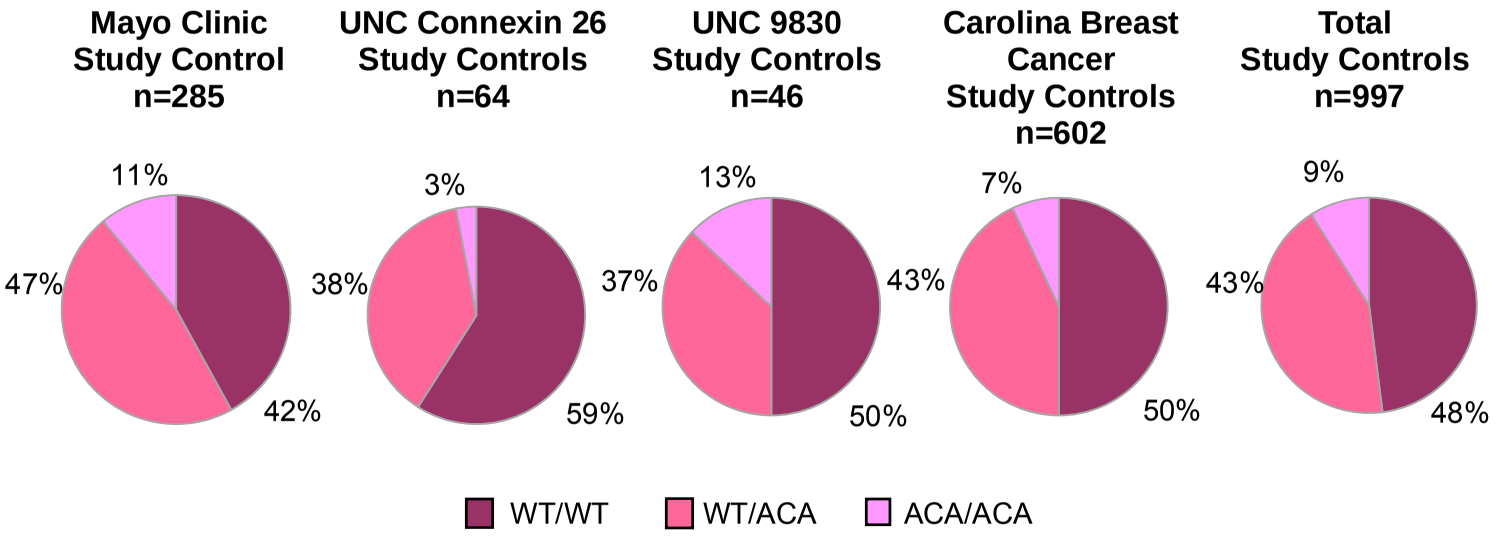

Figure 10. Frequency of *BRCA1* Promoter Genotypes in Unaffected Individuals. Illustration of the frequency of the +ACA *BRCA1* promoter polymorphism in individual control subject groups and the total control subjects +ACA *BRCA1* promoter distribution (Mayo Clinic Study, UNC Hospital Connexin 26, UNC 9830 study, and the Carolina Breast Cancer Study control subjects).

Table 8. Hardy-Weinberg Distribution of the +ACA *BRCA1* Promoter Polymorphism. Expected (Top) and Observed (Bottom)

Genotype	DCIS Patients n=14	Sporadic Breast Cancer Patients n=707	BRCAx Patients n=25	BRCA1 Mutant Patients n=17	Total Breast Disease Groups n=763	Unaffected Individuals n= 997	CBCS African-American Cases n=248	CBCS African-American Controls n=258	CBCS Caucasian Cases n=372	CBCS Caucasian Controls n=344
WT/WT	36%	47%	44%	53%	47%	49%	51%	62%	45%	43%
WT/ACA	50%	43%	44%	41%	43%	42%	41%	34%	44%	45%
ACA/ACA	14%	10%	12%	6%	10%	9%	8%	4%	11%	12%

Genotype	DCIS Patients n=14	Sporadic Breast Cancer Patients n=707	BRCAx Patients n=25	BRCA1 Mutant Patients n=17	Total Breast Disease Groups n=763	Unaffected Individuals n= 997	CBCS African-American Cases n=248	CBCS African-American Controls n=258	CBCS Caucasian Cases n=372	CBCS Caucasian Controls n=344
WT/WT	43%	45%	48%	53%	46%	48%	49%	60%	44%	43%
WT/ACA	36%	46%	36%	41%	45%	43%	45%	38%	46%	46%
ACA/ACA	21%	9%	16%	6%	9%	9%	6%	2%	10%	11%

We analyzed the genotypic distribution of the *BRCA1* promoter polymorphism in individuals with breast disease from four breast disease classifications (DCIS patients; BRCAx subjects; *BRCA1* mutant patients; and sporadic breast cancer patients from the UNC archival tumor bank, the UNC 9830 Study, and the Carolina Breast Cancer Study).

The genotypic frequency distribution of the *BRCA1* promoter polymorphism among DCIS [6/14 (43%) WT/WT, 5/14 (36%) WT/ACA, and 3/14 (21%) ACA/ACA] and BRCAx patient cohorts [12/25 (48%) WT/WT, 9/25 (36%) WT/ACA, and 4/25 (16%) ACA/ACA] had a statistically significant variation compared to the overall control cohort genotypic frequency distribution (Chi-squared p-value, p= 0.0001 and p=0.04, respectively). In contrast, there were no significant genotypic frequency distribution changes between the controls and *BRCA1* mutants [9/17 (53%) WT/WT, 7/17 (41%) WT/ACA, and 1/17 (6%) ACA/ACA] or sporadic breast cancer cases [321/707 (45%) WT/WT, 322/707 (46%) WT/ACA, and 64/707 (9%) ACA/ACA].

The allelic frequency of the +ACA *BRCA1* promoter polymorphism among different breast disease groups were analyzed and compared to the allelic frequency of the controls. The +ACA allelic distribution corresponding to the DCIS [5/8 (63%) WT/ACA and 3/8 (37%) ACA/ACA, p=0.002] and BRCAx [9/13 (69%) WT/ACA and 4/13 (31%) ACA/ACA, p=0.03] cohorts varied significantly from the control +ACA allelic distribution [432/523 (83%) WT/ACA and 91/523 (17%) ACA/ACA] (Figures 10 and 11). The allelic frequency of the +ACA *BRCA1* promoter polymorphism among *BRCA1* mutant [7/8 (88%) WT/ACA and 1/8 (12%) ACA/ACA, p=0.4] and sporadic breast cancer [322/386 (83%) WT/ACA and 64/386 (17%) ACA/ACA, p=1.0]

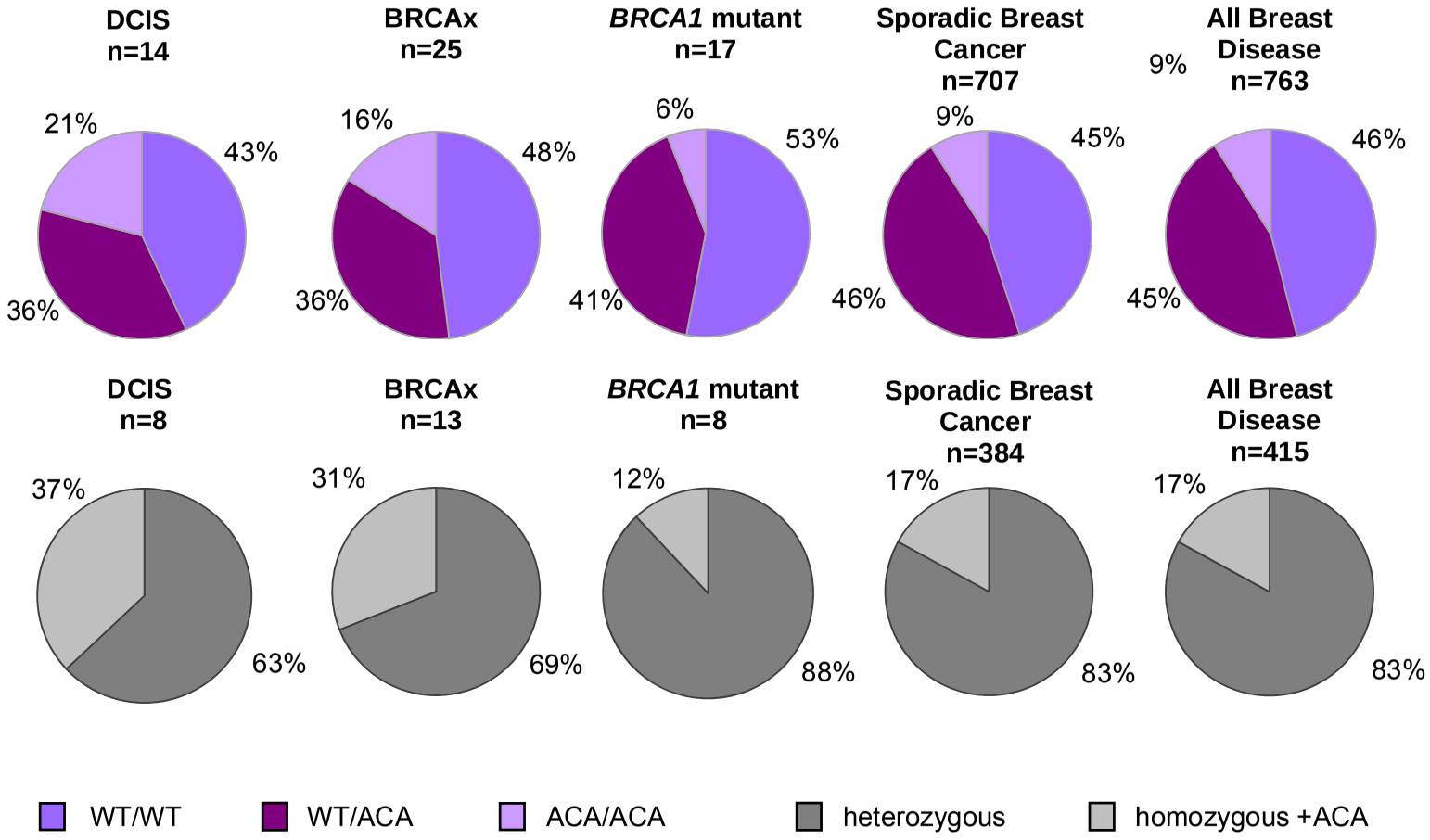
DCIS
n=14
21%
43%
36%
BRCAx
n=25
16%
48%
36%
BRCA1 mutant
n=17
6%
53%
41%
Sporadic Breast Cancer
n=707
9%
45%
46%
All Breast Disease
n=763
9%
46%
45%
DCIS
n=8
37%
63%
BRCAx
n=13
31%
69%
BRCA1 mutant
n=8
12%
88%
Sporadic Breast Cancer
n=384
17%
83%
All Breast Disease
n=415
17%
83%
WT/WT
WT/ACA
ACA/ACA
heterozygous
homozygous +ACA

Figure 11. Frequency of *BRCA1* Promoter Genotypes in Ductal Carcinoma *In Situ* (DCIS), Breast Cancer Patients, and All Breast Disease. Top. Illustration of the frequency of the +ACA *BRCA1* promoter polymorphism in patients with DCIS, patients lacking a *BRCA1* mutation (BRCAx), patients having a *BRCA1* mutation; patients with sporadic breast cancer, and all patients with breast disease (DCIS, BRCAx patients, *BRCA1* mutant subjects, sporadic breast cancer patients). Bottom: Illustration of the frequency of homozygous versus heterozygous ACA polymorphism in patients having at least one polymorphic allele (WT/ACA versus ACA/ACA) in DCIS, BRCAx, *BRCA1* mutants, sporadic breast cancer, and all breast disease.

subjects did not deviate from the control group allelic distribution (Figures 10 and 11). No deviations from the Hardy-Weinberg equilibrium were detected between observed and expected values for the total breast disease group (p=0.6) or any of the four individual breast disease groups (DCIS p=0.5, BRCAx p=0.7, *BRCA1* mutant p=1.0, sporadic breast cancer p=0.4) (Table 8).

Frequency of the +ACA *BRCA1* Promoter Polymorphism in the Carolina Breast Cancer Study (CBCS)

***BRCA1* Promoter Genotypic Distribution in the CBCS.** We analyzed the *BRCA1* promoter polymorphism genotypic distribution in the CBCS study independently of the other data sets described. The CBCS has a large sample size, age and race matched controls, and oversampled for African-American subjects in order to equally represent African-American and Caucasian cases and controls. Additionally, pre-menopausal and post-menopausal cases/controls were selected to achieve equal representation in both racial categories. We performed genotypic analysis on 620 sporadic breast cancer cases and 602 controls. The genotypic frequency of the *BRCA1* promoter polymorphism distribution of the sporadic breast cancer cases [286/620 (46%) WT/WT, 283/620 (46%) WT/ACA, and 51/620 (8%) ACA/ACA] does not significantly deviate from that of the control population [301/602 (50%) WT/WT, 258/602 (43%) WT/ACA, and 43/602 (7%) ACA/ACA] (Figure 12).

***BRCA1* Promoter Genotypic Distribution Comparison of African-American Women to Caucasian Women.** We examined variation in the genotypic distribution

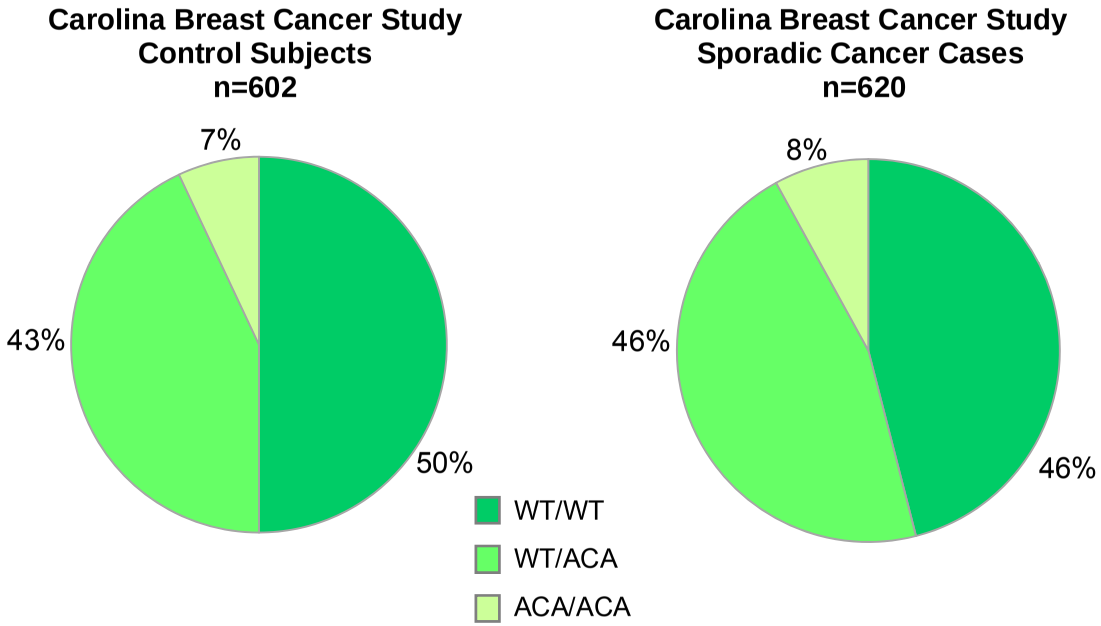

Figure 12. Frequency of *BRCA1* Promoter Genotypes in Unaffected Individuals and Breast Cancer Patients of the Carolina Breast Cancer Study (CBCS). Illustration of the frequency of the +ACA *BRCA1* promoter polymorphism in CBCS age-matched controls and CBCS patients with primary invasive breast cancer. Overall frequencies between controls and cases do not significantly deviate.

of the *BRCA1* promoter polymorphism stratified by Caucasian cases and controls, versus African-American cases and controls. The *BRCA1* promoter genotypes among Caucasian women with sporadic breast cancer [165/372 (44%) WT/WT, 170/372 (46%) WT/ACA, and 37/372 (10%) ACA/ACA] and the Caucasian women controls [147/344 (43%) WT/WT, 160/344 (47%) WT/ACA, and 37/344 (10%) ACA/ACA] were similar. However, the frequency of the homozygous +ACA genotype and +ACA allelic frequency is significantly increased among the African-American women with sporadic breast cancer [121/248 (49%) WT/WT, 113/248 (45%) WT/ACA, and 14/248 (6%) ACA/ACA] compared to the African-American control cohort [154/258 (60%) WT/WT, 98/258 (38%) WT/ACA, and 6/258 (2%) ACA/ACA] (Fisher's Exact two-tailed p-value, p=0.02 and p=0.01, respectively) (Figure 13). Homozygous +ACA African-American females have a 3-fold increased relative risk (p=0.03) for breast cancer development and heterozygous African-American women have a 1.5-fold increased breast cancer risk (p=0.03) compared to wild-type African-American individuals. The relative risk for breast cancer development among Caucasian subjects that are homozygous (OR=0.9, p=0.8) and heterozygous (OR=1.0, p=0.95) for the +ACA allele did not significantly deviate from WT/WT Caucasian subjects.

For the CBCS, the Hardy-Weinberg Equilbrilium was calculated for African-American and Caucasian sporadic breast cancer cases and African-American and Caucasian controls. Of these four groups, the African-American controls observed values were the only group to conflict with the expected Hardy-Weinberg equilibrium values (Table 8). This divergence would be expected if there was a shift in

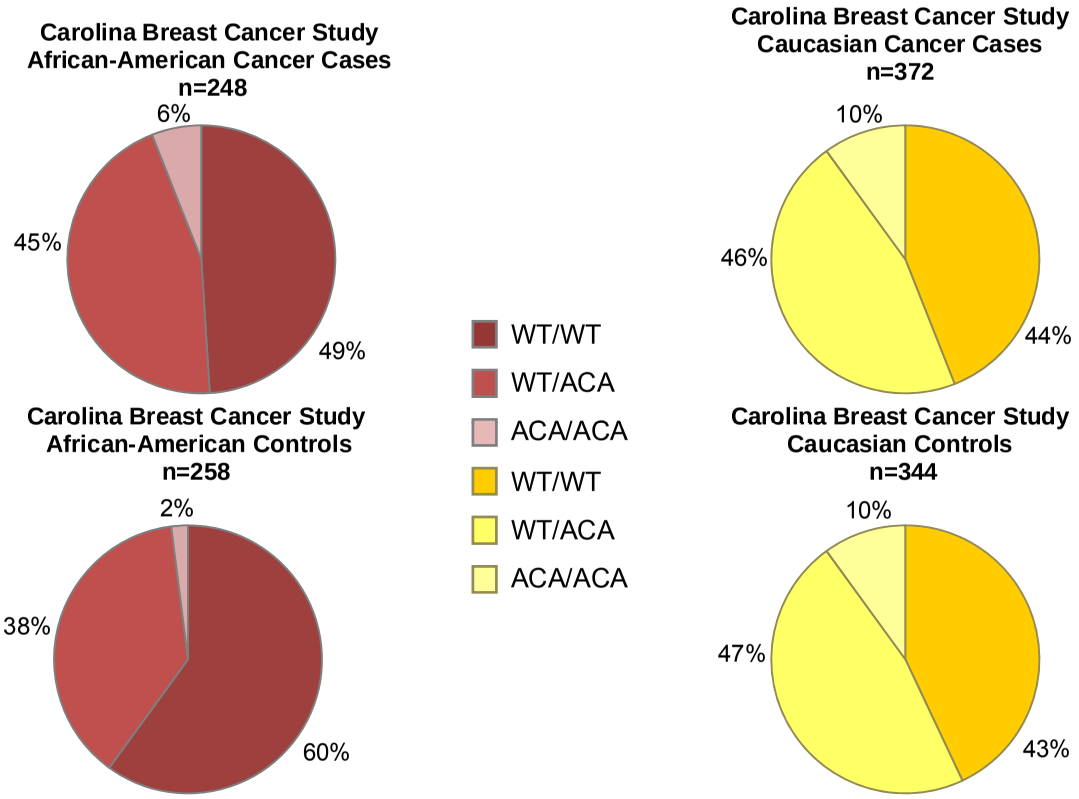

Figure 13. Comparison of *BRCA1* Promoter Genotypes Between African-American and Caucasian Breast Cancer Subjects and Controls from the Carolina Breast Cancer Study. Top: Comparison of the frequency of the +ACA *BRCA1* promoter polymorphism in African-American and Caucasian women with primary invasive breast cancer. Bottom: Comparison of the frequency of the +ACA *BRCA1* promoter polymorphism in African-American and Caucasian age-matched controls.

population dynamics that reflects the movement of homozygous for the + ACA *BRCA1* promoter polymorphism from the African-American control group to the African-American sporadic breast cancer case group.

***BRCA1* Promoter Genotypic Distribution Comparison with Pre-Menopausal and Post-Menopausal Women.** To examine possible relationships between the +ACA *BRCA1* promoter polymorphism and age of breast cancer onset, we compared the variation in genotypic distribution for the *BRCA1* +ACA promoter polymorphism among pre-menopausal cases and pre-menopausal controls, and post-menopausal cases and post-menopausal controls. The *BRCA1* promoter genotypes for pre-menopausal sporadic breast cancer cases [143/313 (46%) WT/WT, 140/313 (45%) WT/ACA, and 30/313 (9%) ACA/ACA] and post-menopausal sporadic breast cancer cases [143/307 (47%) WT/WT, 143/307 (47%) WT/ACA, and 21/307 (6%) ACA/ACA] did not deviate from the pre-menopausal control population [149/283 (53%) WT/WT, 112/283 (40%) WT/ACA, and 22/283 (7%) ACA/ACA] or post-menopausal control population [152/319 (48%) WT/WT, 146/319 (46%) WT/ACA, and 21/319 (6%) ACA/ACA] (Figure 14). The relative risk for breast cancer development among pre-menopausal (OR=1.4, p=0.3) or post-menopausal (OR=1.1, p=0.8) cases that are homozygous for the +ACA allele did not significantly deviate from WT/WT pre-menopausal and post-menopausal controls. Likewise, the breast cancer risk among pre-menopausal (OR=1.3, p=0.1) or post-menopausal (OR=1.1, p=0.7) cases that are heterozygous compared to the WT/WT pre-menopausal and post-menopausal controls did not significantly deviate. Hardy-

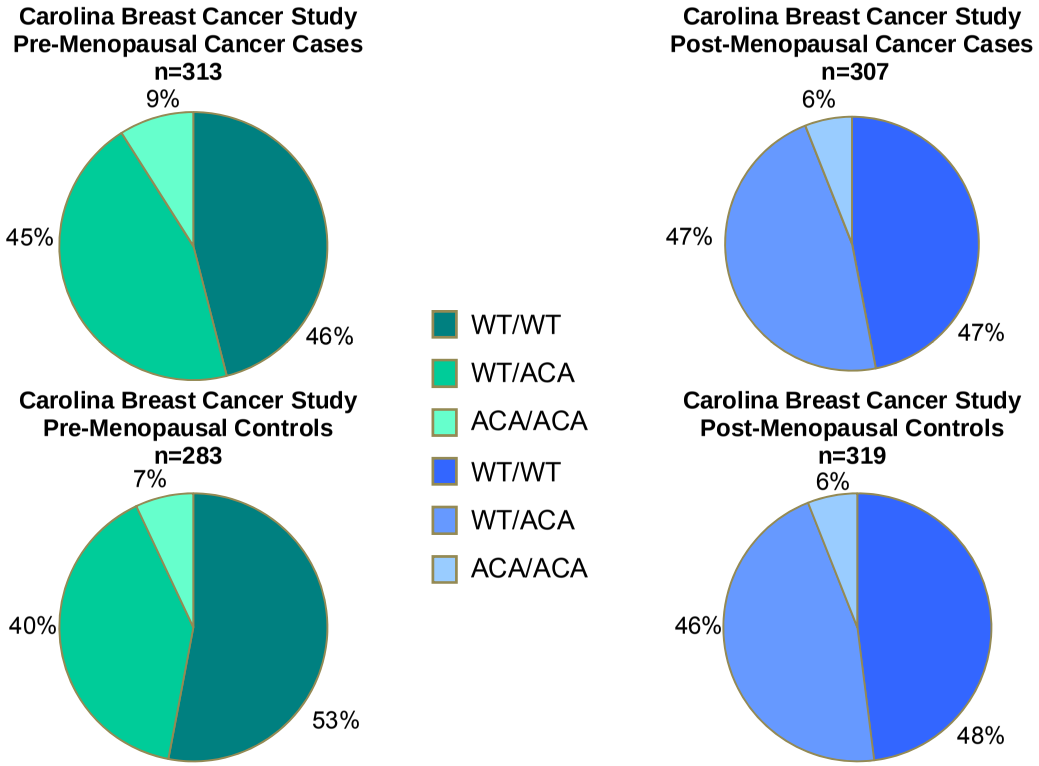

Figure 14. Comparison of the +ACA Genotypic Distribution Between Pre-Menopausal and Post-Menopausal Women from the Carolina Breast Cancer Study (CBCS). Top: Comparison of the frequency of the +ACA *BRCA1* promoter polymorphism in pre-menopausal and post-menopausal women with primary invasive breast cancer. Bottom: Comparison of the frequency of the +ACA *BRCA1* promoter polymorphism in pre-menopausal and post-menopausal age-matched and race-matched female controls.

Weinberg Equilbrilium was not calculated for the pre-menopausal or post-menopausal cases or controls.

***BRCA1* Promoter Genotypic Distribution Comparison of Breast Cancer Molecular Classification.** To determine if the +ACA *BRCA1* promoter polymorphism segregated with any of the molecular subtypes of breast tumors we evaluated the variation in genotypic distribution for the +ACA *BRCA1* promoter polymorphism among luminal A, luminal B, basal, and Her2+ sporadic breast cancer tumors. We did not observe any significant distribution changes between the controls and luminal A [82/179 (46%) WT/WT, 83/179 (46%) WT/ACA, and 14/179 (8%) ACA/ACA], luminal B [26/53 (49%) WT/WT, 25/53 (47%) WT/ACA, and 2/53 (4%) ACA/ACA], basal [37/74 (50%) WT/WT, 31/74 (42%) WT/ACA, and 6/74 (8%) ACA/ACA], and Her2+ [15/24 (62%) WT/WT, 7/24 (30%) WT/ACA, and 2/24 (8%) ACA/ACA] breast tumors (Figure 15). A subset of breast cancers from the CBCS was not classifiable. These unclassified breast tumors were distributed 11/20 (55%) WT/WT, 6/20 (30%) WT/ACA, and 3/20 (15%) ACA/ACA. The relative risk for breast cancer development among the molecular subtypes that are homozygous for the +ACA allele did not significantly deviate from the WT/WT controls [luminal A (OR=1.16, p=0.7), luminal B (OR=0.48, p=0.3), basal (OR=1.63, p=0.3), and Her2+ (OR=1.1, p=0.9)]. Hardy-Weinberg Equilibrium was not calculated for any of the breast cancer molecular subtypes.

FAC1 Binds to the +ACA BRCA1 Promoter

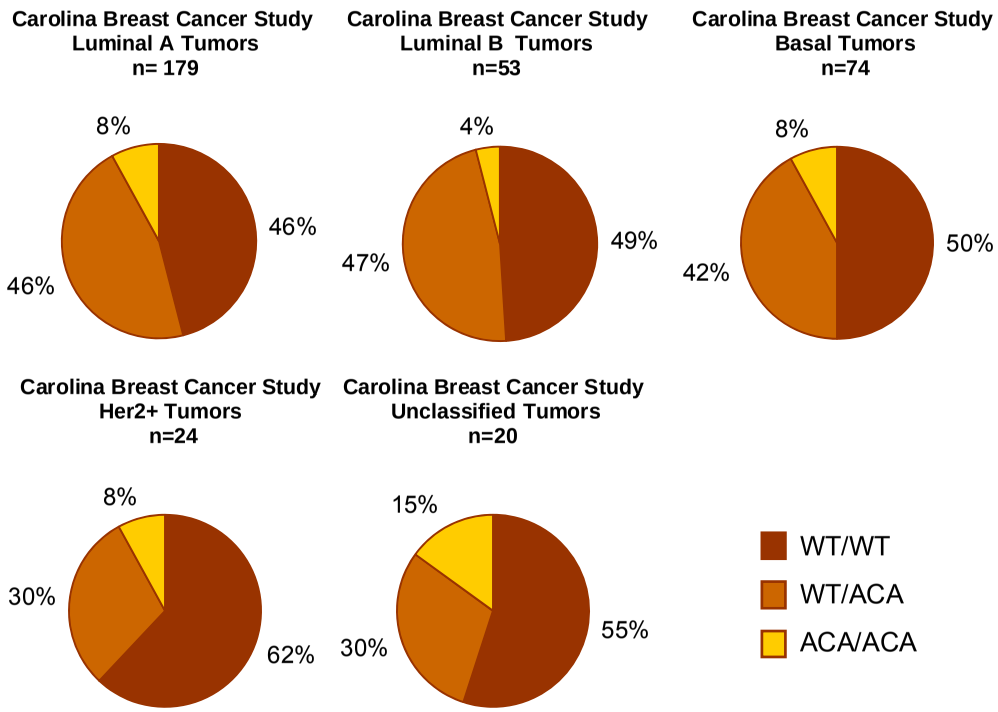

Figure 15. Comparison of *BRCA1* Promoter Distribution Among Molecular Subtypes in the Carolina Breast Cancer Study (CBCS). Comparison of BRCA1 promoter polymorphism genotype in women with primary invasive breast cancer, segregated by molecular subtype (luminal A, luminal B, basal, Her2+, and unclassified).

The +ACA *BRCA1* promoter polymorphism introduces a recognized consensus binding sequence for the fetal ALZ-50 reactive clone 1 (FAC1) transcriptional repressor protein (Figure 16) (Bowser, 1996; Jordan-Sciutto et al, 1999a; Jordan-Sciutto et al, 1999b). The +ACA *BRCA1* promoter polymorphism varies from the FAC1 consensus binding site by one base pair on the 5' end. In collaboration with Dr. Robert Bowser (University of Pittsburgh, Pittsburgh, PA) we have shown that FAC1 binds to the +ACA *BRCA1* promoter sequence (Figure 17). In contrast, FAC1 does not bind the wild-type *BRCA1* promoter sequence with any appreciable affinity. The specific binding of FAC1 to the +ACA *BRCA1* promoter sequence was sensitive to competition using a cold competitor (Figure 17). These results suggest that the +ACA insertion in the *BRCA1* promoter creates a functional and specific FAC1 binding site.

BRCA1 and FAC1 Protein Expression in Breast Cancer Samples

BRCA1 Protein Expression

BRCA1 expression in tumors was scored based on expression in normal breast epithelial cells. The majority of normal breast tissues [7/8, (88%)] stained ≥2+ and staining was primarily localized to the nucleus of the epithelial cells. After the exclusion of tumor and normal breast tissue cores that were negative for the tissue quality control antibody or lacked data for either the BRCA1 or FAC1 antibody, the protein expression analysis included 111/121 luminal A tumor cores, 21/23 basal tumor cores,15/18 Her2+ tumor cores, and 6/9 normal tissues. Examples of BRCA1-positive staining tumors are shown in Figures 18 and 19 (B5-B8). 69%

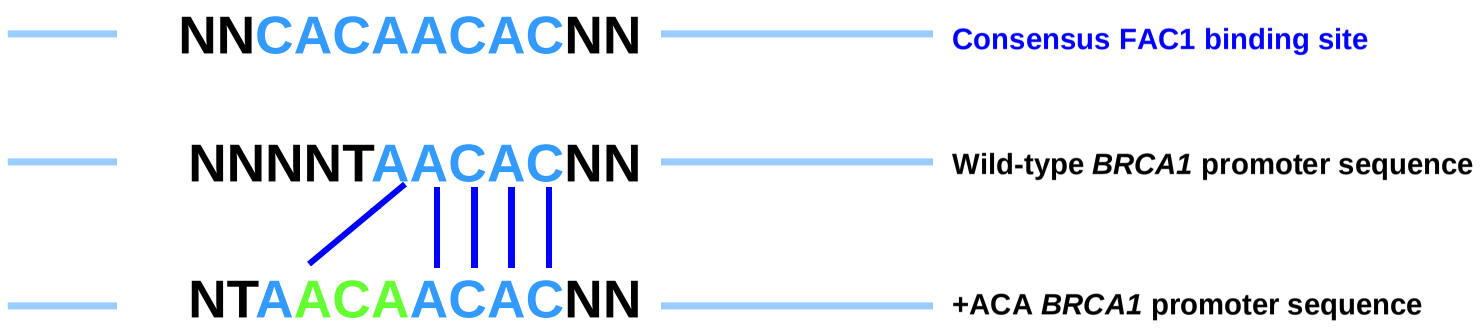

Figure 16. FAC1 Transcriptional Regulator Consensus Binding Site. The alignment of the consensus FAC1 repressor binding site sequence is shown with the putative binding site of the wild-type and +ACA polymorphic *BRCA1* promoter. The +ACA *BRCA1* promoter varies by one base pair on the 5' end. The wild-type *BRCA1* promoter has less homology than the +ACA *BRCA1* promoter with 5/8 base pair matches.

BRCA1 wild-type probe
BRCA1 +ACA probe
Control probe
BRCA1 wt probe
BRCA1 wt FAC1
BRCA1 +ACA probe
BRCA1 +ACA FAC1
FAC binding element control probe
Cold competitor
Cold competitor
Cold competitor

Figure 17. Evidence for FAC1 Binding to the +ACA *BRCA1* Promoter. An electrophoretic mobility shift assay is shown. FAC1 is allowed to bind to either the wild-type or +ACA *BRCA1* promoter probe. To demonstrate specificity, a cold competitor is added in increasing quantity (1 ng, 10 ng, 50 ng) to either of the *BRCA1* promoter probes or the FAC1 control probe. The positive control probe contains a FAC1 binding element. FAC1 binds the +ACA *BRCA1* promoter with greater affinity than the wild-type *BRCA1* promoter as designated by the red arrows. This analysis was provided by Courtney Wilson and Dr. Robert Bowser (University of Pittsburgh, Pittsburgh, PA).

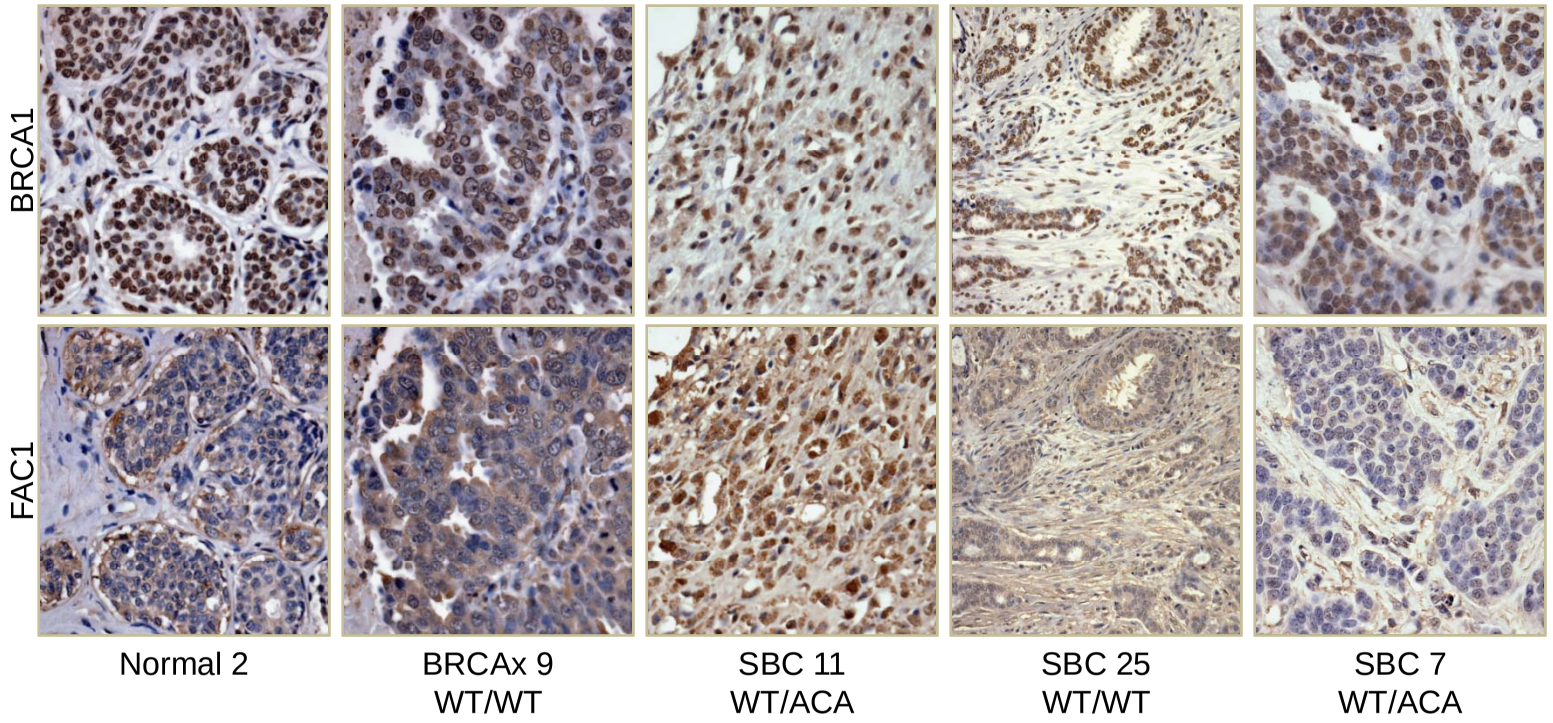

Figure 18. FAC1 Localization Varies Among Breast Tumors with Positive BRCA1 Expression. Representative examples of BRCA1-positive tumors and the diverse localization patterns of FAC1. Normal 2, BRCAx 9, and SBC 11 are examples of BRCA1 positivity with cytoplasmic FAC1 (cFAC1). SBC 25 and SBC 7 are examples of BRCA1-positive tumors with a nuclear FAC1 (nFAC1) pattern. Genotypes are given if known.

B4
F3
B8
F8
B3
F2
B7
F7
B2
F2
B6
F6
B1
F1
B5
F5
BRCA1
FAC1
BRCA1
FAC1

Figure 19. FAC1 Localization Varies Among Sporadic Breast Tumors. Representative examples of BRCA1-positive and BRCA1-negative sporadic breast tumors and the diverse localization patterns of FAC1. B1-B4 are BRCA1-negative/reduced expression tumors and F1-F4 sections are the corresponding matched sections of FAC1 expression. F1 represents an example of a tumor with a nuclear FAC1 staining pattern, F2 represent an example of a tumor with mixed nuclear and cytoplasmic FAC1 expression pattern, F3 represents an example of a tumor with predominantly cytoplasmic FAC1 staining pattern, and F4 represents an example of a tumor with FAC1 negative/reduced expression pattern. B5-B8 are BRCA1-positive stained tumors and F5-F8 are the corresponding matched sections of FAC1 expression. F5 represents an example of a tumor with a nuclear FAC1 staining pattern, F6 represent an example of a tumor with mixed nuclear and cytoplasmic FAC1 expression pattern, F7 represents an example of a tumor with predominantly cytoplasmic FAC1 staining pattern, and F8 represents an example of a tumor with FAC1 negative/reduced expression pattern.

(25/36) of UNC archive tumors and 63% (102/161) DTMA tumors were positive for BRCA1 expression. Examples of negative/reduced staining for BRCA1 protein in tumors are shown in Figures 19 and 20 (B1-B4).

FAC1 Protein Expression

FAC1 expression was categorized by subcellular localization: nuclear (n), cytoplasmic (c), or equal nuclear and cytoplasmic localization (n=c). FAC1 expression was observed in 86% (31/36) of UNC tumors and 85% (137/161) of DTMA tumors. Conversely, the remaining 5/36 (14%) of the UNC archive tumors and 24/161 (15%) of the DTMA tumors were FAC1 negative/reduced. FAC1 expression was observed in 4/8 (50%) normal breast tissue sections. In 3/4 FAC1-positive normal breast tissue sections, the localization of FAC1 was primarily in the cytoplasm, while 1/4 showed equal levels of nuclear and cytoplasmic FAC1. Examples of FAC1 expression in UNC archive tumors are shown in Figures 18 and 20, and selected FAC1 expression examples in DTMA tumors are shown in Figure 19. In 16/36 (44%) of the UNC archive tumors and 19/161 (12%) of the DTMA tumors, FAC1 was localized to the nucleus. In 15/36 (42%) of the UNC archive tumors and 51/161 (32%) of the DTMA tumors FAC1 was primarily localized in the cytoplasm. Additionally, 41% (67/161) of the DTMA tumors were also categorized as equal nuclear and cytoplasmic localization.

Among DTMA sporadic tumors, nFAC1-positivity was observed in 29/59 (49%) of tumors with reduced or negative BRCA1 expression, and 57/102 (56%) of BRCA1-positive tumors. The association of FAC1 localization and BRCA1 expression

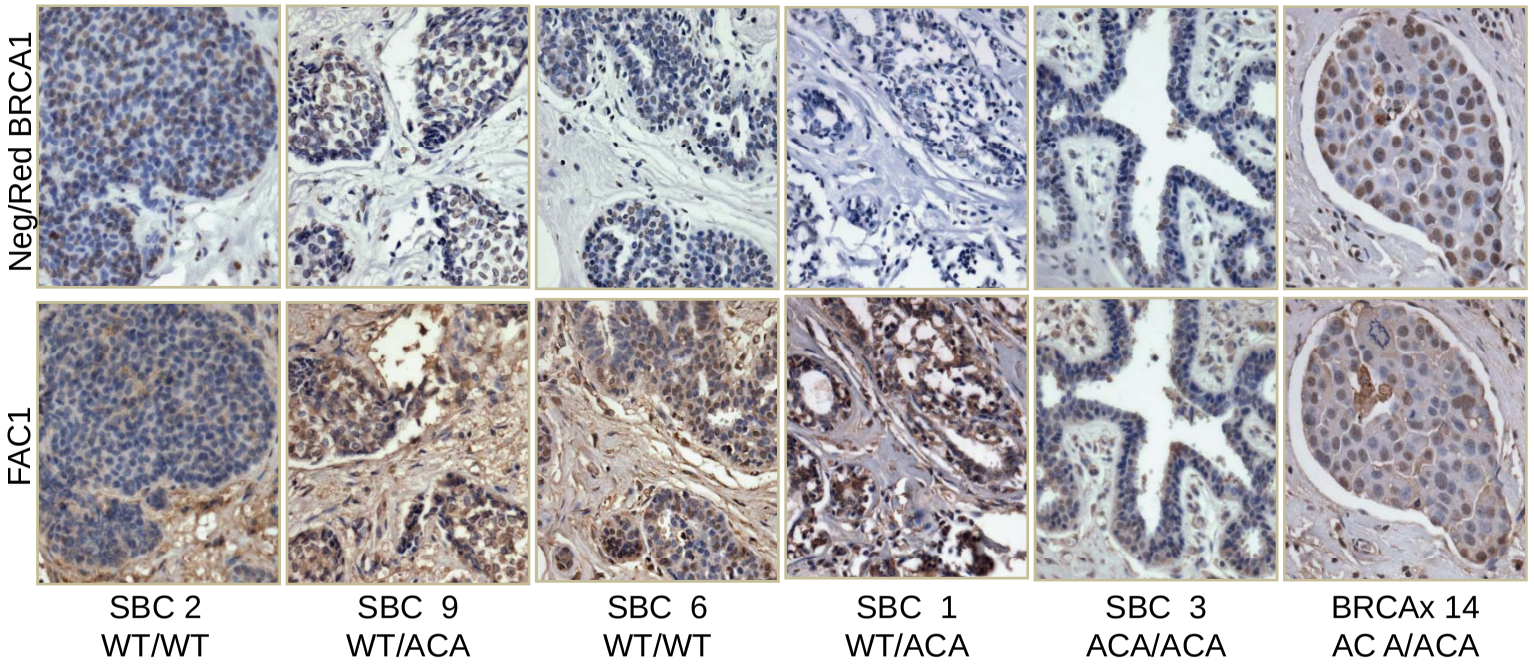

Figure 20. FAC1 Localization Varies Among Breast Tumors with Reduced or Negative BRCA1 Expression. Representative examples of BRCA1-negative/reduced expressing tumors (Neg/Red BRCA1) and the diverse localization patterns of FAC1. SBC 2 and SBC 9 are examples of BRCA1-negative/low expressing tumors with cytoplasmic FAC1 (cFAC1). SBC 6, SBC 1, SBC 3, and BRCAx 14 are examples of BRCA1 negative tumors with a nuclear FAC1 (nFAC1) pattern.

stratified by breast cancer molecular subtype is illustrated in Figure 21. No clear patterns emerged from the analysis of the DTMA tumors in comparing protein expression with molecular breast tumor classification. Summaries of the distribution of FAC1 localization in BRCA1-positive and BRCA1-negative normal breast tissue and DTMA sporadic breast tumors are provided in Tables 9-13.

Correlative Analysis of *BRCA1* Genotype and Protein Expression Status in Breast Tumors

Thirty-one sporadic tumors from UNC (unknown molecular classification) and five BRCAx tumor sections were examined to determine if there is an association between tumor genotype and BRCA1 protein expression. We observed an association in the UNC sporadic tumors between genotype, BRCA1 expression, and localization of FAC1. Of the 16 sporadic breast cancer tumors with strong nFAC1 expression, eight (50%) had low levels of BRCA1 expression. Additionally, of the nine tumors genotyped +ACA/+ACA, 4/9 (44%) expressed normal BRCA1 and 5/9 (56%) expressed low levels of BRCA1, compared to 4/14 (29%) and 2/13 (15%) of the tumors genotype WT/WT and WT/ACA, respectively (Figure 22).

BRCA1 and FAC1 mRNA and Protein Expression in Cell Lines

BRCA1 and *FAC1* Real-time PCR analysis in Breast Cancer Cell Lines

In order to determine if genotype, *BRCA1* expression, and *FAC1* localization are correlated, mRNA from twelve breast cell lines with known genotypes were analyzed for the expression of *BRCA1* and *FAC1* by Real-time PCR. Relative quantitation of

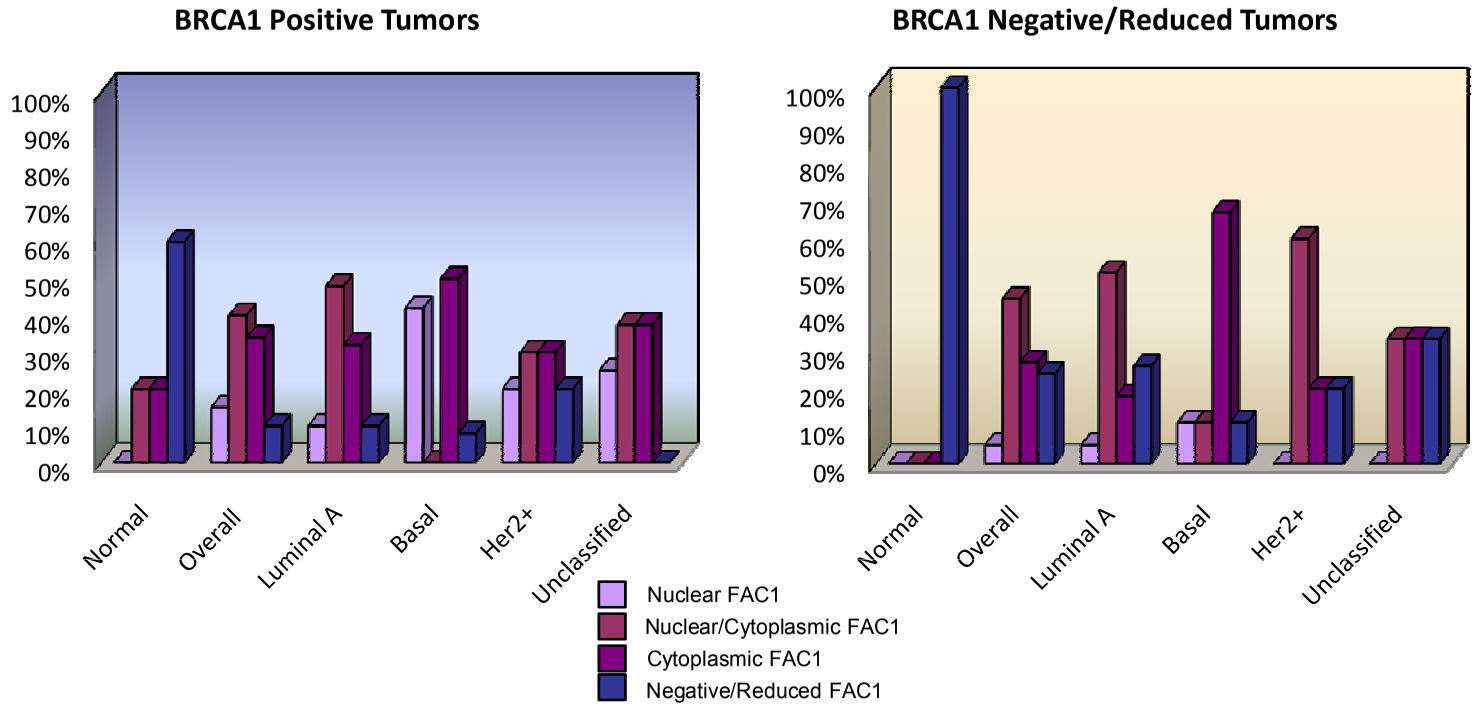

Figure 21. Association of FAC1 Protein Localization and BRCA1 Expression Stratified by Breast Cancer Molecular Subtypes and Normal Breast. Percentage of normal breast and sporadic breast cancers (Dartmouth-Hitchcock Study) in each subtype distributed by FAC1 localization for positively expressing BRCA1 tumors and negative/reduced expressing BRCA1 tumors.

Table 9. Summary of the Distribution of FAC1 Localization in BRCA1-Positive and BRCA1-Negative Normal Breast Tissue from the Dartmouth-Hitchcock Study.

Normal [1] N = 6	BRCA1 negative/reduced N = 1 (16.7%)	BRCA1 positive N = 5 (83.3%)
nFAC1 N=0 (0%)	N = 0 (0%)	N = 0 (0%)
n=c FAC1 N=1 (16.7%)	N = 0 (0%)	N = 1 (20%)
cFAC1 N = 1 (16.7%)	N = 0 (0%)	N = 1 (20%)
FAC1 negative/reduced N = 4 (66.6%)	N = 1 (100%)	N = 3 (60%)

[1] n= nuclear localization, c=cytoplasmic localization, n=c equal nuclear and cytoplasmic localization

Table 10. Summary of the Distribution of FAC1 Localization in BRCA1-Positive and BRCA1-Negative Luminal A Subtype Sporadic Breast Tumors from the Dartmouth-Hitchcock Study.

Luminal A[1] N = 111	BRCA1 negative/reduced N =39 (35%)	BRCA1 positive N = 72 (65%)
nFAC1 N=9 (8%)	N = 2 (5%)	N = 7 (10%)
n=c FAC1 N = 55 (50%)	N = 20 (51%)	N = 35 (48%)
cFAC1 N = 30 (27%)	N = 7 (18%)	N =23 (32%)
FAC1 negative/reduced N = 17 (15%)	N = 10 (26%)	N = 7 (10%)

[1] n= nuclear localization, c=cytoplasmic localization, n=c equal nuclear and cytoplasmic localization

Table 11. Summary of the Distribution of FAC1 Localization in BRCA1-Positive and BRCA1-Negative Basal Subtype Sporadic Breast Tumors from the Dartmouth-Hitchcock Study.

Basal[1] N = 21	**BRCA1 negative/reduced N = 9 (43%)**	**BRCA1 positive N = 12 (57%)**
nFAC1 N=6 (29%)	N = 1 (11.1%)	N = 5 (42%)
n=c FAC1 N=1 (5%)	N = 1 (11.1%)	N = 0 (0%)
cFAC1 N = 12 (56%)	N = 6 (66.7%)	N = 6 (50%)
FAC1 negative/reduced N = 2 (10%)	N = 1 (11.1%)	N = 1 (8%)

[1] n= nuclear localization, c=cytoplasmic localization, n=c equal nuclear and cytoplasmic localization

Table 12. Summary of the Distribution of FAC1 Localization in BRCA1-Positive and BRCA1-Negative Her2 + Subtype Sporadic Breast Tumors from the Dartmouth-Hitchcock Study.

Her2 +[1] N = 15	BRCA1 negative/reduced N = 5 (33.3%)	BRCA1 positive N = 10 (66.7%)
nFAC1 N=2 (13.3%)	N = 0 (0%)	N = 2 (20%)
N = C FAC1 N = 6 (40%)	N = 3 (60%)	N = 3 (30%)
cFAC1 N = 4 (26.7%)	N = 1 (20%)	N = 3 (30%)
FAC1 negative/reduced N = 3 (20%)	N = 1 (20%)	N = 2 (20%)

[1] n= nuclear localization, c=cytoplasmic localization, n=c equal nuclear and cytoplasmic localization

Table 13. Summary of the Distribution of FAC1 Localization in BRCA1-Positive and BRCA1-Negative Sporadic Breast Tumors (combined totals of Luminal A, Basal, Her2+ and Unclassified) from the Dartmouth-Hitchcock Study.

All tumors[1] N = 161	**BRCA1 negative/reduced N = 59 (37%)**	**BRCA1 positive N = 102 (63%)**
nFAC1 N=19 (12%)	N = 3 (5%)	N = 16 (15%)
n=c FAC1 N = 67 (41%)	N = 26 (44%)	N = 41 (40%)
cFAC1 N = 51 (32%)	N = 16 (27%)	N = 35 (34%)
FAC1 negative /reduced N = 24 (15%)	N = 14 (24%)	N = 10 (10%)

[1] n= nuclear localization, c=cytoplasmic localization, n=c equal nuclear and cytoplasmic localization

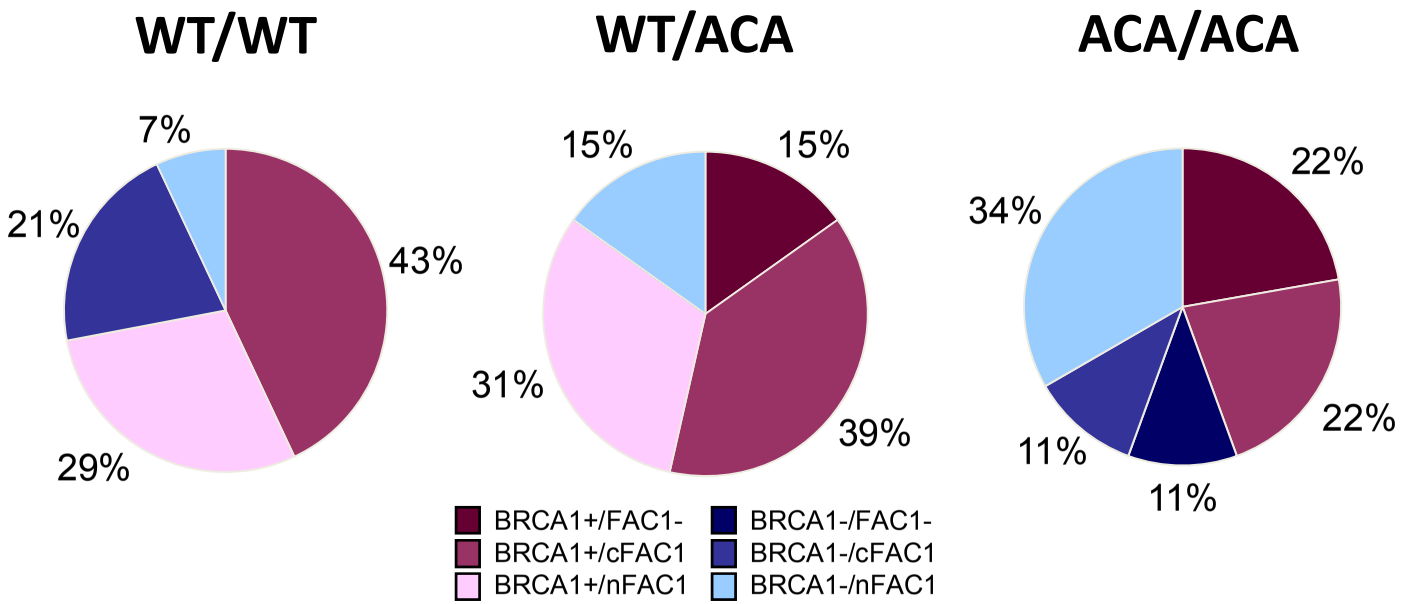

Figure 22. Summary of BRCA1/FAC1 Immunohistological Staining Patterns in Breast Cancer Tissue. UNC breast tumors (n=36) segregated by *BRCA1* promoter genotype (WT/WT, WT/ACA, and ACA/ACA) correlated to BRCA1 expression and FAC1 localization. (BRCA1+ ≥40% staining based on both percentage of tumor cells and intensity of the stain compared to normal breast tissue, BRCA1- = BRCA1 negative/reduced <40% staining based on both percentage of tumor cells and intensity of the stain compared to normal breast tissue, FAC1- = FAC1 negative/reduced <40% based on both percentage of tumor cells and intensity of the stain , nFAC1 = majority of nuclear FAC1 localization with >40% tumor staining, and cFAC1 = majority of cytoplasmic FAC1 localization with >40% tumor staining). Of the sixteen tumors with nFAC1 expression, 50% had BRCA1 negative/reduced expression.

BRCA1 and *FAC1* from the breast cancer cells line mRNA was normalized to the mRNA from MCF12a normal breast cell line, a WT/ACA cell line. Interestingly, 11/12 and 10/12 breast cancer cell lines exhibit *BRCA1* and *FAC1* expression levels that are greater than that observed in MCF12a normal breast epithelial cell line, respectively. Notably, all cell lines having one or more +ACA allele are on the low end of *BRCA1* expression, although none less than MCF12a (Figure 23). MDA-MB-436, an ACA/ACA cell line, had relatively low levels of both *BRCA1* and *FAC1* mRNA expression. SKBR3, an ACA/ACA cell line, had the third highest level of FAC1 mRNA expression and the third lowest levels of *BRCA1* expression. These results support the suggestion that the homozygous +ACA *BRCA1* promoter polymorphism in the presence of increased FAC1 expression could result in a reduction of BRCA1 expression.

BRCA1 Protein Expression

MCF12a cells (WT/ACA) stained positively for BRCA1, which was primarily localized to the nucleus. Among breast cancer cell lines, 14/16 (88%) were positive for nuclear BRCA1 expression (Table 14) suggesting that BRCA1-negative cells are underrepresented compared to the general BRCA1-negative tumor population (31% of the UNC archive tumors and 37% of the DTMA tumors). BRCA1 protein expression results for breast cell lines having one or more +ACA *BRCA1* promoter polymorphic alleles are shown in Figure 24. There were three breast cancer cells lines that have at least one +ACA allele. SUM102 is a heterozygous cell line that stained positively for BRCA1 expression. Two homozygous +ACA cell lines, SKBR3

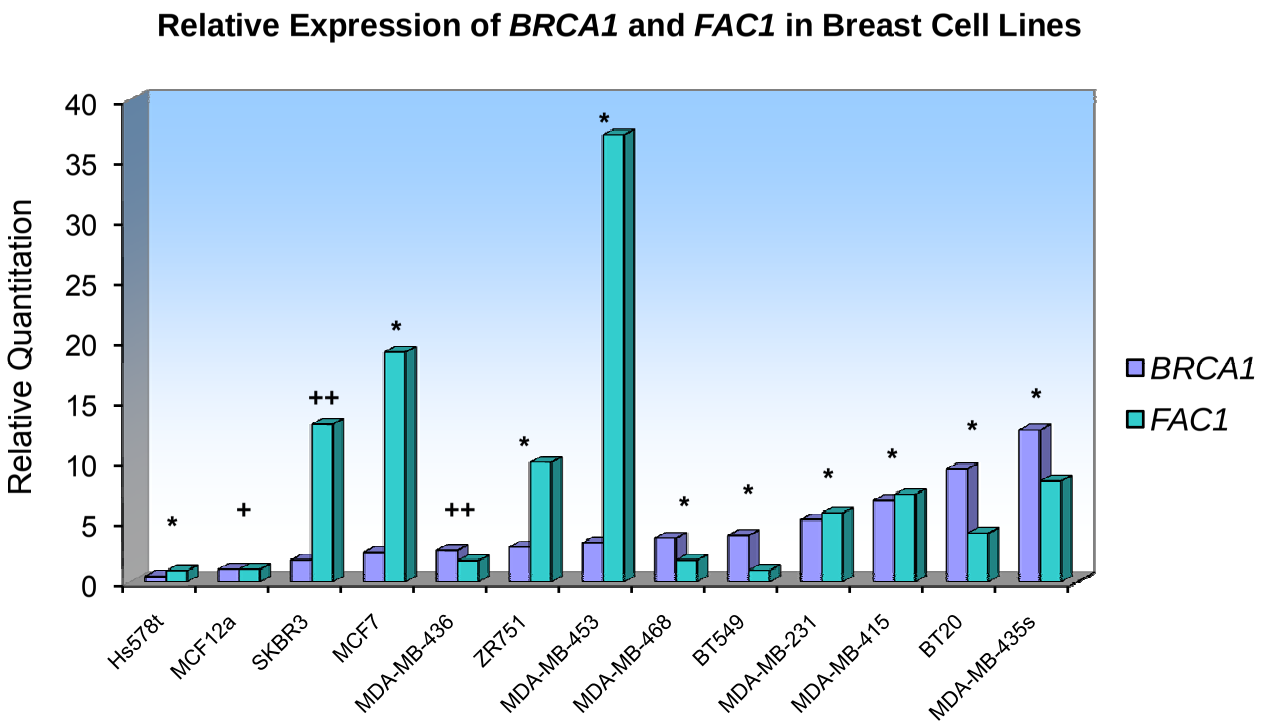

Figure 23. *BRCA1* and *FAC1* Relative Expression in Breast Cancer Cell Lines. *BRCA1* and *FAC1* relative expression shown in increasing levels of *BRCA1* expression. The expression level of each gene was normalized to MCF12a breast epithelial cell line. Genotypes are denoted as the following: *=WT/WT, +=WT/ACA, ++=ACA/ACA

Table 14. Correlation of Genotype, *BRCA1* Expression, and FAC1 Localization in Breast Cancer Cell Lines Normalized to MCF12a.

Cell line	Genotype	*BRCA1* RT-PCR[1]	*FAC1* RT-PCR	*BRCA1* qPCR[2]	*FAC1* qPCR	BRCA1 IHC[3,4]	FAC1 IHC[3,4]
BT20	WT/WT	++	w+	9.3	4.0	POS	N>
BT549	WT/WT	+	+	3.8	0.9	POS	N=C
Hs578t	WT/WT	++	++	0.4	0.9	NEG	N=C
MCF7	WT/WT	+	-	2.4	19.1	POS	N=C
MDA-MB-231	WT/WT	++	-	5.1	5.7	POS	C>
MDA-MB-415	WT/WT	++	-	6.7	7.2	POS	N=C
MDA-MB-435s	WT/WT	++	+	12.6	8.3	POS	N=C
MDA-MB-436	ACA/ACA	w+	w+	2.6	1.7	POS	N=C
MDA-MB-453	WT/WT	+	+	3.2	37.1	POS	N>
MDA-MB-468	WT/WT	++	+	3.6	1.8	POS	N=C
SKBR3	ACA/ACA	++	w+	1.8	13.0	POS	C>
UACC812	WT/WT	+	-	NT[5]	NT	NT	NT
ZR751	WT/WT	++	+	2.8	9.9	POS	C>
SUM102	WT/ACA	NT	NT	NT	NT	POS	C>
SUM149	WT/WT	NT	NT	NT	NT	POS	N=C
SUM185	WT/WT	NT	NT	NT	NT	POS	N>
HCC1937	WT/WT	NT	NT	NT	NT	NEG	C>
MCF12a[6] (Normal)	WT/ACA	++	++	1.0	1.0	POS	C>

[1] RT-PCR = reverse transcriptase PCR
[2] qPCR = real-time PCR
[3] IHC = Immmunohistochemisty
[4] BRCA1 POS > 40% staining, NEG <40% staining; FAC1 N> =staining is predominantly nuclear; C>= staining is predominantly cytoplasmic; and N= C, staining localization is both nuclear and cytoplasmic
[5] NT = not tested
[6] Normal breast epithelial cells

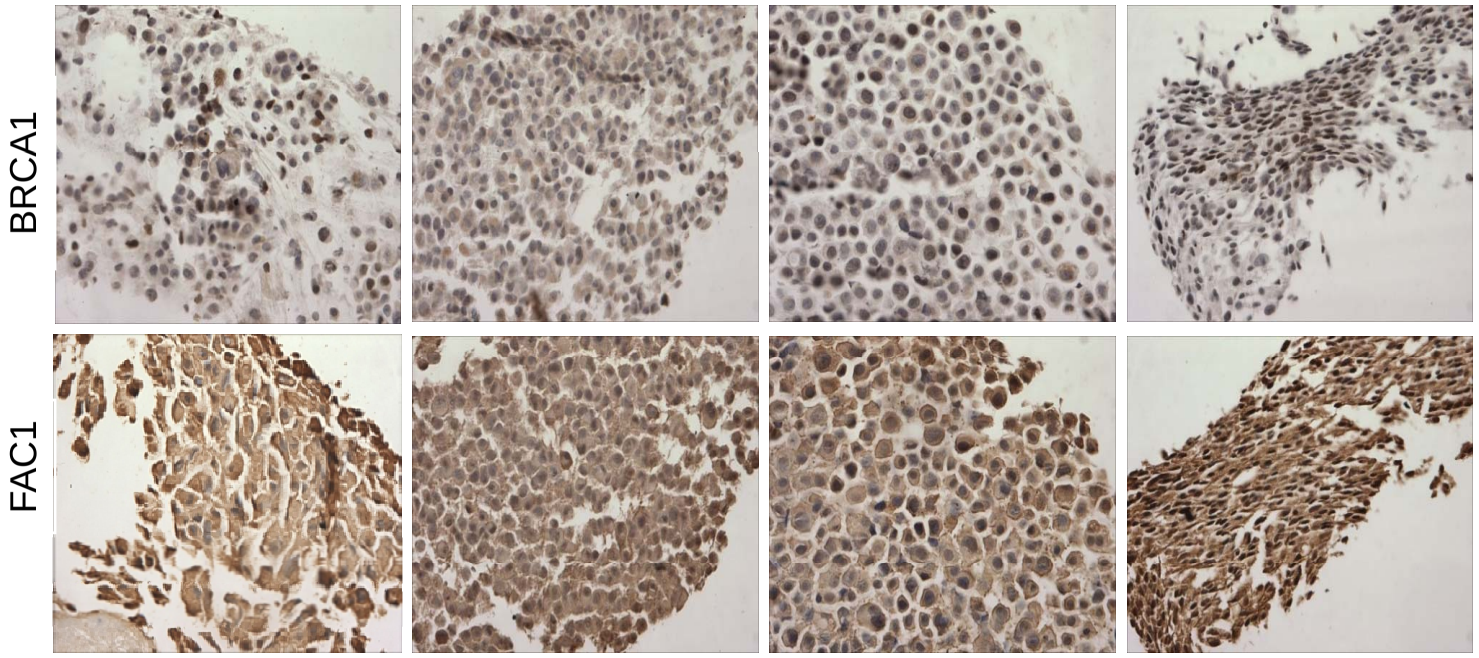

Figure 24. BRCA1 and FAC1 Protein Expression in Breast Cell Lines Having One or More +ACA *BRCA1* Promoter Polymorphic Alleles. Of the seventeen breast cell lines analyzed, 4 cell lines have one or more +ACA alleles. MCF12a is a normal breast epithelial cell line, with positive BRCA1 expression and cytoplasmic FAC1 (cFAC1) expression. SUM102 and SKBR3 have positive BRCA1 expression and cFAC1 expression. MDA-MB-436 has positive BRCA1 expression and mixed nuclear and cytoplasmic FAC1 expression.

and MDA-MB-436, were also positive for BRCA1 expression. The two cells lines that were BRCA1-negative, Hs578t and HCC1937, were both WT/WT genotype.

FAC1 Protein Expression

MCF12a cells were positive for cytoplasmic FAC1 expression. 16/16 (100%) of the breast cancer cells lines stained positively for FAC1 (Table 14). Images of BRCA1 and FAC1 protein expression in breast cell lines having one of more +ACA allele are shown in Figure 24. SUM102 and SKBR3 were positive for cytoplasmic FAC1 expression, while MDA-MB-436 displayed equal nuclear and cytoplasmic FAC1 distribution (Figure 24). Overall the FAC1 localization for the breast cell lines (n=17) was distributed: 3 nFAC1, 8 n=c FAC1, 6 cFAC1.

Correlative Analysis of *BRCA1* Promoter Genotype and Protein Expression Status in Breast Cancer Cell Lines

The three breast cancer cell lines containing a +ACA allele were all positive for BRCA1 expression as analyzed by immunohistochemistry, real-time PCR, and RT-PCR. However, in each of these cancer cell lines, FAC1 expression was either relatively low or localization was in the cytoplasm (Table 14). 18% of the breast cell lines have one or more +ACA allele. Unfortunately, this suggests the +ACA allele is underrepresented in the cell lines we have analyzed.

Functional Analysis

FAC1 mRNA Analysis in Transfected NIH-3T3 Cell Lines

NIH-3T3 cells do not express detectable levels of *FAC1* mRNA (Jordan-Sciuttoet al, 1999b). To verify the successful transfection of NIH3T3 cells with the *FAC1* expression vector, all transfected cell lines were analyzed for *FAC1* mRNA expression by RT-PCR (Figure 25). NIH-3T3 cells transfected with luciferase vectors show little to no detectable *FAC1* mRNA expression. This result is consistent with previous reports that NIH-3T3 cells do not express *FAC1*. Hs578t breast cancer cell line previously had detectable FAC1 by RT-PCR. Therefore, we utilized Hs578t cells as a positive control for RT-PCR analysis of *FAC1* mRNA (Figure 25). A RT-PCR using primers for GAPDH amplification was used for a quality and loading control for the NIH-3T3 transfected cell lines (Figure 25).

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Wild-type and +ACA *BRCA1* Luciferase Promoter Activity in the Absence of Exogenous FAC1

We utilized a cell culture model system to examine basal levels of transcriptional activity from wild-type or +ACA *BRCA1* promoter sequences in NIH-3T3 cell lines. The NIH-3T3 mouse fibroblast cell line was selected because it has been reported to be FAC1-negative (Jordan-Sciuttoet al, 1999b). NIH-3T3 cells and NIH-3T3 cells containing control promoter-less luciferase vector had very low levels of luciferase activity, demonstrating that the vector itself was not generating luciferase protein in the absence of a functional promoter (Figures 26 and 27**).** Both the wild-type and +ACA *BRCA1* promoter-driven luciferase constructs demonstrated transcriptional activity in the NIH-3T3 cells. The wild-type *BRCA1* promoter-driven luciferase construct produced 64,448 ± 9,125 units of luciferase activity (relative light units),

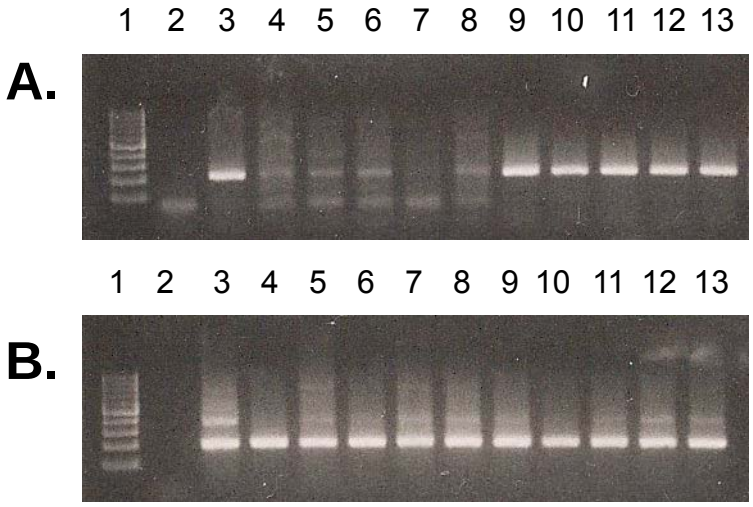
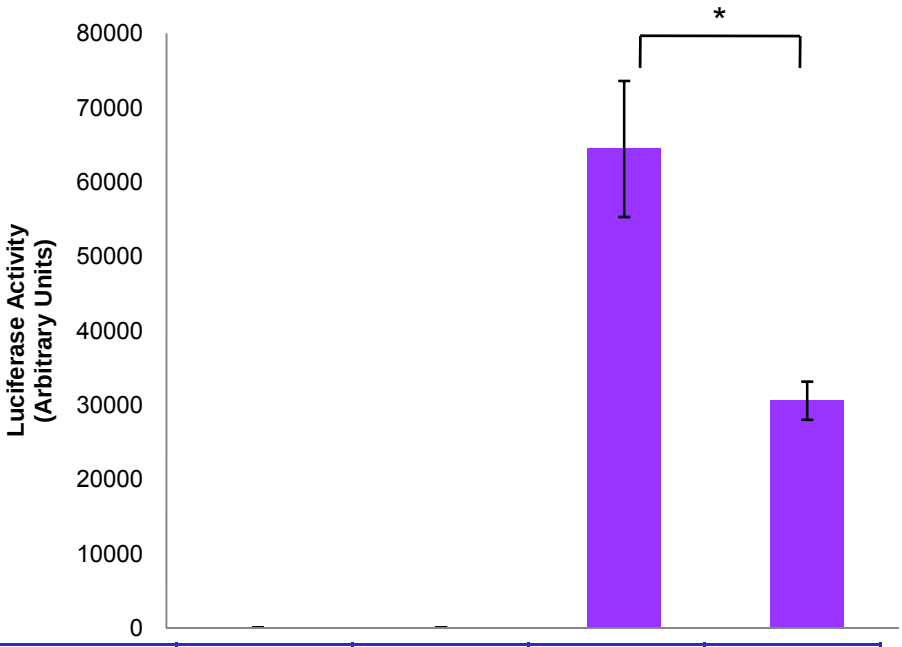
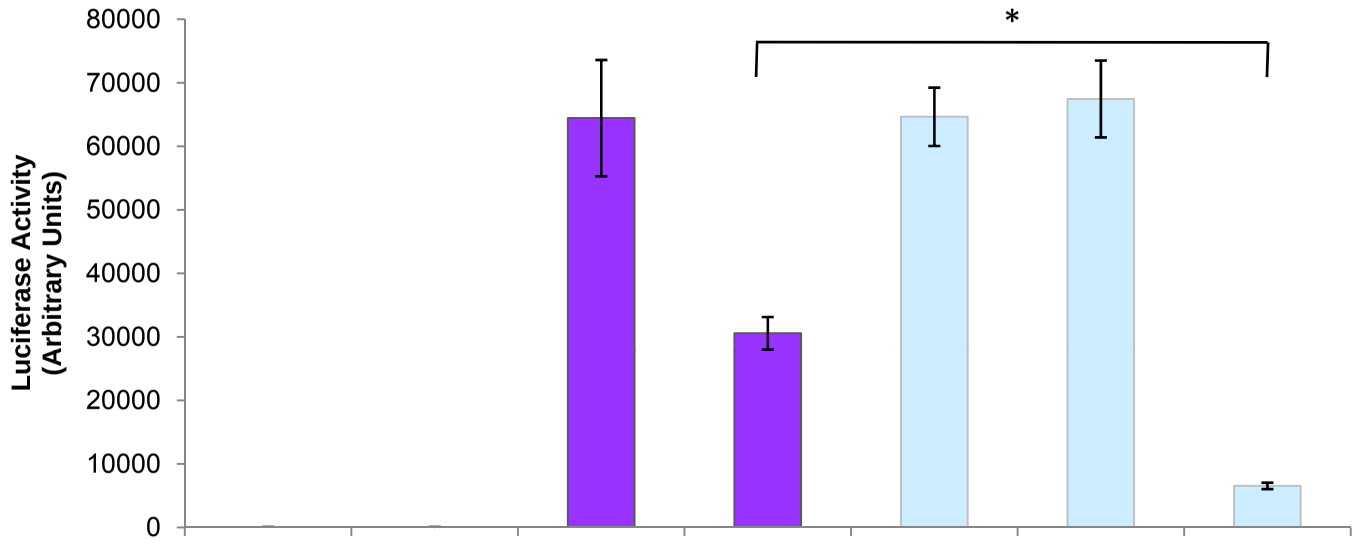


Figure 25. *FAC1* mRNA Expression in Transfected NIH-3T3 Cells. A. RT-PCR for *FAC1* mRNA expression in NIH-3T3 transfected cell lines. B. Control RT-PCR for *GAPDH* mRNA expression in NIH-3T3 transfected cell lines. Lane 1, Molecular size ladder (100 bp-1 kb range, 100 bp increments); Lane 2, No DNA template PCR control, Lane 3, *FAC1*-positive control (Hs578t breast cancer cell line); Lane 4, NIH-3T3 cells; Lane 5, NIH-3T3 promoter-less luciferase vector cells; Lane 6, NIH-3T3 wild-type *BRCA1* luciferase vector cells; Lane 7, NIH-3T3 +ACA *BRCA1* luciferase vector cells; Lane 8, NIH-3T3 empty expression vector cells; Lane 9, NIH-3T3 empty luciferase vector with *FAC1* expression vector cells; Lanes 10-12, NIH-3T3 wild-type *BRCA1* luciferase vector with *FAC1* expression vector-clone 1, clone 2, clone 3 cells; Lane 13, NIH-3T3 +ACA *BRCA1* luciferase vector with *FAC1* expression vector cells.



NIH-3T3 Cells and	1	2	3	4
pGL4.17 Control Luciferase Construct	-	+	-	-
WT Luciferase Construct	-	-	+	-
+ACA Luciferase Construct	-	-	-	+

Figure 26. Transcriptional Activity of Wild-type and +ACA *BRCA1* Promoter-Driven Luciferase Reporter Constructs in NIH-3T3 Cell Lines (FAC1 Negative). Averaged luciferase activity of untransfected NIH-3T3 cells, NIH-3T3 cells containing control pGL4.17 luciferase construct, NIH-3T3 cells containing the wild-type and +ACA *BRCA1* promoters (n = 12 for each cell type). There is a 53% reduction of transcriptional activity in the +ACA *BRCA1* promoter compared to the WT promoter (*P = 0.004). Error bars show standard error of the mean.



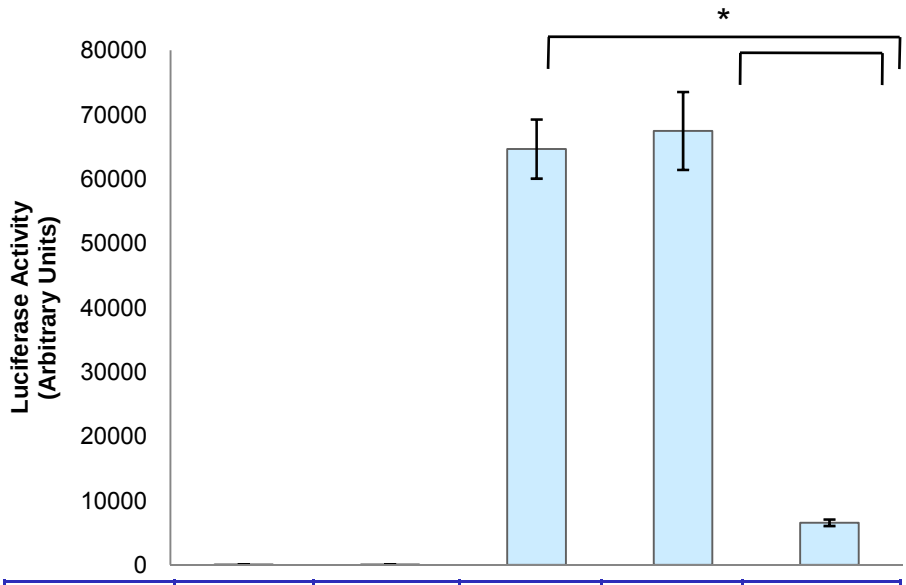
NIH-3T3 Cells and	1	2	3	4	5	6	7
pGL4.17 Empty Luciferase Construct	-	+	-	-	-	-	-
WT Luciferase Construct	-	-	+	-	+	+	-
ACA Luciferase Construct	-	-	-	+	-	-	+
FAC1 Expression Construct	-	-	-	-	+	+	+

Figure 27. Transcription Activity of NIH-3T3 fibroblast Cells Containing Wild-type and +ACA *BRCA1* Promoter-Driven Luciferase Reporter Constructs in the Absence and Presence of FAC1 Expression Construct. Averaged luciferase activity levels for the NIH-3T3 cells containing wild-type *BRCA1* promoter luciferase construct with FAC1 expression construct clones 2 and 3 do not significantly differ from those of NIH-3T3 cells containing with wild-type *BRCA1* promoter luciferase construct alone (n = 12 for each cell type). The NIH-3T3 cells containing +ACA *BRCA1* promoter luciferase construct with FAC1 expression construct luciferase activity is 79% reduced from NIH-3T3 cells containing +ACA *BRCA1* promoter luciferase construct alone (*P < 0.0001). Error bars show standard error of the mean.

and the +ACA *BRCA1* promoter-driven luciferase construct produced 30,567 ± 2,568 units. Notably, the +ACA *BRCA1* promoter-driven luciferase construct expressed a significantly reduced level of transcriptional activity (53% reduction, p=0.004) compared to the wild-type *BRCA1* promoter-driven luciferase construct (Figures 26 and 27). These results suggest that the basal transcriptional activity of the +ACA *BRCA1* promoter allele is approximately half that observed with the wild-type *BRCA1* promoter allele.

Wild-type and +ACA *BRCA1* Luciferase Promoter Activity in the Presence of Exogenous FAC1

We utilized the NIH-3T3 *BRCA1* promoter-driven luciferase construct cell lines, to examine the effects of exogenous FAC1 protein expression on the wild-type and the +ACA *BRCA1* promoter sequence. There was no significant difference in the luciferase activity between either the wild-type *BRCA1* promoter-driven luciferase construct with FAC1 expression vector clones producing 64,655 ±4,592 and 67,466 ± 6,057 relative light units, respectively. Likewise, there was no significant difference from the wild-type *BRCA1* promoter-driven luciferase construct cell line compared to the wild-type *BRCA1* promoter-driven luciferase construct with FAC1 expression vector (Figures 27 and 28). Strikingly, we observed a 79% and 90% reduction of luciferase activity ($p<0.0001$ for both) in NIH-3T3 cells containing the +ACA *BRCA1* promoter-driven luciferase and FAC1 expression vectors compared to the transcriptional activity levels in +ACA and wild-type *BRCA1* promoter-driven luciferase constructs, respectively (Figures 27 and 28). These results suggest that



NIH-3T3 Cells and	1	2	3	4	5
pGL4.17 Empty Luciferase Construct	-	+	-	-	-
WT Luciferase Construct	-	-	+	+	-
+ACA Luciferase Construct	-	-	-	-	+
FAC1 Expression Construct	-	-	+	+	+

Figure 28. Transcriptional Activity of Wild-type and +ACA *BRCA1* Promoter-Driven Luciferase Reporter Constructs in NIH-3T3 Cell Lines Co-Transfected with FAC1 Expression Construct. Averaged luciferase activity of untransfected NIH-3T3 cells, NIH-3T3 cells containing control pGL4.17 luciferase construct, NIH-3T3 cells containing the wild-type *BRCA1* promoter with the FAC1 expression construct clone 2, NIH-3T3 cells containing wild-type *BRCA1* promoter with the FAC1 expression construct clone 3, and NIH-3T3 cells containing the +ACA *BRCA1* promoter with the FAC1 expression construct (n = 12 for each cell type). The +ACA *BRCA1* promoter with FAC1 compared to both wild-type *BRCA1* promoter with FAC1 clone 2 and 3 has statistically significant reduction of transcriptional activity (*P < 0.0001). Error bars show standard error of the mean.

the basal transcriptional activity of the +ACA *BRCA1* promoter allele in the presence of FAC1 is functioning at 10% capacity the wild-type *BRCA1* promoter allele. These results strongly suggest that FAC1 can silence the +ACA *BRCA1* promoter allele.

Localization and Expression of FAC1 in *BRCA1* Luciferase Promoter NIH-3T3 Cell Lines Co-Transfected with FAC1 Expression Vector

We performed an immunohistochemical analysis to observe the presence and localization of FAC1 protein in the NIH-3T3 cell lines co-transfected with expression vectors for FAC1 and luciferase reporter constructs. NIH-3T3 cells containing the FAC1 expression vector expressed nFAC1 very intensely and displayed increased levels of cFAC1. We observed negligible levels of cFAC1 staining in the control NIH-3T3 cells. Additionally, the sections that lacked primary antibody, which controlled for nonspecific background staining (negative control) was negative (Figure 29).

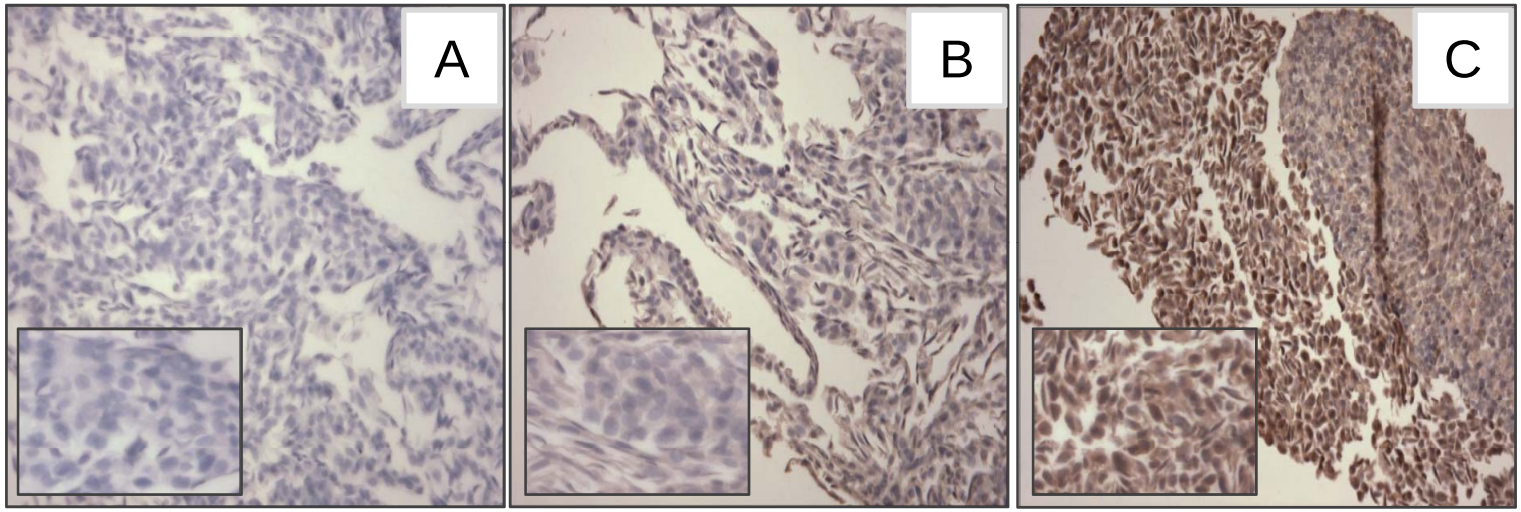


Figure 29. FAC1 Protein Expression in NIH-3T3 Mouse Fibroblast Cell Lines Transfected with FAC1-Expression Vector. A. NIH-3T3 cells lacking primary FAC1 antibody exposure was used as the negative staining reference. **B.** NIH-3T3 cells with FAC1 antibody showing a negligible amount of cytoplasmic FAC1 staining. **C.** NIH-3T3 cells co-transfected with *BRCA1* promoter-driven luciferase vector and *FAC1* expression vector confirm that the FAC1 expression vector is generating FAC1 protein and that it demonstrates intense nuclear and moderate cytoplasmic staining.

DISCUSSION

Summary of Findings

We identified a +ACA insertional polymorphism in the *BRCA1* promoter. This +ACA insertion is located -600bp from the *BRCA1* exon1a transcriptional start site. The +ACA insertion creates a consensus binding site (AACAACAC) for the transcriptional repressor, FAC1. The frequency of the +ACA allele was analyzed in 1760 DNA samples from the general population and breast disease patients. African-American cases had a significantly higher allelic frequency of the +ACA *BRCA1* promoter (27%) compared to African-American controls (17%, P=0.0005), No significant difference were observed between Caucasian cases and controls (34% versus 37%, P=0.50). Statistically significant reduction in functional activity in the +ACA polymorphic promoter in both the absence and presence of exogeneous FAC1 was observed compared to the wild-type *BRCA1* promoter. These results suggest that +ACA *BRCA1* promoter is susceptible to FAC1 transcriptional repression.

Mechanism for Loss of BRCA1 and Breast Cancer

Hereditary breast cancer makes up approximately 10% of all breast cancer and the remaining 90% are classified as sporadic. Loss of BRCA1 expression occurs in more than half of the hereditary breast cancer cases and in approximately 30% of sporadic breast cancers (Couch and Weber, 1998; Hedenfalk et al, 2001;

Hedenfalk et al, 2003; Lacroix and Leclercq, 2005; Thompson et al, 1995; Wilson et al, 1999; Yoshikawa et al, 1999). Germline mutations in *BRCA1* account for 40-50% of the BRCA1 expression loss in hereditary breast cancer (Couch, and Weber, 1998). The majority of *BRCA1* coding region genetic errors create frameshift or nonsense mutations that result in an absent or trunctated protein in 87% of cases. On the other hand, very few *BRCA1* mutations have been detected in sporadic breast cancer (Catteau and Morris, 2002; Dobrovic and Simpfendorfer, 1997; Futreal et al, 1994; Khoo et al, 1999; Merajver et al, 1995; Uhrhammer et al, 2008; van der Looij et al, 2000). However, *BRCA1* frameshift mutations and deletions have been reported to contribute to loss of *BRCA1* expression in both hereditary and sporadic breast cancers (Catteau and Morris, 2002; Couch and Weber, 1998). *BRCA1* loss of heterogosity (LOH) occurs as a result of a deletion of a portion of chromosome 17 that generates a loss of the wild-type allele, secondary to the other allele having already been inactivated (Couch and Weber, 1998). LOH has been observed and contributes to loss of *BRCA1* both hereditary and sporadic breast cancer. *BRCA1* methylation gene-silencing occurs in 10-30% of sporadic breast cancers (Catteau and Morris, 2002). Until recently, few studies had investigated hypermethylation of *BRCA1* in hereditary breast tumors. However, Tapia *et al* reported hypermethylation in hereditary breast cancer with a correlative loss of BRCA1 expression. These results suggest that hypermethylation of *BRCA1* could be contributing to the second *BRCA1* allelic loss in hereditary breast cancers and the loss of one or both *BRCA1* alleles in sporadic breast cancers (Tapia et al, 2008).

BRCA1 expression is lost in approximately 35% of all breast cancers (~5% in hereditary and ~30% in sporadic). In a recent study BRCA1 expression was examined in 1,940 consecutive cases of invasive breast tumors that were collected from 1986-1998. 15% of these tumors had loss of nuclear *BRCA1* expression, while an additional 37% exhibited cytoplasmic *BRCA1* localization. This observation supports that negative, reduced or aberrantly localized *BRCA1* expression may be contributing to the genesis of 52% of breast tumors (Rakha et al, 2008). Rakha *et al.* reported that alteration in BRCA1 localization (absence, reduction or cytoplasmic translocation) was found to be associated a shorten interval until recurrence. cBRCA1 expression correlated with recurrence of breast cancer, and a decrease in survival, most specifically in patients with low-grade, small in size, and ER+ tumors (Rakha et al, 2008). A portion, perhaps even as high as 17%, of breast cancers with loss of BRCA1 expression do not have clear mechanism to account for the loss of BRCA1 expression. The evidence to date suggests that genetic variants (mutation or LOH) do not account for these cases. Thus, it is likely that some alternative mechanism may govern loss of *BRCA1* in this subset of patients. We have suggested that FAC1-mediated silencing of the +ACA *BRCA1* promoter may account for some of these patients. In fact, the +ACA *BRCA1* promoter polymorphism produces lower than normal levels of *BRCA1* expression even in the absence of FAC1. The reduced activity of the +ACA *BRCA1* promoter appears to represent an interesting characteristic of this sequence. This may be especially true for the homozygous +ACA individual, who may have BRCA1 expression that is functionally equivalent to an individual with a *BRCA1* allele loss. Strikingly, in an *in*

vitro model, the +ACA *BRCA1* promoter polymorphism in the presence of exogeneous FAC1, had greatly reduced functional levels of the *BRCA1* promoter compared to the wild-type *BRCA1* promoter in both the absence and presence of exogeneous FAC1. This result strongly suggests that a homozygous +ACA individual in the presence of FAC1 would exhibit negligible levels of *BRCA1* expression and very low levels of BRCA1 protein. Such an individual may be phenotypically similar to a patient with two affected (mutated) alleles of *BRCA1*.

BRCA1 Promoter Polymorphisms

To date, few *BRCA1* promoter polymorphisms have been reported or investigated. An evaluation of a C to G base pair substitution polymorphism in the *BRCA1* promoter located at nucleotide 1802 based upon sequence of GenBank accession number U37574 (Catteau et al, 1999; Xu et al, 1995). This study found the allelic frequency of the G to be 35% (101/292) (Catteau et al, 1999). However, no correlation between the C/G *BRCA1* promoter polymorphism and decreased *BRCA1* expression was observed (Catteau et al, 1999). Notably, the C/G (heterozygote) closely associates with another mutation Pro871Leu, that does not confer increase breast or ovarian cancer risk, but allowed for its use as a deletion screening tool (Catteau et al, 1999).

More recently, Chan et al. reported four *BRCA1* promoter polymorphisms [c.-2804T>C, c.-2265C>T, c.-2004A>G, and c.-1896(ACA1)/(ACA2)], located at -1508, -969, -708, and -600 bp from the *BRCA1* transcriptional exon1a start site, respectively. c.-1896(ACA1)/(ACA2) represents the same +ACA three base pair

insert that we report in this thesis and their study confirms its presence in Chinese women (Chan et al, 2008). The genotypic frequency for the c.-1896(ACA1)/(ACA2) among 375 Hong Kong Chinese women with breast cancer was [126/375 (34%) WT/WT, 185/375 (49%) WT/ACA, and 64/375 (17%) ACA/ACA], suggesting the genotypic distribution in Chinese women, differs from the distribution we have observed in both Caucasian and African-American cases and controls (Chan et al, 2008). However, their study focused on the c.-2265C>T SNP, in the context of haplotypes with the other 3 polymorphisms. Functional studies were performed using constructs with the 4 polymorphism haplotype promoter model (Chan et al, 2008). Haplotypes containing the +ACA allele had higher *BRCA1* promoter-driven luciferase activity than haplotypes containing the wild-type *BRCA1* promoter. Unfortunately, it is difficult to compare these two studies functional activity results, because the lengths of the promoter are so variable. Many promoter-related regulators could be altered by the addition of promoter sequence. Interestingly, the haplotypes that are present in Chinese women may be unique or occur at a different frequency in the Chinese population. Further studies evaluating the presence, prevalence, and function of these haplotypes in Caucasian and African-American women would be interesting to perform. Notably, Chan *et al.* suggested that the c.-2265C>T SNP to confer a decrease associated risk for breast cancer in Chinese women, while the other three polymorphisms weren't fully evaluated (Chan et al, 2008).

We have investigated a novel +ACA *BRCA1* promoter polymorphism, which has recently been confirmed by Chan *et al.* (Chanet al, 2008). Overall, the +ACA allele

occurred with high prevalence in the general population and we observed 931/1760 (53%) individuals that had one or more +ACA allele. Strikingly, we observed a significantly higher genotypic and allelic frequency of the +ACA *BRCA1* promoter in African-American cases compared to the African-American controls. This is interesting, since the African-American women are the demographic that is more frequently diagnosed with the basal subtype breast cancer which is often associated with loss of *BRCA1* expression. We hypothesize that the +ACA *BRCA1* promoter polymorphism could negatively affect BRCA1 expression, contributing to loss of BRCA1 function and breast cancer induction. However, due to the high frequency in the general population we do not expect that it would inactivate promoter function, but rather that it renders the *BRCA1* promoter susceptible to *cis*-acting elements that could lead to changes in transcriptional regulation. The +ACA *BRCA1* promoter polymorphism that we have identified may contribute to inactivation of the *BRCA1* gene and loss of BRCA1 protein function in several different ways. It is possible that the +ACA insertion into the *BRCA1* promoter has created or deleted a transcriptional regulator binding site. A deletion of a transcriptional activator or the creation of a transcriptional repressor binding site would directly inhibit transcription. Additionally, if the transcriptional regulator directs methylation machinery, it is plausible that aberrant promoter methylation could prevent transcription, indirectly.

Inactivation of the +ACA BRCA1 Promoter Through FAC1-Mediated Transcriptional Repression in Human Breast Cancer

The +ACA *BRCA1* promoter polymorphism creates a binding site for the fetal ALZ-50 reactive clone 1 (FAC1) transcriptional repressor protein (Bowser, 1996; Jordan-Sciutto et al, 1999a; Jordan-Sciutto et al, 1999b). In studies aimed at identifying the FAC1 binding element, a GST-FAC1 pull down assay was utilized (Jordan-Sciutto et al, 1999b). The majority of the sequences that were analyzed contained an AACA core and revealed that the CACAACAC sequence was the consensus site. While occasionally there was a single bp change, overall there was no more than three nonconsecutive single bp changes (Jordan-Sciutto et al, 1999b). *FAC1* is a member of the PHD/LAP zinc finger family and its nuclear expression has been observed in the developing fetal brain (Jordan-Sciutto et al, 1999b). There is a translocation of FAC1 to the cytoplasm in the healthy adult brain, but in Alzeheimer's progression, FAC1 gets localized to dystrophic neurites and neuritic components of the β-amyloid plaques (Jordan-Sciutto et al, 1999b). In patients that have both a wild-type and +ACA *BRCA1* promoter alleles, the loss of expression of the +ACA *BRCA1* allele from FAC1 repression increases the risk of breast carcinogenesis. A breast tumorigenesis prone status would result from loss of function from the remaining wild-type allele (through mutation, deletion, or methylation). Likewise, in homozygous +ACA *BRCA1* polymorphic patients, both *BRCA1* alleles would be sensitive to FAC1-mediated repression. Thus, in the presence of nuclear FAC1 expression (or overexpression), the homozygous patient may be rendered functionally BRCA1-negative (Figure 30). Additionally, the polymorphic allele may be more susceptible to methylation silencing. These observations suggest a putative novel mechanism for *BRCA1* gene silencing.

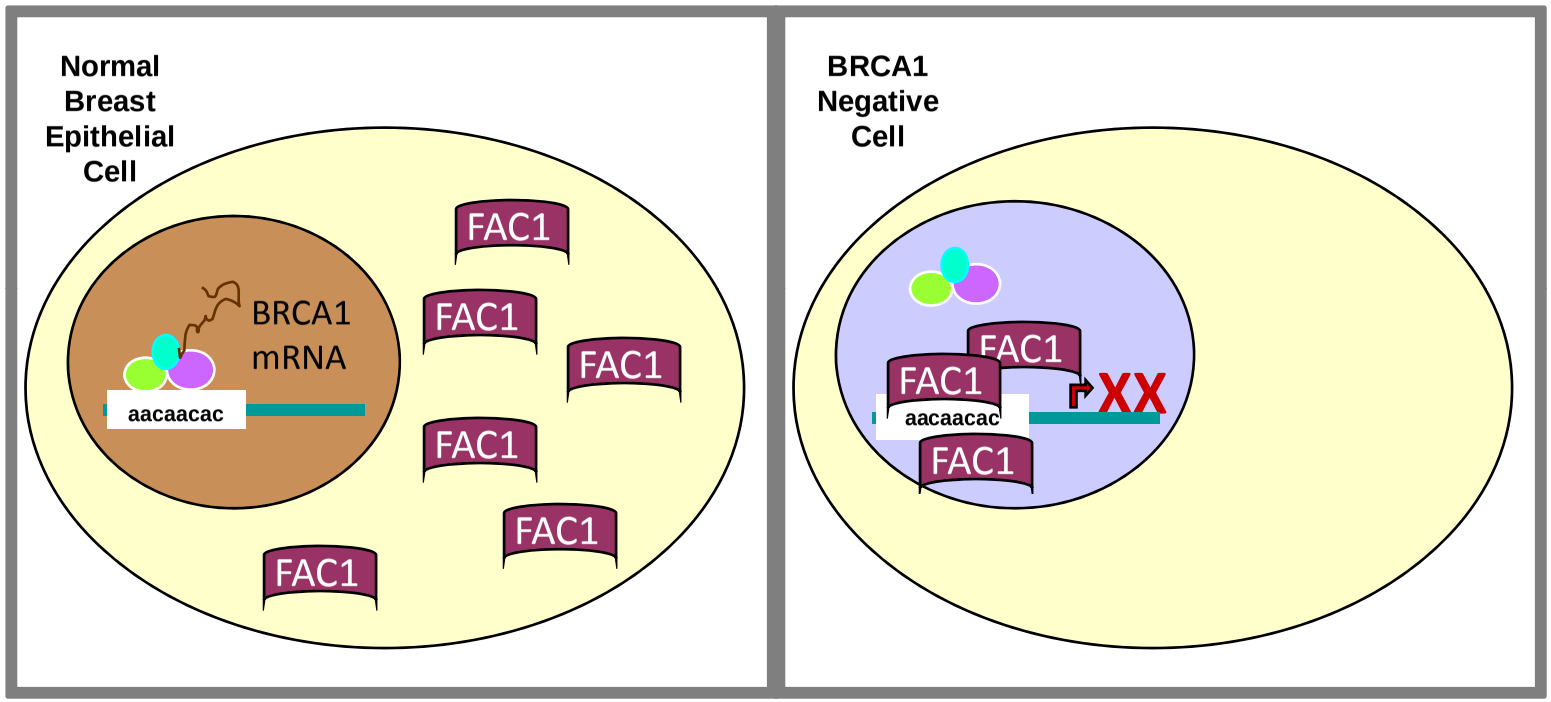


Figure 30. Proposed Model for Inactivation of the +ACA *BRCA1* Promoter in the Presence of Nuclear FAC1. In this model, activation or overexpression of nFAC1 in a breast cell of an individual that harbors the +ACA *BRCA1* promoter allele (or alleles) represses the *BRCA1* promoter resulting in the loss of BRCA1 function.

With the exception of microarray gene expression data mining, FAC1 status in breast tissue has not been reported. We observed that FAC1 expression in normal breast and breast tumor is quite common and like its expression in the brain, is variable. Although there are studies, that have documented FAC1 protein interactions, there is not much data about the regulation of FAC1 intracellular transport or by what mechanism it becomes dysregulated. Investigation of the mechanism by which FAC1 translocates from the cytoplasm to the nucleus, may begin to elucidate targets for monitoring or treating, not only in individuals with breast cancer but perhaps in neurodegenerative disorders as well.

Knudson's Two-Hit Hypothesis

For a normal breast epithelial cell to convert to a neoplastic cell, multiple aberrant genetic and/or epigenetic events must occur. Knudson's two hit hypothesis suggests that the general progression of a healthy cell to a cancer cell, must sustain a minimal of two damaging events. Loss of function in one allele does not assure tumor initiation, only confers an increased susceptibility of carcinogenesis (Carter, 2001; Knudson, 2001). *BRCA1* has been defined as a tumor suppressor gene, and loss of function of both alleles is thought to be necessary in order for a cell to transition to a malignant state (Carter, 2001). For individuals with a BRCA1 mutation, every cell starts out containing only one copy of *BRCA1*, otherwise in a sporadic event, a deletion, frameshift or point mutations, methylation or a functionally-detrimental polymorphism contributes to the first copy or hit of *BRCA1*. Cells that are derived from a cell with a *BRCA1* hit, will also have one remaining

BRCA1 allele, and are considered breast cancer susceptible. Should any cell from the *BRCA1* mutant individual or a descendant of a cell with loss of *BRCA1* expression acquire a second *BRCA1* allele loss, through LOH, frameshift or point mutations, methylation or a functionally-detrimental *BRCA1* polymorphism, then that cell and its descendants will be breast cancer prone. The risk of tumorigenesis becomes greatly increased through dysregulation of the pathways where *BRCA1* is not present to interact with other proteins that maintain genetic stability through cell-cycle checkpoints, DNA damage repair, and transcription regulation (Couch and Weber, 1998; Deng, 2006).

We have observed that FAC1 is capable and preferentially binds to the +ACA *BRCA1* promoter, that FAC1 is expressed in our FAC1-expression vector transfected cell lines, and that FAC1 is localized to the nucleus in our transfected cell lines. Our functional studies suggest that a patient with one +ACA allele, even in the absence of nuclear FAC1, may functionally be equal to quarter less than a homozygous wild-type patient. Likewise, a homozygous +ACA may effectively be similar to a patient that has a *BRCA1* mutation, deletion, or promoter methylation gene-silencing. Additionally, the heterozygous individual in the presence of nuclear FAC1 may be functionally equivalent to a *BRCA1* mutation carrier or the homozygous +ACA patient in the absence of FAC1. A homozygous +ACA individual, in the presence of nuclear FAC1 potentially be functionally null (Figure 31). Additionally, the functional data suggests a patient with homozygous wild-type *BRCA1* promoter alleles will not be functional different in the presences of FAC1. Together these results suggest that a WT/ACA or ACA/ACA individual may be

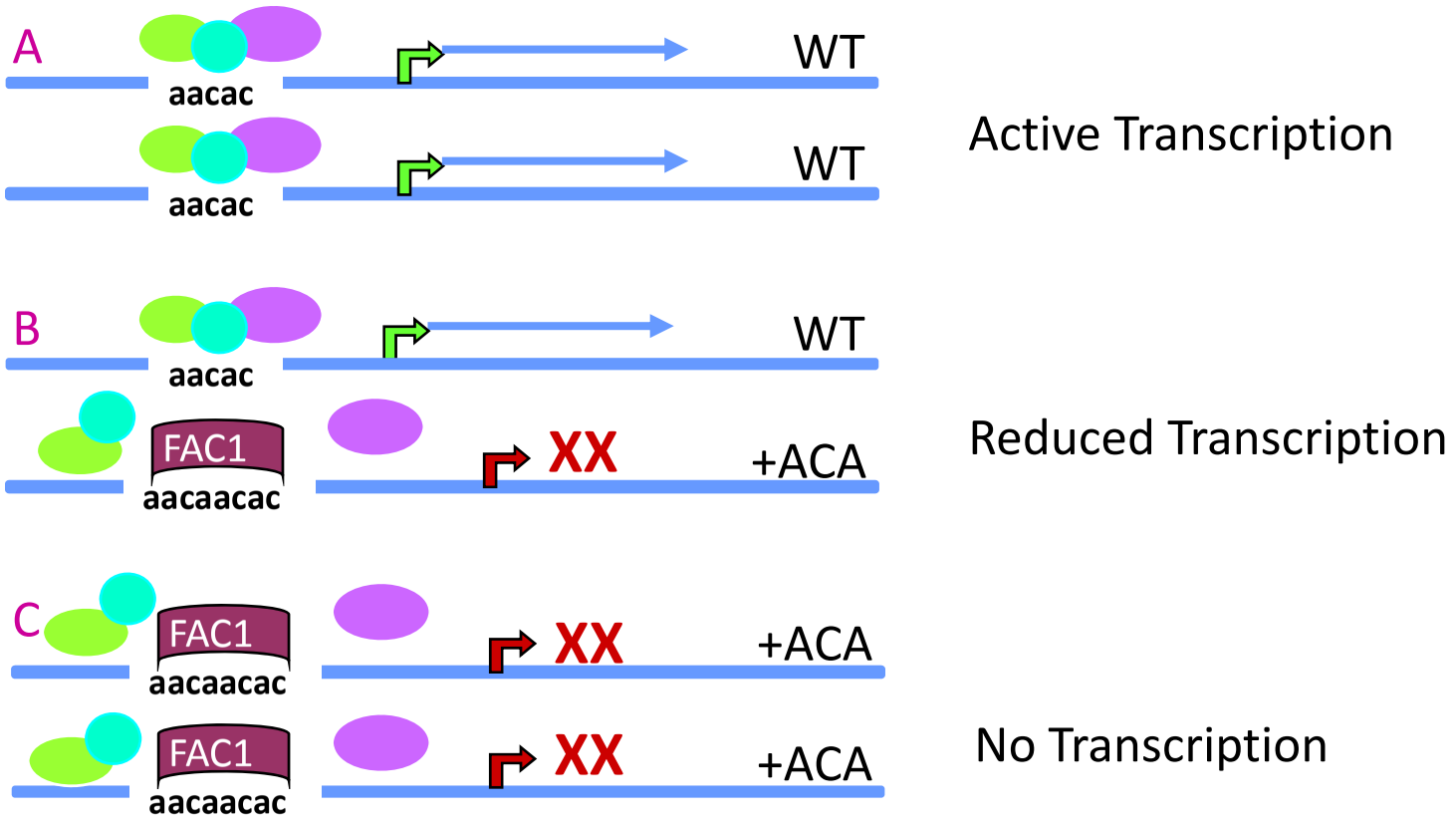


Figure 31. Proposed Model for Inactivation of the *BRCA1* Promoter in the Presence of FAC1. In this model, A.) an individual with the homozygous wild-type *BRCA1* promoter is actively transcribed even in the presence of nFAC1. B.) An individual that is heterozgyous for the *BRCA1* promoter will exhibit reduced levels of *BRCA1* transcription in the presence of nFAC1 and is partially reduced in the absence of nFAC1. C.) Individuals that are homozygous for the +ACA *BRCA1* promoter, would have reduced *BRCA1* transcription in the absence of nFAC1, while this same individual may be functionally *BRCA1*-negative in the presence of nFAC1.

breast cancer susceptible even in the absence of nuclear FAC1. Additionally, a heterozygous or homozygous +ACA individual in the presence of aberrant FAC1 may result in the FAC 1-mediated silencing of the +ACA *BRCA1* promoter. We suggest that this mechanism of FAC1-dependant silencing of the +ACA *BRCA1* promoter can be included in an expanded version of Knudson's two hit hypothesis and putative mechanisms, and a adapted flow chart illustrates this in Figure 32.

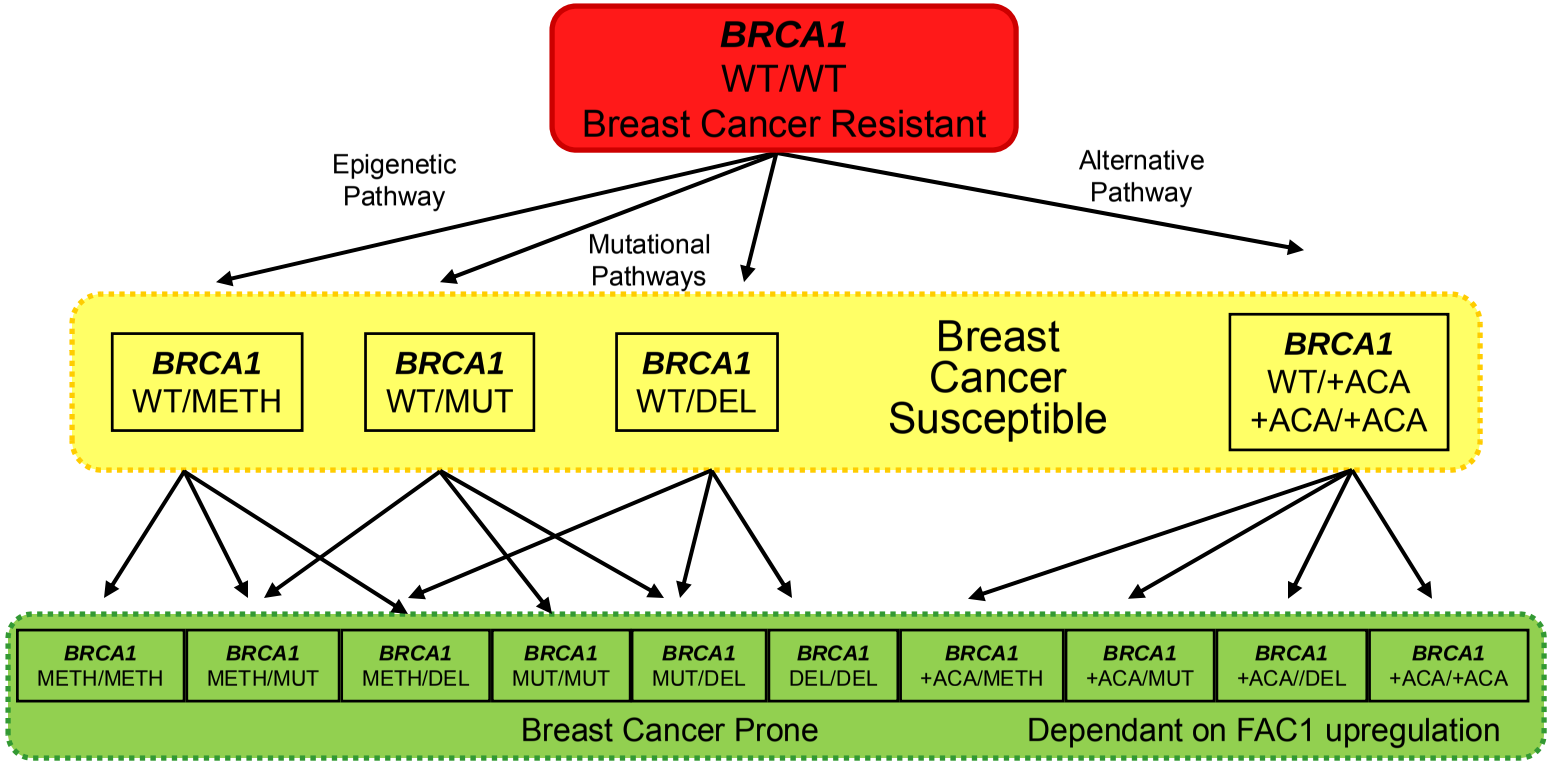


Figure 32. Molecular Pathways to Breast Cancer Through Inactivation of *BRCA1*. This flowchart expands on Knudson's Two-Hit Hypothesis model to include the +ACA *BRCA1* promoter polymorphic allele. A WT/WT cell can become breast cancer susceptible through epigenetic (methylation), mutational (mutations or deletions) or alternative (+ACA *BRCA1* promoter) pathways. Individuals born with a germline *BRCA1* mutation and one or more +ACA *BRCA1* promoter allele, will start out in the breast cancer susceptible category. If a cell that has attained a first hit or loss of one *BRCA1* allele, acquires an additional *BRCA1* allelic loss through methylation, mutation, deletion or has two copies of the +ACA *BRCA1* promoter allele that resulting cell is breast cancer prone. Notably, the loss of function of the +ACA *BRCA1* promoter is dependant on the aberrant presence of nFAC1

REFERENCES

American Cancer Society. (2008a). Overview: Breast Cancer: How Many Women Get Breast Cancer? http://www.cancer.org/docroot/CRI/content/CRI_2_2_1X_How_many_people_get_breast_cancer_5.asp?sitearea=

American Cancer Society. (2008b). Overview: Breast Cancer: What Causes Breast Cancer? http://www.cancer.org/docroot/CRI/content/CRI_2_2_2X_What_causes_breast_cancer_5.asp?sitearea=

American Cancer Society. (2007). Breast Cancer Facts and Figures 2007-2008. http://www.cancer.org/docroot/STT/stt_0_2007.asp?sitearea=STT&level=1

Berry, D.A., Iversen, E.S.,Jr, Gudbjartsson, D.F., Hiller, E.H., Garber, J.E., Peshkin, B.N., Lerman, C., Watson, P., Lynch, H.T., Hilsenbeck, S.G. *et al*. (2002). BRCAPRO validation, sensitivity of genetic testing of BRCA1/BRCA2, and prevalence of other breast cancer susceptibility genes. J. Clin. Oncol. *11,* 2701-2712.

Bianco, T., Chenevix-Trench, G., Walsh, D.C., Cooper, J.E., and Dobrovic, A. (2000). Tumour-specific distribution of BRCA1 promoter region methylation supports a pathogenetic role in breast and ovarian cancer. Carcinogenesis *2,* 147-51.

Bishop, J.M. (1991). Molecular themes in oncogenesis. Cell *2,* 235-248.

Bowser, R. (1996). Assignment of the human FAC1 gene to chromosome 17q24 by fluorescence in situ hybridization. Genomics *3,* 455-7.

Brenton, J.D., Carey, L.A., Ahmed, A.A., and Caldas, C. (2005). Molecular classification and molecular forecasting of breast cancer: ready for clinical application? J. Clin. Oncol. *29,* 7350-7360.

Brodie, S.G., and Deng, C.X. (2001). BRCA1-associated tumorigenesis: what have we learned from knockout mice? Trends Genet. *10,* S18-22.

Buckland, P.R., Hoogendoorn, B., Guy, C.A., Coleman, S.L., Smith, S.K., Buxbaum, J.D., Haroutunian, V., and O'Donovan, M.C. (2004). A high proportion of polymorphisms in the promoters of brain expressed genes influences transcriptional activity. Biochim. Biophys. Acta *3,* 238-249.

Callebaut, I., and Mornon, J.P. (1997). From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. FEBS Lett. *1,* 25-30.

Carey, L.A., Perou, C.M., Livasy, C.A., Dressler, L.G., Cowan, D., Conway, K., Karaca, G., Troester, M.A., Tse, C.K., Edmiston, S. *et al*. (2006). Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA *21,* 2492-502.

Carter, R.F. (2001). BRCA1, BRCA2 and breast cancer: a concise clinical review. Clin. Invest. Med. *3,* 147-157.

Catteau, A., Harris, W.H., Xu, C.F., and Solomon, E. (1999). Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. Oncogene *11,* 1957-65.

Catteau, A., and Morris, J.R. (2002). BRCA1 methylation: a significant role in tumour development? Semin Cancer Biol *5,* 359-371.

Catteau, A., Xu, C.F., Brown, M.A., Hodgson, S., Greenman, J., Mathew, C.G., Dunning, A.M., and Solomon, E. (1999). Identification of a C/G polymorphism in the promoter region of the BRCA1 gene and its use as a marker for rapid detection of promoter deletions. Br J Cancer *5-6,* 759-63.

Cebrian, A., Pharoah, P.D., Ahmed, S., Ropero, S., Fraga, M.F., Smith, P.L., Conroy, D., Luben, R., Perkins, B., Easton, D.F. *et al*. (2006). Genetic variants in epigenetic genes and breast cancer risk. Carcinogenesis *8,* 1661-1669.

Chan, K.Y., Liu, W., Long, J.R., Yip, S.P., Chan, S.Y., Shu, X.O., Chua, D.T., Cheung, A.N., Ching, J.C., Cai, H. *et al*. (2008). Functional polymorphisms in the promoter of BRCA1 influences transcription and are associated with decreased risk for breast cancer in Chinese women. J. Med. Genet. Epub ahead of print.

Chapentier, A.C. (2002). The molecular basis of breast carcinogenesis. In The molecular basis of human cancer, Coleman, W. B. and Tsongalis, G.J. ed., (Totowa, NJ: Humana Press) pp. 347-363.

Chapman, M.S., and Verma, I.M. (1996). Transcriptional activation by BRCA1. Nature *6593,* 678-679.

Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F., Tomlinson, G., Couch, F.J., Weber, B.L., Ashley, T., Livingston, D.M., and Scully, R. (1998). Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. Mol. Cell *3,* 317-328.

Cohen, A.M., Minsky, B.D., and Schilsky, R.L. (1997). *Cancer of the colon* . In *Cancer: Principles and Practice of Oncology*, DeVita, V. T., Hellman, S. and Rosenberg, S. A. eds., Lippincott-Raven) pp. 1144-1197.

Cortez, D., Wang, Y., Qin, J., and Elledge, S.J. (1999). Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science *5442,* 1162-1166.

Couch, F., and Weber, B.L. (1998). Breast Cancer. In: Volgelstein B, Kinzler K (eds) The *Genetic Basis of Human Cancer*. In *The Genetic Basis of Human Cancer*, Volgelstein B and K, Kinzler eds., (New York, NY: McGraw-Hill, Health Professions Division, pp: 537-563)

Cropp, C.S., Champeme, M.H., Lidereau, R., and Callahan, R. (1993). Identification of three regions on chromosome 17q in primary human breast carcinomas which are frequently deleted. Cancer Res. *23,* 5617-5619.

Deng, C.X. (2006). BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. Nucleic Acids Res. *5,* 1416-1426.

Deng, C.X. (2002). Tumor formation in Brca1 conditional mutant mice. Environ. Mol. Mutagen. *2-3,* 171-177.

Deng, C.X., and Brodie, S.G. (2000). Roles of BRCA1 and its interacting proteins. Bioessays *8,* 728-737.

Dobrovic, A., and Simpfendorfer, D. (1997). Methylation of the BRCA1 gene in sporadic breast cancer. Cancer Res *16,* 3347-50.

Dragani, T.A., Canzian, F., and Pierotti, M.A. (1996). A polygenic model of inherited predisposition to cancer. FASEB J. *8,* 865-870.

Esteller, M., Silva, J.M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I.C., Repasky, E.A. *et al*. (2000). Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J. Natl. Cancer Inst. *7,* 564-569.

Ford, D., Easton, D.F., Bishop, D.T., Narod, S.A., and Goldgar, D.E. (1994). Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Lancet *8899,* 692-695.

Friedman, L.S., Ostermeyer, E.A., Lynch, E.D., Szabo, C.I., Anderson, L.A., Dowd, P., Lee, M.K., Rowell, S.E., Boyd, J., and King, M.C. (1994a). The search for BRCA1. Cancer Res. *24,* 6374-6382.

Friedman, L.S., Ostermeyer, E.A., Szabo, C.I., Dowd, P., Lynch, E.D., Rowell, S.E., and King, M.C. (1994b). Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. Nat. Genet. *4,* 399-404.

Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., and Miki, Y. (1994). BRCA1 mutations in primary breast and ovarian carcinomas. Science *5182,* 120-122.

Hakem, R., de la Pompa, J.L., and Mak, T.W. (1998). Developmental studies of Brca1 and Brca2 knock-out mice. J. Mammary Gland Biol. Neoplasia *4,* 431-445.

Hedenfalk, I., Duggan, D., Chen, Y., Radmacher, M., Bittner, M., Simon, R., Meltzer, P., Gusterson, B., Esteller, M., Kallioniemi, O.P. *et al*. (2001). Gene-expression profiles in hereditary breast cancer. N Engl J Med *8,* 539-48.

Hedenfalk, I., Ringner, M., Ben-Dor, A., Yakhini, Z., Chen, Y., Chebil, G., Ach, R., Loman, N., Olsson, H., Meltzer, P., Borg, A., and Trent, J. (2003). Molecular classification of familial non-BRCA1/BRCA2 breast cancer. Proc Natl Acad Sci U S A *5,* 2532-7.

Hu, Z., Fan, C., Oh, D.S., Marron, J.S., He, X., Qaqish, B.F., Livasy, C., Carey, L.A., Reynolds, E., Dressler, L. *et al*. (2006). The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 7, 96.

Jensen, D.E., Proctor, M., Marquis, S.T., Gardner, H.P., Ha, S.I., Chodosh, L.A., Ishov, A.M., Tommerup, N., Vissing, H., Sekido, Y. *et al*. (1998). BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. Oncogene *9,* 1097-1112.

Jordan-Sciutto, K.L., Dragich, J.M., and Bowser, R. (1999a). DNA binding activity of the fetal Alz-50 clone 1 (FAC1) protein is enhanced by phosphorylation. Biochem Biophys Res Commun *3,* 785-9.

Jordan-Sciutto, K.L., Dragich, J.M., Rhodes, J.L., and Bowser, R. (1999b). Fetal Alz-50 clone 1, a novel zinc finger protein, binds a specific DNA sequence and acts as a transcriptional regulator. J Biol Chem *49,* 35262-8.

Kawahara, M., Sakayori, M., Shiraishi, K., Nomizu, T., Takeda, M., Abe, R., Ohuchi, N., Takenoshita, S., and Ishioka, C. (2004). Identification and evaluation of 55 genetic variations in the BRCA1 and the BRCA2 genes of patients from 50 Japanese breast cancer families. J. Hum. Genet. *7,* 391-395.

Kerr, P., and Ashworth, A. (2001). New complexities for BRCA1 and BRCA2. Curr. Biol. *16,* R668-76.

Khoo, U.S., Ozcelik, H., Cheung, A.N., Chow, L.W., Ngan, H.Y., Done, S.J., Liang, A.C., Chan, V.W., Au, G.K., Ng, W.F. *et al*. (1999). Somatic mutations in the BRCA1 gene in Chinese sporadic breast and ovarian cancer. Oncogene *32,* 4643-4646.

Kinzler, K.W., and Vogelstein, B. (2001). Colorectal tumors. In The metabolic & molecular bases of inherited disease, Scriver, Charles R., Beaudet, A. L., Sly, W. S. and Valle, D. eds., (New York: McGraw-Hill) pp. 1033-1062.

Knudson, A.G. (2001). Two genetic hits (more or less) to cancer. Nat. Rev. Cancer. *2,* 157-162.

Lacroix, M., and Leclercq, G. (2005). The "portrait" of hereditary breast cancer. Breast Cancer Res. Treat. *3,* 297-304.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. Nature *6712,* 643-649.

Loeb, K.R., and Loeb, L.A. (2000). Significance of multiple mutations in cancer. Carcinogenesis *3,* 379-385.

Lovering, R., Hanson, I.M., Borden, K.L., Martin, S., O'Reilly, N.J., Evan, G.I., Rahman, D., Pappin, D.J., Trowsdale, J., and Freemont, P.S. (1993). Identification and preliminary characterization of a protein motif related to the zinc finger. Proc. Natl. Acad. Sci. U. S. A. *6,* 2112-2116.

Magdinier, F., Billard, L.M., Wittmann, G., Frappart, L., Benchaib, M., Lenoir, G.M., Guerin, J.F., and Dante, R. (2000). Regional methylation of the 5' end CpG island of BRCA1 is associated with reduced gene expression in human somatic cells. Faseb J *11,* 1585-94.

Mancini, D.N., Rodenhiser, D.I., Ainsworth, P.J., O'Malley, F.P., Singh, S.M., Xing, W., and Archer, T.K. (1998). CpG methylation within the 5' regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site. Oncogene *9,* 1161-9.

Mao, L., and Sidransky, D. (1994). Cancer screening based on genetic alterations in human tumors. Cancer Res. *7 Suppl,* 1939s-1940s.

Merajver, S.D., Frank, T.S., Xu, J., Pham, T.M., Calzone, K.A., Bennett-Baker, P., Chamberlain, J., Boyd, J., Garber, J.E., and Collins, F.S. (1995). Germline BRCA1 mutations and loss of the wild-type allele in tumors from families with early onset breast and ovarian cancer. Clin. Cancer Res. *5,* 539-544.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., and Ding, W. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science *5182,* 66-71.

Millikan, R.C., Newman, B., Tse, C.K., Moorman, P.G., Conway, K., Smith, L.V., Labbok, M.H., Geradts, J., Bensen, J.T., Jackson, S. *et al*. (2007). Epidemiology of basal-like breast cancer. Breast Cancer Res Treat 109(1):123-39

Montgomery, K.G., Liu, M.C., Eccles, D.M., and Campbell, I.G. (2004). The DNMT3B C-->T promoter polymorphism and risk of breast cancer in a British population: a case-control study. Breast Cancer Res. *4,* R390-4.

Niwa, Y., Oyama, T., and Nakajima, T. (2000). BRCA1 expression status in relation to DNA methylation of the BRCA1 promoter region in sporadic breast cancers. Jpn J Cancer Res *5,* 519-26.

Ouchi, T., Monteiro, A.N., August, A., Aaronson, S.A., and Hanafusa, H. (1998). BRCA1 regulates p53-dependent gene expression. Proc. Natl. Acad. Sci. U. S. A. *5,* 2302-2306.

Perou, C.M., Jeffrey, S.S., van de Rijn, M., Rees, C.A., Eisen, M.B., Ross, D.T., Pergamenschikov, A., Williams, C.F., Zhu, S.X., Lee, J.C. *et al*. (1999). Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc. Natl. Acad. Sci. U. S. A *16,* 9212-9217.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A. *et al*. (2000). Molecular portraits of human breast tumours. Nature *6797,* 747-752.

Pharoah, P.D., Antoniou, A.C., Easton, D.F., and Ponder, B.A. (2008). Polygenes, risk prediction, and targeted prevention of breast cancer. N. Engl. J. Med. *26,* 2796-2803.

Pharoah, P.D., Dunning, A.M., Ponder, B.A., and Easton, D.F. (2004). Association studies for finding cancer-susceptibility genetic variants. Nat. Rev. Cancer. *11,* 850-860.

Rakha, E.A., El-Sheikh, S.E., Kandil, M.A., El-Sayed, M.E., Green, A.R., and Ellis, I.O. (2008). Expression of BRCA1 protein in breast cancer and its prognostic significance. Hum. Pathol. *6,* 857-865.

Renan, M.J. (1993). How many mutations are required for tumorigenesis? Implications from human cancer data. Mol. Carcinog. *3,* 139-146.

Rice, J.C., Ozcelik, H., Maxeiner, P., Andrulis, I., and Futscher, B.W. (2000). Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis *9,* 1761-5.

Ries, L.A.G., Melbert, D., Krapcho, M., Stinchcomb, D.G., Howlader, N., Horner, M.J., Mariotto, A., Miller, B.A., Feuer, E.J., Altekruse, S.F. *et al*. (2007). *SEER Cancer Statistics Review, 1975-2005*
http://seer.cancer.gov/csr/ 1975_2005/results_single/ sect_01_table.13_2pgs.pdf

Roll, J.D., Rivenbark, A.G., Jones, W.D., and Coleman, W.B. (2008). DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Mol. Cancer. 7,15.

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 365-386.

Ryan, J.L., Kaufmann, W.K., Raab-Traub, N., Oglesbee, S.E., Carey, L.A., and Gulley, M.L. (2006). Clonal evolution of lymphoblastoid cell lines. Lab. Invest. *11,* 1193-1200.

Scully, R., Anderson, S.F., Chao, D.M., Wei, W., Ye, L., Young, R.A., Livingston, D.M., and Parvin, J.D. (1997a). BRCA1 is a component of the RNA polymerase II holoenzyme. Proc. Natl. Acad. Sci. U. S. A. *11,* 5605-5610.

Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D.M. (1997b). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell *3,* 425-435.

Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D.M. (1997c). Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell *2,* 265-275.

Scully, R., Ganesan, S., Vlasakova, K., Chen, J., Socolovsky, M., and Livingston, D.M. (1999). Genetic analysis of BRCA1 function in a defined tumor cell line. Mol. Cell *6,* 1093-1099.

Shen, H., Wang, L., Spitz, M.R., Hong, W.K., Mao, L., and Wei, Q. (2002). A novel polymorphism in human cytosine DNA-methyltransferase-3B promoter is associated with an increased risk of lung cancer. Cancer Res. *17,* 4992-4995.

Sidransky, D. (1995). Molecular markers in cancer: can we make better predictions? Int J Cancer 64, 1.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S. *et al*. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U. S. A. *19,* 10869-10874.

Sorlie, T., Wang, Y., Xiao, C., Johnsen, H., Naume, B., Samaha, R.R., and Borresen-Dale, A.L. (2006). Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms. BMC Genomics 7, 127.

Starita, L.M., and Parvin, J.D. (2003). The multiple nuclear functions of BRCA1: transcription, ubiquitination and DNA repair. Curr. Opin. Cell Biol. *3,* 345-350.

Tapia, T., Smalley, S.V., Kohen, P., Munoz, A., Solis, L.M., Corvalan, A., Faundez, P., Devoto, L., Camus, M., Alvarez, M., and Carvallo, P. (2008). Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. Epigenetics *3,* 157-163.

Thakur, S., and Croce, C.M. (1999). Positive regulation of the BRCA1 promoter. J Biol Chem *13,* 8837-43.

Thompson, M.E., Jensen, R.A., Obermiller, P.S., Page, D.L., and Holt, J.T. (1995). Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat. Genet. *4,* 444-450.

Uhrhammer, N., Abdelouahab, A., Lafarge, L., Feillel, V., Ben Dib, A., and Bignon, Y.J. (2008). BRCA1 mutations in Algerian breast cancer patients: high frequency in young, sporadic cases. Int. J. Med. Sci. *4,* 197-202.

van der Looij, M., Cleton-Jansen, A.M., van Eijk, R., Morreau, H., van Vliet, M., Kuipers-Dijkshoorn, N., Olah, E., Cornelisse, C.J., and Devilee, P. (2000). A sporadic breast tumor with a somatically acquired complex genomic rearrangement in BRCA1. Genes Chromosomes Cancer *3,* 295-302.

Venkitaraman, A.R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell *2,* 171-182.

Venkitaraman, A.R. (2001). Functions of BRCA1 and BRCA2 in the biological response to DNA damage. J. Cell. Sci. *Pt 20,* 3591-3598.

Vissac, C., Peffault De Latour, M., Communal, Y., Bignon, Y.J., and Bernard-Gallon, D.J. (2002). Expression of BRCA1 and BRCA2 in different tumor cell lines with various growth status. Clin. Chim. Acta *1-2,* 101-110.

Wang, L., Liu, Z., Mao, L., Spitz, M.R., and Wei, Q. (2004). Functional relevance of C46359T in the promoter region of human DNMT3B6. Proceed. Am. Asso. Cancer Res. 2913.45

Welcsh, P.L., Owens, K.N., and King, M.C. (2000). Insights into the functions of BRCA1 and BRCA2. Trends Genet. *2,* 69-74.

Wetzels, R.H., Holland, R., van Haelst, U.J., Lane, E.B., Leigh, I.M., and Ramaekers, F.C. (1989). Detection of basement membrane components and basal cell keratin 14 in noninvasive and invasive carcinomas of the breast. Am. J. Pathol 134(*3),* 571-579.

White, K.K., Wilson, C., Belloni, D.R., Booker, J., Silverman, L.M., Gardner, K.L., Tsongalis, G.J., Bowser, R., and and Coleman, W.B. (2006). A novel +ACA BRCA1 promoter polymorphism creastes a binding site for the FAC1 transcriptional repressor. FASEB J. 20:A1324.

White, K.K., Belloni, D.R., Booker, J., Silverman, L.M., Tsongalis, G.J., and Coleman, W.B. (2005). Identification of a novel +ACA polymorphism in the BRCA1 promoter of hereditary breast cancer patients. FASEB J. 19:A1509-1510.

Wilson, C.A., Ramos, L., Villasenor, M.R., Anders, K.H., Press, M.F., Clarke, K., Karlan, B., Chen, J.J., Scully, R., Livingston, D. *et al*. (1999). Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. Nat. Genet. *2,* 236-240.

Xu, C.F., Brown, M.A., Chambers, J.A., Griffiths, B., Nicolai, H., and Solomon, E. (1995). Distinct transcription start sites generate two forms of BRCA1 mRNA. Hum Mol Genet *12,* 2259-64.

Yang, X., and Lippman, M.E. (1999). BRCA1 and BRCA2 in breast cancer. Breast Cancer Res. Treat. *1,* 1-10.

Yoshikawa, K., Honda, K., Inamoto, T., Shinohara, H., Yamauchi, A., Suga, K., Okuyama, T., Shimada, T., Kodama, H., Noguchi, S. *et al*. (1999). Reduction of BRCA1 protein expression in Japanese sporadic breast carcinomas and its frequent loss in BRCA1-associated cases. Clin. Cancer Res. *6,* 1249-1261.

Zhong, Q., Chen, C.F., Li, S., Chen, Y., Wang, C.C., Xiao, J., Chen, P.L., Sharp, Z.D., and Lee, W.H. (1999). Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. Science *5428,* 747-750.