Methylation of the TumorSuppressor Protein, BRCA1, Influences its Transcriptional Cofactor Function

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Dedication

To my family and friends who supported me while designing, executing, and conveying this thesis; in particular to my aunt who is a breast cancer survivor herself.

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Abstract

Methylation of the TumorSuppressor Protein, BRCA1, Influences its Transcriptional Cofactor Function

Breast cancer is one of the most frequently occurring cancers in women. Approximately half of hereditary breast cancers have mutations in either BRCA1 or BRCA2. BRCA1 is a multifaceted tumor suppressor protein that has implications in processes such as cell cycle, transcription, DNA damage response and chromatin remodeling. This multifunctional nature of BRCA1 is achieved by exerting its many effects through modulation of transcription of various factors. Many cellular modulatory events are dictated by covalent modification of proteins, an important mechanism in regulating protein and genome function; of which protein methylation is an important posttranslational modification with activating or repressive effects. Here we demonstrate that BRCA1 is methylated both in breast cancer cell lines and breast cancer tumor samples at both arginine and lysine residues. Arginine methylation by PRMT1 was observed *in vitro* and the region of BRCA1 504-802 showed to be highly methylated. Furthermore, we observed the functional role of BRCA1 methylation in protein-DNA interactions *in vivo* when methylation inhibition resulted in differential BRCA1 binding to several DNA damage response gene promoters. These results suggest that methylation may influence either the ability of BRCA1 to bind to specific promoters or protein-protein interactions which alters the recruitment of BRCA1 to these promoters. Thus, given the importance of BRCA1 to genomic stability, methylation of BRCA1 may ultimately affect the tumor suppressor ability of BRCA1.

Table of Contents

	Page
Dedication	**ii**
Acknowledgment	**iii**
Abstract	**iv**
List of Figures	**vii**
List of Abbreviations	**viii**
CHAPTER I: INTRODUCTION	1
A. The global impact of breast cancer	1
B. Breast cancer biology and etiology	1
C. Functional significance of breast cancer protein 1 (BRCA1)	2
D. Post-translational modifications of BRCA1	4
CHAPTER II: RESEARCH OBJECTIVES AND SPECIFIC AIMS	7
CHAPTER III: MATERIALS AND METHODS	8
A. *In silico* BRCA1 analysis	8
B. Cell culture and siRNA-mediated knockdown of PRMT1	8
C. Western blot analysis	9
D. Immunoprecipitation assay	9
E. GST pull-down and in vitro methyltransferase assay	9
F. siRNA-mediated knockdown of PRMT1 in HeLa cells	10
G. Chromatin immunoprecipitation assay (ChIP)	10
CHAPTER IV: RESULTS	12
A. BRCA1 is methylated in breast cancer cell lines	12
B. BRCA1 is methylated in *ex vivo* patient samples	16

C. PRMT1 methylates and associates with BRCA1 19

D. Methylation of BRCA1 alters promoter binding *in vivo* 23

E. Decreased levels of PRMT1 alters promoter binding *in vivo* 26

F. Methylation of BRCA1 alters protein-protein interactions 29

CHAPTER V: DISCUSSION 32

CHAPTER VI: REFERENCES 38

List of Figures

Page

Figure 1: BRCA1 is methylated at both arginine and lysine residues in breast cancer cell lines. 14

Figure 2: Methylation of BRCA1 in *ex vivo* breast tumor samples. 17

Figure 3: BRCA1 504-802 is methylated by PRMT1 *in vitro*. 21

Figure 4: Methylation status of BRCA1 alters BRCA1-DNA interactions at specific promoters *in vivo*. 24

Figure 5: Decreased levels of PRMT1 alters BRCA1 promoter binding *in vivo*. 27

Figure 6: BRCA1 methylation status alters protein-protein interactions at the 504-802 region. 30

List of Abbreviations

BRCA1, BRCA 2	Breast cancer protein 1, 2
HER2	Human epidermal growth factor receptor 2
ER	Estrogen receptor
PR	Progesterone receptor
aa	Amino acid
TAD	Transactivation domain
BRCT	BRCA1 C-terminus
PTM	Post-translational modification
PRMT	Protein arginine methyltransferase
PKMT	Protein lysine methyltransferase
R	Arginine
K	Lysine
MMA	Monomethylarginine
aDMA	Asymmetric dimethylarginine
sDMA	Symmetric dimethylarginine
MeMo	Methylation Modification Prediction Server
GST	Glutathione S transferase
AdOx	Adenosine dialdehyde methyltransferase inhibitor

CHAPTER I: INTRODUCTION

A. The global impact of breast cancer

Breast cancer is one of the three most frequently diagnosed cancers in women. General trends suggest a decrease of breast cancer mortality in industrialized countries, which have increased access to mammographic screening programs and improved therapies. In contrast, the lowest mortality rates have been reported in Asian countries thus suggesting the implication of dietary, cultural and environmental factors may play a role in the incidence and progression of the disease (Mettlin 1999).

In the United States during 2008 alone, the expected number of new cases for female invasive carcinoma of the breast neared 26% of total cancers in women (Jemal et al 2008, Jemal et al 2009), while the National Cancer Institute reported an estimate of 40,000 female deaths (http://www.cancer.gov/cancer topics/types/breast), with case projections increasing in 2009 (Jemal et al 2009).

B. Breast cancer biology and etiology

The breast and ovarian cancer susceptibility gene, breast cancer protein 1 or BRCA1, was firstly proposed only a decade ago as a result of linkage analysis in large cohorts of hereditary breast cancer families (Hall et al 1990). Although successfully cloned in 1994 and BRCA1 has been one of the most intensively studied genes, its mechanism of action is not yet fully elucidated but it is assumed that alongside breast cancer protein 2 (BRCA2) it plays a primordial role in important cellular pathways involving DNA damage repair, apoptosis and

transcriptional regulation (Hu 2009, Miki et al 1994, Narod and Foulkes 2004, Somasundaram 2003, Starita and Parvin 2003). Interestingly, loss of heterozygosity in tumor samples of BRCA1-related breast and ovarian cancer results in invariable loss of the wild type allele and thus the retention of the mutant copy, qualifying it as a tumor suppressor gene (Clarke et al 2006, Hu 2009). Furthermore, BRCA1 tumor suppression functions in a gender and tissue specific manner, where germline mutations of the autosomal dominant BRCA1 gene lead to breast and ovarian cancer primarily in women while somatic mutations in the BRCA1 coding region are scarcely associated with sporadic breast and ovarian cancers (Hall et al 1990, Miki et al 1994). Early studies established that BRCA1 mutations account for roughly 50% of hereditary breast cancer and approximately 85% of hereditary breast-ovarian cancers (Ford et al 1998, Hu 2009, Miki et al 1994). The course of disease, tumor characteristics and responses to chemotherapy are determined by the breast cancer subtype which exhibit varying properties. These subtypes were identified through gene expression studies and include the luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) over-expressing, and the basal-like subtype (Perou et al 2000). Basal-like breast tumors while distinct from triple negative breast cancer often cluster together with this group. Triple negative breast cancers lack estrogen receptor (ER) and progesterone receptor (PR) expression and do not have HER2 amplification. Due to these characteristics, hormone therapy (tamoxifen and aromatase inhibitors) and HER2-targeted approaches (trastuzumab) are not options for this tumor type and standard chemotherapy must be used. Approximately 45% of hereditary breast cancers account for mutations in either BRCA1 or BRCA2 (Ford et al 1998). Breast cancers that have mutations in BRCA1 are mostly basal-like and triple negative (Foulkes et al 2003, Sorlie et al 2003). On the other hand, sporadic breast cancer rarely exhibits BRCA1 mutations; however the BRCA1 protein may be functionally inactivated in these cases.

C. Functional significance of breast cancer protein 1 (BRCA1)

BRCA1 gene encodes a large protein of 1,863 aa with only a few conserved familiar motifs, whose gene product is a tumor suppressor protein that has implications in processes such

as cell cycle, transcription, DNA damage response, and chromatin remodeling (Rosen et al 2003). One way to explain the multifaceted nature of BRCA1 is that it exerts its many effects through modulating transcription of various factors. BRCA1 was first implicated in transcription when the C-terminus (amino acid, aa, 1560-1863) fused to Gal4 was able to activate transcription (Monteiro et al 1996), with aa 1760-1863 being the minimal transactivation domain (TAD). Within this TAD are two BRCA1 C-terminus (BRCT) motifs that are found in a large family of proteins important for DNA damage response, such as DNA ligase IV, p53BP1, and base excision response scaffold protein XRCC1 (Glover et al 2004). Since that time numerous other findings have served to strengthen the connection between transcription and BRCA1. For instance, BRCA1 is part of the RNA polymerase II holoenzyme complex (Anderson et al 1998, Krum et al 2003, Scully et al 1997). One way BRCA1 may be recruited to this complex is through binding to RNA Helicase A, an enzyme that unwinds RNA and DNA molecules (Anderson et al 1998). The C-terminus of BRCA1 interacts with CDK-activating kinase and inhibits the phosphorylation of the RNA polymerase II (RNAPII) CTD by competing with ATP (86). BRCA1/BARD1 (N-terminus binding partner) also ubiquitinates the hyper-phosphorylated form of RNAPII (Smith et al 1999), resulting in the targeted proteasomal degradation of RNAPII following DNA damage (Somasundaram 2003). A recent study demonstrated that in order for BRCA1 to directly stimulate transcription, both the N-and C-termini of BRCA1, but not BARD1, are required (Somasundaram et al 1997).

Beyond the RNA polymerase II complex, BRCA1 also interacts with multiple cofactors and transcription factors. BRCA1 binds to the co-activators CBP/p300 in a phosphorylation independent manner, which resulted in the cooperative activation of the Rous sarcoma virus-long terminal repeat promoter (Pao et al 2000). Conversely, another study found that BRCA1 downregulated p300 expression and that p300 expression rescued BRCA1 inhibition of ER mediated transcription (Fan et al 2002). BRCA1 stimulates STAT1 in response to IFN-γ (Ouchi et al 2000), NF-κB in response to tumor necrosis factor-alpha or interleukin-1beta (Benezra et al 2003), and p53 responsive promoters (Ouchi et al 1998). Among the genes found to be transactivated by BRCA1 are MDM2, BAX, p21/WAF1, p27/KIP1, and GADD45ɑ with p21/WAF1

and GADD45α transactivation being independent of p53 (Harkin et al 1999, Jin et al 2000, Ouchi et al 1998, Somasundaram et al 1997, Williamson et al 2002, Zhang et al 1998). BRCA1 can also act as a CTD kinase inhibitor contributing to the activation of p21/WAF1 gene expression (90). BRCA1 is able to inhibit transcription of estrogen responsive genes as well as telomerase reverse transcriptase gene expression. These studies also demonstrated BRCA1 on pS2 (estrogen responsive gene) and hTERT promoters *in vivo* (Xiong et al 2003, Zheng et al 2001, Zhou and Liu 2003). Therefore, the current literature suggests that based on the promoter of interest, BRCA1 can have either activating or inhibitory effects through multiple mechanisms.

D. Post-translational modifications of BRCA1

Covalent protein modifications create regulatory and functional mechanisms that waive the requirement for new protein synthesis. Proteins involved in transcription are often regulated through post-translational modifications (PTMs), such as acetylation and methylation. Acetylation is broadly linked with activation, whereas methylation can have either activating or repressing effects depending on the amino acid that is modified and the nature of the modification (mono-, di-, or tri-methylation). One well known example of this is the transcription factor p53, where K372 monomethylation and K370 dimethylation result in stabilization and activation of p53 versus K370 and K382 monomethylation, which results in transcriptional repression (Sims and Reinberg 2008). To date, the known PTMs of BRCA1 include phosphorylation and ubiquitinylation (Rosen et al 2003). The phospho-BRCA1 predominates in S phase and subsequently becomes dephosphorylated after M phase (Rosen et al 2003). This cell cycle dependent phosphorylation occurs in the absence of DNA damage and is accomplished by multiple protein kinases including cyclin A/cdk2, cyclin E/cdk2, cyclin D/cdk4, and aurora-A (Rosen et al 2003). In addition, BRCA1 is phosphorylated following DNA damage by ATR and ATM kinases (Rosen et al 2003). Although epigenetic changes to the BRCA1 gene have been extensively researched at the DNA-level, proteome research has focused on phosphorylation of BRCA1 by DNA damage protein kinases, while little information is known about the effects of other PTM events.

Protein methylation can occur on both lysine and arginine residues. Arginine methylation is carried out by a family of protein arginine methyltransferases (PRMT), which contains eleven family members to date (Herrmann et al 2009). These enzymes utilize S-adenosyl methionine (AdoMet) as a methyl donor (McBride and Silver 2001), and can be further subdivided into type I and type II enzymes. Type I enzymes form monomethylarginine (MMA) and asymmetric dimethylarginine (aDMA) and type II enzymes catalyze the formation of MMA and symmetric dimethylarginine (sDMA) (Pahlich et al 2006). PRMT1, PRMT2, PRMT3, PRMT4/CARM1, PRMT6, and PRMT8 are type I enzymes; while PRMT5, PRMT7, and PRMT9 are type II enzymes. PRMT1 is the most predominant methyltransferase in mammalian cells (Tang et al 2000) and is responsible for the majority of arginine methylation, thus the vast majority of research has been focused on this enzyme (Gary et al 1996). A number of PRMT1 substrates have been identified including 53BP1, histone H4, MRE11, nucleolin, RNA helicase A, SAM68, and ER□ (Boisvert et al 2005a, Boisvert et al 2005b, Le Romancer et al 2008, Pahlich et al 2005). Arginine methylation can regulate multiple cellular processes including transcription (Chen et al 1999, Cuthbert et al 2004, Kwak et al 2003, Rezai-Zadeh et al 2003, Wang et al 2001, Wang et al 2004), protein-protein interactions (Bedford et al 2000), nuclear trafficking (Yu et al 2004, Yun and Fu 2000), transcriptional elongation, DNA damage response (Boisvert et al 2005a), and cell cycle checkpoints (Boisvert et al 2005a). Lysine methylation occurs mainly through the SET domain family of proteins. These enzymes were originally termed histone methyltransferases due to their ability to methylate various histone protein residues (Dillon et al 2005), but in light of the identification of many non-histone protein substrates they are now referred to as protein lysine methyltransferases (PKMTs). The exception to this is the DOT1 family of lysine methyltransferases. Lysine residues can be mono-, di-, or tri-methylated (Kakimoto and Akazawa 1970, Kakimoto 1971).

In this study, we have identified for the first time the arginine and lysine methylation of BRCA1 in tissue culture cell lines as well as breast tumor tissue samples. We show that BRCA1 is methylated by PRMT1 *in vitro* within the 504-802 region. PRMT1 was detected in complex with BRCA1 504-802 through *in vitro* binding assays and co-immunoprecipitated with BRCA1.

Inhibition of methylation resulted in decreased BRCA1 methylation and alteration of BRCA1 binding to promoters *in vivo*. Knockdown of PRMT1 also resulted in increased BRCA1 binding to particular promoters *in vivo*. Finally, following methylation inhibition, Sp1 was found to preferentially associate with hypo-methylated BRCA1 and STAT1 was found to preferentially associate with hyper-methylated BRCA1. These findings indicate that BRCA1 is post-translationally modified through methylation by PRMT1 and that this methylation influences its ability to bind to different promoters *in vivo*.

CHAPTER II: RESEARCH OBJECTIVES AND SPECIFIC AIMS

The aim of the work described in this manuscript is to characterize BRCA1 methylation and determine how this post-translational modification affects its function. This hypothesis assumed that BRCA1 is differentially methylated in immortalized cancerous conditions and that BRCA1 methylation status would alter its tumor suppressor activity. As with other characterized post-translational modifications of BRCA1, methylation of the protein is expected to modulate the ability of BRCA1 to bind DNA, either as a transcription factor or as part of DNA damage repair complex. Given the importance of BRCA1 to genomic stability, methylation of BRCA1 may ultimately affect the tumor suppressor ability by changing dynamics of protein-DNA or protein-protein interactions. This is an attractive concept because it is a novel description of potential tumorigenesis mechanisms and regulation. Particularly, this study was aimed to identify BRCA1 arginine and lysine methylation in breast cancer cell lines and tumor samples at various stages of carcinogenesis, identify a potential candidate for methylation, determine the functional consequence of BRCA1 methylation and determine the influence of methylation in BRCA1 DNA damage response activity and interaction with known proteins that are known to bind BRCA1. Future studies would be able to investigate in more detail the findings of this characterization, in particular the specific sites of methylation within this region as well as further characterization of methylation status in a broader population of tumors involving different variables such as tumor stage, age and race.

CHAPTER III: MATERIALS AND METHODS

A. *In silico* BRCA1 analysis. Predicted methylation sites from the full length primary amino acid sequence of BRCA1 were generated by the freeware MeMo: Methylation Modification Prediction Server (http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo.html).

B. Cell culture and siRNA-mediated knockdown of PRMT1. MDA-MD-231 and MCF-7 are epithelial breast carcinoma cells derived from pleural effusions (Lacroix and Leclercq 2004). HeLa is a cervical carcinoma cell line. All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1% L-glutamine, and 1% streptomycin/penicillin (Quality Biological). All cells were incubated at 37°C and 5% CO_2. Cells were cultured to confluency , washed and pelleted at 4°C for 15 minutes at 3,000 rpm. Pellets were lysed in a buffer containing Tris-HCl pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM DTT and one tablet complete protease inhibitor cocktail per 50 ml. Lysis was performed under ice-cold conditions, incubated on ice for 30 minutes and spun at 4°C for 5 minutes at 14,000 rpm. Supernatant was transferred to a new tube and protein was quantitated with Bradford protein assay (BioRad, Hercules, CA, USA). For the breast tumor tissue lysis, the tissue was ground with a mortar and pestle in the presence of liquid nitrogen to create a powdered tissue. RIPA buffer (50 mM Tris-HCl pH 7.5, 105 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) was added to the powdered tissue, the sample vortexed for 60 seconds, and placed on ice for 45 minutes. Samples were homogenized with a syringe

and needle, followed by centrifugation at 14,000 g for 10 minutes. Supernatants containing the lysate were quantitated with Bradford protein assay (BioRad).

C. Western blot analysis. Cell extracts were resolved by SDS PAGE on a 4-20% tris-glycine gel (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to polyvinylidene difluoride microporous membranes using the iBlot dry blotting system as described by the manufacturer (Invitrogen). Membranes were blocked with Dulbecco's phosphate-buffered saline (PBS) 0.1% Tween-20 + 3% BSA. Primary antibody against specified proteins was incubated with the membrane in blocking solution overnight at 4℃. Western blots were performed with anti- methyl mono/di arginine, anti-methyl lysine (AbCam, Cambridge, MA, USA), anti-BRCA1(C-20), anti-Sp1 and anti-STAT1 (Santa Cruz, Santa Cruz, CA, USA), anti-PRMT1 (Cell Signaling, Danvers, MA, USA) antibodies. Membranes were washed twice with PBS + 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody for one hour in blocking solution. Presence of secondary antibody was detected by SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). Luminescence was visualized on a Kodak 1D image station.

D. Immunoprecipitation assay. For immunoprecipitation (IP) 2 mg of whole cell protein or tumor tissue extracts were brought up to a final volume of 500 µl with lysis buffer and precleared for 30 minutes with 50 µl of 30% A/G agarose bead slurry (CalBioChem, La Jolla, CA). Supernatants were transferred to a new tube with 10 µg of BRCA1 or normal rabbit IgG antibodies (Santa Cruz), and the solution was rotated overnight at 4℃. The next day complexes were precipitated with A/G beads for 90 minutes. Beads were washed twice with TNE_{150} + 0.1% NP-40 and once with TNE_{50} + 0.1% NP-40.

E. GST pull-down and in vitro methyltransferase assay. GST tagged proteins were purified as described previously [53]. Constructs were washed three times with PBS + 1% Triton X-100, pelleted and resuspended in the methyltransferase buffer reaction. Five hundred nanograms of GST-BRCA1 1-500, 452-1079, 504-802, 697-1276, 1021-1552, 1501-1861 and core histones

were incubated with 0.2 µg of recombinant purified PRMT1 (Active Motif, Carlsbad, CA, USA) in the presence of 0.55 µCi S-Adenosyl-L-[*methyl*-^{3}H] methionine (GE Healthcare, Piscataway, NJ, USA) and reaction buffer (50 mM Tris-HCl pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose, 10 µM β-mercaptoethanol) overnight at 37℃ in a final reaction volume of 20 µl. The overnight methylation reactions (beads containing substrate) were spun, washed three times in excess cold 10% TCA, 1% sodium phosphate followed by once with 100% ethanol. Control samples were spotted on GF/C membranes (Millipore, Bedford, MA, USA), allowed to dry and processed equally as beads alone. Both beads and filters were counted in Beckman Coulter LS6001C scintillation counter in 2 ml of scintillation fluid (Beckman Coulter, Fullerton, CA, USA). For the protein GST-BRCA1 pull-down, 2 mg of HeLa whole cell protein extract were brought up to a final volume of 500 µl with lysis buffer and 500 ng of GST-BRCA1 constructs were rotated at 4℃ overnight. Beads were washed twice with TNE_{150} + 0.1% NP-40 and once with TNE_{50} + 0.1% NP-40.

F. siRNA-mediated knockdown of PRMT1 in HeLa cells. HeLa cells (1.8 x 10^7 cells) were transfected with double-stranded duplex with Hs_HRMT1L2_7 HP Validated siRNA (Qiagen, Valencia, CA, USA) or luciferase (Dharmacon, Lafayette, CO, USA) using Lipofectamine reagent according to the manufacturer's recommendations (Invitrogen). Initial transfections were carried out in 24-well plates in order to establish optimal knockdown conditions by titrating siRNA at 10, 25 and 50 nM. Transfections for chromatin immunoprecipitation assays were conducted at 50 nM final siRNA concentration.

G. Chromatin immunoprecipitation assay (ChIP). MDA-MB-231 cells were treated with 30 µM adenosine periodate methyltransferase inhibitor (AdOx, Sigma, St Louis, MO, USA) and processed 48 hours later for ChIP using an established protocol. Approximately 5x10^6 cells were used per IP. Cells were cross-linked with 1% formaldehyde at 37ºC for 10 minutes, pelleted, washed, and cells lysed using SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, one tablet complete protease inhibitor cocktail per 50 ml) on ice for 10 minutes. Cells were

sonicated on ice for 6 cycles to obtain an average DNA length of 500 to 1200 bp. Lysate was clarified by centrifugation at 4℃ for 10 minutes at 14,000 rpm. Supernatant was then diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) and pre-cleared with a mixture of protein A/G agarose (blocked previously with 1 mg/ml salmon sperm DNA and 1 mg/ml BSA, Stratagene, La Jolla, CA, USA) at 4℃ for 1 hour. Pre-cleared chromatin was incubated with 10 µg of antibody at 4℃ overnight. Next day, 60 µl of 30% slurry of blocked protein A/G agarose was added and complexes incubated for 2 hours. Immune complexes were recovered by centrifugation and washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), twice with high salt buffer (0.1% SDS, 1% Triton X-100 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and once with TE buffer. Immune complexes were eluted twice with elution buffer (1% SDS, 0.1 M NaHCO3) and incubating at room temperature for 15 minutes on a rotating wheel. Cross-links were reversed by adding 20 µl of 5M NaCl and incubating elutes at 65℃ overnight. The next day, proteinase K (100 µg/ml final concentration) was added and samples incubated at 55℃ for 1 hour. Samples were extracted with phenol:chloroform twice and ethanol precipitated overnight. Pellets were then washed with 70% ethanol, dried, resuspended in 50 µl of TE and assayed by PCR. Thirty-five cycles of PCR were performed in 50 µl with 10 µl of immunoprecipitated material, 0.1 µM of primers, 0.2 mM dNTPs, and 1 unit of Taq DNA polymerase. Finally, PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

CHAPTER IV: RESULTS

A. BRCA1 is methylated in breast cancer cell lines. Methylation of proteins can have either activating or repressive effects depending on the amino acid that is modified (arginine-R or lysine-K) and whether it is mono-, di- ,or tri-methylated (Bhaumik et al 2007). For example, K4 methylation on histone H3 is an indicator of actively transcribed genes (Bhaumik et al 2007). In contrast, methylation of histone H3K9, H3K27, and H4K20 is involved in heterochromatin formation and gene silencing (Bhaumik et al 2007). To date there are no reports of BRCA1 protein methylation. Based on the role of BRCA1 in transcription and the influence of methylation on various transcription factors, we speculated that BRCA1 may be methylated. According to the bioinformatic tool MeMo, there are a total of seven R and ten K residues in BRCA1 that could potentially be methylated (Figure 1a). Interestingly, two of these residues, R1076 and R1751 have known BRCA1 mutations, R1076T, R1751Q and R1751P, according to the Breast Care Information Core (http://research.nhgri.nih.gov/bic/). To determine if methylated BRCA1 could be detected in breast cancer cell lines, two cell lines MCF-7 and MDA-MB-231 were analyzed. BRCA1 was immunoprecipitated and western blot analysis performed with anti-K methyl, anti-R-methyl and anti-BRCA1 antibodies. Results in Figure 1b indicated that BRCA1 is methylated on both K and R residues in MDA-MB-231 cells, but only R methylation could be detected in MCF-7 cells. These cell lines have very distinct characteristics, with MDA-MB-231 being triple negative and MCF-7 being ER and PR positive. In addition, MDA-MB-231 is a highly metastatic cell line, whereas MCF-7 is not. Collectively, these results indicate that BRCA1 is methylated in breast

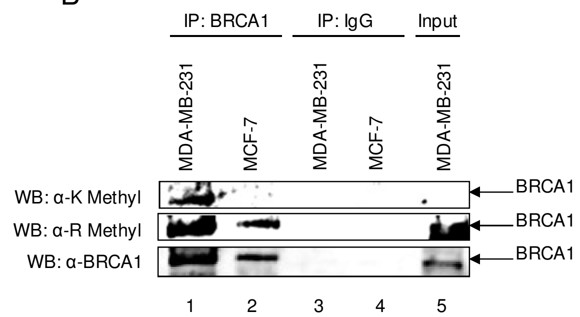
cancer cell lines. This is the first time to our knowledge that BRCA1 has been shown to be methylated.

Figure 1: BRCA1 is methylated at both arginine and lysine residues in breast cancer cell lines. (A) Predicted BRCA1 methylation sites generated by Memo: Methylation Modification Prediction Server (http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo.html) R=Arginine and K=Lysine. **(B)** Two milligram of whole cell protein extracts from MCF-7 and MDA-MB-231 cells were immunoprecipitated with either BRCA1(C-20) or rabbit normal IgG antibody, separated on a 4-20% gel by SDS-PAGE, and western blotted with antibodies against K-methyl, R-methyl and BRCA1(C-20). Input correlates to whole cell extract in order to confirm proteins probed.

A

Residue	Position	Residue	Position
R	610	K	56
R	959	K	505
R	1076	K	581
R	1204	K	748
R	1737	K	912
R	1751	K	935
R	1762	K	1459
		K	1489
		K	1671
		K	1759

B



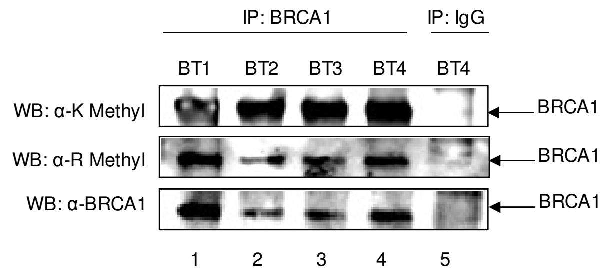
B. BRCA1 is methylated in *ex vivo* patient samples. To determine if BRCA1 is also methylated in *ex vivo* patient samples, four different breast tumor tissue samples (BT1-4) were tested. These samples were randomly chosen and their characteristics are shown in Figure 2a. Interestingly, 3 of the 4 samples were triple negative breast cancers (BT1, 3, and 4), which are extremely difficult to treat. BT3 was ER positive, PR negative and does not have HER-2 overexpression. We immunoprecipitated BRCA1 from BT1-4 and western blotted with with anti-K methyl, anti-R-methyl and anti-BRCA1(C-20) antibodies (Figure 2b). Our results indicate that BRCA1 is methylated at both K and R residues (lanes 1-4) in all four breast tumor patient samples, while no methylation was observed with the negative control IgG IP (lane 5). Larger sample sizes are necessary to determine if BRCA1 methylation occurs more frequently in particular types of breast cancer. These results demonstrate that both K and R residues of BRCA1 are methylated in breast tumor tissue samples.

Figure 2: Methylation of BRCA1 in *ex vivo* breast tumor samples. (A) Characteristics of the four different breast tumor samples tested in panel B. **(B)** Solid breast tumor tissue was ground with a mortar and pestle in the presence of liquid nitrogen to create a powdered tissue. RIPA buffer (50 mM Tris-HCl pH 7.5, 105 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) was added to the powdered tissue, the sample vortexed for 60 seconds, and placed on ice for 45 minutes. Samples were homogenized with a syringe and needle, followed by centrifugation at 14,000 g for 10 minutes. Two milligram of whole cell protein extracts from four different breast tumor samples (BT1-4) were immunoprecipitated with either BRCA1(C-20) or rabbit normal IgG antibody, separated on a 4-20% gel by SDS-PAGE, and western blotted with an anti-K methyl, anti-R methyl and anti-BRCA1 antibodies.

A

Sample Name	Age	Race	ER	PR	HER-2	p53	Histology
BT1	55	AA	neg	neg	neg	neg	Infiltrating ductal carcinoma
BT2	83	C	pos	neg	neg	neg	Infiltrating lobular carcinoma
BT3	54	AA	neg	neg	neg	neg	Variant papillary serous type of ductal carcinoma
BT4	68	C	neg	neg	neg	pos	Infiltrating ductal carcinoma

B



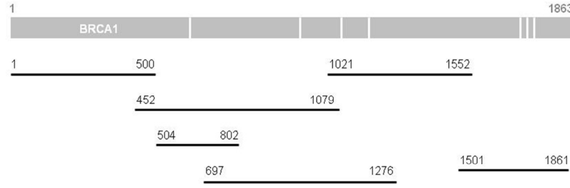
C. PRMT1 methylates and associates with BRCA1. Results above indicate that BRCA1 protein is methylated in both cancer cell lines and patient samples. We were interested in the site of BRCA1 arginine methylation and for that purpose, GST-BRCA1 constructs spanning BRCA1 protein were used for *in vitro* methyltransferase assays. Due to the lack of a previously identified enzyme, GST-PRMT1 was chosen for this analysis because PRMT1 is responsible for approximately 85% of all arginine methylation (Gary et al 1996). GST-BRCA1 constructs spanning the protein (Figure 3a) were incubated in a reaction mixture containing GST-PRMT1 enzyme, buffer, and *S*-Adenosyl-L [*methyl*-3H] methionine as a source of radio-labeled methyl groups. The predicted methylation sites are shown in Figure 3a for each of the GST-BRCA1 fragments. Core histones were used as a positive control, as PRMT1 has been shown to methylate histone H4 (Pahlich et al 2006, Paik and Kim 1967) and GST was used as negative control. BRCA1 fragment 504-802 was consistently the most highly methylated in repeated experiments, and no methylation was detected for BRCA1 1501-1861 and GST-BRCA1 697-1276 (Figure 3b). Lower levels of methylation were observed with BRCA1 1-500, 1021-1552, and 452-1079. The loss of methylation observed with 452-1070 could be due to a change in protein confirmation masking the methylation site. Interestingly, there are no predicted methylation sites within the 1-500 region, indicating that BRCA1 1-500 may contain a site of methylation that does not follow the standard consensus site. As BRCA1 504-802 overlaps with GST-BRCA1 697-1276, which exhibited no methylation, amino acids 504-696 are the minimal area necessary for the observed methylation. According to the methylation prediction software MeMo (Figure 3a) the only arginine predicted to be methylated within the 504-802 region is R610 (highlighted in red in Figure 3c). R610 is not the typical GAR motif present in many PRMT1 substrates, but is an RXR sequence, which is methylated in Poly(A)-binding protein II (PABPII) by PRMT1 (Smith et al 1999). However, it is possible that other arginine residues within BRCA1 504-802 are methylated (highlighted in blue in Figure 3c).

To confirm an interaction between PRMT1 and BRCA1, all the GST-BRCA1 constructs utilized for the methyltransferase assay were used for a pull-down assay with total cell lysates from HeLa cells. HeLa cells were chosen as they display high levels of PRMT1 expression in

contrast to other breast cancer cell lines (data not shown). An anti-PRMT1 western blot of the GST pull-down revealed that the only detectable interaction between PRMT1 and BRCA1 was occurring at the previously identified region of 504-802 (Figure 3d). To further corroborate this interaction, whole cell extracts were prepared and immunoprecipitated with BRCA1 or IgG and western blotted with anti-PRMT1. BRCA1-PRMT1 interaction was observed specifically with the BRCA1 immunoprecipitation and not with the IgG (Figure 3e). Collectively, these results indicate that PRMT1 methylates BRCA1 *in vitro*, that the region of 504-802 is methylated, and that physical interaction of PRMT1-BRCA1 is only detectable at the 504-802 region of BRCA1.

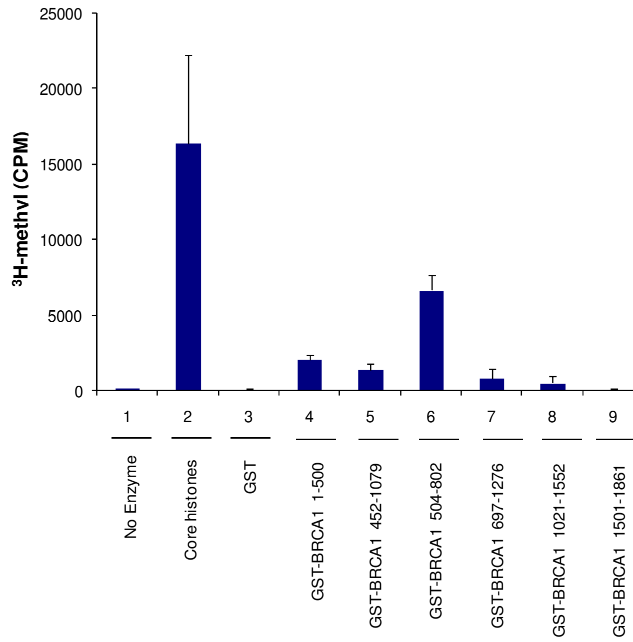
Figure 3: PRMT1 methylates and associates with BRCA1. **(A)** Layout of GST-BRCA1 constructs spanning the length of BRCA1 primary sequence and the predicted arginine methylation sites in the respective fragments. **(B)** GST-BRCA1 constructs (0.5 ug) and core histones (1 ug) were incubated with purified recombinant PRMT1 enzyme (0.2 μg) in the presence of 0.55 μCi *S*Adenosyl- L-[*methyl*-3H] methionine. GST was used as negative control and all GST-BRCA1 construct methylation levels normalized to background GST methylation levels. An average result of three replicates is shown. **(C)** The amino acid sequence of BRCA1 504-802 with R residues within the 540-696 minimal region highlighted in blue and bolded, and the predicted methylated arginine residue highlighted in red, underlined and bolded. **(D)** Two milligram of HeLa whole cell protein extract was incubated with 0.5 ug GST-BRCA1 constructs, beads were washed twice with TNE_{150} + 0.1% NP-40 and once with TNE_{50} + 0.1% NP-40, separated on a 4-20% gel by SDS-PAGE, and probed with an antibody against PRMT1. **(E)** Two milligram of HeLa whole cell protein extract were immunoprecipitated with anti-BRCA1 and anti-IgG antibodies, beads were washed twice with TNE_{150} + 0.1% NP-40 and once with TNE_{50} + 0.1% NP-40, separated on a 4-20% by SDS-PAGE, and probed with an anti-PRMT1 antibody. Input correlates to whole cell extract in order to confirm proteins probed.

A

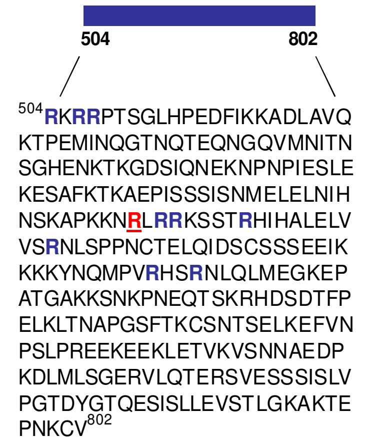


BRCA1 Constructs						
Predicted Methylation Sites	1-500	452-1079	504-802	697-1276	1021-1552	1501-1861
R610		1	1			
R959		1		1		
R1076		1		1	1	
R1204				1	1	
R1737						1
R1751						1
R1762						1
Total Sites	-	3	1	3	2	3

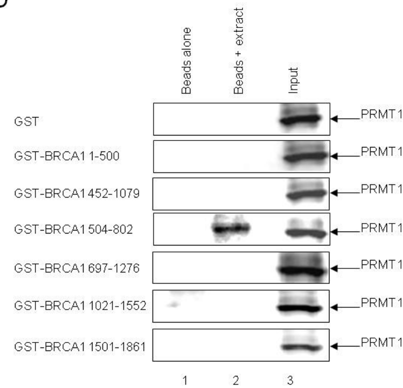
B



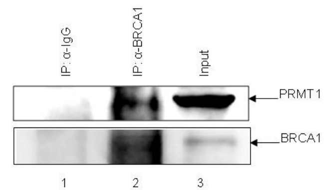
C



D

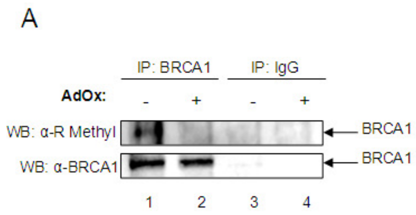


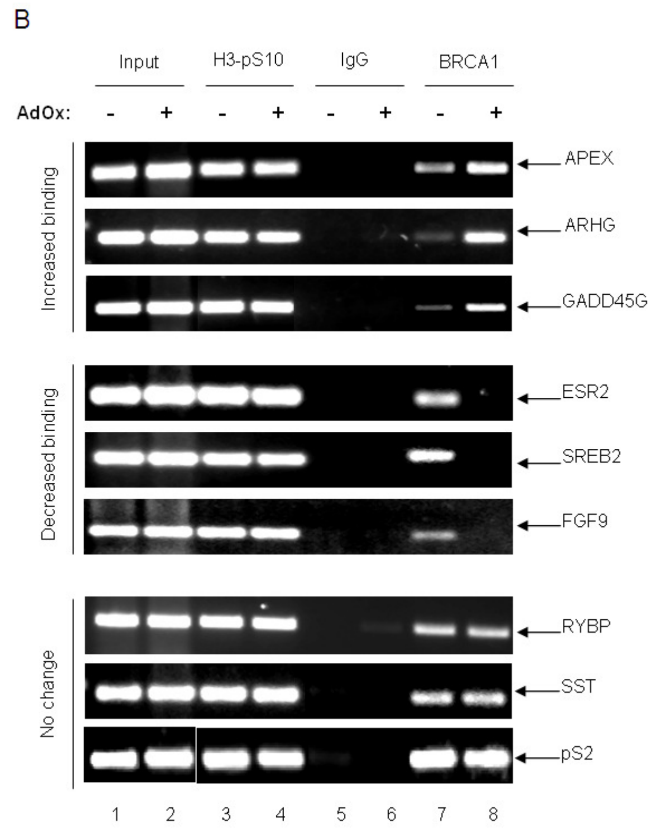
E



D. Methylation of BRCA1 alters promoter binding *in vivo*. The internal region of BRCA1 (aa 452-1079) contains two DNA binding domains, DB1 at aa 498-663 and DB2 at aa 936–1057 (Mark et al 2005). BRCA1 binds to branched DNA structures (Paull et al 2001) as well as the TTC(G/T)GTTG consensus sequence (Cable et al 2003). Therefore, it can influence DNA damage repair pathways such as homologous recombination as well as being a transcriptional cofactor. We have previously published that BRCA1 can bind to eight novel promoters containing the TTC(G/T)GTTG consensus site (Kehn et al 2007) and were interested if methylation of BRCA1 influences the binding of BRCA1 to these promoters. To this end, MDA-MB-231 cells were treated with the methyltransferase inhibitor adenosine dialdehyde (AdOx). AdOx is an indirect methyltransferase inhibitor that is vastly used for *in vitro* methylation analysis (Chen et al 2004). AdOx treatment resulted in decreased BRCA1 arginine methylation (Figure 4a), further verifying that BRCA1 is methylated and providing a system to test the influence of methylation on the function of BRCA1. ChIP assays from untreated and AdOx treated cells were performed using antibodies against histone H3-pS10 (positive control), IgG (negative control) and BRCA1. Interestingly, BRCA1 binding was increased at the AP endonuclease (APEX), Ras homolog gene family member G (ARHG) and the growth arrest and DNA damage inducible family member G (GADD45G) when BRCA1 is hypomethylated (Figure 4b). Conversely, estrogen receptor beta (ESR2), G-protein coupled receptor 85 (SREB2) and the mesothelial fibroblast growth factor 9 (FGF9) exhibited a marked decrease of BRCA1 binding upon protein methylation inhibition. Lastly, the RING1 and YY1 binding protein (RYBP) promoter, somatostatin (SST) and the estrogen regulated gene, pS2 displayed no change in promoter binding between BRCA1 methylation states (Figure 4b). These results indicate that methylation of BRCA1 could play an important role in BRCA1 DNA binding at specific promoters *in vivo*.

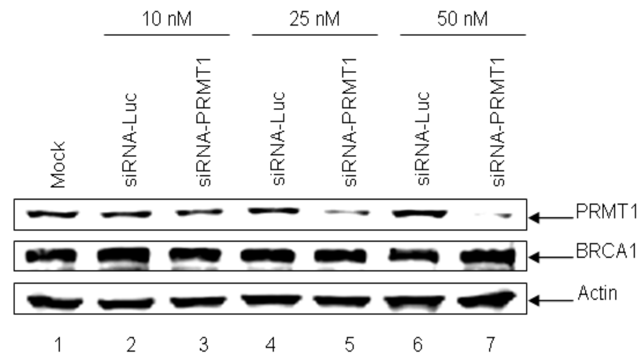
Figure 4: Methylation status of BRCA1 alters BRCA1-DNA interactions at specific promoters *in vivo.* **(A)** MDA-MB-231 cells were treated with AdOx (30 uM) in order to observe BRCA1 methylation inhibition upon treatment. Two milligram of MDA-MB-231 whole cell protein extract was immunoprecipitated with anti-BRCA1 and anti-IgG antibodies, separated on a 4-20% gel by SDS-PAGE, and probed with anti-K methyl antibody. Blot was stripped and reprobed with anti-BRCA1 antibody. **(B)** MDA-MB-231 cells were treated with AdOx (30 uM) for 48 hours prior to being collected for ChIP analysis. Antibodies used for ChIP were anti-BRCA1 (10 ug), anti-IgG (10 ug), and anti-histone H3-phosphorylated at S10 (H3-pS10, 5 ug). PCR products were run on a 2% agarose gel and visualized with ethidium bromide staining.

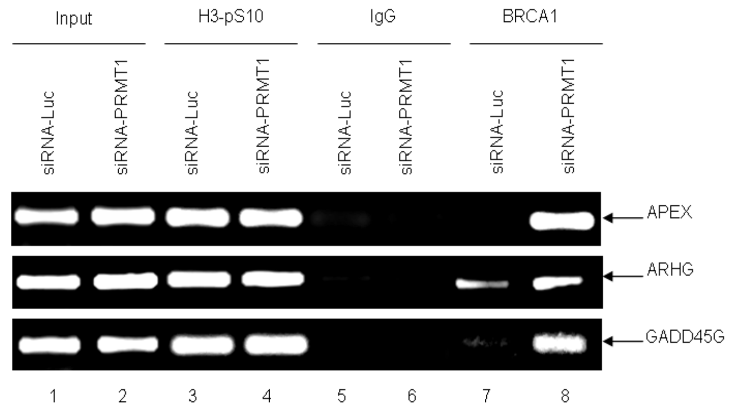

A
IP: BRCA1
IP: IgG
AdOx:
-
+
-
+
WB: α-R Methyl
WB: α-BRCA1
BRCA1
BRCA1
1
2
3
4


B
Input
H3-pS10
IgG
BRCA1
AdOx:
Increased binding
Decreased binding
No change
APEX
ARHG
GADD45G
ESR2
SREB2
FGF9
RYBP
SST
pS2
1
2
3
4
5
6
7
8

E. Decreased levels of PRMT1 alters BRCA1 promoter binding *in vivo*. To further characterize the specific impact of BRCA1 methylation by PRMT1, PRMT1 cellular levels were decreased by means of RNAi. A titration of PRMT1 siRNA indicated that at 50 nM, PRMT1 protein levels were decreased by 90% (Figure 5a). Therefore, 50 nM PRMT1 siRNA was utilized for ChIP assays to determine the influence of PRMT1 on BRCA1 DNA binding. Consistent with ChIP results from the AdOx treated cells, PRMT1 knockdown resulted in a dramatic increase of BRCA1 DNA binding to the APEX and GADD45G promoters (Figure 5b). A modest increase in binding was also observed at the ARHG gene. No change in H3-pS10 was observed following PRMT1 knockdown. These results further suggest that PRMT1 methylates BRCA1 and that its capacity to methylate affects binding of BRCA1 to its responsive promoters.

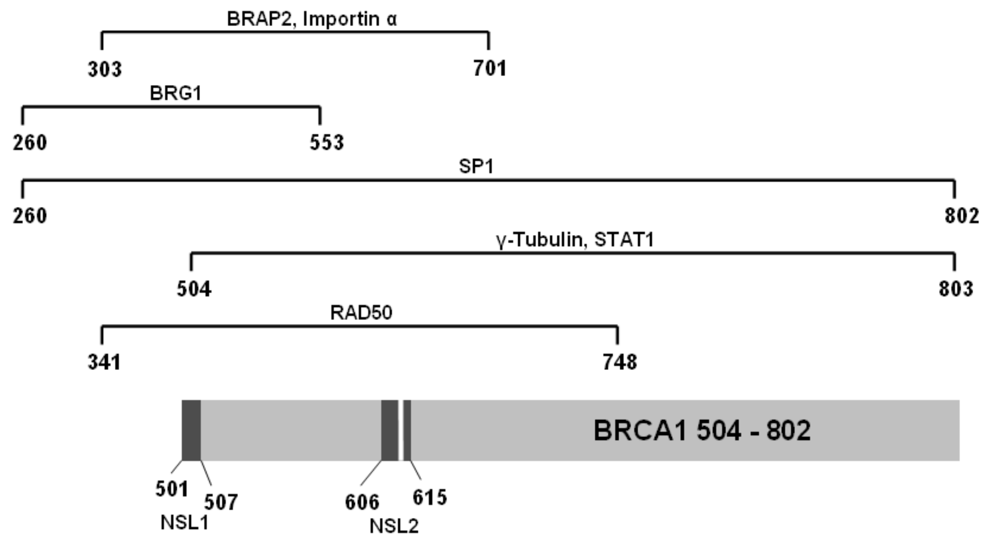
Figure 5: Decreased levels of PRMT1 alters BRCA1 promoter binding *in vivo.* (A) HeLa cells were transfected with different concentrations of PRMT1 siRNA (10, 25, 50 nM) following manufacturer's instructions. **(B)** HeLa cells transfected with 50 nM Luc or PRMT1 siRNA were collected for ChIP analysis. Anti-BRCA1 (10 ug), anti-IgG (10 ug), and anti-histone H3-phosphorylated at S10 (H3-pS10, 5 ug) antibodies were used for ChIP analysis. PCR products were run on a 2% agarose gel and visualized with ethidium bromide staining.

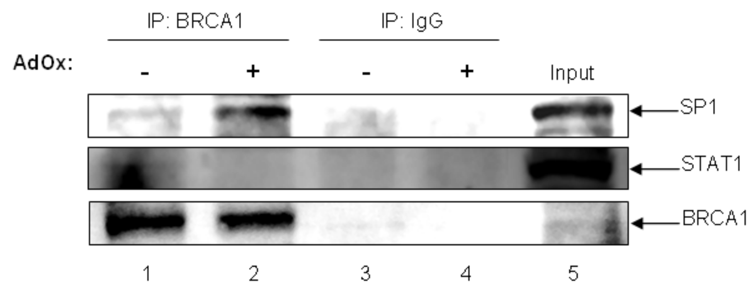

A
10 nM
25 nM
50 nM
Mock
siRNA-Luc
siRNA-PRMT1
siRNA-Luc
siRNA-PRMT1
siRNA-Luc
siRNA-PRMT1
PRMT1
BRCA1
Actin
1
2
3
4
5
6
7


B
Input
H3-pS10
IgG
BRCA1
siRNA-Luc
siRNA-PRMT1
siRNA-Luc
siRNA-PRMT1
siRNA-Luc
siRNA-PRMT1
siRNA-Luc
siRNA-PRMT1
APEX
ARHG
GADD45G
1
2
3
4
5
6
7
8

F. Methylation of BRCA1 alters protein-protein interactions. BRCA1 is known to participate in many protein-protein interactions. A schematic diagram of the BRCA1 504-802 protein region is displayed in Figure 6a to indicate protein-protein interactions that have been reported within the region and thus interactions that may be affected by the methylation status of BRCA1. These include transcriptional coactivator BRG1 (Bochar et al 2000), DNA repair protein RAD50 (Zhong et al 1999), regulators of nuclear BRCA1 transport importin-α and BRAP2 (Feng et al 2004, Li et al 1998a), centrosome and microtubule component γ-tubulin (Parvin 2009), and transcription factors Sp1 and STAT1 (Abramovitch et al 2003, Ouchi et al 2000). To investigate whether hyper- or hypo-methylation status of BRCA1 interfered with protein-protein interactions, AdOx treated MDA-MB-231 whole cell protein extracts were immunoprecipitated with anti-BRCA1 or anti-IgG antibodies. Immunoblotting of electrophoresed proteins revealed Sp1 preferentially bound to hypo-methylated BRCA1 (Figure 6b, compare lanes 1 and 2). In contrast STAT1 preferentially associated with hyper-methylated BRCA1. These results suggest that methylation of BRCA1 affects protein-protein interactions.

Figure 6: BRCA1 methylation status alters protein-protein interactions at the 504-802 region. (A) Schematic of BRCA1 504-802 primary sequence depicting important protein-protein interactions and domains that could be affected by the methylation of this region. **(B)** MDA-MB-231 cells were treated with AdOx (30 uM) in order to observe BRCA1 methylation inhibition upon treatment. Two milligram of MDA-MB-231 whole cell protein extract was immunoprecipitated with anti-BRCA1 or anti-IgG antibodies, separated on a 4-20% gel by SDS-PAGE, and western blotted using antibodies against Sp1 and BRCA1.


A
BRAP2, Importin α
303
701
BRG1
260
553
SP1
260
802
γ-Tubulin, STAT1
504
803
RAD50
341
748
BRCA1 504 - 802
501
507
NSL1
606
615
NSL2


B
IP: BRCA1
IP: IgG
AdOx:
-
+
-
+
Input
SP1
STAT1
BRCA1
1
2
3
4
5

CHAPTER V: DISCUSSION

The molecular function of BRCA1 has been subject of focused studies since it was cloned in 1994 (Miki et al 1994). Extensive studies have characterized BRCA1 as a multifaceted tumor suppressor protein due to its role in cell cycle progression, DNA repair and DNA damage response processes, transcriptional pathway regulation and apoptosis (Rosen et al 2003). BRCA1 is regulated through phosphorylation by the DNA damage response kinases, hCds1/Chk2, ATM, and ATR, following DNA damage produced by ionizing radiation, UV, or DNA damaging inducing chemicals such as mitomycin C (Cortez et al 1999, Gatei et al 2000, Gatei et al 2001, Lee et al 2000, Tibbetts et al 2000). Our *in vitro* and *in vivo* analyses indicate another avenue for BRCA1 regulation through arginine methylation, and PRMT1 as a cellular arginine methyltransferase candidate for this methylation. Interestingly, methylation of BRCA1 by PRMT1 indicates a regulatory mechanism for BRCA1 binding to particular promoters as well as protein-protein interactions.

We have demonstrated that BRCA1 is methylated both in breast cancer cell lines and breast cancer tumor samples. Both arginine and lysine methylation was detected. Interestingly, lysine methylation was only detected in MDA-MB-231 cells but not MCF-7, while arginine methylation was detected in both. Both cell lines were obtained from pleural effusions, but differ in their characteristics. *In vitro*, MDA-MB-231 cells display a highly invasive phenotype in contrast to MCF-7 cells, while they both have the ability to form *in vivo* tumors in mice (Hotary et al 2006, Man et al 2007). In correlation with breast cancer, MDA-MB-231 cells are triple negative and posses a mutant p53. MCF-7 cells are double positive, negative for HER2 and posses wild-

type p53 (Arteaga et al 1993, Lacroix and Leclercq 2004, Man et al 2007). It is tempting to speculate that methylation patterns for both lysine and arginine may be linked to phenotypical characterization of breast cancer types. However, a much larger sample size is needed to draw a clear conclusion in this regard. Arginine methylation by PRMT1 was observed *in vitro* and the region of BRCA1 504-802 was highly methylated. It is important to note that significant methylation of the BRCA1 1-500 fragment was also observed, a region that was not predicted to have arginine methylation sites by MeMo. This is indicative that PRMT1 may be recognizing novel arginine-binding motifs that are not yet documented by the program or in the literature. One well known PRMT consensus methylation sequence is the arginine and glycine-rich (GAR) motif (i.e. repeating RGG sequences), which are recognized by PRMT1, 3, 5, 6, and 8 (Bedford and Richard 2005). However, more recently a focused peptide library screen was used to identify additional sequences methylated by PRMT1 (Wooderchak et al 2008). The authors demonstrated that additional sequences such as “RLG”, “RYG”, “RFG”, “RTG”, and “RKG” were substrates for PRMT1. In addition, other PRMTs, such as PRMT4 have no known consensus site, which hinders the identity of arginine methylated proteins. The predicted methylation site at residue 610 harbors a “RXR” sequence, where X is occupied by a leucine, making it the most likely candidate for methylation within that area.

Upon methylation inhibition, *in vivo* BRCA1 binding to the APEX, ARHG and GADD45G promoters was increased. BRCA1 binding to the ESR2, SREB and FGF9 gene promoters was hindered. In addition, BRCA1 binding to RYBP, SST and pS2 gene promoters was unaffected. These results suggest that methylation may influence either the ability of BRCA1 to bind to specific promoter or protein-protein interactions which alters the recruitment of BRCA1 to these promoters. As can be observed in Figure 4a, AdOx treatment abolished detectable levels of arginine methylation in BRCA1. AdOx inhibits activity of all cellular methyltransferases, thus its effect regarding PRMT1 is non-specific. However, BRCA1-DNA interaction at the APEX, ARHG and GADD45G promoters upon siRNA-mediated knockdown of PRMT1 mimicked the results observed when cells were treated with the methyltransferase inhibitor. The difference between the levels of increased binding observed in AdOx-treated versus PRMT1 knockout samples may

be due to compensatory mechanisms by other PRMTs or lysine methylation. Preliminary studies demonstrated that SETDB1, a PKMT member of the SUV39 family of SET-domain containing proteins, methylated BRCA1 *in vitro* (data not shown). Neither the site of methylation nor the influence of lysine methylation on BRCA1 is known, but will be the focus of future studies.

Protein-protein interactions at the BRCA1 504-802 region involve several proteins that are important for transcription pathways and in particular, protein localization. These include BRAP2, importin-α, BRG1, Sp1, STAT1 and γ-tubulin. BRCA1 localization also plays an important role in protein function, with both cytoplasmic and nuclear targets. Subsequently, BRCA1 has been found to posses two nuclear localizations signals (NLS) as well as one nuclear export sequence (NES) that guide the shuttling process of BRCA1 (Chen et al 1996, Rodriguez and Henderson 2000, Wilson et al 1997). Albeit, mechanisms for this shuttling process are not clearly understood. Interestingly, both NLS are located in the vicinity of the identified BRCA1 region that is being methylated. Specifically, NSL1 is located at residues 501-507 and NSL2 at residues 606-615. Furthermore, phosphorylation of T508 at the Akt concensus phosphorylation motif immediately adjacent NSL1 resulted in cytoplasmic accumulation of BRCA1 (Altiok et al 1999), suggesting that methylation of this region may possess similar shuttling regulatory mechanisms. PRMT1 is also regulated through nucleo-cytoplasmic shuttling (Herrmann et al 2005, Herrmann and Fackelmayer 2009). Importantly, enzymatic activity is required for this shuttling process where a catalytically inactive mutant of PRMT1, rapidly accumulates in the nucleus. The nuclear export of PRMT1 is dependent on the release of the enzyme from its substrates following methylation (Herrmann and Fackelmayer 2009). These findings suggest a dynamic mechanism for the regulation of substrate methylation that is dependent on the methylation status of its substrates, in this case, BRCA1.

Upon hypomethylation of BRCA1, increased binding to Sp1 protein was observed. The Sp1 transcription factor is a potent transactivator of the insulin-like growth factor-I receptor (IGF-I-R) gene. Initially, the functional interaction between BRCA1 and Sp1 was suggested to regulate the IGF-I-R, a receptor overexpressed in most breast cancers that serves as an antiapoptotic factor (Maor et al 2007b, Maor et al 2000). Later, the same group showed that BRCA1 itself

does not exhibit any specific binding to the IGF-I-R promoter but instead, it prevented Sp1 binding the promoter by BRCA1-Sp1 interaction at the BRCA1 260-802 region (Abramovitch et al 2003). Moreover, it has been found that BRCA1 gene expression is regulated by the IGF-I signaling pathway where IGF-I enhances BRCA1 promoter activity and that Sp1 is directly involved in BRCA1 gene transactivation (Maor et al 2007a). Thus, it is possible that the status of BRCA1 methylation plays a role in the transcriptional regulation of the IGF-I-R gene by BRCA1 and Sp1. Because BRCA1 has an inhibitory control of the IGF-I-R promoter as well as repressing the Sp1-induced transactivation of the IGF-I-R gene, this suggests that methylated BRCA1 state (as observed in our cancer cell lines and breast tumor tissues) allows for decreased BRCA1-Sp1 binding which could be part of a regulatory mechanism of the IGF-I-R gene expression. The *in vivo* interaction of Sp1 with the IGF-I-R promoter in MDA-MB-231 cells treated or untreated with AdOx still needs to be characterized. We have however, observed decreased BRCA1 binding at APEX promoter, which is regulated by Sp1. APEX participates in base excision repair through the recognition and the initial step toward removing abasic sites (Wilson and Thompson 1997). The APEX promoter contains Sp1 binding sites both upstream and downstream of the transcriptional start site (Akiyama et al 1994, Fung et al 2001, Ikeda et al 2002). Sp1 binding to the downstream site regulates the expression of APEX in a cell cycle dependent fashion (Fung et al 2001). Interestingly, our results indicate that hypomethylated BRCA1 binds to the APEX promoter and that this form of BRCA1 has increased binding to Sp1. Therefore it is possible that BRCA1 is acting as a transcriptional coactivator for Sp1 mediated APEX transcription. The precise role of BRCA1 in APEX transcription will be the focus of future studies.

On the other hand, upon hypomethylation of BRCA1, decreased binding to the STAT1 protein was observed. STAT1 or the signal transducer and activator of transcription 1, is a member of the STAT protein family and a transcription transactivator. Protein-protein interactions between STAT1 and BRCA1 were mapped to the BRCA1 502-802 region when it was shown that STAT1 acts synergistically with BRCA1 to both differentially activate transcription of a subset of interferon gamma (IFN-γ) genes and mediate growth inhibition by the cytokine (Ouchi et al 2000). The majority of functional mechanisms of IFN-γ are STAT1-mediated transcriptional responses.

These include growth arrest at G1 phase of the cell cycle upon viral infection (Chesler and Reiss 2002), but most important, it has been proposed to play an important role as a tumor surveillance system (Coughlin et al 1998, Kaplan et al 1998, Ouchi et al 2000). It is suggested by Ouchi et al. (Ouchi et al 2000) that this IFN-γ antitumor response requires BRCA1 as a critical component due to the promoter specific STAT1-BRCA1 dependent transcriptional activation. This differential activation was observed by the requirement of BRCA1 protein for IFN-γ activation of the p21/WAF1 gene but not the interferon regulatory factor 1 gene. The p21/WAF1 gene product is a cyclin-dependent kinase inhibitor and is a functional paradox to the extent that it can both induce cell cycle arrest and act as a tumor suppressor (Kuljaca et al 2009, Li et al 1998b) or promote tumor growth due to its antiapoptotic capacity (Gartel and Tyner 2002, Gartel and Radhakrishnan 2005). To date, it is not known how BRCA1 achieves promoter specificity for the differential coactivation of the proposed STAT1-BRCA1 complex of the IFN-γ genes. As we have shown in our study, BRCA1 methylation affixes differing promoter specificities. BRCA1 methylation may imply the recruitment of several factors that may alter chromatin structure and thus accessibility to gene promoters, an issue that requires further investigation. For example, a factor known to be recruited by both STAT1 and BRCA1 is CBP/p300 (Pao et al 2000), functionally linked to DNA topology. p300 expression has been found to be downregulated by BRCA1 and p300 expression rescues BRCA1 inhibition of ER, a critical regulator of estrogen-dependent breast cancer (Fan et al 2002, Wojciak et al 2009). Moreover, BRCA1 has been linked to chromatin remodeling in a number of reports that include functional remodeling by BRCA1 BRCT domains and activation domain (Ye et al 2001) and formation of a histone acetyltransferase complex requiring wild-type BRCA1 that may play a role in recognition of damaged DNA (Oishi et al 2006). Collectively, we propose that a hypomethylated BRCA1 inhibits STAT1-BRCA1 complex formation and thus affects important anticancer responses by IFN-γ sensitive genes that require BRCA1 for transactivation.

In our current study we have identified a novel posttranslational modification of BRCA1, methylation. We have only begun to elucidate the role of arginine methylation in terms of regulating BRCA1 protein-protein interactions and its transcriptional coactivator function. Future

studies will be focused on the identification of the methylated residues, study of lysine methylation, and determining if there is a correlation between BRCA1 methylation and tumor progression. As was the case for BRCA1 phosphorylation, this study is the beginning and multiple future research efforts are needed to uncover the intricate workings of BRCA1 methylation.

CHAPTER VI: REFERENCES

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