The Biological Significance of BRG1 Mutations

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ABSTRACT
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"Under the direction of Dr. Bernard Weissman"

Eukaryotic organisms package DNA into chromatin for compact storage in the cell

nucleus, but this packaging process results in transcriptional repression of genes. Chromatin

remodeling complexes have evolved to overcome the transcriptional repression caused by

chromatin packaging of DNA into nucleosomes by histones. One example of a chromatin

remodeling complex is the SWI/SNF complex in yeast which uses ATP to drive the

chromatin apart and make DNA accessible to transcription factors. The yeast SWI2 protein

was discovered as the catalytic subunit of the yeast SWI/SNF chromatin remodeling complex

and is required for the complex to counteract the repressive nature of chromatin. BRG1 and

BRM, SWI2 homologs, are part of human chromatin remodeling complexes and have been

shown to play a redundant role in the regulation of certain cell cycle and cellular adhesion

genes, as well as cellular pathways. Recent studies showing loss of BRG1 in human tumor

cell lines and primary tissue samples, BRG1 mutations in human tumor cell lines, a

requirement for BRG1 in Rb mediated arrest, and development of apocrine like tumors by

BRG1 heterozygous mice, have implicated a role for BRG1 in cancer development.

However, little is known about BRG1's role in the cell and the subsequent mechanistic

changes cells experience after loss of BRG1 function.   To better understand the role of

BRG1 in cancer development we studied previously characterized human tumor cell lines

with BRG1 point mutations. We found that the mutations in BRG1 resulted in the loss of

CD44 and E-cadherin gene regulation by the complex and disruption of Rb mediated arrest.

We next wanted to investigate the mechanism by which loss of BRG1 function effected gene

regulation and Rb mediated arrest. We observed that reintroduction of BRG1 into the cells,

or treatment with 5-azacytidine, demethylated bases in the CD44 and E-cadherin promoter,

leading to re-expression of the genes. Loss of functional BRG1 may lead to aberrant

methylation of target gene promoters and cancer development and/or progression through

silencing of tumor suppressor genes.

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LIST OF ABBREVIATIONS

DNA - deoxyribonucleic acid

bp -basepairs

nm – nanometers

ATP – adenosine triphosphate

HAT – histone acetyltransferase

HDAC – histone deacetylase

SWI/SNF – Switching/Sucrose Non-fermenting

BRM - Brahma

BRG1 – Brahma-related gene

BAF – BRG1 associated factors

DNMT – DNA methyltransferases

LOI – Loss of Imprinting

5-azaC – 5-azacytidine

RNA – ribonucleic acid

AR – androgen receptor

ER – estrogen receptor

EMT – epithelial-mesenchymal transition

ChIP – Chromatin Immunoprecipitation

DNBRG1 – dominant-negative BRG1

RB – retinoblastoma gene

Rb – retinoblastoma protein

PSM–RB – phosphorylation-site-mutated retinoblastoma protein

NSCLC – Non-small cell lung cancer

TMA – tissue microarray

BRDU – bromo-deoxyuridine

PCR – polymerase chain reaction

CHAPTER 1

INTRODUCTION

A. Significance

Cancer accounts for 557,271 deaths a year, nearly one quarter of all deaths in the United States (1). Since 1950, the death rate for other major diseases such as, heart disease, cerebrovascular disease, and pneumonia/influenza, have decreased while the death rate for cancer has remained the same (1). Progress in diagnosis and treatment of cancer has been slower because of the many types of cancer and the multiple factors involved in development of cancer. Therefore, research on the mechanisms involved in cancer initiation and progression can lead to breakthroughs in the diagnosis of cancer and potential therapeutic targets for treatment of cancer.

B. Chromatin

To fit cellular deoxyribonucleic acid (DNA) into a cell, DNA must be compacted by nucleosomes into a complex tertiary structure called chromatin. Each nucleosome consists of 146bp of DNA wrapped 1.65 turns around a nucleosome core, which consists of 2 copies each of histones H2A, H2B, H3, and H4 (2). This initial compaction of chromatin, called the extended form, has a diameter of ~11nm. This form of chromatin is also referred to as the "beads on a string form" of chromatin since the nucleosomes connected by DNA resemble beads on a string (Figure 1). Each histone has N-terminal tails and some have C-terminal tails that extend from the nucleosome structure. The histone tails are required for further

compaction of the chromatin structure since proteolytic cleavage of the tails abolishes this process (3). Compaction of chromatin into a solenoid arrangement is stabilized by "linker" histone H1, which along with 22bp of DNA is responsible for connecting the nucleosome cores to each other (2). The solenoid structure contains six nucleosomes per turn creating a condensed chromatin fiber approximately 30nm in diameter. According to the radial-loop model, the solenoid structure is further compacted by the formation of looped domains of DNA by non-histone proteins attached to a chromosome scaffold (3). In non-dividing cells, chromosomes are not visible even with the aid of DNA stains or electron microscopy. Chromosome condensation due to helical folding of looped DNA attached to a protein scaffold occurs during mitosis and meiosis in dividing cells to create a visible structure (3) (Figure 1).

C. Chromatin Remodeling

One problem associated with compaction of DNA into condensed solenoid chromatin is the repression of transcription. Therefore mechanisms of chromatin remodeling have evolved to overcome this repressive nature of chromatin and make DNA accessible to sequence-specific transcription factors and transcription machinery (4, 5). Chromatin remodeling results in an alteration of nucleosomes to allow the binding of transcription factors and initiation of transcription (4-7). Two classes of enzymes exist, histone modifying enzymes and ATP-dependent chromatin remodeling enzymes. Histone modifying enzymes alter chromatin structure by directly adding or removing posttranslational modifications to amino acids in various histone proteins (8). A variety of histone modifications occur and distinct

short region of
DNA double helix
2 nm

"beads-on-a-string"
form of chromatin
11 nm

30-nm chromatin
fiber of
packed nucleosomes
30 nm

section of
chromosome in an
extended form
300 nm

condensed section
of metaphase
chromosome
700 nm

entire
metaphase
chromosome
1400 nm
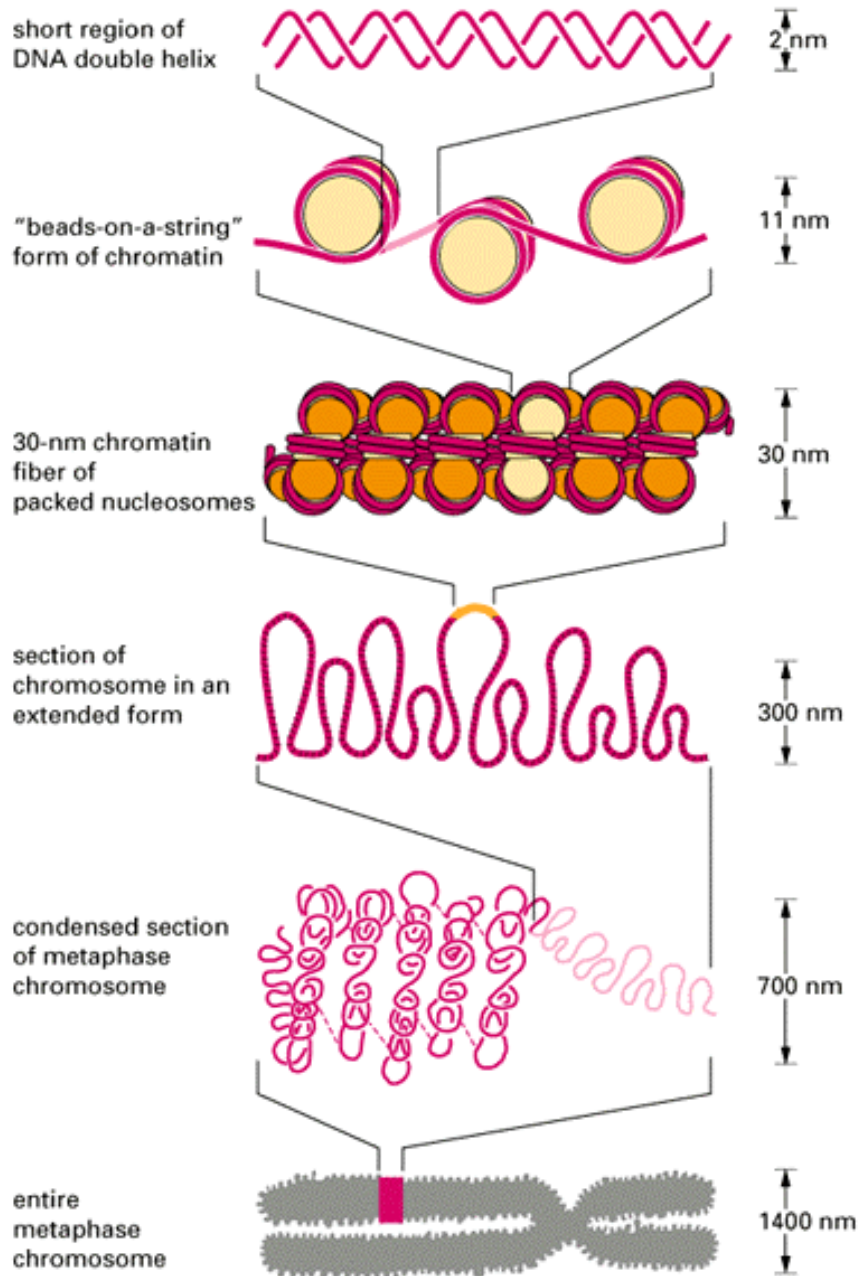
Figure 1.1: Compaction of DNA. This shows the compaction of DNA from the condensed form to the visible chromosome. From Alberts et al, Molecular Biology of the Cell, Figure 8-30.

modifications represent distinct chromatin states (Table 1). A common enzymatic alteration of histones is by histone acetyltransferase (HAT) complexes, which decrease the affinity of histones for DNA upon acetylation of lysines on histone tails, and histone deacetylase (HDAC) complexes, which reverse the effects of HATs (9). Hyperacetylation of histones is indicative of transcriptionally active genes, while hypoacetylation of histones is characteristic of inactive regions of transcription.

The other class of chromatin remodeling enzymes, ATP-dependent chromatin remodeling enzymes, are multi-protein complexes that use the energy of adenosine triphosphate (ATP) to remodel nucleosomes. The ATP-dependent movement of nucleosomes in cis along a DNA fragment result in enhanced accessibility of nucleosomal DNA (2). ATP-dependent chromatin remodeling complexes are conserved in species ranging from yeast to humans (Table 2) (6, 7). In each ATP-dependent chromatin remodeling complex there is a helicase-like subunit of the switching/sucrose non-fermenting (SWI2/SNF2) family of SF2 helicases (14). Three major subfamilies of ATP-dependent chromatin remodeling complexes exist based on sequence homology within the catalytic subunit: SWI2/SNF2, Mi-2/CHD, and ISWI families (14). The unique domains characteristic of the SWI/SNF complex catalytic subunits are the bromodomain and an AT-hook region (14). Bromodomains interact with acetylated lysines and the AT-hook region binds to AT-rich regions of DNA. These domains may help target the complex to histones and DNA helping the SWI/SNF complex in its main role of transcriptional regulation. The Mi-2 complex catalytic subunits contain chromodomains, which also bind nucleosomal DNA (14). Conversely, these complexes appear to play a role in transcriptional repression due to their association with histone

Table 1.1: Histone Modifications

Modification	Amino Acids	General Function	Reference
Acetylation	Lysine and Arginine	Gene Regulation (Activation)	(8, 9)
Methylation	Lysine and Arginine	Gene Regulation (Activation and Repression)	(8, 9)
Phosphorylation	Serine and Threonine	Chromosome Condensation	(10)
Ubiquination	Lysine	Maintenance of Histone Methylation	(11)
Sumoylation	Lysine	Maintenance of Heterochromatin	(12)
Ribosylation	Lysine	Marker of Histone in Excision DNA Repair	(13)

Table 1.2: Human ATP-dependent Chromatin Remodeling Complexes

	BAF	**PBAF**	**EBAF**	**NURD**	**RSF/CHRAC/ACF**
Type	ATP	ATP	ATP	ATP	ATP
Homology	Yeast SWI/SNF Drosophila BRM	Yeast SWI/SNF Or Yeast RSC	Yeast SWI/SNF	Xenopus Mi-2	Yeast ISWI Drosophila CHRAC ACF NURF
Complex Members	BRG1 or BRM BAF250 BAF170 BAF155 BAF110 BAF47/hSNF5 BAF60 BAF57 BAF53	BRG1 BAF180 BAF170 BAF155 BAF110 BAF47/hSNF5 BAF60 BAF57 BAF53	BRG1 BAF250 BAF170 BAF155 BAF47/hSNF5 BAF60 BAF57 BAF53 ENL EBAF140 EBAF100 EBAF 70	CHD4 HDAC1 HDAC2 RbAp48 RbAp46 MTA1/2 MBD3	hSNF2h p325

deacetylases. The ISWI complex catalytic subunits also contain unique domains that interact with histone tails called SANT domains, and SLIDE domains, that interact with nucleosomal DNA (14). ISWI complexes are involved in transcriptional regulation, chromatin assembly, and nucleosome spacing (14). These unique domains may indicate a functional specificity of different classes of SWI2/SNF2 ATP-dependent chromatin remodelers.

D. Epigenetics

The function of chromatin remodeling complexes is an essential part of the epigenetic machinery responsible for maintaining proper genome regulation. Each cell in an organism has basically identical genomes, but each cell has a distinct structure and function. In a single cell, the majority of genes are inactivated and the structure and function of the cell is defined by a few selectively activated genes. The difference in gene expression is established in a cell by the epigenetic machinery composed of DNA methyltransferases, methyl-CpG binding proteins, histone modifying enzymes, chromatin remodeling factors, and transcriptional factors (15). The unique gene expression of cells is maintained through replication by epigenetics, which is defined as "heritable changes in gene expression that occur without a change in DNA sequence" (15).

DNA Methylation

The three main types of epigenetic inheritance are DNA methylation, genomic imprinting, and histone modifications (16). The first type of inheritable epigenetics to be discovered was alteration of DNA methylation. Southern blotting of DNA digested with methylation-sensitive restriction enzymes found hypomethylation of CpG dinucleotides in cancer cells when compared to normal tissue (17). Hypomethylation can lead to activation of

genes, and subsequent expression of oncogenes in cancer cells. HRAS is an example of an oncogene activated by hypomethylation in human cancer (18). The mechanism by which demethylation occurs is still unknown. Two possible mechanisms are either a passive mechanism whereby methylation patterns are not maintained during DNA replication, or by an active mechanism which would be catalyzed by an unidentified DNA demethylase (15). Research into how global hypomethylation in human cancer occurs has revealed two possible links between chromatin remodeling and maintenance of DNA methylation. Patients with the developmental disorder ATRX have mutations in the ATRX gene, a SNF2 DNA helicase involved in chromatin remodeling (19). These patients have hypomethylation of ribosomal DNA repeats (19). Lsh, a SNF2 family member, was found to be required for maintenance of normal methylation, since gene knockout in mice of Lsh leads to a global defect in methylation (20, 21).

In contrast to hypomethylation, hypermethylation or the covalent addition of methylation to cytosines by DNA methyltransferase (DNMT), is repressive to gene activation (22). Methylation patterns are maintained by DNMT1, which fully methylates the two hemi-methylated DNA strands formed during DNA replication (23). Recently, de novo methylases (DNMT3a and DNMT3b) have been found that add methyl groups to unmethylated DNA (23). Interestingly, proteins in the DNMT3 family have a region of homology to the ATRX gene, which may partially explain how loss of ATRX may lead to loss of methylation (23). The silencing of specific tumor suppressor genes, such as p16, MLH1, VHL, and E-cadherin, by hypermethylation has been identified in human cancer cells (24-27).

Other Forms of Epigenetics

Another form of heritable epigenetics is genomic imprinting, which is the silencing of one parent allele, partially regulated by methylation (28). Imprinting results in silencing of a specific parental allele and loss of imprinting (LOI) can lead to increased expression of a gene and subsequent genomic effects. IGF2 is an imprinted gene commonly associated with LOI (29). LOI of IGF2, may lead to the development of Wilms tumors. This observation along with other LOI studies shows aberrations in imprinting contribute to human disease (30).

A point of ongoing investigation is whether the hypermethylation of DNA is the initial silencing event or whether it helps maintain the silenced state. Evidence that indicates DNA methylation is a secondary event in gene silencing includes studies that show activation of MHL1 by demethylating agent 5-azacytidine (5-azaC) is rapidly reversed (31). Also histone modifications, another main form of heritable epigenetics, are shown to be involved in silencing of genes. In fact, methylation of histone H3 lysine 9, occurred in re-silencing of CDKN2A in the absence of DNA methylation (32). Similarly, CDKN2A was found to be silenced, in the absence of DNA methylation, in proliferating colonies of mammary epithelial cells that escape senescence (33). Conversely, recent biochemical studies show DNA methylation precedes H3K9 methylation of the GSTP1 gene (34). While the issue of whether DNA methylation is an initial silencing event or helps maintain silencing is still unresolved, it is clear that DNA methylation and other forms of heritable epigenetics work together to determine gene expression patterns.

Chromatin and DNA Methylation

The link between chromatin and DNA methylation was discovered when DNA templates pre-methylated in vivo only became transcriptionally silenced after packaging into repressive chromatin states (35).   Repressive chromatin states are passed along by histone modifications, such as histone methylation, which is critical to a gene's repressed state and is catalyzed by SUV39H1 (36). As mentioned before, methylation of H3 lysine 9 is critical to cytosine methylation-independent resilencing of the CDKN2A gene (32). Studies showing histone modifications leading to gene silencing independent of DNA methylation, and the involvement of ATRX and Lsh in the maintenance of DNA methylation, establish an indirect link between chromatin modification and DNA methylation. Chromatin remodeling and subsequent DNA methylation may occur in two ways. DNA methylation may occur on a gene promoter after chromatin remodeling of nucleosomes increased accessibility to the promoter. Alternatively, chromatin remodeling and DNA methylation may be linked by a chromatin remodeling enzyme directly associating with a DNMT.   The complex may first remodel and then methylate the DNA. Interestingly, a direct interaction has been shown between SNF2H and DNMT3B (37). DNMTs and other proteins that associate with methylated cytosines, such as MBD and MeCP2, have been found to associate with HDACs a common member in some chromatin remodeling complexes (38). MBD and MeCP2 associate with methylated DNA and mediate dynamic repression of gene expression (39). Research into epigenetics has revealed a complex system of gene activation and repression mediated by the cooperative efforts of DNA methylation and chromatin modification.

E. Epigenetics and Human Disease

Aberrations in epigenetic machinery result in altered gene regulation and the development of human disease. Mutations in methyl-CpG binding protein, MeCP2, leads to altered gene expression by aberrant activation of BDNF in Rett Syndrome (40). BDNF plays a crucial role in neuronal survival, development, and plasticity, and dysregulation of it accounts for the neuropathology observed in Rett Syndrome. Mutations in SNF2 family member, ATR-X, leads to changes in methylation patterns and ATR-X syndrome, a severe X-linked form of mental retardation (41). One would expect the alteration of gene expression from aberrations in epigenetics to lead to silencing of tumor suppressor genes and activation of oncogenes leading to neoplastic disease. In fact, cancer development is a common result of aberrations in epigenetic machinery (Table 3). As discussed before, altered DNA methylation patterns can lead to altered gene expression and cancer development (16). Alterations in histone modifications through changes in HDAC expression lead to cancer by increased gene repression in gastrointestinal cancers, and several forms of leukemia (42). Aberrations in ATPase dependent chromatin remodeling complexes have also been implicated in the development of human disease. Findings that loss of SNF5, a member of the human SWI/SNF complex, occurs in 99% of rhabdoid tumor cases, identifies SNF5 as a bona fide tumor suppressor gene (43). Increasing evidence also indicates a role for inactivation of other members of the SWI/SNF complex BRG1, BRM, BAF155, and BAF57 in cancer development and/or cancer progression (44-46). Loss of function of members of the SWI/SNF complex may impair its ability to regulate expression of genes involved in cellular proliferation and adhesion leading to cancer development and progression. Findings that aberrations in chromatin remodeling lead to the development of human disease, has led to

Table 1.3: Chromatin Remodeling and Human Disease

Disease	Link to Chromatin	Reference
Myeloid Leukemias	Translocation of the MOZ HAT	(47)
Promyelocytic Leukemias	Fusion of a HDAC to transcription factors	(48, 49)
Acute Myeloid Leukemia	CBP-HRX fusion	(47)
Rubinstein-Taybi Syndrome	CBP point mutations	(50)
Colorectal and Gastric Carcinomas	p300 missense mutations	(51)
Breast Cancer	Amplification and Overexpression of AIB1/HAT	(52)
ATRT/Rhabdoid	SNF5/INI1 deletion	(43)

increased investigation into the mechanism by which chromatin remodeling complexes work, in hopes of discovering more about the pathogenesis of these diseases.

F. SWI/SNF Chromatin Remodeling Complex

The SWI/SNF chromatin remodeling complex was first identified in *S. cerevisiae* by genetic screens looking at altered gene expression, which identified multiple SWI and SNF genes as positive regulators of the HO and SUC2 genes (53, 54). Mutations in genes encoding histones resulted in suppression of transcriptional defects associated with SWI and SNF mutations, indicating an involvement of these genes in chromatin remodeling (55, 56). Since mutations in multiple SWI and SNF genes resulted in similar phenotypes, it was believed they were together in a complex (57). These studies also suggested that all the members of the complex were required for their function in expression of genes. The SWI2/SNF2 subunit was found to contain a consensus helicase motif and a putative ATP binding site (58). Identification of a protein homologous to the yeast SWI2/SNF2 gene in humans (59) led to studies that purified a human SWI/SNF complex (60). The complex was isolated from multiple cell lines and was approximately 2 Mda and varied from 9-12 members depending on the tissue. Each human SWI/SNF complex contains an ATPase, BRG1 or hBRM, that is a homolog of the yeast SWI2 gene, and is required for remodeling and activation of genes. The catalytic subunit is associated with BRG1 associated factors (BAFS) of different sizes. BAFS may be involved in functional interactions between subunits, recognition of specific gene sequences, interaction with proteins that control three-dimensional nuclear architecture, and modifications to control SWI/SNF activity in different cellular conditions. The intact SWI/SNF complex was shown to remodel nucleosomes in vitro confirming a role for it in

chromatin remodeling (60-62). These findings led to the belief that the main function of the

SWI/SNF complex is gene regulation by relieving compaction of DNA from histones. The

SWI/SNF complex has been shown to associate with ~5% of yeast genes, but the mechanism

of SWI/SNF promoter specificity is not completely understood (63). Several theories exist to

explain SWI/SNF promoter specificity; 1) The "Catalytic Model" explains that SWI/SNF

causes transient changes in chromatin structure in a random manner. 2) The "Holoenzyme

Model" states SWI/SNF is recruited to the promoter of target genes by ribonucleic acid

(RNA) Polymerase II. 3) The most established theory is the "Activator Model" in which

gene-specific transcriptional activators recruit SWI/SNF to the promoter. Once at the

promoter, SWI/SNF, possibly in conjunction with HATs, remodels nucleosomes in a cis

(sliding), or trans (displacement of nucleosome to another DNA strand) manner, to allow

transcription of DNA (7, 64).

SWI/SNF and Gene Regulation in Development and Differentiation

Further studies of the SWI/SNF complex have shown a requirement for the complex

in the regulation of a variety of genes. SWI/SNF was initially found to be involved in

transcriptional regulation when studies found it bound to zinc finger domains of several DNA

binding domains including EKLF, which is necessary for stage-specific expression of the

human B-globin gene (6). BRG1 and BAF155 were found to be necessary for chromatin

remodeling and transcriptional activation of B-globin in vitro (65). These studies indicated

that the complex may be specifically recruited to promoters at specific developmental stages

in order to regulate gene expression. In fact, studies have shown the complex is involved in

development and differentiation. Knockout models of SWI/SNF complex members, BRG1,

BAF155, and BAF47 are all preimplantation embryonic lethal, suggesting a requirement for

the complex in early development (65-69). The SWI/SNF complex has also been shown to be required for transcriptional activation of nuclear receptors, which are critical for homestasis and development. BAF57 has been shown to interact with both the estrogen receptor (ER) and androgen receptor (AR) (70-72). This interaction recruits the SWI/SNF complex to AR and ER target promoters. BRG1 is also required for expression of glucocorticoid receptor target genes (73). CSF-1 was one of 80 genes found to be activated by the mammalian BAF complex by DNA microarray assay (74). It was found prior binding of NFI/CTF transcription factor was required for recruitment of the complex. CSF1 is implicated in proliferation and differentiation of macrophages. CIITA binds SWI/SNF and is responsible for transcriptional activation of MHC class II genes involved in antigen presentation (75). The SWI/SNF complex is also shown to be required for neuronal differentiation (76), thymocyte differentiation (77, 78), vasculogenesis and heart chamber maturation (79-81), osteoblast differentiation (82), and muscle differentiation (83). The SWI/SNF complex is responsible for altering chromatin at promoters of differentiation-specific loci.

The Wnt signaling pathway is essential to a number of development processes and is commonly altered in cancer development (84). B-catenin, a molecule that docks TCF transcription factors to target promoters, was found to interact with BRG1 (85). Reintroduction of BRG1 into BRG1 deficient cells leads to increased activation of a TCF-responsive reporter gene (85). The SWI/SNF complex facilitates chromatin remodeling before transcriptional activation of TCF target promoters. One gene activated by TCF is E-cadherin (86), which we have found to be up-regulated with re-introduction of BRG1 into deficient cells. Loss of E-cadherin is observed in tumors formed by epithelial-meshenchymal

15

transition (EMT) (87). BRG1/BRM deficient cell lines and tumors appear to be dedifferentiated and may be undergoing EMT, due to a loss of regulation of genes involved in development and differentiation.

SWI/SNF and Gene Regulation in Cellular Adhesion and Proliferation

The SWI/SNF complex has also been shown to be involved in the regulation of other genes that may play a role in cellular adhesion. Reexpression of BRG1 in SW13 cells enhances MMP2 expression (88). Chromatin immunoprecipitation (ChIP) demonstrates a requirement of BRG1 in recruitment of transcription factors to the MMP2 promoter. MMP2 is involved in maintaining the extracellular matrix and loss of MMP2 leads to invasion and metastasis. Reexpression of BRG1 or BRM in deficient cells upregulates expression of CD44, a protein involved in cellular adhesion and cellular metastasis (89).

The complex is also involved in controlling cellular proliferation. BRG1 or BRM chromatin remodeling activity has shown to be required for endogenous stress response by hsp70 (90). Expression of BRG1 in SW13 cells was found to affect the RHOA pathway by increasing expression of Rock1 (91). Increased expression of RHOA leads to stress fiber formation in cells. Additional studies have shown that expression of dominant-negative BRG1 (DNBRG1) increases the cell volume, area of attachment, and nuclear size of the cell indicating altered growth. These changes correlate with over-expression of two integrin proteins. The SWI/SNF complex is also found to be involved in the pathway responsible for activation of p53-dependent promoters (92). p53 is commonly mutated in human cancer. Several subunits of the complex have been shown to bind to p53 and chromatin immunoprecipitation assays show SNF5 and BRG1 are recruited to a p53-dependent promoter in vivo. Overexpression of dominant-negative SNF5 or BRG1 blocked p53-

mediated growth suppression and apoptosis. Loss of SWI/SNF function may lead to cancer by altering these important tumor suppressor pathways in cellular adhesion and cellular proliferation.

The SWI/SNF complex has also been found to be involved in transcriptional repression of several genes including c-fos and cyclin E (93, 94). This repression may be carried out by an interaction with MeCP2 and/or mSin3a HDAC complex (95, 96). SWI/SNF also associates with several proteins involved in human disease, BRCA1 (97), FANCA (98), LKB1 (99), and TACC2 (100). These proteins may recruit the complex to target genes, which are critical for normal cellular function. The role of the complex in transcriptional regulation has led to its participation in multiple cellular activities.

Other Roles of SWI/SNF

SWI/SNF modification of mononucleosomes in concert with histone acetylases enhances RSS cleavage in vitro indicating a role for SWI/SNF in recombination (101). Mutations in two homologs of the SWI/SNF complex in *C. elegans* disrupts asymmetric T cell division (102). PBAF is found to localize at kinetochores of mitotic chromosomes during mitosis and may be involved in cell division (103).

Another critical function of the SWI/SNF complex is that it is required for retinoblastoma (RB)-mediated arrest (104). Transfection of p16 or phosphorylation-site-mutated (PSM-RB), coding for a non-phosphorylateable form of Rb, into a cell results in growth arrest. In cells deficient for BRG1 or BRM, this does not occur. Reexpression of either BRG1 or BRM is required for the restoration of RB-mediated arrest. The catalytic subunits interact with Rb through a LXCXE motif and form a complex with HDAC (105). Phosphorylation of Rb by cyclin D/cdk4 disrupts association of HDAC and relieves

repression of cyclin E, allowing progression through G1 arrest (94). The association of SWI/SNF and Rb maintains repression of cyclin A, inhibiting exit from S phase until the cell is ready to progress. BRG1 has been shown to upregulate p21 leading to Rb hypophosphorylation and RB-mediated growth arrest (106). Loss of BRG1 and BRM impairs RB-mediated growth arrest and allows the cell to proliferate. The role of the SWI/SNF complex in gene regulation and cellular proliferation makes it important in maintaining proper gene expression and cellular growth.

G. BRG1

Identification:

SWI2 was identified in yeast as part of a protein complex required for activation of messenger RNA in eukaryotes. SWI2 was one of a group of activators distinct from transcription machinery and transcription factors (57). SWI2 is known to assist binding of DNA-binding regulatory factors, and suppression of SWI2 mutations by certain histone genes suggest SWI2 may aid in overcoming the repressive effects of chromatin. SWI2 is a DNA dependent ATPase that functions as a helicase and interacts with a transcription factor to go to specific DNA sequences and remodel chromatin structure. Genes homologous to SWI2 have been found in Drosophila BRM and higher order eukaryotes (59). Screening of a human HeLa cDNA library with a Drosophila BRM isolated BRG1(BRM-related gene 1), a 1,613 amino acid protein highly related to Drosophila BRM (52% identity) (59). Another homolog to Drosophila BRM has been identified as, hBRM, which is 76% homologous to BRG1 (Figure 2). The catalytic subunit is required for proper functioning of the complex.

Structural Domains:

Little is known about the structure and function of BRG1. Four domains appear to be conserved from Drosophila to humans (59, 107). The proline rich domain is conserved and the function is unknown. Recent studies have discovered the highly charged domain II interacts with transcription factors like B-catenin. The ATPase domain is the third domain and is responsible for the catalytic ability of the subunit. The fourth conserved domain is the bromodomain found to bind acetylated lysines, which may help target the complex to histones (108). BRG1 also contains a Pest-like domain, which can be involved in ubiquination, at the N-terminal end of the protein where most sequence differences with BRM exists. BRG1 also contains a helicase domain that plays a role in binding DNA and helping catalyze ATP. BRM, which has a similar structure to BRG1, has been shown to have a redundant function in terms of gene regulation and RB- mediated arrest (109). Differences in BRG1 and BRM must exist since BRG1 knockout mice are embryonic lethal, while BRM knockout mice survive and are slightly larger with no significant phenotype (66, 110). In fact, differences in the transcription factors BRG1 and BRM associate with, and the genes that they regulate have been identified (111). More research is still needed to help explain how loss of either member contributes to cancer development and cancer progression.

H. BRG1 is implicated in development of human cancer

As mentioned previously, recent studies implicate a role for BRG1 in the development of human cancer. These studies include:

1) <u>Loss of BRG1 in human tumor cell lines and primary human tissue</u>. During initial purification of the SWI/SNF complex, two human cell lines were found to lack BRG1 and BRM (60). Our laboratory screened 83 more cell lines for the presence of BRG1 and found 8

Brg1 A

Brg1 B

Brm

200 400 600 800 1000 1200 1400 1600

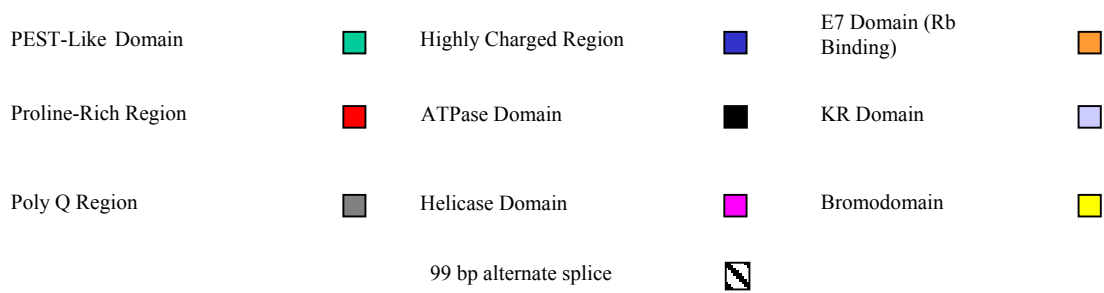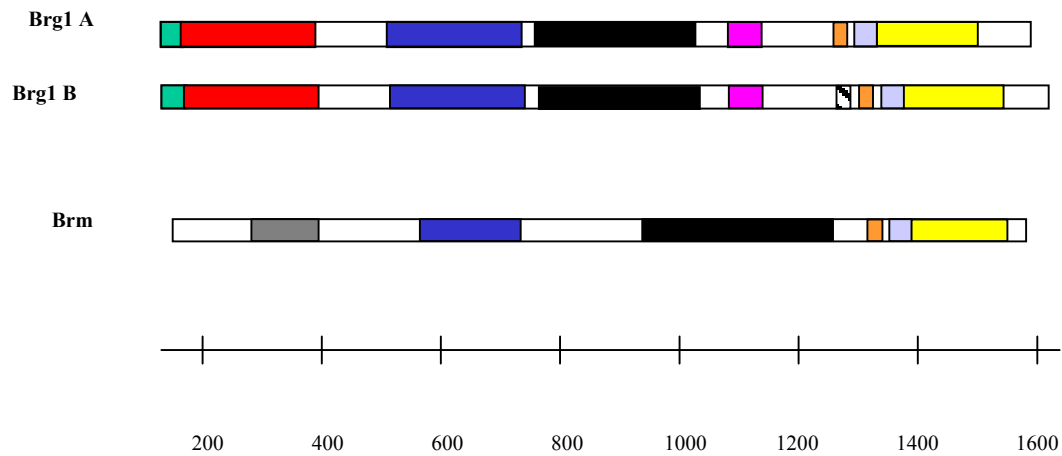| PEST-Like Domain | ■ (teal) | Highly Charged Region | ■ (blue) | E7 Domain (Rb Binding) | ■ (orange) |
Proline-Rich Region	■ (red)	ATPase Domain	■ (black)	KR Domain	■ (light blue)
Poly Q Region	■ (gray)	Helicase Domain	■ (magenta)	Bromodomain	■ (yellow)
		99 bp alternate splice	▨		

Figure 1.2: Structure of Catalytic Subunits of hSWI/SNF Complexes. The two catalytic subunits of the human SWI/SNF complex are BRG1 and BRM. The structure of BRG1 and BRM is 76% homologous. BRG1 has two splice forms, the most common form of BRG1 is Brg1A.

additional cell lines with reduced or absent expression of BRG1 (45, 104), making the total

10/85. All 10 cell lines were derived from adenocarcinoma of the respective tissue (lung,

pancreas, adrenal). Immunhistochemistry on lung adenocarcinoma primary tissue revealed

4/40 lacked expression of BRG1 (46). This may indicate a role for the loss of BRG1 in

progression of adenocarcinoma.

2) <u>The presence of BRG1 mutations in human tumor cell lines</u>. Wong et al. screened a panel

of tumor cell lines to determine if BRG1 is targeted for mutation (44). They identified

16/180 human tumor cell lines that possessed mutations in BRG1. Of these 16 cell lines, 15

were adenocarcinomas, again drawing a correlation between loss of BRG1 function and the

development of adenocarcinomas. Characteristic of tumor suppressor genes, reintroduction

of functional BRG1 into the tumor cell lines resulted in senescence (44). A screen of

primary Non-small cell lung cancer (NSCLC) tumors found two mutations in BRG1 (112).

They are in domain V of the ATPase in a region of BRG1 that also has the point mutation in

BRG1 found in HCT116 cells.

3) <u>BRG1 is required for RB-mediated cell cycle arrest</u>. Upon phosphorylation, Rb releases

E2F transcription factors to allow cell cycle progression (113). Previous studies have shown

the ability of BRG1 to bind Rb (94, 114). C33-A, a cervical adenocarcinoma cell line and

one of the original ones identified as BRG1/BRM deficient, was found resistant to the effects

of PSM-RB transfection. Transfection of PSM-RB, a mutated form of non-phosphorylatable

Rb, should lead to cell cycle arrest (104). When BRG1 and PSM-RB were co-transfected

into C33-A, the cells underwent growth arrest. These studies demonstrated a requirement for

BRG1 in RB-mediated arrest. Expression of DNBRG1 in cell lines resulted in the loss of

RB-mediated cell cycle arrest (104). These results showed a way in which loss of BRG1 function may cause loss of RB tumor suppressor activity.

4) BRG1 heterozygous mice are predisposed to tumor development. BRG1 null mice die early in development during the peri-implantation stage. BRG1 heterozygote mice are predisposed to exencephaly (5/36 mice) and tumors (66). Some BRG1 heterozygote mice (3/20) were found to develop large subcutaneous tumors of the neck and inguinal regions, compared to 0/15 wildtype mice that developed tumors (66). These tumors were epithelial and formed glandular structures (66), similar to adenocarcinomas. These tumors did not appear to have LOH of BRG1 and tumor formation appeared to be due to haploinsufficiency of BRG1. This finding indicated a potential role of BRG1 in mammalian tumor development.

5) BRG1 loss is a poor prognostic marker in NSCLC. NSCLC BRG1/BRM-negative tumor patients have a shorter survival time then NSCLC BRG1/BRM-positive tumor patients (115). NSCLC BRG1/BRM-negative tumor patients of all stages have a shorter survival time then NSCLC BRG1/BRM-positive tumor patients in stage 3 (115). BRG1/BRM loss is a poor prognostic marker for NSCLC. Immunohistochemical examination of 12 core proteins involved in chromatin remodeling on a tissue microarray (TMA) of 150 lung adenocarcinomas and 150 squamous cell carcinomas supported these results. It was found that positive Brm staining lead to 53.5% 5-year survival, compared with 32.3% for tumors negatively stained for BRM (116). It was also determined that patients with both BRG1 and BRM had a 5-year survival of 72%, while only 33.6% had a five year survival if lacking staining of either or both.

I. Specific Aims

Since all members of the SWI/SNF complex are required for proper functioning in yeast, we wanted to see how loss of other complex members besides SNF5 lead to cancer development and/or cancer progression. A logical subunit to investigate is one of the catalytic subunits, BRG1, since loss of the catalytic ability of the complex would most likely result in loss of function of the complex. The specific aims for this research are:

Specific Aim 1. To determine the effects of known BRG1 mutations on BRG1's biological activity. To test the effects of BRG1 mutations from known human tumor cell lines we made stable clones of BRG1 mutant cell lines expressing BRM RNAi and tested them in four ways to determine the functional effects of the BRG1 mutations. 1) We tested the ability of BRG1 to growth arrest cells in the presence of PSM-RB or p16 by bromo-deoxyuridine (BRDU) incorporation assays. 2) We tested if the BRG1 mutants retain the ability to regulate CD44 and E-cadherin protein expression. 3) We tested if BRG1 mutants remained in a complex with other SWI/SNF proteins by immunoprecipitation. 4) We tested if the BRG1 mutants were able to target promoters by chromatin immunoprecipitation

Specific Aim 2. To determine if loss of BRG1 alters promoter methylation patterns.
We screened cell lines with/without BRG1 by western blot for genes known to be inactivated by promoter methylation. Upon identification of CD44 and E-cadherin as candidate genes altered by BRG1 loss, we assessed if transfection of wild type BRG1, and treatment by 5-

azacytidine led to their re-expression. We also examined if cytosine methylation status of

target gene promoters changed in the presence or absence of BRG1 by bisulfite sequencing.

To determine if loss of BRG1 altered promoter methylation globally we ran assays to

determine methylation status of the genome in the presence and absence of BRG1.

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CHAPTER 2

BRG1 Mutations found in Human Cancer Cell Lines Inactivate Rb-mediated Cell Cycle Arrest

A. Introduction

Eukaryotic organisms package DNA into condensed solenoid chromatin for compact storage in the cell nucleus. This packaging results in transcriptional repression of genes. Therefore, mechanisms of chromatin remodeling have evolved to overcome this repressive nature of chromatin and make DNA accessible to sequence-specific transcription factors and transcription machinery (1, 2). Chromatin remodeling results in an alteration of nucleosomes to allow the binding of transcription factors and initiation of transcription (1, 2). Two classes of chromatin remodeling enzymes exist, histone modifying enzymes and ATP-dependent chromatin remodeling enzymes.

The SWI/SNF complex is an ATP-dependent chromatin remodeling complex that is conserved from humans to yeast (3). The complex is approximately 2-Mda in size and consists of 9-12 members in a tissue specific manner (4). To date, six SWI/SNF complexes have been isolated with each complex consisting of one catalytic subunit, either BRG1 or BRM, plus 8-11 BAFs (BRG1 associated factors). A minimum catalytic core of BRG1 or BRM, BAF155 or BAF170, and BAF47 is required for disruption of nucleosome arrays in vitro, but all members of the complex are required for proper functioning in yeast.

The main function of the SWI/SNF complex is transcriptional regulation. SWI/SNF has been shown to be required for expression of a variety of genes involved in cellular

adhesion such as CD44 (5) and E-cadherin (6), and genes involved in cellular proliferation such as cyclin A (7) and cyclin E (7), CSF1 (8), and p53-dependent target promoters (9). SWI/SNF has also been shown to be required for RB-mediated arrest by upregulating p21, which inhibits cyclin-dependent kinases and leads to RB hypophosphorylation (10). Due to the role of the complex in cellular adhesion and growth arrest, it is not surprising evidence has linked loss of SWI/SNF complex members with human disease.

The first association between loss of a SWI/SNF complex member and human disease was established when loss of BAF47 was found to lead to development of rhabdoid tumors (11). Increasing evidence indicates a role for inactivation of other members of the SWI/SNF complex including BRG1, BRM, BAF155, and BAF57 in cancer development and/or cancer progression (12-14). As expected, loss of the catalytic subunits, BRG1 and BRM, leads to loss of function of the complex. In fact, loss of BRG1 and BRM is found in human tumor cell lines and primary tumor of lung, breast, and prostate (15). Wong et al screened a panel of tumor cell lines to determine if BRG1 is targeted for mutation (13). They identified 16/180 human tumor cell lines that possessed mutations in BRG1. We have found loss of expression of BRG1 and BRM in approximately 20% of NSCLC cell lines (13, 15). In most cases, loss of BRG1 protein expression arises from gene deletions or truncating point mutations (13). However, primary NSCLC tumors and several human tumor cell lines contain point mutations in BRG1 (13, 16). Several groups, including our own, have found loss of BRG1 and/or BRM expression in primary NSCLC (15, 17). Most importantly, loss of BRG1/BRM is an indicator of poor prognosis. NSCLC BRG1/BRM-negative tumor patients have a shorter survival time then NSCLC BRG1/BRM-positive tumor patients (15, 17).

In a previous study, tumor cell lines lacking both BRG1 and BRM expression were analyzed for impaired RB-mediated growth arrest and for expression of CD44 (18). It was found that either BRM or BRG1 was sufficient to restore RB-mediated growth arrest and CD44 expression, indicating that BRM can compensate for BRG1 loss. As a further characteristic of tumor suppressor genes, the stable reintroduction of BRG1 into the tumor cell lines resulted in replicative senescence.

In this study we analyzed if the point mutations in BRG1 found in human tumor cell lines resulted in altered functions. Therefore, we suppressed BRM expression in each cell line by stable expression of shRNA and assessed them for impaired RB-mediated arrest, CD44 expression, and E-cadherin expression. We found that 2 of the BRG1 mutations have lost the ability to promote RB-mediated cell cycle arrest and CD44 expression. We also showed that these mutant BRG1 proteins still form a complex with other members by co-immunoprecipitation and were present at target promoters by Chromatin immunoprecipitation. Our results indicate that the point mutations found in these cell lines impair the function of BRG1 by altering the ability of the complex to remodel chromatin. The sites of these point mutations should provide insight into the structure and function of this important cellular regulatory protein.

B. Material and Methods

Cell Lines:

MCF7, HeLa, SW13, OVCAR3, Hs578t, and HCT116 were obtained from ATCC. All the cell lines were grown in RPMI with 10% FBS except HCT116 which was grown in DMEM with 10% FBS.

Immunoblotting:

Protein was collected from cells grown in 100 mm tissue-culture dishes. Detergent-based extraction buffer (6) was added to the dishes and cells were collected with a scraper into an Eppendorf tube. The tube was rocked in the cold room for 10 min and then spun at 14,000 rpm for 10 min. Supernatant was collected and protein concentration was determined by Bradford protein assay. Equal amounts of protein lysate (30 ug) were separated by SDS-polyacrylamide gel electrophoresis (either 7.5% or 4-20% gradient) for 1 hr at 100 V. Protein was then transferred to Immobilon-P membranes (Millipore) for 16-18 hr at 80 mA. Membranes were blocked for 1 hr in 5% non-fat dry milk/0.2% Tween 20 in PBS. Membranes were then incubated for 2 hr in primary antibody at room temperature. Primary antibodies included BRG1 (G-7) (Santa Cruz), BRM (Dr.Yaniv Moshe), E-cadherin (Transduction Labs), Cytokeratin 18 (DC-10) (Santa Cruz), Fhit (Zymed), CD44 (H3) (Dr. Larry Sherman), VHL (G-7) (Santa Cruz), vimentin (Biomeda), p16 (BD PharMingen). Membranes were then washed 3 times for 10 min each in 200 ml of 0.2% Tween 20 in PBS. Membranes were then incubated in a 1:2000 dilution of either anti-mouse or anti-rabbit secondary antibody for 1 hr. Membranes were then washed once again, 3 times for 10 min each time. Bands were detected with ECL chemiluminescence reagent (Amersham) on Biomax ML film (Kodak).

Nuclear Extraction:

Cells were grown in 25, 150 mm dishes to obtain at least 10^9 cells. Cells were trypsinized and a cell pellet was collected. The cell pellet was washed with PBS with 5 mM $MgCl_2$. The pellet was resuspended in hypotonic buffer (10 mM Hepes pH 7.9/10 mM NaCl/1.5 mM $MgCl_2$/2 mM DTT) for 10 min. The pellet was collected and resuspended in 2X original

pellet volume with hypotonic buffer for 10 min. The pellet was Dounce homogenized with a

size A pestel. The nuclei were centrifuged in an SS34 Sorvall rotor at 4K for 10 min. The

supernatant was removed, the tubes were rebalanced and centrifuged again at 18K for 20

min. The pellet was resuspended in Nuclear Extract Buffer (20 mM Hepes ph 7.9/420 mM

NaCl/1.5mM MgCl$_2$/2.0 mM DTT/0.2 mM EDTA/25% glycerol) using 3 ml per 1×10^9 cells.

Nuclei were sonicated on Bronson Digital 1 sonicator at a setting of 20 4 times for 15

seconds each time, then dounce homogenized to release nuclear proteins. The homogenate

was placed into a prechilled plastic beaker and stirred in an ice water bath for 30 minutes.

Nuclear proteins were collected by centrifugation at 14K for 30 min in an Eppendorf

microfuge. The supernatant was then dialyzed against three changes of dialysis buffer (20

mM Hepes pH 7.9/50 mM KCl/1.0 mM DTT/0.2 mM EDTA/10% glycerol) and clarified by

an additional centrifugation in an Eppendorf microfuge at 14,000 rpm for 30 min. Protein

concentrations were determined by Bradford assay.

Immunoprecipitation:

Protein A and G sepharose beads (Amersham) were washed 5X in RIPA buffer (150mM

NaCL/1% NP40/0.5% deoxycholate/0.1% SDS/50 mM Tris pH8/5 mM EDTA/protease

inhibitor cocktail (Sigma)). Forty microliters of beads were added to 300 ug of nuclear

extract per antibody and diluted to 1 ml in RIPA buffer. Five percent of the protein sample

was removed for input control. Samples were rocked for 2 hr at 4°C. At the same time 40 ul

of beads per IP reaction were saturated in 1 mg/ml BSA and 0.3 mg/ml salmon sperm (SS).

Supernatant was collected and 300 ug of protein was diluted to 1 ml in PBS. Primary

antibodies were added (10 ul for commercial, 2 ul for BRG1 (J1)) and rocked at 4°C

overnight. BSA/SS beads were then added to each tube for 2 hr. Beads were collected and

then washed once in RIPA buffer, once in IP wash buffer (100 mM Tris HCl pH 8.5/500 mM

LiCl/1% NP40/1% deoxycholate), and once in PBS. Beads were diluted in 36 ul PBS and 9

ul of western loading buffer. After heating beads at 95°C for 5 min, 20 ul was then loaded

onto a SDS-polyacrylamide gel for immunoblot analysis.

QT-PCR ChIP:

Six 150 mm dishes of each cell line were fixed with Paro-Fixation Solution (PBS with 50

mM Hepes pH 8/1 mM EDTA/0.5 mM EGTA/100 mM NaCl/11% formaldehyde) at 32°C for

20 min. To stop fixation, glycine was added to the dishes to a final concentration of 0.125

M. Plates were washed with PBS and then cells were collected with a scraper into 1X TE.

Samples were freeze-thawed in liquid nitrogen and then sonicated with 15 pulses for 15 sec

at 30% setting. Sonicated sample was spun in a SW55 rotor for 10 min at 12,000 rpm.

Protein was then quantified by microbradford protein assay.

For Chromatin immunoprecipitation, Protein A/Protein G sepharose beads

(Amersham) were washed 5X in RIPA buffer. 40 ul of beads was added to 1 mg of protein

for 2 hr at 4°C for each reaction to preclear the protein-DNA samples. At the same time,

beads were saturated in 1 mg/ml BSA and .3 mg/ml salmon sperm for 2 hr. Nine hundred

and fifty microliters of each precleared sample was incubated overnight at 4°C, with primary

antibody (5 ul-10 ul for commercial antibodies) or PBS control. 100 ul of sample was set

aside for input. BSA-SS beads were then added and rocked for 2 hr at 4°C. To release

crosslinked DNA, beads (at this step start processing the input sample) were washed once in

RIPA buffer, three times in IP wash buffer, once again in RIPA buffer, and then two times in

TE. Two hundred microliters of Elution Buffer (70 mM Tris pH 8/1 mM EDTA/1.5% SDS)

was added to the beads and incubated at 65°C for 10min. Two hundred forty microliters of

sample was added to a fresh tube along with 10 ul 5M NaCl and incubated for 5 hr at 65°C to reverse crosslink. Next, protein was degraded with the addition of 2 ug of Proteinase K and incubation for 30 min at 45°C. DNA was phenol-chloroform extracted and ethanol precipitated and resuspended in 150 ul 0.1X TE. Five microliters of DNA was used in 25 ul QPCR reaction with 12.5 ul SYBER Green (Applied Biosystems), 7 ul ddH$_2$0, and 0.5 ul of 50 uM primer for either CD44 (forward 5'-TCCAGCCGGATTCAGAGAAA-3' reverse 5'-TTCAGCCTTTGGCCTCTCCT-3') or E-cadherin (forward 5'-TCGAACCCAGTGGAATCAGAA-3' reverse 5'-GGGTCTAGGTGGGTTATGGGAC-3'). The fluorescent signal (fluorescent staining by SYBER green of replicated DNA) was detected at each cycle. The fluorescent signal (in the exponential stage of replication) for the samples were subtracted from PBS alone samples to give a signal intensity above background to allow for comparison across samples.

Generation of Stable Clones:

The BRM and BRG1 shRNA expression vectors were previously generated by Dr. Gary Rosson (19, 20). Cell lines were transfected with these shRNAi vectors using Lipofectamine 2000 (Invitrogen) following the manufacturers instructions. Clones were selected using puromycin selection media based on death curves established for each cell line; 0.4ug/ml puromycin in RPMI for Hs578t cells, 1 ug/ml puromycin in RPMI for OVCAR3, and 2 ug/ml puromycin in DMEM for HCT116 cells. Protein was extracted from clones using a urea extraction buffer. Protein levels from the clones were analyzed by immunoblotting.

Brdu Incorporation Assay:

The assay was carried out as previously described (21). Briefly, cells were plated in eight well cover slips and transfected with 1 ug of pcDNA3 or p16/PSMRB and 0.2ug of GFP
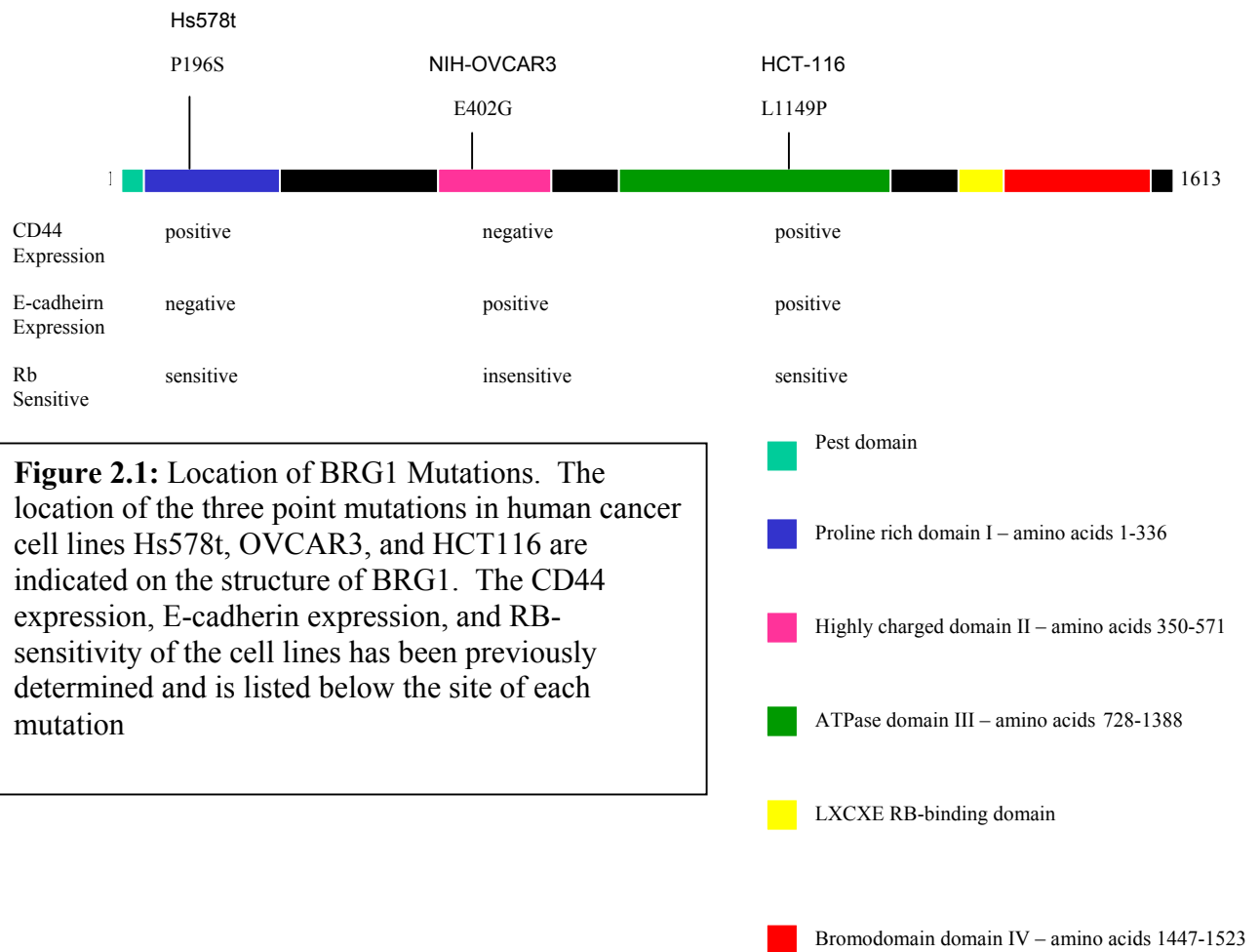
using lipofectamine 2000 (Invitrogen), following the manufacturers instructions. After 36 hr 1 ug/ml Brdu was added for an additional 12 hr. The cells were then fixed in 10% formalin for 15-20 min. Brdu incorporation was detected using mouse anti-Brdu (Amersham) primary antibody followed by alexa fluor 546 anti-mouse IgG secondary antibody (Molecular Probes). Fifty to one hundred GFP positive cells were counted for Brdu incorporation by fluorescent microscopy. The average of three experiments per sample was normalized to the parent cell line transfected with pcDNA3.

Transient Transfection:

Cell lines were transfected with shRNAi vectors using Lipofectamine 2000 (Invitrogen), following the manufacturers instructions. Transfected cells were harvested for protein 48 hours after transfection with detergent based extraction buffer (6).

C. Characterization of BRG1 mutant cell lines

To determine whether point mutations in BRG1 affected its normal functions, we chose 3 human tumor cell lines with known mutations (Figure 1). These cell lines were chosen because the mutations are in three different domains of BRG1. The Hs578t mutation is in the proline rich domain I, near an area shown to be required for B-catenin signaling (22). The OVCAR3 mutation is hemizygous, and in the highly charged domain II, whose function remains unknown (13). The HCT116 mutation is in the ATPase domain, which is responsible for hydrolyzing ATP (13). Previously, our lab determined a functional copy of either BRG1 or BRM was enough for CD44 expression and RB-mediated arrest. We have also shown transfection of BRG1/BRM-deficient cells with either BRG1 or BRM upregulates E-cadherin protein expression (6, 13). These cell lines have previously been described for their CD44 expression, E-cadherin expression, and RB-sensitivity (Figure 1).

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Figure 2.1: Location of BRG1 Mutations. The location of the three point mutations in human cancer cell lines Hs578t, OVCAR3, and HCT116 are indicated on the structure of BRG1. The CD44 expression, E-cadherin expression, and RB-sensitivity of the cell lines has been previously determined and is listed below the site of each mutation

Pest domain

Proline rich domain I – amino acids 1-336

Highly charged domain II – amino acids 350-571

ATPase domain III – amino acids 728-1388

LXCXE RB-binding domain

Bromodomain domain IV – amino acids 1447-1523

Interestingly, a western-blot screen of our BRG1 mutant cell lines shows OVCAR3 lacks CD44 expression and Hs578t lacks E-cadherin expression, even though they presumably contain a functional copy of BRM (Figure 2).

D. Mutant BRG1 proteins still form SWI/SNF complexes

Point mutations in proteins can affect their folding or their binding properties. We wanted to determine if the mutations in BRG1 in HCT116, Hs578t, and OVCAR3 inhibited formation of the SWI/SNF complex. To see if the mutant BRG1s still associated with other members in the complex, we performed immunoprecipitation of nuclear extracts with antibodies against BRG1 and BAF155. Immunoprecipitated protein was analyzed by western-blot analysis (Figure 3a-e). Protein from HeLa, our positive control for an intact SWI/SNF complex, precipitated with either anti-BAF 155 or anti-BRG1, contained other members of the complex including BRG1 or BAF155, and BAF47 (Figure 3b). Protein from SW13, our negative control that lacks BRG1 and BRM, protein, precipitated with BRG1 showed a small amount of BRG1 present by western blot (due to a small sub-population in the cell line) (23). Therefore, the other members of the complex, BAF155, and BAF47 were observed by immunoblotting (Figure 3a). In HCT116, the nuclear extract precipitated with anti-BAF155 showed less BRG1 but more BAF47 than extract precipitated with anti- BRG1 (Figure 3d). Similarly, HCT116 extract precipitated for BRG1 showed low levels of BAF155 (Figure 3d). These results may indicate that the mutant BRG1 is not as efficient at forming a complex. This may also be true for Hs578t, and OVCAR3 since immunoprecipitation with anti-BAF155 resulted in low levels of BRG1 while immunoprecipitation with anti-BRG1 showed low levels of BAF155 (Figure 3c and 3e). OVCAR3 expresses wtBRG1 so the greater BRG1/155 ratio in the BRG1 pull-down lane
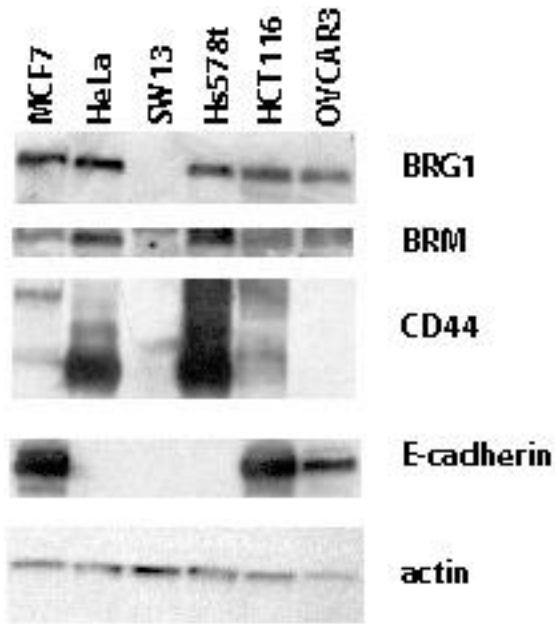
Figure 2.2: Western-blot analysis of BRG1 mutant cell lines. Protein from BRG1 mutant cell lines was separated by SDS-PAGE and immunoblotted for E-cadherin. MCF7 cells served as a positive control for E-cadherin expression. Actin was used as a loading control.
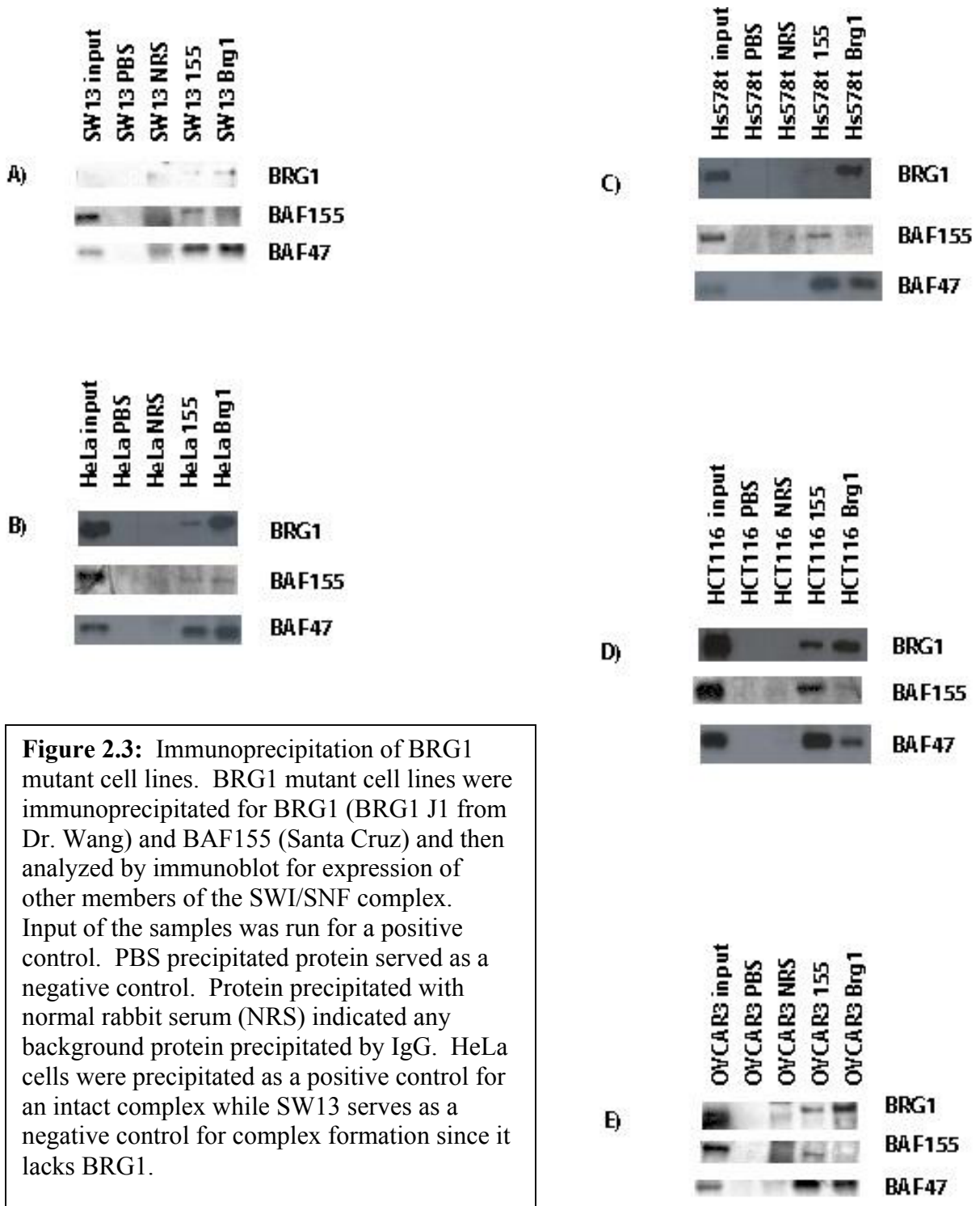
Figure 2.3: Immunoprecipitation of BRG1 mutant cell lines. BRG1 mutant cell lines were immunoprecipitated for BRG1 (BRG1 J1 from Dr. Wang) and BAF155 (Santa Cruz) and then analyzed by immunoblot for expression of other members of the SWI/SNF complex. Input of the samples was run for a positive control. PBS precipitated protein served as a negative control. Protein precipitated with normal rabbit serum (NRS) indicated any background protein precipitated by IgG. HeLa cells were precipitated as a positive control for an intact complex while SW13 serves as a negative control for complex formation since it lacks BRG1.

than the BAF155 pull-down lane may indicate interference with wtBRG1 by the BRG1

mutant protein. These results demonstrate that the mutant BRG1 proteins in the HCT116,

OVCAR3, and Hs578t cell lines might associate with other members of the complex, but

probably in a less efficient manner.

E. Promoter Targeting

For the SWI/SNF complex to regulate gene expression it must be present at target

gene promoters. Since BRG1 mutants appear to be in a complex with other members of the

complex we next determined if the complex went to target gene promoters. CD44 and E-

cadherin are two cellular adhesion proteins know to be regulated by the SWI/SNF complex.

We have previously shown the presence of BRG1 at these promoters by ChIPs (6). To

determine if the complexes containing mutations in BRG1 still localized to these target

promoters, we performed QT-PCR ChIPs on HCT116 and Hs578t cell lines to see if BRG1

was at the promoters of CD44 and E-cadherin. This analysis was not performed on the

OVCAR3 cell line because the presence of wild-type BRG1 protein would not allow us to

distinguish what was binding at the promoter. We examined binding of either BAF155 or

BRG1 to these promoters and included acetylated H3 as a positive control. We also used the

BRG1/BRM-deficient cell line SW13 as a negative control and HeLa as positive control of

BRG1 and BAF155 binding. To determine promoter binding we measured the staining of

DNA by Syber green and analyzed fluorescent intensity at each cycle of a QT-PCR assay.

The value of fluorescence (during the exponential stage of replication) for the samples was

subtracted from the PBS negative control. This gave us a normalized value for signal

intensity that we could compare across samples. As shown in Figure 4, BAF155 and BRG1

localized to the target promoters E-cadherin and CD44 at the same levels in the mutant cell

lines as in HeLa positive control cell. In contrast, the signal for BRG1 was reduced in the

BRG1 deficient-SW13 cell line but was completely reduced either due to background from

the assay or from background levels of BRG1 in SW13 due to a small subpopulation (23)

(Figure 4). These data show that the BRG1 mutations in HCT116 and Hs578t do not prevent

targeting of the complex to the CD44 and E-cadherin promoters.

F. Analysis of BRM-deficient HCT116 and Hs578t cell lines

Since the BRG1 mutant protein appeared to be in a complex with other members of the

SWI/SNF complex and at the promoter of target genes, we wanted to determine if the

function of the BRG1 mutant complexes was impaired. To test this possibility, we needed to

reduce the expression of the BRM protein because of its ability to compensate for BRG1

loss. To create HCT116, OVCAR3 and Hs578t stable knockdowns of BRG1 and BRM we

transfected each cell line with shRNAi vectors against BRM and selected stable clones. We

also isolated cell lines with stable expression of a BRG1 shRNA or vector alone as controls.

Western-blot analysis confirmed that stable expression of the RNAi vectors in the HCT116

and Hs578t cell lines resulted in downregulation of BRG1 by the BRG1 RNAi (Figure 5a

and 5c) and downregulation of BRM by the BRM RNAi vector (Figure 5b and 5d). Attempts

to generate OVCAR3 stable clones expressing a BRM shRNAi vector were unsuccessful.

Although we were able to obtain colonies for vector alone and BRG1 shRNAi transfected

cells, no colonies were able to grow out in BRM shRNAi transfected cells. Therefore, we

chose the cell lines indicated by a star in Figure 5 and tested each one for three phenotypes

associated with SWI/SNF complex function: RB-mediated cell cycle arrest and expression of
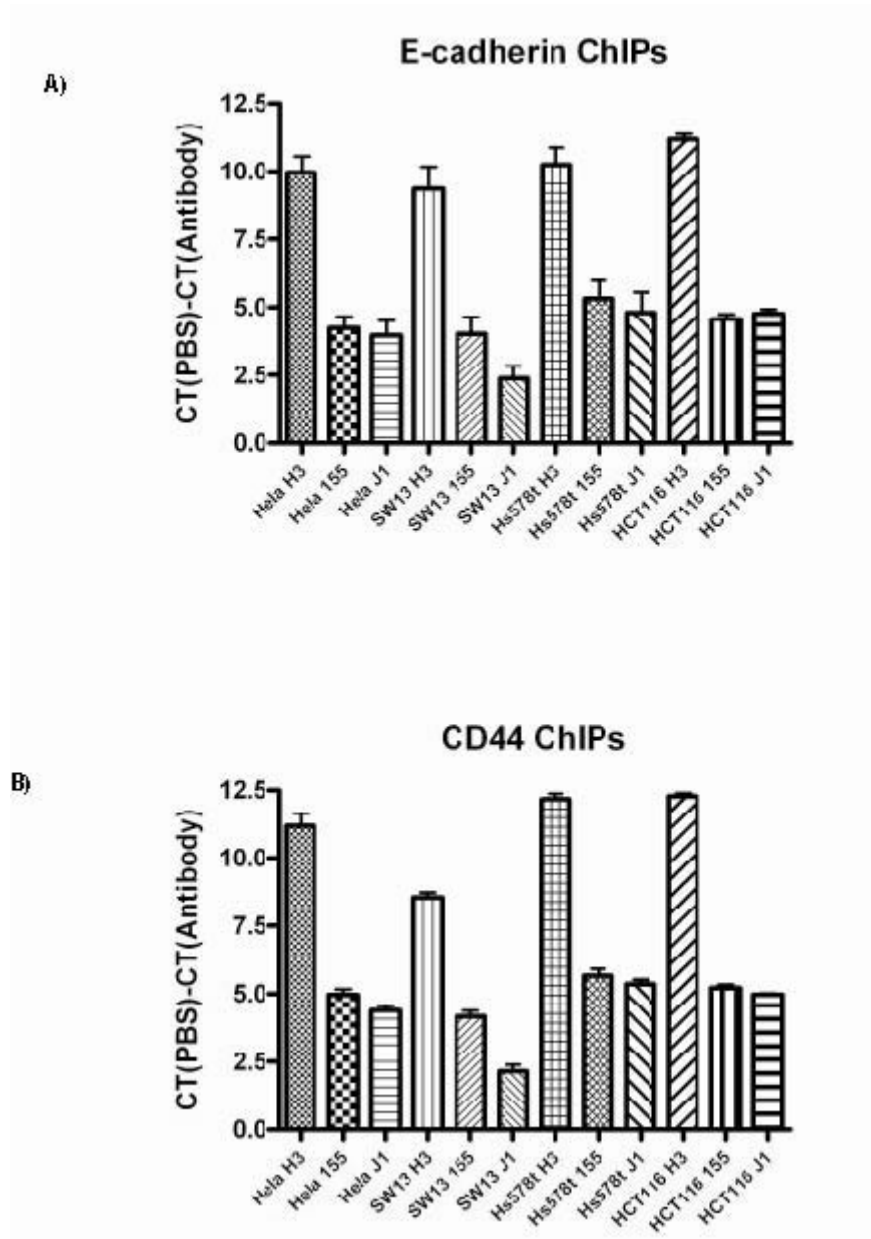
CD44 and E-cadherin protein.

Figure 2.4: Mutant BRG1 protein is at the promoter of E-cadherin and CD44. ChIP assays for interactions between mutant BRG1 and the E-cadherin promoter (A) and the CD44 promoter (B). Cross linked DNA was immunoprecipitated with antibodies against, acetylated histone 3 (H3) (Upstate Biotechnologies), BAF155 and BRG1 (J1). H3 was used as a positive control.

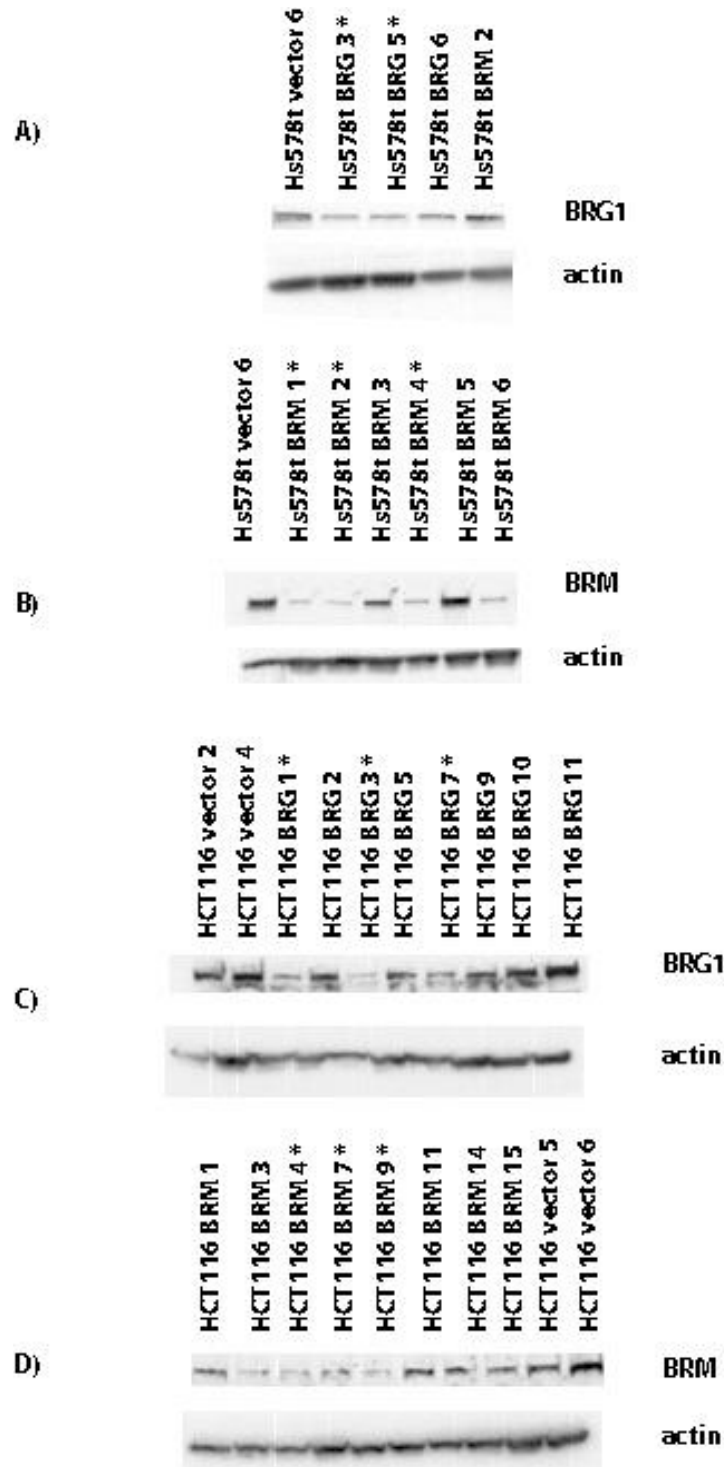**Figure 2.5:** Analysis of RNAi stable clones by immunoblot. Hs578t and HCT116 cells were transfected with shRNAi vectors and selected with puromycin. Protein was extracted from colonies using urea. The protein from colonies was separated by SDS-PAGE and immunoblotted for BRG1 (G-7) (Santa Cruz) in Hs578t cells (A) and HCT116 cells (C) and BRM (Dr. Moshe) in Hs578t cells (B) and HCT116 cells (D). Actin was used as a loading control.

G. Rb sensitivity

Normally, cells transfected with p16^{INK4A} or a constitutively active form of Rb, PSM-RB, undergo growth arrest (18). However, in previous studies, we and others have shown that cell lines without a functional BRM and BRG1 were insensitive to RB-mediated cell cycle arrest (18). Previously, Hs578t was found to be sensitive to RB-mediated arrest due to the presence of functional BRM. In a similar fashion, the p16-deficient HCT116 cell line undergoes cell-cycle arrest when transfected with PSM-RB. To determine if the BRG1 mutations affected RB sensitivity, we analyzed the RNAi stable clones of HCT116 and Hs578t for incorporation of Brdu during the S phase of the cell cycle. In parent cell lines, stable vector clones, and BRG1 RNAi stable clones, transfection of Hs578t with p16, and transfection of HCT116 cells with PSM-RB caused reduction of Brdu incorporation due to growth arrest of the cells (Figure 6). In contrast, the BRM RNAi stable clones became resistant to Rb-mediated growth arrest and continued to incorporate Brdu (Figure 6). These data indicate that the mutations in BRG1 abrogated its ability to regulate the cell cycle.

H. Expression of Target Genes

Since the BRG1 mutant proteins appeared to be in a complex with other members of the SWI/SNF complex and at the promoter of target genes, we assessed if they were still able to regulate gene expression. Western blotting showed that CD44 expression was downregulated and correlated with BRM reduction in BRM RNAi stable clones of HCT116 (Figure 7a). As expected, CD44 expression was not affected in BRG1 RNAi or vector control clones of HCT116. However, the HCT116 BRG1 RNAi stable clones appear to have lost their reduction of BRG1 protein after passage in culture. In Hs578t stable clones, no consistent pattern was found and clonal variaton appears to account for the differences in

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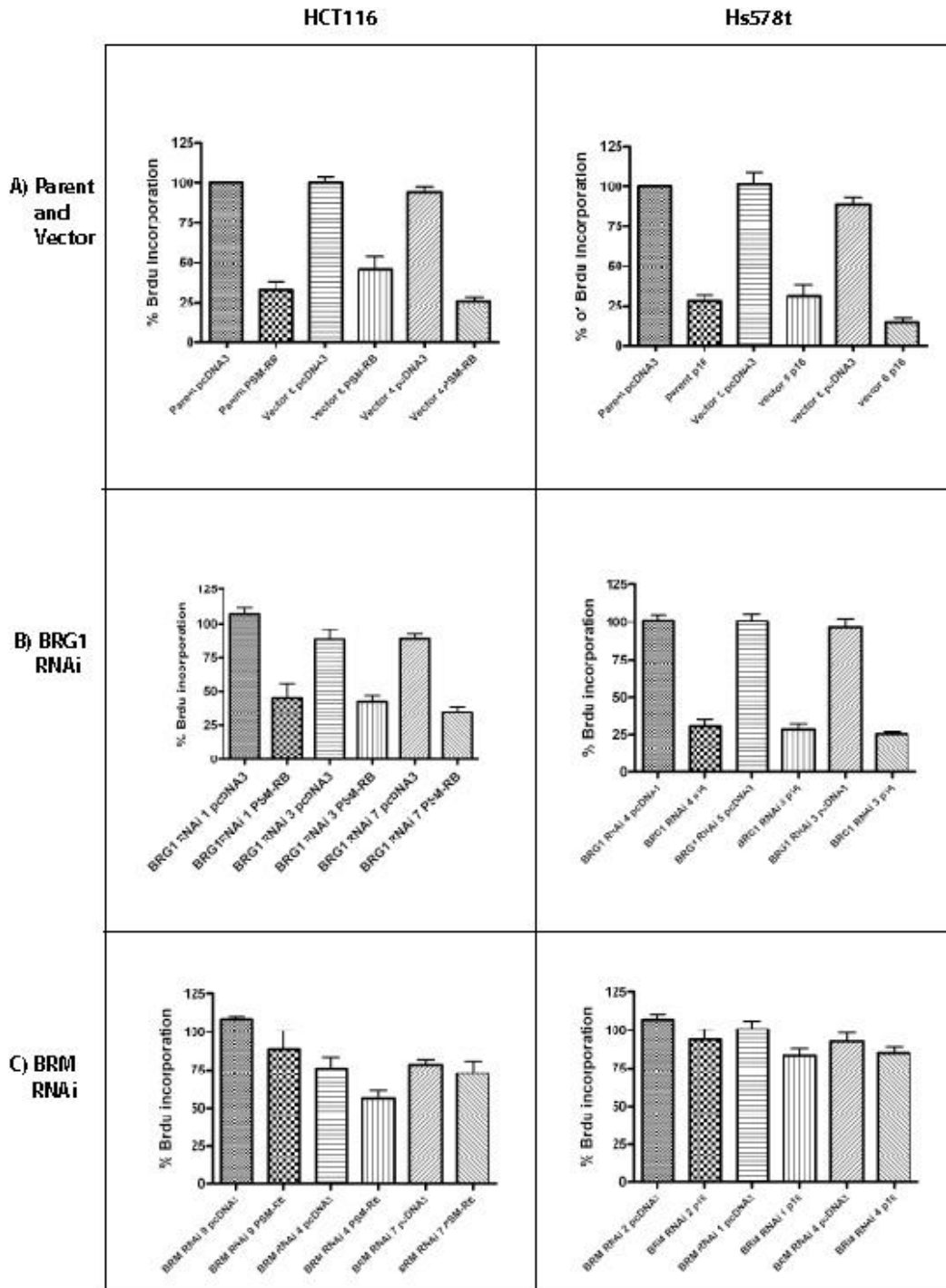**Figure 2.6:** RB sensitivity of HCT116 and Hs578t RNAi stable clones. HCT116 and Hs578t RNAi stable clones. HCT116 and Hs578t RNAi stable clones were transfected with either pcDNA3 or p16 for Hs578t cells, and PSM-RB for HCT116 cells. Brdu incorporation was assessed for Hs578t and HCT116 parent cell lines and vector alone stable clones (A), BRG1 RNAi stable clones (B), and BRM RNAi stable clones (C).

CD44 expression (Figure 7b). The BRG1 mutation in HCT116 may impair CD44 transcriptional regulation.

E-cadherin is another gene regulated by the SWI/SNF complex. Transfection of deficient cell lines with BRG1 or BRM upregulates expression of E-cadherin. The Hs578t cell line doesn't express E-cadherin , possibly due to the mutation near the B-catenin binding domain of the BRG1 protein (22). We therefore determined levels of E-cadherin protein in the HCT116 BRM RNAi cell lines. Interestingly, E-cadherin levels were unaffected in the HCT116 BRM RNAi stable cell lines (Figure 7a). In addition to the genes known to be regulated by BRG1 (CD44 and E-cadherin), we tested other commonly methylated genes (FHIT, MGMT, vimentin, cytokeratin 18), since CD44 and E-cadherin are commonly methylated (6), to see if their expression was affected in the BRM RNAi cell lines. We observed no consistent effects on the other proteins expression levels in the BRM RNAi stable cell lines.

I. Discussion

Research has focused on SWI/SNF due to increasing evidence that aberrations in the complex leads to cancer development. Several lines of evidence implicate loss of catalytic subunits in cancer development. Loss of BRG1 is found in human tumor cell lines, and primary tumors (15). Similarly, mutations in BRG1 are found in human tumor cell lines and primary tumors (13, 16). Interestingly, the presence of point mutations and internal deletions shows that BRG1 loss is not due to the presence of a larger genomic deletion. Loss of BRG1 or BRM most likely contributes to cancer development by altering expression of cellular adhesion proteins and disrupting RB-mediated growth arrest.
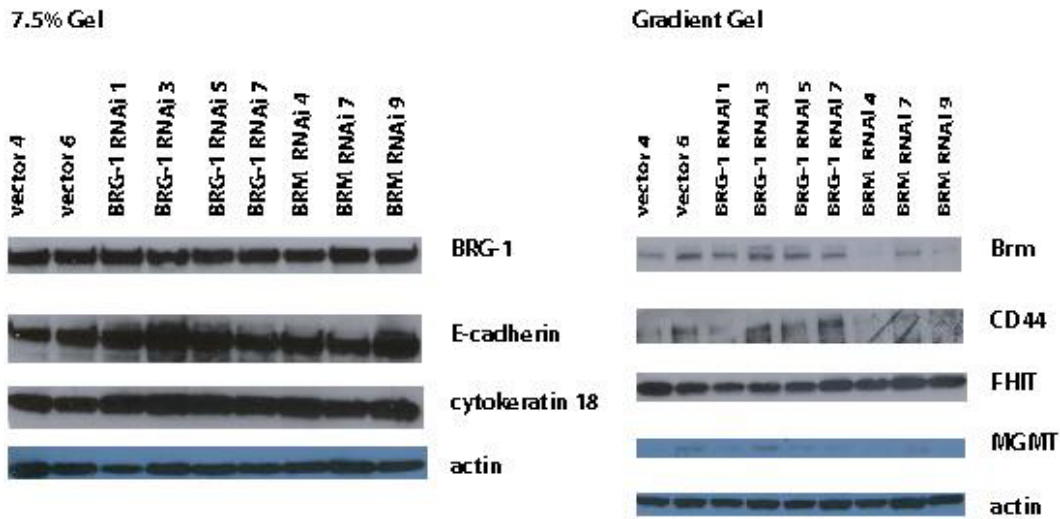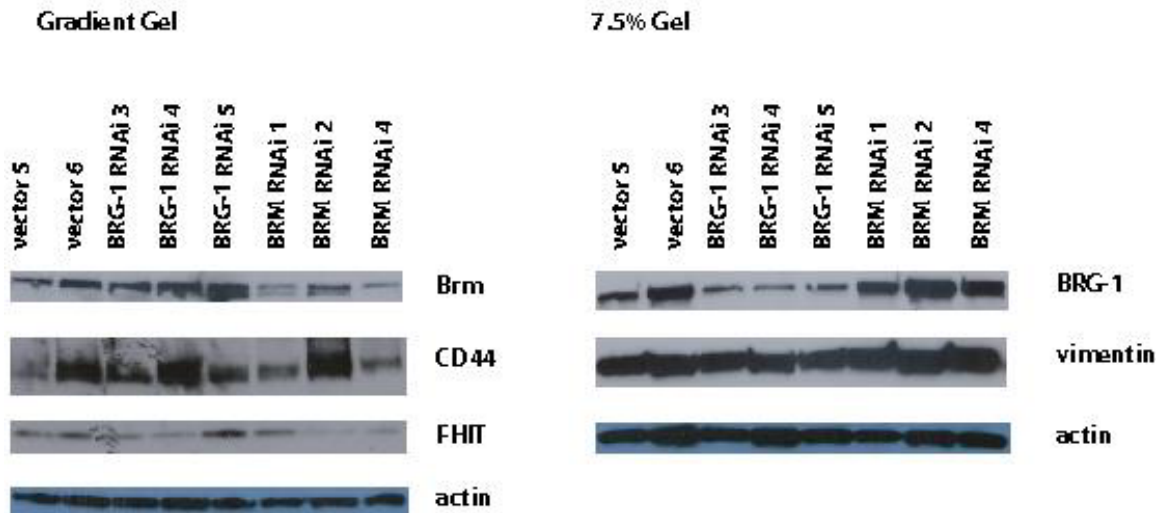
Figure 2.7: Western-blot analysis of Hs578t and HCT116 RNAi stable clones. Hs578t and HCT116 RNAi stable clones were extracted for protein using a detergent based lysis buffer. Protein from HCT116 clones (top) and Hs578t clones (bottom) was separated by SDS-PAGE and immunoblotted for CD44 and E-cadherin. Actin was used as a loading control.

Previous examination of human tumor cell lines revealed that only cell lines deficient for both BRG1/BRM were impaired for their ability to express CD44 protein and were unable to growth arrest through RB indicating compensatory function between the two (18). Expression of BRG1 or BRM has been shown to rescue these defects. HCT116 cells contain a mutation in the ATPase domain in motif V (13, 16, 24). A screen of 20 NSCLC found two mutations in BRG1, both of which are in Motif V of the ATPase domain (16). These findings indicate, although rare, BRG1 mutations may contribute to tumor development. Additional research has indicated how this mutation may impair BRG1 function. Mutations in Motif V don't impair nucleosomal binding but do appear to reduce ATPase activity (24), so mutations in Motif V are believed to alter the coupling of ATPase activity to the specific function of BRG1 (24). The BRG1 mutation in breast cancer cell Hs578t is in domain I (13). Part of domain I has been shown to interact with B-catenin (22). Previous studies have shown that these cell lines are capable of CD44 protein expression and undergo RB-mediated arrest due to presence of functional BRM (18). In this study we wanted to determine if the function of BRG1 mutants in human tumor cell lines was impaired in the absence of BRM using BRM shRNAi. We found that both the mutations found in HCT116 and Hs578t were impaired in terms of RB sensitivity. Neither mutation showed consistent effects on protein expression.

E-cadherin is not expressed in BRG1/BRM deficient cells but can be upregulated by transfection of BRG1 or BRM into deficient cells. In a western-blot screen of Hs578t and HCT116 we found Hs578t doesn't express E-cadherin. Apparently, BRM is unable to compensate for the BRG1 mutation in this cell line. The BRG1 mutation in breast cancer cell Hs578t is in domain I, a domain that is partially required to interact with B-catenin and is

required for transcriptional activation of Tcf dependent promoters (22). E-cadherin is regulated by this pathway, and aberrations in B-catenin signaling and loss of E-cadherin commonly occurs in breast cancer (25). The Hs578t E-cadherin promoter is methylated (25), but treatment with 5-azacytidine does not restore E-cadherin expression, indicating the requirement of other transcriptional factors (26). We found transfection of Hs578t cells with BRG1 doesn't upreguate E-cadherin expression (data not shown). Other labs have demonstrated inducible expression of K789R, a DNBRG1 with no ATPase activity, reduces expression of Tcf target genes (22). The BRG1 mutation in Hs578t may also be acting as a dominant negative and inhibiting BRM from compensating in the absence of functional BRG1. Reduction of BRM in HCT116 stable RNAi clones didn't result in reduction of E-cadherin expression. BRG1 and BRM are approximately 76% homologous but they lack homology in the N-terminal region of their proteins including the area of the Hs578t mutation. So alternatively, Hs578t may not express E-cadherin with functional BRM because it may be a function unique to BRG1, that only over-expression of BRM at non-physiological levels may compensate for.

Redundant functions of BRG1/BRM suggest that cells must lose expression of both genes for tumorigenic effects. Although redundant functions have been shown for the catalytic subunits in *in vitro* experiments, some assays have shown differential functions for the two proteins. Staining of MEFs from BRM knockout mice reveals that CD44 expression is lost even in the presence of BRG1 (27). This is surprising since transfection of BRG1 into deficient cells upregulates CD44 protein expression. Again overexpression of BRG1, at non-physiological levels, may be able to regulate CD44 in cells deficient for BRG1 and BRM. Kadam and Emerson have shown BRG1 and BRM exhibit transcriptional specificity. They

found BRG1 binds zinc finger protein transcription factors while BRM binds ankyrin repeat transcription factors involved in notch signaling (28). Examination of normal human tissues shows differential expression of the proteins, implicating distinct roles for the catalytic subunits in differentiated tissue (29). In fact, homozygous knockout mice indicate differential functions of the two catalytic subunits. Homozygous knockout mice for BRG1 are embryonic lethal and BRG1 heterozygous mice are prone to tumor development by haploinsufficiency (14) while knockout mice for BRM are viable with no significant phenotype (30). Due to evidence indicating differential functions of BRG1 and BRM, inactivating mutations of BRG1 would most likely lead to cancer development or progression.

Loss of BRG1/BRM was found to lead to shorter survival time in patients with NSCLC when compared to patients that had tumors expressing BRG1/BRM. In fact, BRG1 or BRM loss is a poor prognostic indicator with loss of either subunit in a tumor giving patients a 33.6% chance of 5-year survival as opposed to patients whose tumors express BRG1 and BRM having a 5-year survival chance of 72% (15). This indicates that loss of either BRG1 or BRM in differentiated cells might be an event in cancer progression instead of cancer initiation. Certainly loss of cell/cell contact adhesion molecules like CD44 and E-cadherin could lead to invasion of tumorigenic cells while loss of RB-mediated growth arrest results in increased proliferation rates and a selective growth advantage.

BRG1 has been shown to be required for differentiation of neural cells (31), muscle cells (32), and vasculogenesis (33). However once a cell is differentiated, in G0, high levels of BRM are found compared to the rest of the cell cycle when Brm is phosphorylated and degraded during G2/M. BRG1 levels are unaltered throughout the cell cycle. In fact,

omnipotent ES and pluripotent F9 embryonic mouse cells only express BRM after differentiation (30). Consistent with this observation, many normal human differentiated cells don't express BRG1 but do express BRM (29). For loss of BRG1 to contribute to initiation of cancer as opposed to progression, mutations in BRG1 may occur and lead to altered differentiation of the cell type, yielding a cell with a selective growth advantage, and subsequent tumor development. In this instance, BRG1 mutations would be directly leading to development of human cancer.

It is important to study BRG1/BRM mutations to learn more about the function of the proteins and in turn identify unique functions of the proteins. Identification of unique functions of the catalytic subunits may provide insight into how loss of them may lead to cancer progression or development. Investigating the differential expression of the catalytic subunits may lead to information about the role of the complex in different cellular environments. It will also be important to study the temporal expression of the catalytic subunits during development and differentiation to see if alterations in BRG1 during these times result in tumor development. Loss of the catalytic subunits may be more catastrophic during these developmental and differentiation windows leading to tumor development while loss of the catalytic subunits in differentiated tissues may not lead to cancer development but contribute to cancer progression. Future studies will help to define the role of the SWI/SNF complex catalytic subunits and define their importance in cancer development and/or cancer progression.

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CHAPTER 3

SWI/SNF chromatin remodeling factors induce changes in DNA methylation to promote

transcriptional activation

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Abstract

Brahma (*Brm*) and brahma-related gene-1 (*Brg1*) are mammalian homologs of SWI/SNF

chromatin remodeling factor subunits that can regulate both transcriptional activation and

repression. Both *Brg1* and *Brm* are mutated or deleted in numerous cancer cell lines, leading

to the altered expression of genes that influence cell proliferation and metastasis. Here, we

find that the promoters of two such genes, CD44 and E-cadherin, are hypermethylated in

cells that have lost Brg1 or Brm. In two carcinoma cell lines that lack functional Brg1 and

Brm, CD44 and E-cadherin expression are induced by the demethylating agent 5-aza-2'-

deoxycytidine. Transfection with either *Brg1* or *Brm* also induces CD44 and E-cadherin

transcription and protein expression in these cells, as well as loss of methylation at sequences

in the promoters of both genes. Chromatin immunoprecipitation assays show that Brg1 and

Brm associate with these regions of the CD44 and E-cadherin promoters, suggesting that

SWI/SNF protein complexes may directly influence the loss of DNA methylation. In vivo,

Brm-deficient mice also show methylation and silencing of the CD44 promoter.

Collectively, these data implicate loss of SWI/SNF-mediated transcriptional activation as a

novel mechanism to increase DNA methylation in cancer cells and provide insight into the

mechanisms underlying aberrant gene induction and repression during tumor progression.

Introduction

Mammalian SWI/SNF chromatin remodeling complexes, which use energy from ATP hydrolysis to disrupt histone-DNA interactions, contain one of two catalytic ATPase subunits, called Brm (for brahma; also called SNF2α) and Brg1 (for brahma-related gene-1; also called SNF2β). Both Brg1 and Brm have been implicated in transcriptional activation and repression (1). Mutations or deletions of these and related genes lead to altered gene expression in cancer cell lines through largely unknown mechanisms (2). Several SWI/SNF-related factors have been implicated in transcriptional silencing through DNA methylation (3-5). Indeed, DNA methyltransferase 3B (DNMT3B) interacts with the ATP-dependent chromatin remodeling enzyme SNF2H (6). It was unclear, however, if transcriptional activation conversely involved loss of DNA methylation. We previously found that cell lines and tissues lacking functional Brg1 or Brm do not express the CD44 transmembrane glycoprotein (7, 8), a cell adhesion protein whose loss in some cell types has been implicated in tumor progression (9). When these cells were transfected with Brg1 or Brm, endogenous CD44 transcription was induced (7, 8). Interestingly, CD44 transcription is silenced in a number of cancer cell lines by hypermethylation of CpG islands within the CD44 promoter that normally remain unmethylated at all times (10-14). Here, we tested if transcriptional activation by Brg1 and Brm involves loss of methylation of CpG islands in promoter sequences of both the *CD44* gene and another gene whose transcription is often silenced by DNA methylation, *E-cadherin* (15). We find, for the first time, that Brg1- and Brm-mediated transcriptional activation involves direct interactions with promoter sequences of affected genes and loss of DNA methylation.

Materials and Methods

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Plasmids

The pCG-BRM, pBJ5-BRG1, dnBRG1, and dnBRM constructs were described previously (7, 8). Plasmids were transfected using either calcium phosphate or FuGene (Roche, Nutley, NJ) according to the manufacturer's instructions. Cells were analyzed 48 hours following transfection.

Cells

The SW13, C33A, HeLa, and Saos-2 cell lines, and the BO5-1 clone of NIH 3T3 cells, carrying a tetracycline-inducible dominant-negative Brg1 construct, were all grown as previously described (7).

Immunoblotting

Cell lysates were prepared and analyzed by immunoblotting as previously described (7). Mouse anti-Brm (1:250, clone 24) and mouse-anti-E-cadherin (1:1000) were obtained from BD Transduction Laboratories (Lexington, KY); goat-anti-Brm (1:250, N-19), mouse-anti-Brg1 (1:10000, G-7), rabbit anti-Brg1 (1:1000, H-88), and goat anti-actin (1:1000, I-19) were from Santa Cruz Biotechnology (Santa Cruz, CA); the mouse anti-human CD44 (1:50, Hermes-3) was from American Type Culture Collection (Manassas, VA); mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:4000) was from Ambion (Austin, TX); horseradish peroxidase-conjugated secondary antibodies were from Biorad (Richmond, CA) and were used at 1:1000 (for anti-rabbit antibodies) or 1:2000 (for anti-goat and anti-mouse antibodies).

In vitro methylation and luciferase assays

A 0.5 kb CD44 promoter fragment was cloned from rat genomic DNA then subcloned into the PGL2 luciferase vector (Promega, Madison, WI). Plasmid DNA was methylated by incubating 1 μg of DNA with 2.5 units of SssI methylase (New England Biolabs, Beverly, MA) and 160 μmol/L S-adenosylmethionine for 2 hours at 37°C followed by a 20 minute incubation at 65°C. As a control, DNA was incubated in the presence of buffer alone. DNA was precipitated and suspended in 10 μl TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA) and cotransfeceted into cells with a CMV-β-galactosidase expression vector. After 48 hours, cells were washed with PBS, then harvested, and analyzed for β-galactosidase and luciferase activity using a Luciferase Kit (Promega).

PCR analysis of methylated DNA

Genomic DNA from cells and tissues was extracted, precipitated and suspended in TE (16). For each sample, 250 ng of genomic DNA were incubated with HpaII, MspI, or HhaI (New England Biolabs) in the recommended buffer or with buffer alone in a total volume of 20 μl for 2 hours at 37°C. Samples were incubated for 20 minutes at 65°C. DNA was precipitated then suspended in 10 μl water, and used for PCR in a final volume of 20 μl. Amplifications were carried out using a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) using the Advantage-GC genomic PCR Kit (Clontech, Palo Alto, CA) or PureTaq Ready-to-Go PCR beads (Amersham, Arlington Heights, IL). For DNA from human cell lines, the forward CD44 primer was 5'-GGATGGGCGGATGG AAGGAT-3' and the reverse primer was 5'-TCCGCTGGGCAATGAGGCTG-3'; for E-cadherin: 5'-GAACTCAGCCAAGTGTAAAAGCC-3' and 5'-GAGTCTGAACTGA CTTCCGC-3'. For GAPDH: 5'-TCCTGTTTCATCCAAGCGTG-3' and 5'-

GACTGTCGAACAGGAGGAG-3'. For amplification of mouse CD44, 5'-ATAAGTCCAGCTTCCTCAGC-3' and 5'-TCCACAGGCTTCCTACCCTG-3'. DNA was denatured by 4 minutes of incubation at 94°C then amplified using the following conditions: denaturation at 94°C for 40 sec, annealing at 64.9°C (for human CD44), 66.0°C (for mouse CD44), or 60.3°C (for E-cadherin and GAPDH) for 2 minutes; and extension at 72°C for 2 minutes for 40 cycles. Amplification was followed by 10 minutes of incubation at 72°C. Five microliters of the PCR products were then separated in a 0.8% agarose gel. The PCR products were visualized by ethidium bromide staining.

Alterations in DNA mehtylation were confirmed by sequencing DNA (from at least 10 distinct clones) following treatment with sodium bisulfite as previously described (17). For the first CD44 PCR reaction, the forward primer was 5'-TTTGTTTTAGTYGGATTTAG-3' and the reverse primer was 5'-AATAACGAACCGAACCTAAC-3' with an annealing temperature of 64°C. For the second reaction, we used 5'-GAGAAATTTAGYGGGAAAGG-3' and 5'-TCCACTAAACAATAAAACTACCTC-3' with an annealing temperature of 66.5°C. For mouse CD44, the primers used were based on sequence AF069289. For the first PCR, 5'-GGAATGGATGGGTGGATTGT-3' and 5'-AAACACACCARCCTRCTTTC-3'; for the second PCR, 5'-GAGAGYATGAATGAAATGTG-3' and 5'-TCCATCCTAATAAAAAATACTTTCC-3'. The annealing temperatures for these reactions were 56°C and 58°C, respectively. For E-cadherin, the forward primer in the first reaction was 5'-GAATTTAGTTAAGTGTAAAAGT-3'. The reverse primer was 5'-AATCTAAACTAACTTCCRCA-3' with a 50°C annealing temperature. For the second PCR, 5'-AGGTTTTAGTGAGTTATYGG-3' and 5'-CTTTACAATTCCRACRCCACT-3'

with a 60.3°C annealing temperature. PCR products were purified and subcloned into

pGEM-Teasy vector (Promega).

Reverse transciption-PCR

RNA was isolated from cells using Trizol (Invitrogen, San Diego, CA) and reverse

transcribed using a Thermoscript reverse transcription-PCR (RT-PCR) kit (Invitrogen)

according to the manufacturer's instructions. CD44 and E-cadherin sequences were

amplified using the following primers; for human CD44, forward primer 5'-

AGAAGGTGTGGGCAGAAGAAAA-3' and reverse primer 5'-

CATTCTGCAGGTTCCTTGTCT-3'; for human E-cadherin, 5'-

CGACCCAACCCAAGAATCTA-3' and 5'-AATGGCAGGAATTTGCAATC-3'; for

GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-

3'. PCR reactions were run with Platinum Taq polymerase (Invitrogen) at 94°C for 2

minutes, 58°C for 40 seconds, and 72°C for 1 minute, with 28 cycles followed by 5 minutes

at 72°C.

Chromatin Immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were done using the protocol described in a

ChIP Assay Kit from Upstate Cell Signaling Solutions (Lake Placid, NY).

Immunoprecipitations were done using 5μg of either the mouse anti-BRG1 (G-7), goat anti-

Brm (N-19), or rabbit anti-HDAC1 (H-51) antibodies (Santa Cruz Biotechnology). The

primers used to amplify E-cadherin and GAPDH are the same as those described above for

the analysis of methylated DNA. To analyze the human CD44 promoter sequences that

interacted with BRG1 and BRM, we used either the primers used above or the forward

primer 5'-CCTCTCTCCAACTCCTCTCCC-3' and the reverse primer used above. The PCR

conditions were similar to those described above with an annealing temperature of 68ºC.

Results and Discussion

We first examined the possibility that the CD44 promoter is hypermethylated in cell

lines lacking functional Brg1 or Brm and which do not express CD44. The human cervical

carcinoma cell line C33A and the human adenocarcinoma cell line SW13, which lack

functional Brg1 and/or Brm, do not express CD44 (Fig 1A; note that C33A cells express very

low levels of BRM that can be detected following longer exposure; e.g, see Fig. 2A)

compared with Brg1- and Brm-positive cell lines that express CD44, including the

osteogenic sarcoma cell line Saos2 (data not shown) and the mammary carcinoma cell line,

MCF-7 (Fig 1B). However, after ≥ 2 days in the presence of 50 µmol/L 5-aza-2'-

deoxycytidine (5-aza), which causes genomic hypomethylation by interfering with the

activities of DNA methyltransferase 1 (DNMT1), CD44 expression was induced in the C33A

and SW13 cell lines, but was not further induced in cell lines that expressed Brg1 and Brm

(Fig 1A and B). 5-aza did not affect the expression of Brg1 or Brm (Fig 1A and B).

To confirm that hypermethylation inhibits CD44 transcription in the cell lines used in

these studies, we examined whether methylating a stretch of the CD44 promoter was

sufficient to inhibit transcriptional activation. A fragment of the CD44 promoter cloned into a

luciferase reporter construct was treated with the *Sss*I CpG methylase and transfected into

SW13 (Fig. 1C), Saos2 and C33A (data not shown) cells. High levels of luciferase activity

were observed in all three cell lines following transfection with unmethylated constructs,
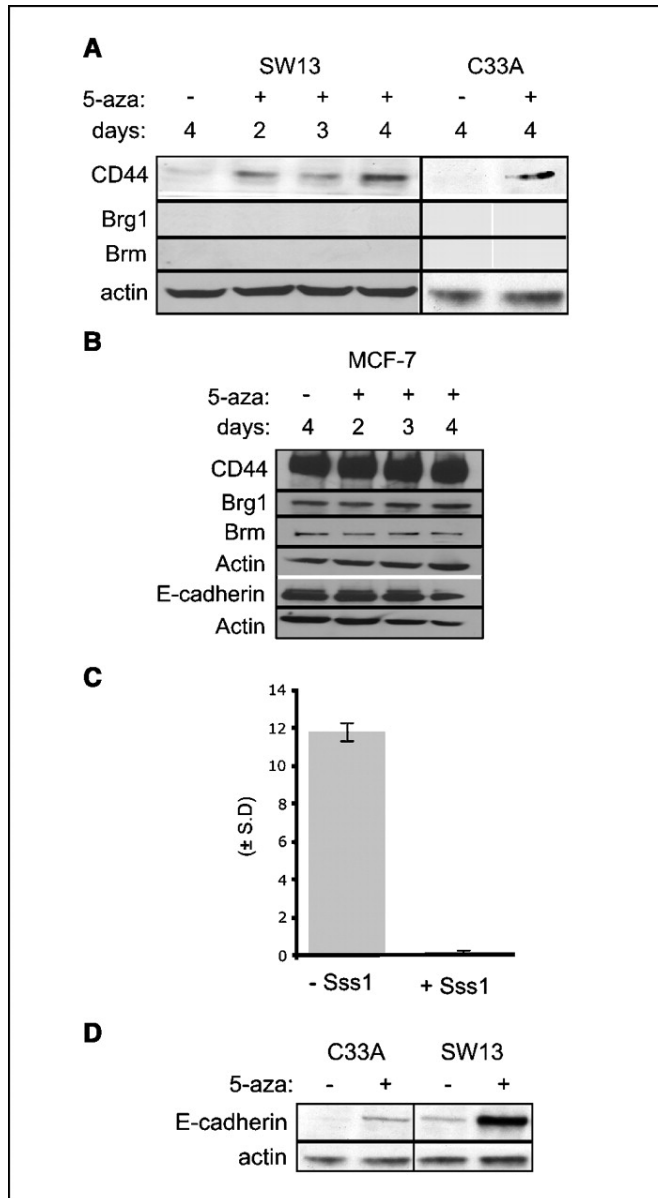
Figure 3.1: CD44 and E-cadherin promoters are hypermethylated in cell lines that lack Brg1 and Brm. *A,* Western blots of the effects of 50 µmol/L 5-aza on CD44, Brg1, and Brm expression in SW13 and C33A cells after different periods of time. Actin was used as a loading control. 5-Aza was added to cultures with fresh medium daily. *B,* effects of 5-aza on CD44, E-cadherin, Brg1, and Brm expression in MCF-7 cells. Note that E-cadherin expression was assayed in a different experiment than CD44. *C,* activity of both unmethylated and *Sss*I methylase–treated CD44 promoter DNA, confirming that SW13 cells possess the necessary transcriptional activity to promote CD44 transcription in the absence of Brg1 and Brm, and that DNA methylation blocks this activity. *D,* Western blot showing that 5-aza induces E-cadherin expression in C33A and SW13 cells.

indicating that Brg1 and Brm function are not required for the transcriptional activation of promoter sequences in plasmid DNA (Fig. 1C). Luciferase activity was abolished, however, in cells transfected with the methylated construct (Fig. 1C), confirming that CD44 transcription is silenced by methylation of promoter sequences.

We next tested the possibility that other genes might also be transcriptionally silenced by DNA methylation in cells lacking Brg1 or Brm. E-cadherin transcription is inhibited in numerous cancer cell lines, including C33A cells (18), and in tumor tissues by hypermethylation (15). We therefore tested whether E-cadherin expression was altered in C33A and SW13 cells by treatment with 5-aza, as above. C33A cells expressed no detectable E-cadherin, whereas SW13 cells expressed only low levels of the protein as determined by Western blotting (Fig. 1D). As with CD44, however, E-cadherin expression was induced in both cell lines following treatment with 5-aza (Fig. 1D). In contrast, 5-aza did not influence the expression of E-cadherin in MCF-7 cells, which express moderate levels of E-cadherin (Fig. 1B).

Consistent with our previous findings (7, 8), CD44 protein expression (Fig. 2A, top) and transcription (Fig. 2A, bottom) was induced in both SW13 and C33A cells following transfection with either Brg1 or Brm. Mutants of either protein that lack the ATPase domain failed to induced CD44 expression, indicating that ATPase activity is required for transcriptional activation (Fig. 2A). Similarly, we found here that Brg1 and Brm both induced E-cadherin expression in these cells in an ATPase-dependent manner (Fig. 2B).

Collectively, these data indicate that the *CD44* and *E-cadherin* genes are both hypermethylated in cells that lack functional Brg1 and Brm and suggest that transcriptional activation by Brm or Brg1 may involve loss of DNA methylation. To test this hypothesis,
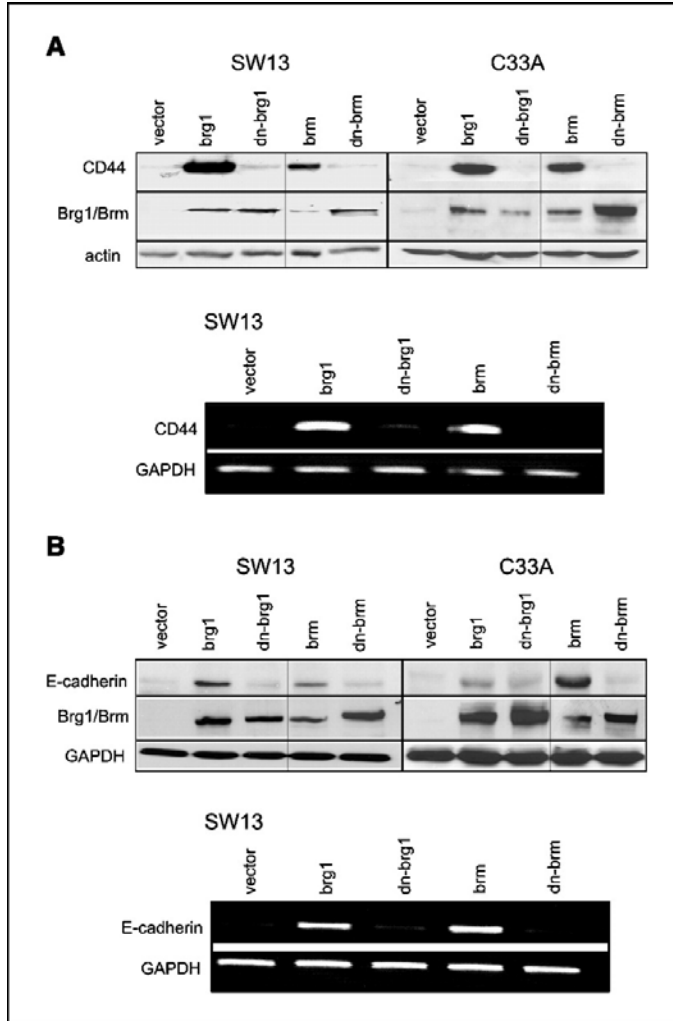
Figure 3.2: Brg1 and Brm induce CD44 and E-cadherin expression in an ATPase-dependent manner. *A,* Western blots (*top*) and RT-PCR analysis (*bottom*) showing that Brg1 and Brm but not mutants of either protein that lack the ATPase domain (dn-Brg1 and dn-Brm), induce CD44 expression in SW13 and C33A cells. Actin expression served as a loading control, while GAPDH served as a control for RT-PCR reactions. *B,* Western blots (*top*) and RT-PCR analysis (*bottom*) showing that Brg1 and Brm induce E-cadherin expression in SW13 and C33A cells. In this experiment, GAPDH protein served as a loading control for Western blots.

C33A and SW13 cells were transfected with Brg1, Brm, or vector alone, or, as a control for

demethylation, treated with 5-aza as above. Genomic DNA was isolated and incubated with

*Hpa*II or *Msp*I, restriction enzymes that are sensitive to cytosine methylation but which have

distinct sensitivities for different methylated sequences (19, 20), and amplified by PCR using

E-cadherin ([Fig. 3*A*](#)) or CD44 ([Fig. 3*C*](#)) promoter-specific primers. *Hpa*II has weak nicking

activity in the unmethylated strand of the hemimethylated sequence ^{m5}CCGG/CCGG,

whereas *Msp*I is sensitive to ^{m5}CCGG sequences (20). In the case of the E-cadherin promoter,

DNA from untreated cells could not be digested by either *Hpa*II or *Msp*I ([Fig. 3*A*](#)). Both

enzymes, however, at least partially digested E-cadherin promoter DNA following

transfection with Brg1 ([Fig. 3*A*](#)). Interestingly, in SW13 cells, which constitutively express

low levels of E-cadherin ([Fig. 1*D*](#)), we did not observe a similar change in methylation

following transfection with Brm, with only slight increases in *Hpa*II digestion ([Fig. 3*A, top*](#)).

In contrast, both enzymes could digest E-cadherin promoter DNA in C33A cells transfected

with Brm ([Fig. 3*A, bottom*](#)) suggesting that Brg1 and Brm may differ in their effects on DNA

methylation in different cells. To verify that Brg1 and Brm induce specific changes in the

methylation of the E-cadherin promoter, we used sodium bisulfite sequencing of the same

promoter region amplified in the PCR assays described above. We consistently (>80% of

clones sequenced) observed changes in three regions of a 5' untranslated region of E-cadherin

encompassing nucleotides 863 to 1138 (Genbank accession no. L34545) and containing CpG

islands at positions 887, 901, and 920 following transfection with *brg1* or *brm* in both C33A

and SW13 cells ([Fig. 3*B*](#)). The cytosines at 901 and 920 had lost methylation in all of the
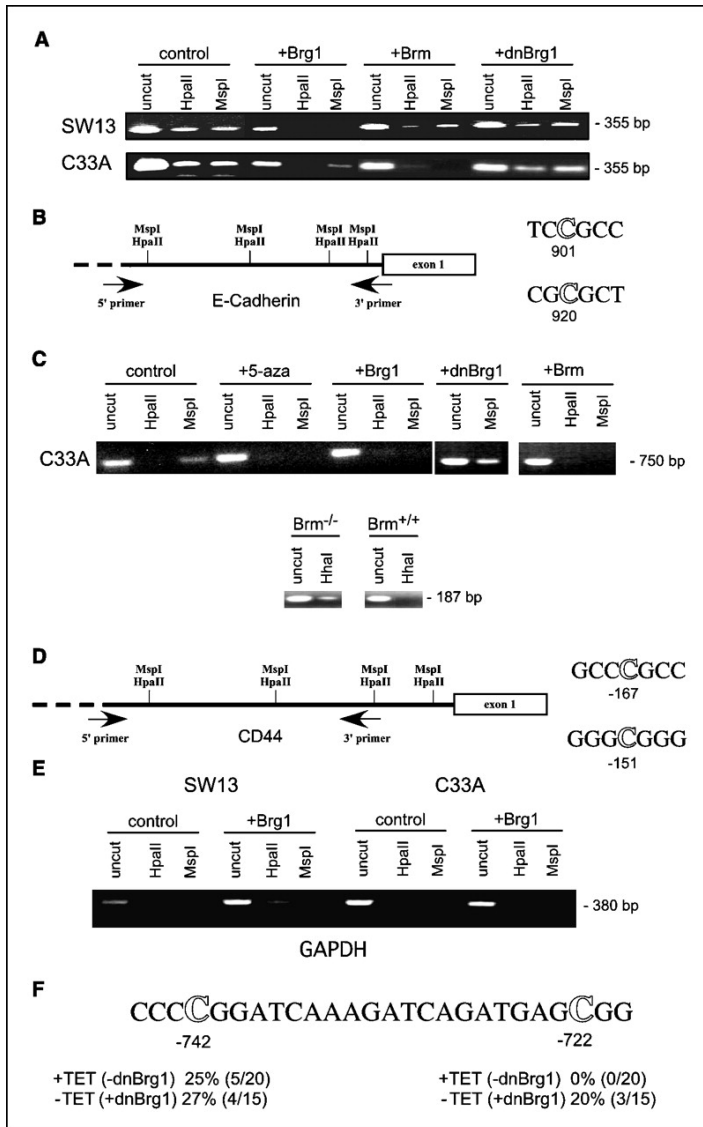
clones sequenced.

Figure 3.3: Brg1 and Brm cause loss of DNA methylation in the 5' untranslated regions of CD44 and E-cadherin. *A,* SW13 and C33A cells were transfected with Brg1, Brm, mutant Brg1 (dnBrg1), or an empty vector. DNA was isolated, incubated with methylation-sensitive restriction enzymes as indicated, and amplified using the primers shown in (*B*). *B,* altered methylation was verified by bisulfite sequencing, which demonstrated a loss of methylation in 100% of clones sequenced at cytosines 901 and 920. These sequences have previously been reported to be hypermethylated in some cancer cell lines. *C,* loss of DNA methylation was also observed in the CD44 promoter following treatment of C33A cells with 5-aza (as a positive control) or transfection with Brg1 or Brm but not mutant Brg1 (*top*). *D,* primers used to amplify enzyme-treated DNA. We also observed consistent loss of DNA methylation in the CD44 promoter in DNA isolated from the brains of adult Brm-null mice (*bottom*). Loss of methylation within the region analyzed using methylation-sensitive enzymes was confirmed by bisulfite sequencing as above, which revealed that the cytosines at positions −167 and −151 had become unmethylated in 100% of the clones analyzed following transfection with Brg1 or Brm. *E,* DNA methylation analysis of a 380-bp stretch of the 5' untranslated region of the GAPDH promoter, showing that neither Brg1 nor Brm influence the methylation of this gene. *F,* results from bisulfite sequencing of genomic DNA isolated from tetracycline-inducible NIH 3T3 cells carrying an expression construct for dnBrg1. After four passages in the presence of tetracycline (*TET*), dnBrg1 expression was repressed and the CD44 promoter was methylated in some clones at −742 but not at −722. In the absence of tetracycline for the same number of passages, there was no significant change in methylation at −742, but 20% of clones demonstrated methylation at −722.

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CD44 promoter sequences from cells transfected with empty vector were always at least partially digested by *Hpa*II but not by *Msp*I (Fig. 3C). Both *Hpa*II and *Msp*I could completely digest CD44 promoter sequences, however, if cells had been treated with 5-aza or were transfected with either Brg1 or Brm (Fig. 3C), suggesting that at least a small number of ^{m5}CCGG sequences in the CD44 promoter may become demethylated in the presence of these SWI/SNF family members. These changes were verified by sodium bisulfite sequencing as above, which revealed consistent (100% of clones sequenced) loss of methylation at two sites in the CD44 promoter (Fig. 3D), one of which included an SP1 site that had previously been reported to be hypermethylated in other cell lines (12). Transfection with mutant Brg1 (Fig. 3A and C) or mutant Brm (data not shown) had no influence on either CD44 or E-cadherin promoter methylation. Furthermore, Brg1 and Brm did not influence the methylation status of another gene, GAPDH (Fig. 3E), whose expression is unaltered in cells lacking either protein (Fig. 2B).

We next determined if loss of SWI/SNF factors results in alterations in DNA methylation *in vivo*. Although mice with targeted *brg1* mutations are embryonic lethal at very early stages of development, *brm*-null mice are viable and show little or no CD44 expression as assessed by immunohistochemistry (8). We therefore isolated genomic DNA from the brains of *brm*-null mice and wild-type littermates and assayed for changes in methylation in the CD44 promoter. For the mouse promoter sequence, we used the *Hha*I methylation-sensitive restriction enzyme, which efficiently digested this CD44 promoter DNA from wild-type mice but only partially digested DNA from *brm*-null mice, indicating that this sequence becomes methylated in the absence of Brm (Fig. 3C, bottom).

We previously found that overexpression of the Brg1 mutant lacking the ATPase domain at least partially inhibited CD44 expression in NIH 3T3 cells (7). Because we found that transcriptional activation of CD44 involves loss of promoter DNA methylation, we tested whether this mutant Brg1 inhibited CD44 transcription through a mechanism that involved increased DNA methylation. NIH 3T3 cells induced to express the mutant Brg1 protein (dnBrg1) showed reductions (20-40% by Western blotting and immunocytochemistry compared with uninduced cells) in CD44 protein expression, but we did not detect any alterations in DNA methylation either by PCR using *Hha*I digestion as above or by sodium bisulfite sequencing (data not shown). However, if cells were grown for four passages with constant induction of dnBrg1, CD44 expression was even more reduced compared with controls (50-70%), and we found increased incidence of DNA methylation in some but not all CpG islands in the CD44 promoter (Fig. 3*F*). These data suggest that although Brg1 and Brm can induce transcription via a mechanism that includes loss of DNA methylation, reducing the activity of Brg1, and possibly Brm, is not sufficient to immediately induce DNA hypermethylation. However, loss of Brg1 or Brm may promote conditions that lead to hypermethylation and transcriptional silencing following multiple rounds of cell division.

To determine if SWI/SNF complexes containing Brg1 or Brm interact directly with the CD44 and E-cadherin promoters, we did ChIP assays using Brg1 and Brm antibodies and amplified DNA sequences using primers within the same regions where we observed Brg1- or Brm-dependent loss of DNA methylation. As shown in Fig. 4*A*, both factors interacted with the CD44 promoter in Saos2 cells, which constitutively express CD44 and express wild-type Brg1 and Brm. Similar results were obtained using MCF-7 and HeLa cells (data not shown). In SW13 cells, only HDAC1 interacted with the CD44 promoter (Fig. 4*A, top*).

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When SW13 cells were transfected with Brg1, however, Brg1 did interact with CD44 promoter sequences (Fig. 4*A, middle*). Brg1 and Brm similarly interacted with the E-cadherin promoter in MCF-7 cells (data not shown) and in SW13 cells transfected with either factor (Fig. 4*B, top* and *middle*) but not with the GAPDH promoter, whose expression is not regulated by Brg1 or Brm (Fig. 4*C*). The dnBrg1 and dnBrm proteins also interacted with the CD44 and E-cadherin promoters (Fig. 4*A* and *B, bottom*), further confirming that the ATPase chromatin remodeling activities of Brg1 and Brm are required for transcriptional activation of CD44 and E-cadherin but not for recruitment to DNA.

Brg1 and Brm could influence the methylation of the E-cadherin and CD44 promoters through either a direct or indirect mechanism. It is possible, for example, that either factor could promote the activities of other genes that in turn alter DNA methylation. However, given our finding that Brg1 and Brm both interact with the CD44 and E-cadherin promoter sequences within the regions of CpG islands that become unmethylated 48 hours following transfection with either factor, we believe it is more likely that SWI/SNF factors can promote the transcription of some genes by either blocking the ability of methyltransferases, like DNMT1, to methylate promoter sequences or by recruiting a demethylase to promoter sequences.

No matter what the mechanism may be, the controlled recruitment and activation of methyltransferases or demethylases to DNA through the activities of chromatin remodeling factors provides a powerful means of regulating tissue-specific gene expression both during development and in disease. This model explains how changes in the transcriptional activation of numerous genes may be altered in cancer cells with Brg1 or Brm mutations, and
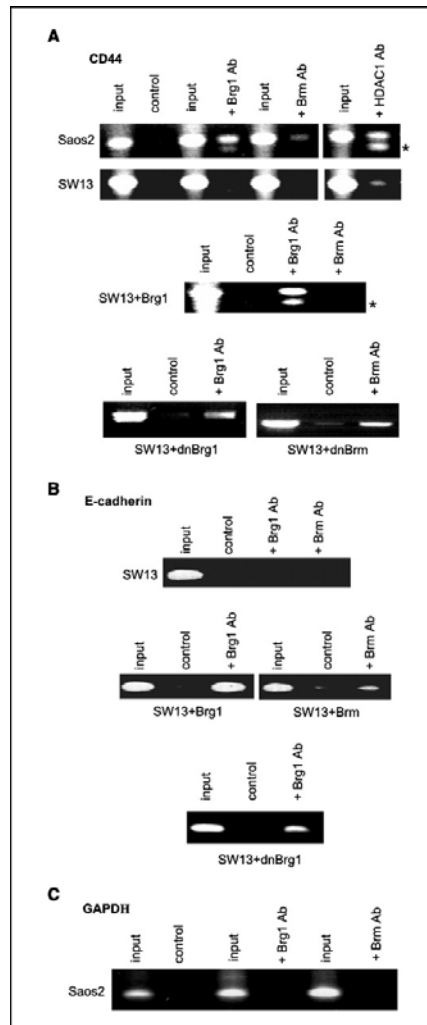
Figure 3.4: Brg1 and Brm interact with the CD44 and E-cadherin promoters. ChIP assays for interactions between Brg1 and Brm with the CD44 (*A*), E-cadherin (*B*), and GAPDH (*C*) promoters. Using primers encompassing the same regions of the promoters analyzed in Fig. 3, cross-linked DNA was isolated from cells and immunoprecipitated with Brg1, Brm or HDAC1 (*A*, as a positive control) antibodies. *A,* ChIP assays were performed using Saos2 cells (*top*), which express endogenous Brg1 and Brm, and SW13 cells that lack both proteins. A nonspecific band (*) was amplified in some experiments. *Middle,* ChIP assays were repeated in SW13 cells transfected with Brg1 to confirm that Brg1 interacts with the CD44 promoter in these cells. *Bottom,* SW13 cells were transfected with dnBrg1 or dnBrm, showing that the mutant proteins, lacking an ATPase domain, still interact with the CD44 promoter. *B,* as above, untransfected SW13 cells (*top*) and cells transfected with Brg1 (*middle left*), Brm (*middle right*), or dnBrg1 (*bottom*) were analyzed by ChIP using primers that amplify the E-cadherin promoter. *C,* ChIP assay of Saos2 cells showing that neither Brg1 nor Brm interact with the 5' untranslated region of GAPDH.

opens a new avenue of research into the link between DNA methylation and chromatin remodeling activities.

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CHAPTER 4

Identification of potential targets of SWI/SNF complex-dependent transcriptional activation due to altered methylation patterns

A. Introduction

The SWI/SNF chromatin-remodeling complexes, are conserved from yeast to mammals, and use the energy from ATP hydrolysis to disrupt histone-DNA interactions to make DNA accessible to transcription factors and transcription machinery (1). The mammalian SWI/SNF complex contains one of two catalytic subunits, either BRM or BRG1, which catalyze ATP (2). Loss of BRG1 and/or BRM has been shown to result in altered gene expression leading to cancer development and/or progression. The mechanism by which altered gene expression occurs upon loss of BRG1/BRM is largely unknown.

Chromatin remodeling complexes are an essential part of the epigenetic machinery responsible for maintaining proper genome regulation. Chromatin remodeling complexes work together with DNA methyltransferases, methyl-CpG binding proteins, histone modifying enzymes, and transcriptional factors (3), to define the structure and function of the cell by inactivating the majority of genes and selectively activating specific genes. The methylation of DNA at cytosines is a covalent modification, in which a methyl group is added to cytosines by DNA methyltransferases (4). Methylation patterns are maintained by DNMT1, which fully methylates the hemi-methylated DNA formed during DNA replication (5). Recently de novo methylases (DNMT3a and DNMT3b) have been found that add

methyl groups to unmethylated DNA (5). ATP-dependent chromatin remodeling enzyme

SNF2H has been shown to interact with DNMT3B (5).   Hypermethylation of cytosines, is

repressive to gene activation. The hypermethylation of specific tumor suppressor genes, such

as p16, MLH1, VHL, and E-cadherin, has been identified in human cancer cells (4). In

contrast to hypermethylation, hypomethylation of CpG dinucleotides, which was found by

southern blotting of cancer cell lines, leads to gene activation (6). Hypomethylation of

oncogenes like HRAS can lead to cancer development (7). The mechanism by which

demethylation occurs is still unknown. There are two possible mechanisms by which

demethylation occurs, either by a passive mechanism whereby methylation patterns aren't

maintained during DNA replication, or by an active mechanism, which would be catalyzed

by an unidentified DNA demethylase (3).

Research into how global hypomethylation in human cancer occurs has revealed two

possible links between chromatin remodeling and maintenance of DNA methylation.

Patients with the developmental disorder ATRX have mutations in the ATRX gene, a SNF2

DNA helicase involved in chromatin remodeling (8). These patients have hypomethylation

of ribosomal DNA repeats (8). Interestingly, proteins in the DNMT3 family have a region of

homology to the ATRX gene, which may partially explain how loss of ATRX may lead to

loss of methylation. Lsh, a SNF2 family member, was found to be required for maintenance

of normal methylation (9, 10), since gene knockout of Lsh leads to a global defect in

methylation.   Research into epigenetics has revealed a complex system of gene activation

and repression mediated by DNA methylation and chromatin modification.

Cellular adhesion proteins, CD44 and E-cadherin, are commonly silenced in cancer

cell lines by hypermethyltion of CpG islands within their promoter region (11, 12). Previous

work by our lab has shown transcriptional activation of SWI/SNF regulated genes, CD44 and E-cadherin, by inducing changes in DNA methylation at their promoters (13). In this study we have identified two additional genes cytokeratin 18 and Fhit that are not expressed or expressed at low levels in BRG1/BRM deficient cells. Unlike CD44 and E-cadherin, cytokeratin 18 appears to be induced upon reintroduction of BRG1, but is not upregulated by treatment with demethylating agent 5-azacytidine. We also ran global assays to try to determine the role of the SWI/SNF complex in altering methylation patterns on promoters to induce transcriptional activation. We found that transfecting BRG1/BRM deficient cells with BRG1 did not result in alterations in global methylation. We are currently looking for more targets of SWI/SNF mediated transcriptional activation by gene expression arrays. These studies may help to determine differences in promoters that are activated by BRG1 due to methylation changes and those that are activated by BRG1 in the absence of methylation changes.

B. Material and Methods

Cell Lines:

MCF7, HeLa, SW13, C33A, A427, and H522 were obtained from the ATCC. All the cell lines were grown in RPMI with 10% FBS except C33A which was grown in DMEM with 10% FBS.

Transfection and Treatment of Cell Lines:

Cell Lines were transfected with either pcDNA3 empty vector, PBJ5-BRG1, DNBRG1, or PCG BRM using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Transfetions were harvested for protein 48 hr after transfection. Cells treated

with 50 uM 5-azacytidine (13) had fresh media added after 48 hours. Twenty four hours

after the addition of fresh media cells were harvested for protein.

Immunoblotting:

Protein was collected from cells grown in 100 mm tissue culture dishes. Detergent based

extraction buffer was added to the dishes and cells were collected with a scraper in an

Eppendorf tube (13). The tube was rocked in the cold room for 10 min and spun at 14,000

rpm for 10 min. Supernatant protein concentration was determined by Bradford protein

assay. Equal amounts of protein lysate (30 ug) were separated by SDS-polyacrylamide gel

electrophoresis (either 7.5% or 4-20% gradient) for 1 hr at 100 volts. Protein was then

transferred to Immobilon-P membranes (Millipore) for 16-18 hr at 80 mA. Membranes were

blocked for 1 hr in 5% non-fat dry milk/0.2% Tween 20 in PBS. Membranes were then

incubated for 2 hr in primary antibody. Primary antibodies included BRG1 (Santa Cruz),

BRM (Dr. Moshe Yaniv), E-cadherin (Transduction Labs), Cytokeratin 18 (Santa Cruz), Fhit

(Zymed), CD44 (Hermes 3), Vhl (Santa Cruz), vimentin (Biomeda), p16 (BD Pharmingen),

actin (Sigma). Membranes were then washed 3 times for 10 min each in 200 ml of 0.2%

Tween 20 in PBS. Membranes were then incubated in a 1:2000 dilution of either mouse or

rabbit secondary antibody for 1 hr. Membranes were then washed again 3 times as before.

Bands were detected with ECL chemiluminescence reagent (Amersham) on Biomax ML film

(Kodak).

Restriction Landmark Genome Scanning:

Forty eight hours after transfection with vector or PBJ5-BRG1 into H522 or A427, cells were

harvested for genomic DNA using DNEasy kit (Qiagen), following the manufacturer's

instructions. RLGS was performed on the DNA as previously described (14). Protein expression was confirmed by western blot.

C. Expression of commonly methylated genes in BRG1/BRM deficient cells

Our previous studies found two genes, CD44 and E-cadherin, silenced by promoter methylation in two cell lines lacking BRG1 and BRM. To further correlate silencing of CD44 and E-cadherin with loss of BRG1/BRM, we ran an immunoblot with two additional BRG1/BRM deficient cell lines. The two additional BRG1/BRM cell lines were lung tumor cell lines, A427 and H522. A427 and H522 were also found to lack CD44 and E-cadherin (Figure 1). On this gel, we also ran three cell lines that contained point mutations in BRG1 (HCT116, Hs578t, OVCAR3), three cell lines that were positive controls containing all the members of the complex (MCF7, HeLa, DLD1), one cell line that expressed BRG1 but lacked BRM (MiaPaca2). On this blot we probed for other commonly methylated genes to determine if they were not expressed in BRG1/BRM deficient cells. We found that along with CD44 and E-cadherin, cytokeratin 18, a marker of epithelial cells, and FHIT, a lung cancer tumor suppressor gene, expression was also lost or greatly reduced in all four double deficient cell lines (Figure 1). None of the other genes we probed for were missing from all 4 double deficient cell lines. Furthemore, we did not observe a correlation between loss of expression of any of these genes and loss of either BRG1 or BRM alone.

D. Transfection and Treatment of BRG1/BRM deficient cells

Previously A427, H522, SW13, and C33A were found to lack protein expression of CD44 and reintroduction of BRG1 results in reexpression of CD44 (15, 16). Next we assessed if reexpression of BRG1 in H522 and A427 cells resulted in re-expression of E-cadherin protein as in the SW13 and C33A cells.   E-cadherin was expressed in H522 cells

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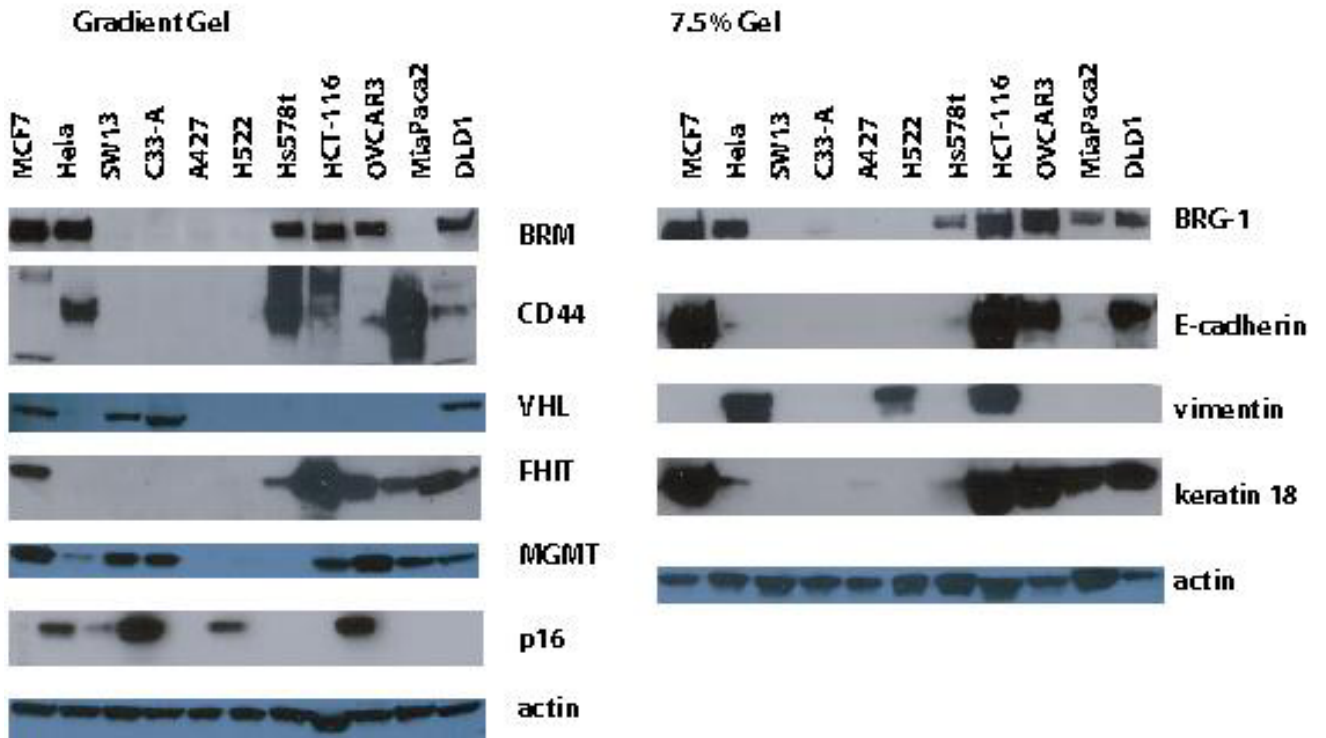**Western Blot Screening Cell Lines for commonly Methylated Genes**

Figure 4.1: Western blot analysis of BRG1/BRM deficient cells. Protein from cells deficient for either BRG1 or BRM or both were separated by SDS-PAGE and immunoblotted for some commonly methylated genes. Actin was used as a loading control.

but not in A427 cells after reintroduction of BRG1 (Figure 2). We also looked to see if

reexpression of BRG1 in H522 and A427 restores expression of cytokeratin 18 and Fhit

(Figure 2). A427 expressed low levels of cytokeratin 18, and H522 expressed low levels of

Fhit. Transfection of H522 cells with BRG1 or BRM leads to expression of cytokeratin 18,

while BRG1 transfection into A427 cells did not induce Fhit (Figure 2). Treatment of the

A427 and H522 with 5-azacytidine was also done to determine if the promoters of

cytokeratin 18 and FHIT were methylated in BRG1/BRM double deficient cells similar to

CD44 and E-cadherin (Figure 2). 5-azacytidine treatment did not turn on either FHIT or

cytokeratin 18, as it does E-cadherin in both A427 and H522. Thus, reintroduction of BRG1

leads to transcriptional activation of cytokeratin 18 in H522 but not apparently by

demethylating bases in the promoter. This is in contrast to the methylated E-cadherin that is

transcriptionally activated in H522. H522 is similar to SW13 in that reexpression of BRG1

into SW13 results in re-expression of the methylated E-cadherin gene and also results in re-

expression of the unmethylated cytokeratin 18 gene (Figure 3).

E. Global Methylation Analysis after transfection of BRG1 into deficient cells

Since the SWI/SNF complex is believed to regulate approximately 6% of the genes in

the human genome, one would assume loss of BRG1 would have global effects. To

determine if loss of SWI/SNF had global effects on promoter methylation, we harvested

DNA from H522 and A427 cells transfected with either BRG1 or vector alone. The DNA

was then subjected to Restriction landmark genomic scanning, an assay that evaluates

promoter methylation globally (14). In this procedure the DNA is first cut with a

methylation sensitive restriction enzyme, NotI, which will cut an unmethylated sequence but

not a methylated sequence. The NotI ends are filled in with ^{32}P to make radiolabeled
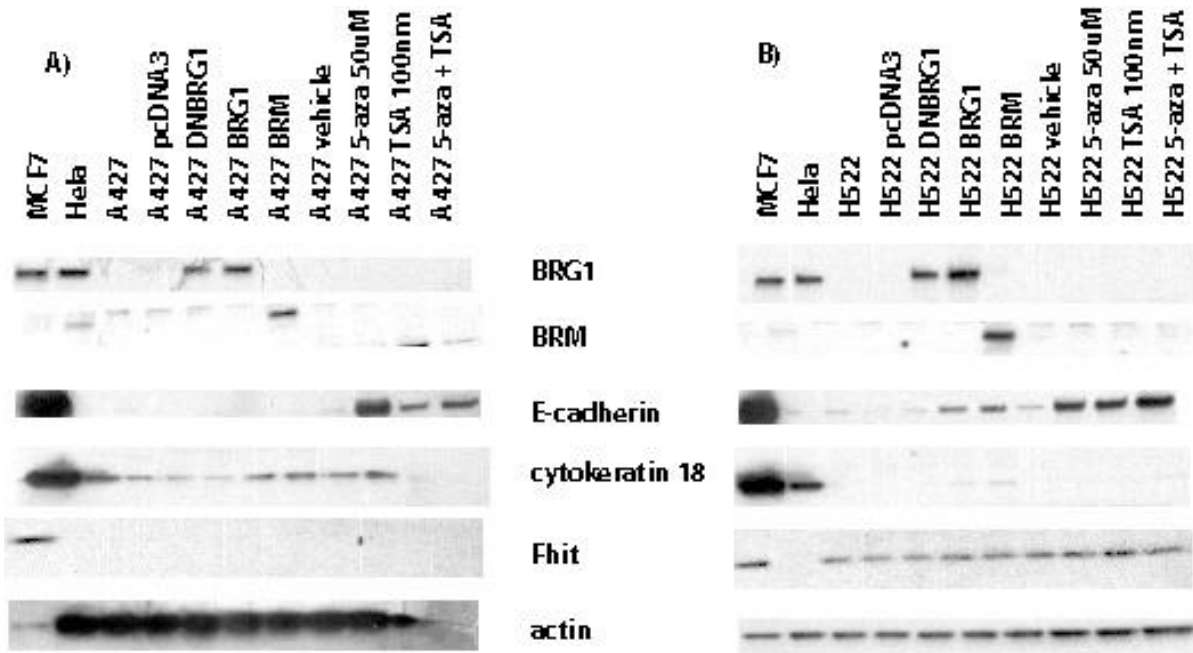
Figure 4.2: Western-blot analysis of A427 cells and H522 cells. A427 cells (A) and H522 cells (B) were transfected with either empty vector, DNBRG1, BRG1, or BRM or treated with 5-azacytidine or trichostatin A. Protein from the cells was separated by SDS-PAGE and immunoblotted for E-cadherin, cytokeratin 18, and Fhit. Actin was used as a loading control.

Figure 4.3: Western blot analysis of SW13 cells. SW13 cells were transfected with either empty vector, DNBRG1, BRG1, or BRM or treated with 5-azacytidine or trichostatin A. Protein from the cells was separated by SDS-PAGE and immunoblotted for E-cadherin, cytokeratin 18, and Fhit. Actin was used as a loading control.
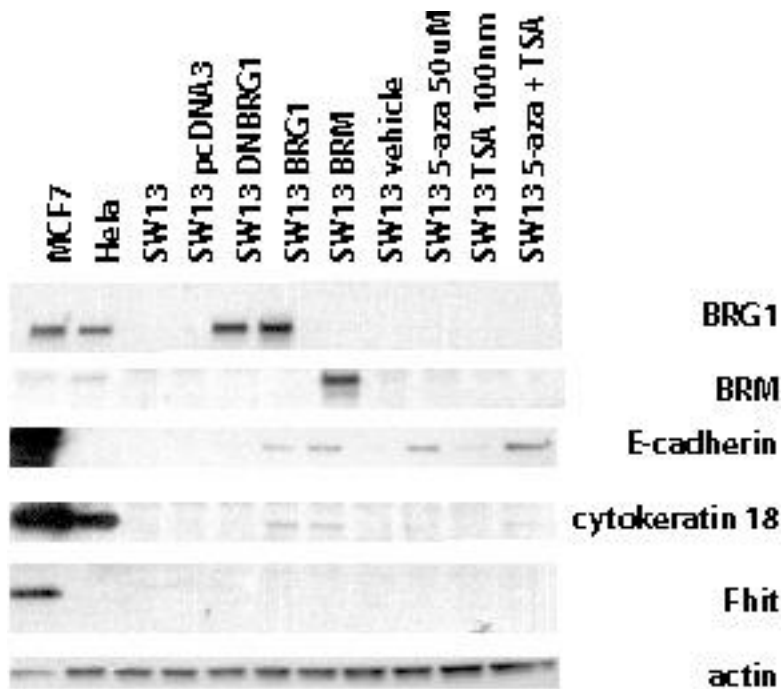
restriction endonuclease sites. Next, a second digestion with EcoRV is done to produce

smaller fragments of DNA. These fragments of DNA then undergo the first dimension

electrophoresis in an agarose tube. The gel is then exposed to a third restriction enzyme,

Hinf I, to make even smaller fragments for the second dimension electrophoresis. After the

second dimension electrophoresis the gel is exposed to a phosphoimager screen for 24 hours

and then scanned. The end result is a highly reproducible RLGS profile with over 2000 Not I

site spots. Comparison of two profiles can determine changes in methylation by the presence

of a new spot in one profile coupled with the absence of another spot in the other profile.

The absence of a RLGS spot is due to methylation that results in failure of the methylation-

sensitive restriction enzyme to digest the DNA. In RLGS profiles of A427 and H522

transfected with either vector alone or BRG1, spots appear in the BRG1 transfected profile.

While these spots could indicate demethylation at a novel site, analysis of the spots revealed

that they were from the BRG1 sequence in the transfected vector. The results for H522 and

A427 showed the same two prominent spots from the BRG1 vector in their profiles.

Comparison of RLGS profiles for A427 and H522 transfected with pcDNA3 or transfected

with BRG1 revealed that BRG1 did not alter promoter methylation patterns globally (Figure

4)

F. Discussion

In this study, we wanted to determine the role BRG1/BRM loss plays in altering gene

expression patterns by aberrant promoter methylation. Our previous studies have shown

CD44 and E-cadherin are silenced by hypermethylation in cancer cell lines deficient for

BRG1/BRM (13). The previous studies found that reexpression of BRG1 or BRM in
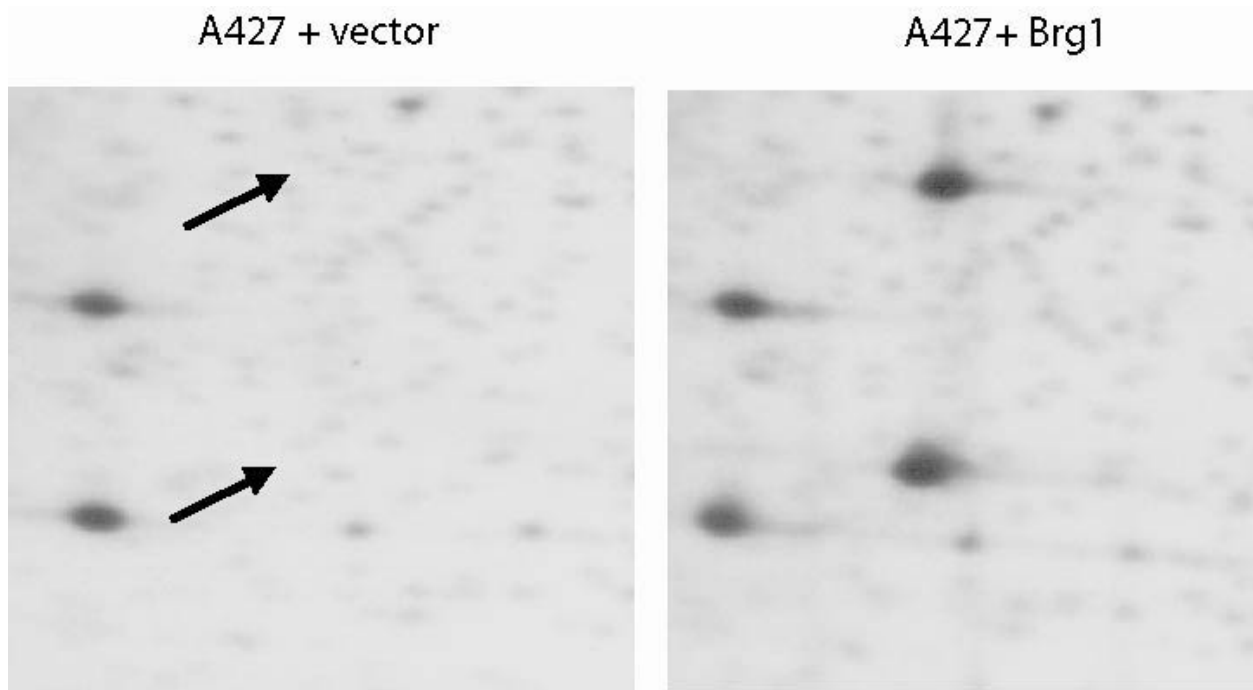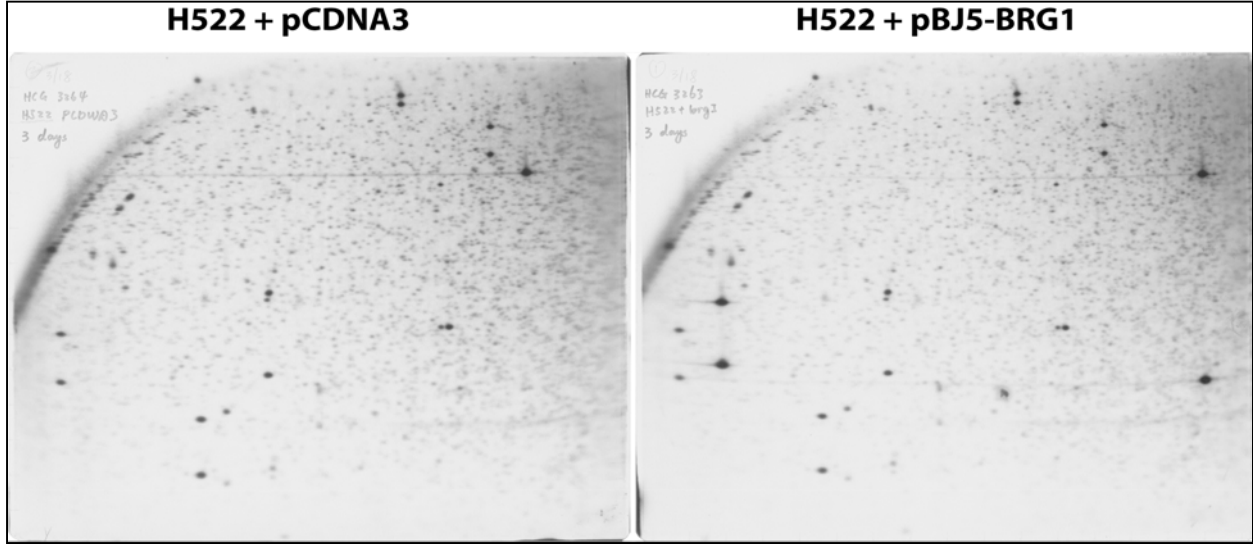
Figure 4.4: Restriction landmark genome scanning (RLGS) of A427 and H522. RLGS was performed on DNA from H522 cells transfected with either empty vector or BRG1 (top) and A427 cells transfected with either empty vector or BRG1(bottom). H522 displays the whole array, while A427 is an enlarged image of the new spots.

deficient cell lines lead to transcriptional activation of CD44 and E-cadherin and correlated with demethylation of specific bases at the E-cadherin and CD44 promoter.

The SWI/SNF complex could influence the methylation of specific genes through either a direct or indirect mechanism. Due to the SWI/SNF complex role in transcriptional activation, it may activate other genes responsible for the demethylation of specific promoters. However, it is more likely the SWI/SNF complex plays a more active role in the demethylation of promoters, by blocking DNMT1's ability to methylate promoter sequences or by recruiting a yet undetermined demethylase to the promoter, since the complex interacts with the promoters to activate transcription of the genes. It is not unexpected that chromatin remodeling complexes would recruit methyltransferases or demethylases to DNA when trying to establish tissue-specific gene expression during development and differentiation. This recruitment by chromatin remodeling complexes may help explain how transcriptional activation of a variety of genes may be altered in cancer cells that lack functional BRG1 and BRM leading to altered cellular programming. Loss of BRG1/BRM is most commonly linked to tumor development in lung cancer cell lines, and lung primary tumor, occurring at a frequency of 10%. Interestingly E-cadherin expression is lost in 10% of NSCLC (17). E-cadherin loss, similar to loss of BRG1/BRM is associated with poor prognosis in patients with NSCLC, and leads to tumor cell dedifferentiation and invasion (17). E-cadherin is found to be hypermethylated twice as frequently in NSCLC compared to non-neoplastic lung tissue (17). In fact, DNA hypermethylation profiles reveal distinct methylation patterns can distinguish between SCC and NSCLC (18). We believe that loss of BRG1/BRM may lead to global alteration of cellular programming, and dedifferentiation of cells perhaps undergoing an epithelial-mesenchymal transition. When we assessed if BRG1 reintroduction lead to

global demethylation we surprisingly didn't find global changes. Therefore, any methylation changes associated with BRG1/BRM loss appears to be at a few specific promoters. To determine specific promoters whose methylation patterns may be altered by loss of BRG1/BRM, microarrays on the BRG1/BRM-deficient cell line, SW13, should be done to compare the genes activated by re-introduction of BRG1, and the genes activated by treatment with demethylating agent 5-azacytidine. One question to be determined is how these specific promoters are targeted for methylation in the absence of BRG1/BRM. One other issue is whether the methylation accumulates in the absence of BRG1/BRM due to a loss of transcriptional activation. This would be consistent with studies that showed increased methylation of the CD44 and E-cadherin promoter in cells expressing a tet inducible DNBRG1 after several passages (13).

The finding that BRG1/BRM loss may contribute to altered DNA methylation may be helpful to the diagnosis and treatment of lung cancer. Loss of the SWI/SNF complex may be a precursor to establishing a distinct hypermethylation profile in lung cancer, therefore making it easier to identify the poor prognostic tumors and treat them accordingly. Studies that inhibit DNMT1 and HDAC in mice prevent murine lung cancer in tobacco carcinogen-induced lung cancer. Treatments involved in reduction of DNMT1 and HDAC activity may help block epigentically mediated gene silencing. Defining the role of SWI/SNF in the pathogenesis of lung tumorigenesis will be important in diagnosis of lung tumors and may provide more targets for therapeutic treatments.

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CHAPTER 5

SUMMARY AND PERSPECTIVES

A. Summary

BRG1 is the catalytic subunit of the SWI/SNF chromatin-remodeling complex (1). The main function of the SWI/SNF complex is transcriptional regulation (2). The complex has been shown to regulate the expression of many genes critical to controlling cellular proliferation and cellular adhesion. Subsequently, loss of BRG1 has been implicated in the development and/or progression of human cancer. The exact mechanism by which loss of BRG1 contributes to cancer development and/or progression remains unclear.

These studies aimed to answer two questions. The first question was- Do mutations in BRG1 alter BRG1 function? The second question was- How does BRG1 loss alter gene expression and ultimately lead to cancer development and/or cancer progression? To answer the first question, we isolated the expression of BRG1 mutant proteins found in human cancer cell lines by reducing endogenous BRM expression with shRNAi. These stable clones were analyzed to see if BRG1 function was impaired. To answer the second question we analyzed if loss of BRG1 altered promoter methylation patterns. Results of these studies allowed us to conclude the following:

- E-cadherin is regulated by the SWI/SNF complex. Transfection of BRG1/BRM deficient cells with either BRG1 or BRM induces E-cadherin protein expression.

- BRG1 is at the promoter of CD44 and E-cadherin. Chromatin Immunoprecipitation assays show BRG1 at the promoter of CD44 and E-cadherin.

- BRG1 mutant proteins are in the SWI/SNF complex and at target promoters. Immunoprecipitation of BRG1 mutant protein found in human cancer cell lines reveals that the BRG1 mutant proteins still associate with other members of the complex.

- SWI/SNF dependent genes protein expression is unaffected in BRM RNAi stable cells with BRG1 mutations. Stable clones of human cancer cell lines with mutations in BRG1 and reduced expression of BRM due to expression of shRNAi vectors reveal little effect on expression of CD44 and E-cadherin protein by western-blot.

- BRG1 mutant proteins impair RB-mediated growth arrest. The BRM RNAi stable clones of HCT116 and Hs578t were resistant to RB-mediated growth arrest when analyzed by Brdu-incorporation assays.

- Transcriptional activation of genes by SWI/SNF is a result of changes in DNA methylation at promoters. Changes in CD44 and E-cadherin protein expression after transfection of BRG1 or BRM into deficient cells correlates with changes in DNA methylation at their promoters

- <u>Loss of SWI/SNF does not result in global methylation changes</u>. Restriction landmark genome scanning shows reintroduction of BRG1 into deficient cells does not result in global methylation changes.

B. Perspectives

BRG1 and BRM are both thought to be tumor suppressor genes since they have been shown to be involved in RB-mediated arrest and in the regulation of cellular adhesion proteins. The redundant function between the two genes in tissue culture experiments is not surprising due to the similar structure of the two proteins. In tissue culture models both BRG1 and BRM are required to be lost to see aberrations in RB-mediated arrest or aberrations in transcriptional regulation of certain cellular adhesion genes.

The discovery that two mutations in BRG1 impair RB-mediated arrest in the absence of BRM supports recent evidence that BRG1 mutations may contribute to human cancer. Other studies have found BRG1 mutations in human cancer cell lines and human primary tumor (3, 4). One of the mutations we examined in HCT116 is in motif V and another group has shown that mutations in motif V uncouple the ATPase activity from BRG1 (5). Our studies performed in human cancer cell lines show a redundant function of BRM and BRG1 since BRM needs to be reduced by shRNAi to see impairment of mutant BRG1 function.

Knockout mice of BRG1 are embryonic lethal and heterozygous BRG1 mice are prone to tumor development (6), while BRM knockout mice have no phenotype (7). These results imply differential function of BRG1 and BRM in development. In fact,

studies have shown that BRG1 and BRM interact with different proteins, indicating a difference in function (8). BRG1 and BRM also show differential expression in differentiated tissues, suggesting a difference in function in differentiated cells (9). It is not clear whether BRG1 and BRM have unique functions *in vivo*. More studies are needed to determine the unique functions of BRG1 and BRM to understand their role in the cell. If BRG1 and BRM are found to have unique functions in vivo then loss of BRG1 function by mutation could result in cancer development/progression.

BRG1 mutations that impair BRG1 function like the ones we examined may play a role in development of human cancer but the cellular environment and the temporal expression, may also be critical to determining the effects of the mutation. In a tissue culture cell line that has both BRG1 and BRM, the effects of a mutation in BRG1 may be compensated for by BRM. In early development when BRM is not expressed or in a differentiated cell that does not express BRM the effects of the BRG1 mutation will not be compensated for and RB-mediated growth arrest may be impaired. Determining the role of BRG1 and BRM in the cell will help to determine how loss of BRG1 may contribute to cancer.

In our studies, we found that loss of BRG1 and BRM is associated with aberrant promoter methylation. This indicates loss of BRG1/BRM may lead to silencing of tumor suppressor genes and contribute to tumor development. Complicating BRG1's role in methylation patterns is that it appears that not all genes regulated by SWI/SNF are methylated in the absence of BRG1/BRM since transfection of BRG1/BRM-deficient cells with BRG1 activates cytokeratin 18 while demethylating agent 5-azacytidine does not. Research is still needed to determine the exact role of BRG1 in maintenance of

methylation patterns. We have not determined whether the demethylation effects observed after reintroduction of BRG1 into BRG1/BRM-deficient cells is indirect or direct. BRG1 may be indirectly playing a role by activating a gene that is playing a direct role. It is also possible BRG1 is playing a direct role by acting in a complex with proteins not yet determined to associate with BRG1. It will also be important to determine what specific promoters regulated by BRG1 are regulated by methylation. The role of BRG1 in transcriptional activation of genes by alteration of promoter methylation still needs to be defined to determine the consequences loss of BRG1 may have on a cell. Determining a role for BRG1 loss in altered methylation patterns will help to diagnose and treat BRG1/BRM deficient tumors. Recent therapies for cancer have included the use of demethylating agents (10).

When we examined the BRG1 mutant cell lines with reduced BRM expression due to shRNAi we expected to see reduced expression of SWI/SNF regulated proteins. We didn't observe a consistent reduced expression of CD44 or E-cadherin. Since we see a loss of RB-mediated arrest in these cells, we would assume this is due to the inability to inhibit expression of genes required for cell cycle progression such as cyclin A, and cyclin E. CD44 and E-cadherin were actively expressed in the cells before reduction of BRM. This may indicate that the BRG1 mutations are not impaired in the regulation of these genes although they are impaired in their ability to alter chromatin to inhibit the expression of cyclin A and cyclin E. This may also indicate that once the chromatin is in a certain conformation, like the CD44 gene is activated, BRG1 is not needed to sustain that conformation but is required to change the conformation. Further investigation will help to determine how BRG1 regulates genes.

Determining the role of the complex will also help in our understanding of epigenetics. Epigenetics is composed of many factors that determine the expression patterns of genes throughout all stages of life. An understanding of epigenetics could help treat many types of diseases that alter the epigenetic machinery. Research is beginning to focus more on epigenetics. Toxicology is a field that historically looked at alterations of the human genome by chemicals, in terms of mutagenesis, or direct changes in DNA. Recent toxicological studies have started to examine epigenetic changes with the emergence of non-genotoxic carcinogens. An understanding of epigenetics would help to characterize changes in gene expression induced by chemicals that are not due to alterations in DNA. In fact, recent studies have shown that cocaine induces acute and chronic changes in histone modifications at promoters of genes (11). The studies performed in this dissertation not only help to define the molecular mechanisms that lead to tumor development or progression when BRG1/BRM is lost, but also help to contribute to the understanding of epigenetics.

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Appendix

Table A.1: Cell Lines

Cell Line	Tissue	Cell Type	Source
SW13	adrenal gland	adenocarcinoma	ATCC
MCF7	breast	adenocarcinoma	ATCC
HeLa	cervix	epitheloid carcinoma	ATCC
Hs578t	breast	ductal carcinoma	ATCC
HCT116	colon	adenocarcinoma	ATCC
OVCAR3	ovarian	adenocarcinoma	ATCC
C33-A	cervix	cervical carcinoma	ATCC
A427	lung	adenocarcinoma	ATCC
H522	lung	adenocarcinoma	ATCC
MiaPaca2	pancreas	pancreatic carcinoma	ATCC
DLD1	colon	adenocarcinoma	ATCC

Table A.2: Antibodies

Antibody	species	source
CD44 (Hermes 3)	mouse	Dr. Larry Sherman
BRG1 (G-7)	mouse	Santa Cruz
BRM (y)	rabbit	Dr. Moshe Yaniv
E-cadherin	mouse	Transduction Labs
Actin	rabbit	Sigma
BAF155	mouse	Santa Cruz
BAF57	rabbit	Dr. Karen Knudsen
BAF47	mouse	Transduction Labs
Brdu	rat	Accurate Chemicals
cytokeratin 18	mouse	Santa Cruz
FHIT	rabbit	Zymed
MGMT	mouse	Chemicon
vimentin	rabbit	Biomeda
VHL	mouse	Santa Cruz
p16	mouse	BD Pharmingen
BRG1 (J1)	rabbit	Dr. Weidong Wang
BRM (ab)	rabbit	Abcam
acetylated histone 3 (H3)	rabbit	Upstate Biotechnologies