Virginia Commonwealth University

VCU Scholars Compass

Theses and Dissertations   Graduate School

2010

The Role of the Methyl DNA Binding Domain Protein 2 (MBD2) in Breast Cancer

Omar Mian
Virginia Commonwealth University

The Role of the Methyl DNA Binding Domain Protein 2 (MBD2) in Breast Cancer

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by
Omar Y. Mian
B.S. James Madison University
M.S. Johns Hopkins University

Thesis Advisor: Gordon D. Ginder, M.D.
Director, Massey Cancer Center
Professor, Internal Medicine, Human Genetics, and Microbiology and Immunology
Molecular Biology and Genetics Program

Virginia Commonwealth University
Richmond, Virginia
January 22, 2010

Acknowledgements

I would like to recognize several people for their gifts of knowledge and friendship.

First and foremost, I would like to thank my advisor, Dr. Gordon D. Ginder, MD. His unique mix of intellect, humor, and kindness make Dr. Ginder an exceptional mentor. He has provided me with an ideal mix of independence and insightful redirection when necessary and continues to be a patient and supportive advisor. His investment in my development as a scientist will remain a source of guidance to me throughout my career and I am immeasurably grateful to him.

I would also like to thank the many teachers, instructors, professors, and advisors I have had at VCU. Among this distinguished group are the members of my thesis advisory committee: Drs. Tomasz Kordula, Joyce Lloyd, Shirley Taylor, and Jolene Windle. In addition, I am grateful for the support of my program directors: Dr. Gail Christie of Molecular Biology and Genetics and Dr. Gordon Archer of the MD/PhD program.

I have been fortunate throughout my time in Dr. Ginder's Lab to be surrounded by talented individuals who were giving with their time and expertise. In particular, Shou Zhen Wang and Sheng Zu Zhu have been a continuous source of wisdom and moral support. They are wonderful people and I am indebted to them for all their help.

In addition I would like to thank my fellow graduate students in the Ginder Lab for their advice, assistance, and camaraderie: Merlin Gnanapragasam, Quintesia Grant, and Maria Amaya. I would also like to thank several other individuals who were essential to the completion of this work: Drs. Jim Roesser, Zendra Zehner, Alexandra Racanelli, and Laura Graham.

Finally, I would like to express my gratitude to the many friends and family who have offered me inspiration and encouragement through the years. In particular I would like to recognize my parents and siblings, Yusuf, Qudsia, Ali and Aisha. Lastly I thank my wife, Lisa, who shares in all my successes and failures and whose enduring support makes everything possible.

Dedication

     I dedicate this work to my father, Yusuf Mian, who lost his battle with cancer in 1998. He was always devoted to his family and in particular to his children's realization of a richer life through the pursuit of knowledge.

iii

Table of Contents

v

vii

List of Tables

List of Figures

xi

List of Abbreviations

(Abbreviations appear in alphabetical order)

3D lrECM	Three Dimensional Laminin-Rich Extracellular Matrix
ADCC	Antibody Dependent Cell-mediated Cytotoxicity
ATCC	American Type-Culture Collection
BME	Basement Membrane Extract (lrECM) for 3D culture
cDNA	Complementary DNA generated by reverse transcriptase PCR
ChIP	Chromatin Immuno-Precipitation assay
CpG	Cytosine-Guanine dinucleotide (5-position of the cytosine ring is methylated by DNA methyl-transferases within the symmetric context of CpG dinucleotides in the genome)
DAR	Division of Animal Research
DCIS	Ductal Carcinoma *in situ*
DMSO	Di-Methyl Sulfoxide
EMT	Epithelial to Mesenchymal Transition
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
G418	aminoglycoside antibiotic (Geneticin, Gibco)
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
H3K27me	methyl Histone H3 monomer Lysine 27 (inhibitory)
H3K36me	methyl Histone H3 monomer Lysine 36 (activating)
H3K4me3	Tri-methyl Histone H3 monomer Lysine 4 (activating)
H3K9,14ac	Acetyl-Histone H3 monomer Lysine 9 and 14 (activating)
H3K9me	methyl Histone H3 monomer Lysine 9 (inhibitory)
HAT	Histone Acetyl-Transferase
HDAC	Histone Deacetylase
HMT	Histone Methyltransferase

IACUC	Institutional Animal Care and Use Committee
IgG	Immunoglobulin G
iPS	Induced pluripotent stem (cells)
KD	Knockdown (by shRNA or siRNA)
Maspin	Mammary Associated Serine Protease Inhibitor (also called SerpinB5)
MBD	Methyl Binding Domain
MBD1	Methyl Binding Domain Protein 1
MBD2	Methyl Binding Domain Protein 2
MBD3	Methyl Binding Domain Protein 3
MBD4	Methyl Binding Domain Protein 4
MCBP	Methyl-CpG Binding Protein
ml	milli-liter (10^{-3} Liter)
ncRNA	Non-Coding Ribonucleic Acid
ng	Nano gram
nM	nano-Molar (10^{-9} Molar)
NURF	Nucleosome Remodeling Factor
Oligo	Oligonucleotide (DNA and RNA)
PBS	Phosphate Buffered Saline (at pH 7.4 unless otherwise indicated)
PCR	Polymerase Chain Reaction
pLV-THM	Lentiviral plasmid vector for delivering stable polymerase III promoter driven shRNA
qPCR	Quantitative (real-time) Polymerase Chain Reaction
RT-PCR	Reverse Transcriptace Polymerase Chain Reaction
SERM	Selective Estrogen Receptor Modulators (e.g., Tamoxifen)
shRNA	Short hairpin (double-stranded) Ribonucleic Acid
siRNA	Short interfering (double-stranded) Ribonucleic Acid
ug	micro-grams (10^{-6} grams)
uM	micro Molar(10^{-6} molar)

Abstract

The Role of the Methyl DNA Binding Domain Protein 2 (MBD2) in Breast Cancer

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by
Omar Y. Mian
B.S. James Madison University
M.S. Johns Hopkins University

Major Advisor: Gordon D. Ginder, M.D.
Director, Massey Cancer Center
Professor, Internal Medicine, Human Genetics, and Microbiology and Immunology
Molecular Biology and Genetics Program
Virginia Commonwealth University, Richmond, Virginia

Methyl-CpG Binding Proteins (MCBPs) are thought to function as the interpreters of

epigenetic information encoded in cytosine methylation. Their ability to translate DNA

methylation into local transcriptional repression has sparked interest in the role of

Methyl-Binding Domain Proteins (MBDs) in cancer, where repatterning of CpG

methylation is common. In this dissertation I summarize and discuss observations made

in the Ginder Lab linking MCBPs to the progression of neoplastic disease. It is clear

from our work that the Methyl Binding Domain Protein 2 (MBD2) is necessary for the

persistent repression of critical tumor suppressor genes in breast cancer. We show that

stable knockdown of MBD2 also leads to growth suppression in cultured human

mammary epithelial cancer lines (MCF-7, 49% suppression; MDA-MB-231, 77%; MDA-

MB-435, 94%; SK-BR-3, 92%) with the peak cytotoxicity and anti-proliferative effect

occurring as late as 2-3 weeks after knockdown. MBD2 knockdown also led to a

decrease in viable tumor cells at equivalent doses of the histone deacetylase inhibitor, SAHA (Vorinostat™), and chemotherapeutic agents Doxorubicin, and Paclitaxel. Stable MBD2 knockdown in MCF7 cells led to an increased proportion of normal epithelial structures in 3D culture (70%, [CI=0.55-0.83]) when compared to untransfected (46%, [CI=0.39-0.53], p≤0.038) or scrambled shRNA transfected (37%, [CI=0.29-0.45], p≤0.012) controls. *In vivo* xenograft studies show tumor growth in BALB/C nu/nu mice was significantly impaired when mice were implanted with human breast cancer cells harboring MBD2 targeted shRNA. Following MBD2 knockdown, tumor suppressor promoter methylation remained unchanged despite sustained increases in gene expression, arguing against the convention that passive demethylation occurs with increased transcription. Our data suggest that uncoupling CpG methylation from histone modifications or other repressor functions by removing MBD2 is sufficient to initiate and maintain anti-tumor gene transcription in the absence of secondary changes in DNA methylation. In this dissertation I present evidence for the pathologic role of MBD2 in breast cancer and provide mechanistic support for the prospect of targeting MBDs in neoplastic disease.

Overview

Carcinoma of the breast remains the most common cancer in women. Great strides have been made in early detection and treatment of primary non-invasive lesions; however treatment options for advanced, aggressive, and disseminated breast cancers remain woefully inadequate. It is estimated by the National Cancer Institute that there will be 192,370 new cases and 40,170 deaths from breast cancer in the United States in 2009 (Horner et al. 2009).

While specific concepts are dealt with in greater detail at appropriate points in the sections that follow, some broad and thematic ideas require introduction. To begin with, cancer is a complex collection of hyper-proliferative disorders with simultaneous and indissoluble genetic and environmental components (Hanahan and Weinberg 2000). One fundamental hallmark of tumors is the loss of appropriate gene regulation, particularly the inactivation of protective genes within critical gene networks. A common and reversible mechanism by which this gene inactivation occurs is a chemical modification of the DNA known as CpG-methylation (Egger et al. 2004; Jones and Baylin 2007; Widschwendter and Jones 2002; Esteller 2008). Abnormal DNA methylation is an early event in carcinogenesis; as such it represents a target for preventing the onset and progression of disease. For some time we have been exploring strategies to reverse the pathological effects of DNA methylation in tumors while preserving this necessary modification in normal tissues. A promising approach has recently emerged involving the inactivation of Methyl CpG Binding Proteins (MCBPs) which "read" and translate DNA methylation into changes in local gene expression (Sansom, Maddison, and Clarke 2007b; Ballestar and Esteller 2005; Hendrich and Bird 1998c). We originally set out to

1

explore the general role that MCBPs were playing in human neoplastic disease. Guided by promising results, the work narrowed in focus to the pathological role of MBD2 in breast cancer.

Rationale:

It is widely accepted that global hypomethylation of chromosomal DNA with selective hypermethylation of specific gene promoters leads to chromosomal instability and down regulation of tumor suppressor genes, both of which can promote cancer development and progression (Jones and Baylin 2007; Esteller 2008). The observation that CpG islands at the promoters of protective genes are often methylated led to the clinical development of DNA methylation inhibitors such as decitabine and azacytidine (Grant 2009; Jabbour et al. 2008). Unfortunately, the clinical utility of these agents has been limited by the potential for carcinogenicity and toxicity associated with non-specific global demethylation (Appleton et al. 2007; Oki and Issa 2006). An alternate strategy for mitigating the effects of abnormal DNA methylation in tumors is needed.

The effects of DNA methylation on gene expression are mediated in large part by MCBPs (Boyes and Bird 1991; Hendrich and Bird 1998; Klose and Bird 2006; Ballestar and Esteller 2005; Prokhortchouk et al. 2001). There has been interest in exploiting the function of MCBPs to selectively interrupt DNA methylation dependent changes in gene expression in tumors. However, in order to validate their utility as therapeutic targets, research to adequately characterize the function of MCBPs in human neoplasia is required.

One member of the MCBP family, Methyl Binding Domain Protein 2 (MBD2), has emerged as particularly noteworthy in the context of cancer for several reasons. MBD2

2

binds methylated CpGs with higher affinity than any known MCBP and has been shown to act upon the highest number of tumor suppressor targets (Lopez-Serra et al. 2008a; Berger and Bird 2005a; Fraga et al. 2003). In addition, this protein has been shown to exacerbate mouse models of intestinal carcinogenesis by increasing tumor size and number (Sansom et al. 2003a). Importantly, complete loss of MBD2 in the mouse does not generate any significant deleterious effect (Hendrich et al. 2001). This finding suggests that MBD2 is not absolutely required for survival either before or after development and therefore is a potentially selective target for therapy in tumors, where MBD2 may be playing an acquired pathologic role (Sansom, Maddison, and Clarke 2007a).

Abnormal DNA methylation has been reported in virtually all human cancers. Breast cancer, however, was of distinct interest to us for several reasons. It is now well established that CpG island hypermethylation is an early event in mammary epithelial cell transformation (Corn 2009; Futscher et al. 2004; Honorio et al. 2003b). At such early time points, prevention is the main therapeutic goal and arresting progression of the primary lesion remains a curative strategy. Furthermore, genetic testing and improved screening methods for breast cancer have dramatically increased the probability of successfully implementing an early intervention strategy. Finally, aggressive forms of breast cancer (e.g., inflammatory carcinoma, triple negative breast cancer) are poorly responsive to conventional treatment and alternate tactics, for example effective epigenetic therapy, are needed (Corn 2009; Lustberg and Ramaswamy 2009).

Therefore, we were very intrigued by the potential of MBD2 as an epigenetic target in the unique and potentially amenable setting of breast cancer. Hence, the primary

3

rationale behind this body of work was to explore the role that MBD2 plays in cancer, specifically in mammary tumor initiation and progression.

4

Hypotheses:

We sought to confirm whether the Methyl DNA Binding Protein 2 (MBD2) acquires a novel pathologic role within the atypical environment of mammary epithelial tumors. We asked whether cancer cells which become dependent on DNA methylation abnormalities simultaneously become 'addicted' to MCBPs and to MBD2 in particular.

1: We hypothesized that **inhibiting the function of MBD2 in tumor cells would lead to transcriptional reactivation of pathologically silenced tumor suppressor genes**.

2: We further hypothesized that any **transcriptional changes induced by MBD2 inhibition would be deleterious to tumor cells**; leading, for example, to growth inhibition and death.

3: Since abnormal methylation is restricted to tumor cells, and since no human pathology we are aware of is linked to loss of function of MBD2, we hypothesized that **the deleterious effects of MBD2 inhibition would remain restricted to tumor cells**; normal tissues would not be affected to the same degree or at all by inactivation of MBD2.

4: Given the redundancy within the MCBP family of proteins, we hypothesized that individual MCBPs might play discrete roles in tumor suppressor silencing. Therefore, we hypothesized that **inhibition of multiple MCBPs, for instance MBD2 and MECP2, in combination may offer superior anti-tumor activity and/or diminished off target toxicity in normal tissues**.

5

Objectives:

Briefly consider the context in which we initiated this line of investigation. When we undertook this project, it was unclear to what extent any individual methyl binding domain protein was required for the pathological behavior of human breast tumors, i.e., hyper-proliferation, failure of apoptosis, increased propensity for metastasis, resistance to therapy, etc. It was reported, however, that MBD2 antagonized several extra-mammary cancers (Sansom et al. 2003a; Slack et al. 2002). Furthermore, it was established that MBDs were bound directly to methylated DNA targets and that the gene expression of these target genes could be changed by perturbing MBD levels (Klose and Bird 2006; Berger and Bird 2005b; Kransdorf et al. 2006b; Rupon et al. 2006). The nature of MBD involvement in cancer pathology was to a large extent unknown and the notion of therapeutic inhibition of MBDs in human disease remained largely speculative. In fact given what was known about MBD function, it was reasonable to infer that any interference with these proteins might have a greater destabilizing effect than a therapeutic one in certain tissues.

Therefore, the chief objective of the studies described in this thesis was to determine whether the Methyl CpG binding domain proteins were functioning as aggravating factors in human neoplasia in general and breast cancer in particular. Pursuant to this objective, we initially sought to identify and characterize any phenotypic changes that resulted from knocking down the methyl binding domain protein 2 (MBD2) in human breast cancer cells.

Our prospective goal in performing the experiments outlined in this thesis was to validate methyl binding proteins as therapeutic targets in human mammary malignancies.

6

An important follow up objective was to build a case for one or more predominant mechanisms by which MBD2 inhibition resulted in any anti-tumor effects. We asked: what are the genes that are regulated by MBD2 in our breast cancer models, i.e., induced by MBD2 knockdown? What cellular programs are invoked by MBD2 knockdown and how specific are these programs to individual MCBPs? How do these effects differ from those induced by general methylation inhibitors, e.g., 5-azaC, and other epigenetic therapies, e.g. histone deacetylase inhibitors? Our hope in seeking the answers to these questions was to reach a point where the entire constellation of MBD2 dependent changes pointed to a consistent model for the function of this protein in breast cancer.

7

Chapter I: MBD2 inhibition alters the phenotype of breast cancer in culture and in the mouse

Introduction:

Epigenetics in Mammals

Deoxyribonucleic acid (DNA) is an elegant molecular database capable of efficiently storing, duplicating, and transferring genetic information (Watson and Crick 1953). However, it has become increasingly clear over the past half century that DNA alone does not provide the complexity required to generate the phenotypic diversity observed within and between higher order species. Epigenetic phenomena fill this gap by significantly expanding the storage capacity and regulatory complexity of the genome. Conrad H. Waddington, who coined the term, initially defined epigenetics as, "the causal relationships between genes and their products which bring the phenotype into being." (Waddington 1957; Goldberg, Allis, and Bernstein 2007) Modern epigenetics is generally defined as the study of those occurrences which generate stable, heritable changes in phenotype without a corresponding change in the sequence of DNA (Ginder, Gnanapragasam, and Mian 2008). An important qualification for epigenetic information is that it can be passed horizontally between cells by somatic transfer or vertically between parent and offspring through the germ line. In vertebrates epigenetic information is transmitted through several principal modes which are discussed below: Ribonucleic Acid (RNA) intermediates, post-translational modification (PTM) of histone

8

proteins (both covalent and non-covalent), nucleosome repositioning, and finally cytosine methylation.

Non-coding RNAs:

Much recent attention has been paid to small RNAs (miRNA, RNAi) that operate at post-transcriptional and translational stages to affect gene regulation. These molecules, however, are not epigenetic modalities in the strictest sense but rather represent alternate trans-acting regulatory elements within the cell. There are, however, several examples of bona fide epigenetic phenomena that are mediate by RNA in mammals. The first to be described and the best characterized among these is dosage compensation of the X chromosome in eutherian females mediated by the Xist family of small non-coding RNAs (Payer and Lee 2008; Boumil and Lee 2001). Recently several other non-coding RNAs have been described that drive analogous gene dosing and imprinting effects at non-sex linked loci, for example at the imprinted IGF2 locus (Mohammad, Mondal, and Kanduri 2009). These RNAs generally work by nucleating changes in chromatin architecture which are, once established, reinforced by DNA methylation and histone modifications.

Another recent report describes transgenerational epigenetic inheritance of a paramutant phenotype of the cKit gene in the mouse, mediated by non-coding RNA packaged into spermatozoa (Rassoulzadegan et al. 2006). It is clear from these studies that non-coding RNAs are emerging as an important, multi-tiered regulatory modality for effecting genetic and epigenetic gene regulation in mammals. These reports also highlight an important concept I will refer to thoughout this dissertation, i.e., the integrative nature of epigenetic regulation. It is evident that significant overlap exists between RNA, DNA methylation, and chromatin biology with respect to initiating and

9

reinforcing epigenetic signals and therefore considering these processes in isolation often crafts an incomplete understanding of their combinatorial nature.

Covalent Histone Modifications:

DNA methylation and ncRNAs alike mediate their effects on chromatin structure and function in large part by initiating the 'rewriting' of histone signals which in turn reinforce a particular epigenetic transcriptional effect (Kouzarides 2007; Li, Carey, and Workman 2007). Histone changes occur in two general varieties: covalent modifications of residues on the exposed N-terminal histone monomer tails and the non-covalent addition or removal of variant histone monomers into the macromolecular nucleosome. While there remains some controversy as to the primary heritability of histone modifications in mammals, it is clear that these marks confer stable transcriptional states to regional chromatin. Covalent histone marks consist of a heterogeneous collection of chemical modifications, some of which are assembled in Table 1 along with their putative functions.

Table 1. Histone Modifications and Their Function

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1, **K**-me2, **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1, **R**-me2a, **R**-me2s	Transcription
Phosphorylation	**S**-ph, **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R** > Cit	Transcription
Proline Isomerization	**P**-cis > **P**-trans	Transcription

Table 1. A list of modifications to histone proteins and their regulatory functions. Amino acid abbreviations: K: Lysine, R: Arginine, S: Serine, T: Threonine, E: Glutamate, P: Proline. Adapted from: (Kouzarides 2007)

10

Importantly, these modifications can be arranged in a myriad of permutations across a given region of chromatin, placing an enormous degree of regulatory complexity at the disposal of the epigenetic machinery which establishes, edits, and maintains these marks. Although many modifications are known to be enriched at either actively transcribed or stably silenced regions of chromatin (see the Table 2 below), in actual fact it is often difficult to ascertain the transcriptional state of a particular locus based on histone modifications alone. Moreover, many so called "bi-valent" domains exist which bear both marks of active and repressed transcription. It is thought that such regions represent loci which are variable with respect to spatial or temporal gene expression and are poised for silencing or activation depending on secondary factors such as the presence or absence of the sequence specific transcription factors. It is worthwhile to note that the inherent plasticity of bivalent chromatin domains may be critical for establishing pluripotency and pathologically in the development of neoplasia (Boyer, Mathur, and Jaenisch 2006; Keenen and de la Serna 2009; Surani, Hayashi, and Hajkova 2007).

11

Table 2. Covalent Histone Modifications and Their Association with Transcription

Modifications	Position		Mammalian Enzymes	Functions in Transcription
Methylation	H3	K4	MLL, ALL-1, Set9/7, ALR-1/2, ALR, Set1	Activation
		K9	Suv39h, G9a, Eu-HMTase I, ESET, SETBD1	Repression, activation
		K27	E(Z)	Repression
		K36	HYPB, Smyd2, NSD1	Recruiting the Rpd3S to repress internal initiation
		K79	Dot1L	Activation
	H4	K20	PR-Set7, SET8	Silencing
Arg Methylation	H3	R2	CARM1	Activation
		R17	CARM1	Activation
		R26	CARM1	Activation
	H4	R3	PRMT1	Activation
Phosphorylation	H3	S10		Activation
Ubiquitination	H2B	K120/123	UbcH6, RNF20/40	Activation
	H2A	K119	hPRC1L	Repression
Acetylation	H3	K56		Activation
	H4	K16	hMOF	Activation
	Htz1	K14		Activation

Table 2. Several well characterized histone modifications and their positions on the N-terminal tail residues of histone monomers are listed on the left. The mammalian isoforms of enzymes that catalyze the placement and/or removal these marks are listed to the right of the corresponding modifications. The predominant effect of each mark on transcription (activation/repression) is listed on the far right. Table adapted from: (Li, Carey, and Workman 2007)

Non-covalent Histone Modifications:

Another layer of epigenetic programming appears to have arisen from the evolutionary divergence of the genes encoding histone proteins. The discovery of histone variants overturned the long held idea that all nucleosomes are composed of a matching complement of the eight core histone proteins (Old and Woodland 1984). Instead it seems that the nucleosome octamer is fairly modular; its individual monomer constituents are interchangeable with functionally diverse variant proteins that confer a regulatory multiplicity to the assembled macromolecule. It follows, therefore, that the placement of nucleosomes in their chromosomal locations is not arbitrary and involves a carefully orchestrated collaboration between the S-phase machinery and histone chaperone proteins (Kamakaka and Biggins 2005). Numerous histone variants have now been described and the list of specialized histone proteins and their functions continues to grow. A brief list of histone variants involved in transcriptional regulation was compiled by Li, B. et al., and is given in Table 3.

Nucleosome Position:

In addition to the composition of histone multimers and their covalent modifications, nucleosome placement and density has profound effects on the epigenetic landscape of a particular region (Saha, Wittmeyer, and Cairns 2006). Nucleosomes can be partially dissociated, shifted along the DNA strand, or entirely 'evicted' from a particular region by the transcription machinery, histone chaperone proteins, and/or ATP dependent nucleosome remodeling complexes such as Swi/Snf (Workman 2006). The result is the transient formation of nucleosome depleted regions which can alter local transcription dramatically by changing the dynamics of DNA-protein interactions.

13

Table 3. Histone Variants Involved in Transcriptional Regulation: Structure and Function

Histone	Variant Forms	Role(s) in Transcription	Localization	Structural Features	Functions
H3	H3.3	Transcription activation	Transcribing region	Different from canonical H3 in only four amino acids.	Active transcription triggers deposition and removal.
H2A	macroH2A	X chromosome inactivation	Inactive X chromosome	C-term nonhistone-like region is responsible for most of functions; histone-fold prevents sliding; prefers to form hybrid nucleosome.	Repressing initiation but not elongation; interfering histone acetylation by p300; it blocks sliding by ACF and remodeling by Swi/Snf; it inhibits transcription factor binding (NFkB).
	macroH2A1	Autosomal gene repression	Repressed gene promoters and a subset of active genes	Variant of the macrodomain superfamily, shares 60% homology with canonical H2A, C-terminal macrodomain is responsible for unique functions	macroH2A1 marks repressed autosomal chromatin, it positively regulates transcription when located in the transcribed regions of a subset of its target genes (Gamble et al. 2010)
	H2AZ	Transcription activation/re-pression	Promoter, heterochromatin boundary	Loop1 differs from H2A, disfavors formation of hybrid nucleosome; C-term α helix is essential for recognition.	Facilitates TBP binding; is evicted upon activation; prevents elongation-associated modification and remodeling at promoter
	H2ABbd	Transcription activation	Active X chromosome and autosomes	Lack of C term; it only organizes 118–130 bp pf DNA and leaves each side 10 bp free DNA.	Swi and ACF fail to mobilize the H2ABbd nucleosome but can increase its accessibility. p300- and Gal4-VP16-activated transcription is more robust on H2ABbd nucleosomes; H2A.Bbd histone fold domain is responsible for the unusual properties of the H2A.Bbd nucleosome.
	H2A.X	Repression	Canonical in yeast, generally distributed	A conserved C-term SQ(E/D) motif that becomes phosphorylated upon DNA damage.	

Table 3. A list of histone monomer variants involved in transcriptional regulation and their function. Table data from: (Li, Carey, and Workman 2007) and (Gamble et al. 2010)

14

DNA Methylation

Perhaps the best characterized and most stable epigenetic mark in mammals is cytosine methylation. In the human genome, 70-80% of cytosines in the context of a CpG dinucleotide are methylated at the C-5 position (Ehrlich et al. 1982). Patterns of CpG methylation are established and maintained faithfully by the DNA methyltransferases (DNMTs), enzymes which catalyze the transfer of a methyl group from S-adenosyl-methionine to cytosine (Taylor and Jones 1982).

Figure 1. Pyrimidine Methylation and Hydrolytic Deamination Reactions
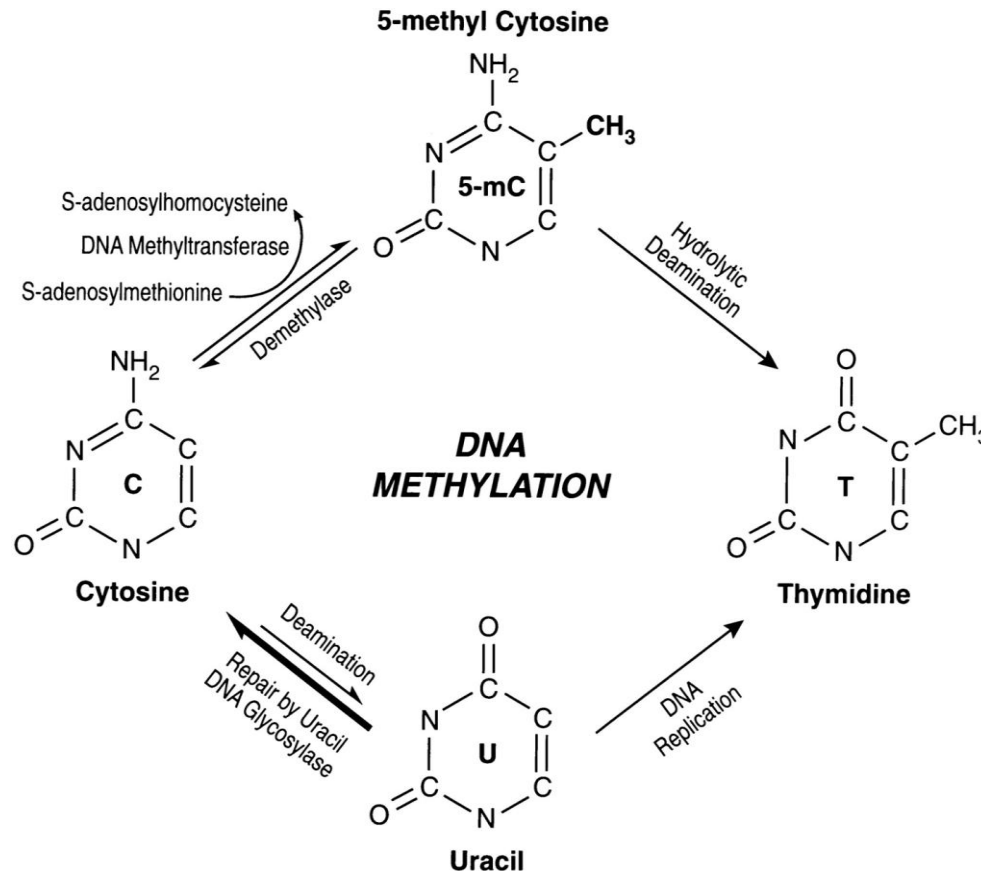
Figure 1: Shown above are the core reactions in the synthesis and loss of 5-methyl Cytosine in vertebrates. 5-Methyl Cytosine is generated from cytosine by the action of DNA methyl transferase enzymes with S-adenosylmethionine as the methyl donor molecule. Hydrolytic deamination of cytosine produces uracil where as deamination of 5-methyl cytosine generates thymidine. (R. Singal and G. Ginder, Blood, 1999)

15

Three functional DNA methyltransferases are recognized in mammals: DNMT3a and DNMT3b, which establish *de novo* patterns of methylation during development (Okano et al. 1999) and DNMT1, which maintains these patterns of methylation through replication of the DNA (Leonhardt et al. 1992). However, evidence suggests some overlap in *de novo*/maintenance activity exists between the methyltransferases (Liang et al. 2002; Jair et al. 2006).

DNA methylation is posited to have a number of important functions (Singal and Ginder 1999). Among these, methylation inhibits the destabilizing proliferation of parasitic elements in the genome (i.e., transposons, repetitive elements) (Robertson and Wolffe 2000). CpG methylation also maintains the mono-allelic expression of imprinted and X-inactivated genes. At imprinted loci DNA methylation insures a single, parentally determined allele is expressed (Feinberg 2007). Similarly, inactivation of a single X chromosome mediated by Xist ncRNAs drives monoallelic DNA methylation which is thought to stabilize heterochromatinization and transcriptional silencing. (Wutz and Gribnau 2007).

As discussed above, histone modifications can also maintain transcriptional states; however, CpG methylation is intuitively more fixed through DNA replication and cell division than histone modifications or occupancy. As such it is considered an important medium for secure, long-term preservation of epigenetic information in the genome (Bernstein, Meissner, and Lander 2007). Though its primary role in developmental gene expression patterning is an area of active investigation, it is clear that intact CpG methylation is indispensable for normal mammalian development (Hendrich et al. 2001;

16

Okano et al. 1999; Li, Bestor, and Jaenisch 1992a). In addition, suggestive differences in CpG methylation are observed in terminally differentiated cells in contrast to the corresponding loci in histologically distinct cells and pluripotent precursors. This CpG methylation is thought to provide a "lock off" mechanism for tight control of tissue restricted patterns of expression in differentiated tissues (Bird 2002a) and perturbation of this pattern in primary and cultured cells is sufficient to cause phenotypic conversion (Taylor and Jones 1979; Reik, Dean, and Walter 2001a).

Consistent with their well-described functional importance, the distribution of CpG sites in the genome is highly non-random. Across most of the genome, CpG dinucleotides are present at a fraction of the expected frequency due to the unchecked deamination of 5-methyl cytosine to thymidine over evolutionary time (Cooper and Krawczak 1989). CpG islands are an important exception; these are pockets of higher than expected CpG density, presumably due to a combination of selection forces and the absence of cytosine methylation within these regions (Cross and Bird 1995; Bird 1986). They tend to occur close to or overlapping the transcription start sites of protein coding genes. In fact, more than half of the known coding genes have promoter associated CpG islands (Jones and Baylin 2002).

CpG Methylation Changes in Cancer:

The normal pattern of CpG methylation is profoundly altered in cancer (Jones and Baylin 2007; Esteller 2008). Breast cancer is of particular significance because this differential methylation is known to be an early event in neoplastic transformation (Futscher et al. 2004; Umbricht et al. 2001; Honorio et al. 2003a; Muggerud et al. 2010).

Moreover, neoplastic mammary epithelium is clinically distinguishable from normal tissue simply on the basis of aberrant DNA methylation (Evron et al. 2001; Krassenstein et al. 2004). This suggestive observation has led to the postulation of a seminal role for abnormal methylation in the initiation or stabilization of early, preneoplastic and preinvasive breast cancer (Berman et al. 2005).

Abnormal CpG methylation in the cancer genome can be categorized into two groups: an overall decrease in widespread DNA methylation (Riggs and Jones 1983) and a local increase in CpG island associated methylation (see Figure 2). Though opposite in character, both hypo- and hypermethylation are consistent with a pro-neoplastic role. Global hypomethylation leads to deregulated oncogene expression, e.g., reactivation of proto-oncogenes such as R-Ras and loss of imprinting (Nishigaki et al. 2005). Loss of global methylation may also lead to widespread genomic instability due to de-repression of transposable elements (Feinberg 2007; Ehrlich 2002).

Conversely, hypermethylation of CpG islands is associated with tumor suppressor gene inactivation. The list of genes know to be silenced by promoter hypermethylation in cancer is large and growing rapidly. Though debate exists as to whether CpG island hypermethylation is an initiating event and the mechanisms directing the hypermethylation of particular loci are poorly understood, it is now widely believed that this alteration stabilizes the transcriptional changes implicated in the onset and progression of cancer (Jones and Baylin 2007; Feinberg 2007; Jones and Baylin 2002; Jones and Laird 1999; Futscher et al. 2002a; Ting, McGarvey, and Baylin 2006).

18

Figure 2. CpG Methylation: Changes in Cancer
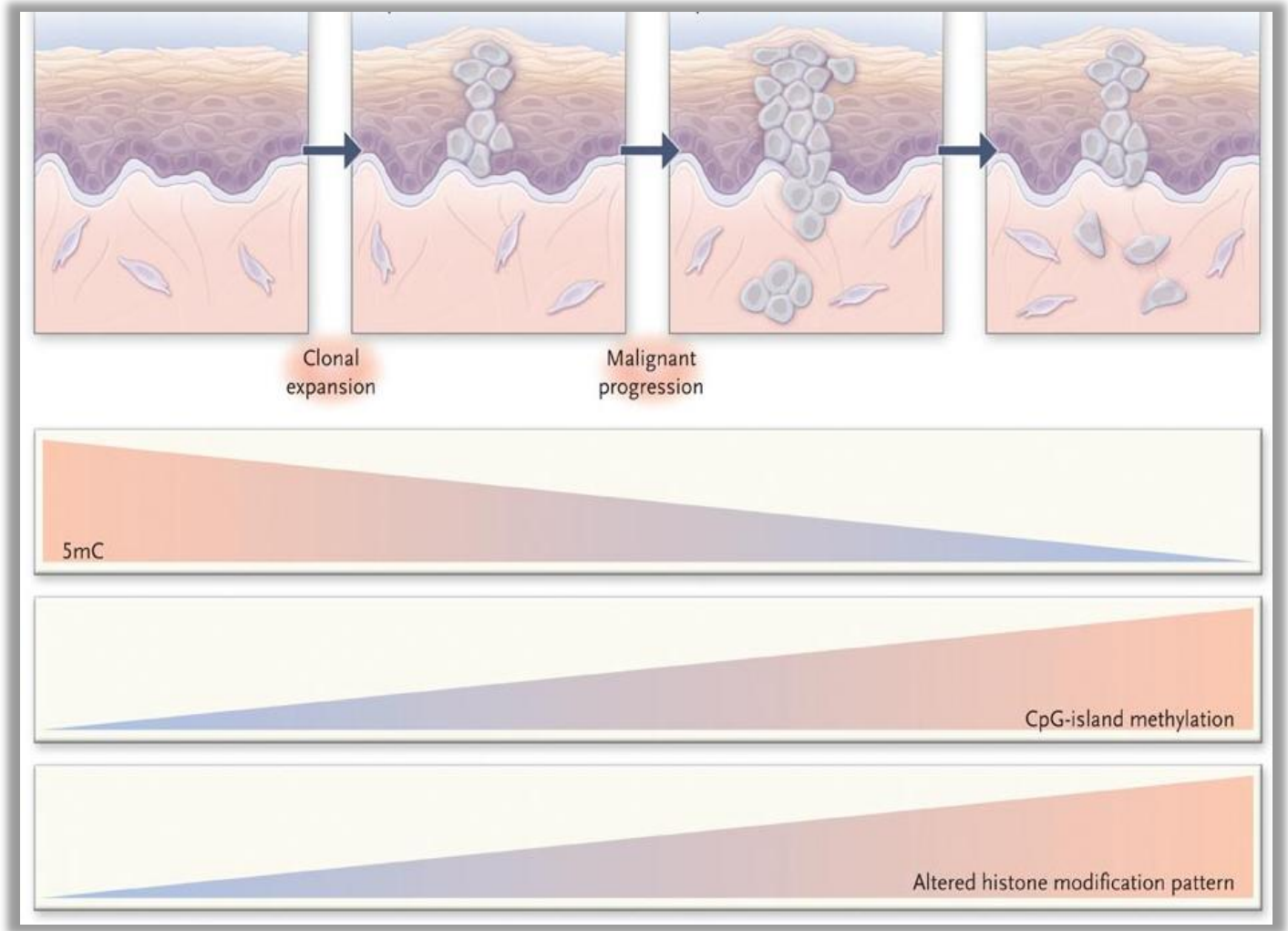
Figure 2. CpG methylation changes occur in tumor cells in two major categories: global hypo-methylation and local (CpG island associated) hyper-methylation. This figure shows that these changes occur progressively as cells transition from normal epithelium to invasive neoplasia. Abnormalities in DNA methylation lead to an altered histone modification pattern and ultimately contribute to stable gene expression changes. Image: (Esteller 2008).

19

Epigenetic Therapy of Cancer

Genetic abnormalities are frequent in tumors; these include DNA deletions, amplifications, mutations, large scale chromosomal abnormalities, and translocation events. Of immediate significance when considering epigenetic pathobiology in cancer is the fact that epigenetic modifications and their effects are more readily reversed than genetic lesions. A variety of strategies are currently used specifically to target epigenetic changes in cancer and more are in development. In the clinical setting these strategies fall into two major classes, DNA methylation inhibitors and histone deacetylase inhibitors (HDAC). A list of epigenetic drugs currently in use was compiled by Cortez and Jones, 2008 and is listed in Table 4 below (Cortez and Jones 2008).

Table 4. Epigenetic Drugs Currently Used in the Treatment of Cancer

Drug name	Cancer
DNA methylation inhibitors	
5-azacytidine (FDA approved)	MDS, AML, CML
5-aza-2-deoxycytidine (FDA approved)	AML, CML, MDS
MG98	Renal cell carcinoma
RG108	Colon cancer cell line
Procainamide	Colon cancer cell line
HDAC inhibitors	
SAHA (FDA approved)	CTCL, various solid tumors
PXD101	Various solid tumors
LBH589	CTCL
Depsipeptide	Multiple cancer cell lines, MDS, AML
Phenylbutyrate	MDS
Valproic acid	Neuroblastoma cells
MS-275	Prostate cancer cell lines, various sold tumors and lymphoid malignancies
CI-994	Various solid tumors

Table 4. A list of the compounds currently used to target epigenetic changes in tumors and the cancer sub-types for which they are used. Table was adapted from Cortez, C. and Jones, P., 2008. (Cortez and Jones 2008)

Demethylating agents such as 5-aza-2-deoxycytidine (Decitabine) are thought to elicit their therapeutic effects through passive demethylation mediated by DNMT inhibition leading to reactivation of tumor suppressor genes, though a clear link between their benefit and changes in methylation has yet to be established (Cortez and Jones 2008). Similarly, HDACs such as suberoylanilide hydroxamic acid (SAHA, Vorinostat) restore activating histone acetylation marks, thereby restoring gene expression and resensitizing tumors to treatment (Karagiannis and El-Osta 2006).

While epigenetic therapy has shown promise, particularly for certain hematopoetic malignancies, the utility of this approach in treating solid tumors remains to be determined. Solid tumors, such as breast cancer, have so far been relatively refractory to available epigenetic therapies (Appleton et al. 2007; Schrump et al. 2006). This limited efficacy could be due in part to the fact that strategies inducing widespread CpG de-methylation generate dose-limiting acute toxicities and are themselves carcinogenic through the mechanisms described above. As such, effort is being invested in finding alternate approaches capable of reactivating tumor suppressor genes in cancer that do not rely on global DNA de-methylation or on the widespread effects of pleotropic compounds such as HDACs. One such approach is inhibiting the function of MCBPs, the proteins which bind to methylated DNA and mediate transcriptional suppression (Figure 3).

21

Figure 3. Breaking the Epigenetic Cycle
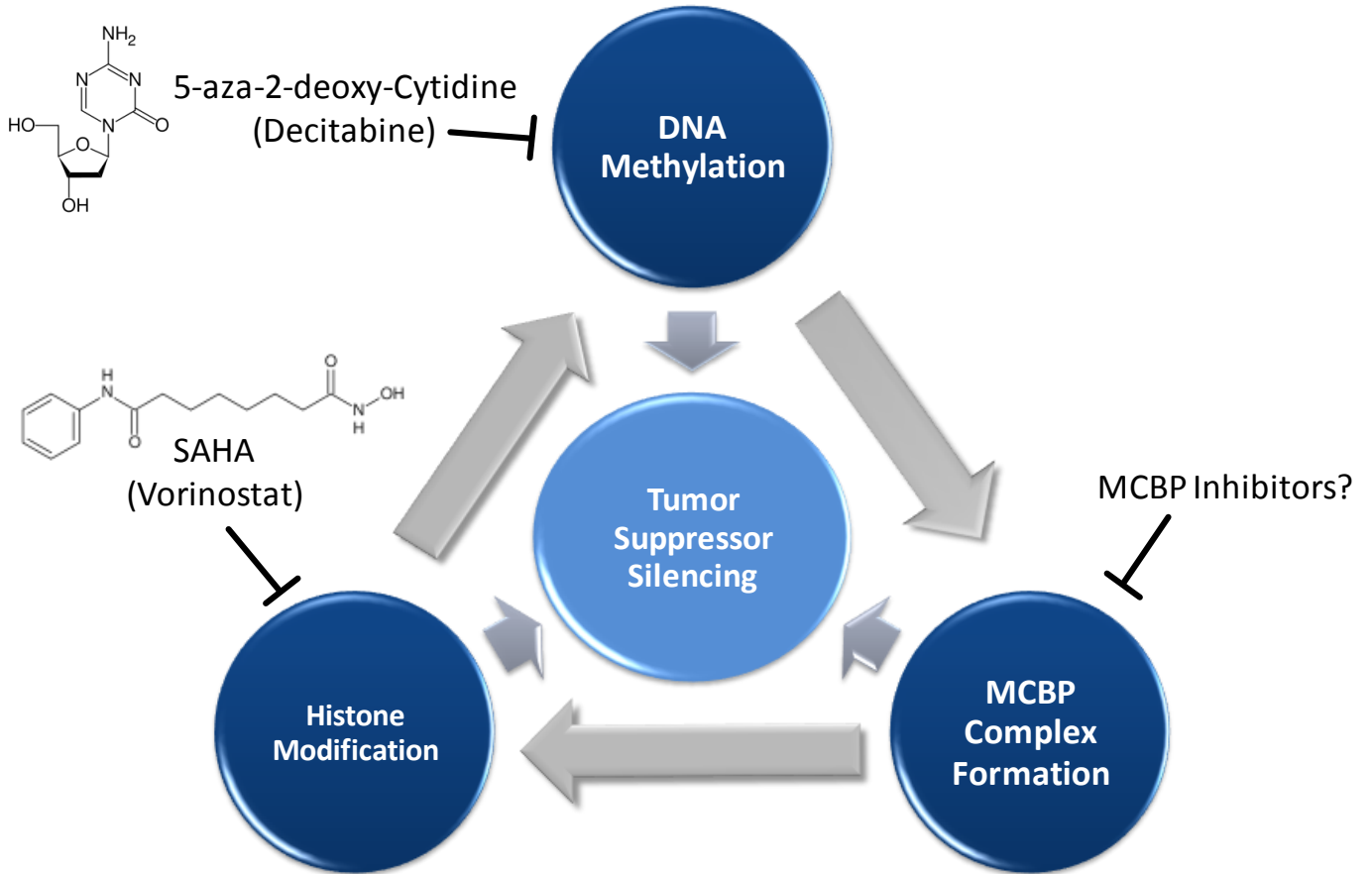
Figure 3. Emerging models suggest DNA methylation and post synthetic histone modifications depend on one another and reinforce each other. Through recruitment of enzymes and complexes, either mark appears capable of recapitulating the other, initiating a cycle which stabilizes gene expression silencing. Vorinostat and Decitabine are inhibitors that act at the indicated points in the cycle. We are investigating the role of MBD2 as a proof of principle that MCBP inhibition may be an alternate, and perhaps preferable, strategy for breaking this cycle.

Methyl CpG Binding Proteins:

The effects of DNA methylation on gene expression (Singal and Ginder 1999; McGhee and Ginder 1979) are mediated in large part by methyl-CpG binding proteins (MCBPs) (Hendrich et al. 2001; Rupon et al. 2006; Nan et al. 1998a; Kransdorf et al. 2006a), making these a promising class of proteins to explore for therapeutic targeting strategies (Lopez-Serra et al. 2008a; Sansom, Maddison, and Clarke 2007a). The MCBPs encompass several distinct sub classes of proteins, including the MBD family and the Kaiso protein (Klose and Bird 2006).

The MBD protein family has five members, assembled based on the presence of a conserved methyl-binding domain (MBD): MeCP2, MBD1, MBD2, MBD3 and MBD4 (Hendrich and Bird 1998a). The MBD motif allows these proteins to recognize and specifically bind methylated CpGs with one exception, MBD3. Mutations within its MBD have rendered MBD3 unable to preferentially bind methylated CpGs (Saito and Ishikawa 2002). MBD3 does retain functional protein interaction and transcriptional repression capability and, interestingly, this protein is the only member of the MBD family which is essential for life (Hendrich et al. 2001). MBD4 preferentially binds to methylated DNA, however this protein is more commonly associated with DNA repair/glycosylase activity in mammals (Hendrich et al. 1999). (Figure 4)

Unlike the MBD family, the Kaiso protein associates with methyl-CpG containing DNA through a zinc finger binding domain (Prokhortchouk et al. 2001a) which confers a degree of sequence specificity to this protein. Similarly, MeCP2 has a preference for methyl-CpGs flanked by short A-T rich stretches (Klose et al. 2005), however the remaining MBD proteins do not seem to exhibit any sequence preference beyond their

23

affinity for methylated DNA. MeCP2 is further unique among the MBD proteins because of its association with a debilitating human neurodevelopmental disorder, Rett syndrome (Amir et al. 1999).

The MCBPs mediate transcriptional repression by recruiting chromatin modifying protein complexes to sites of increased methyl-CpG density (Klose and Bird 2006). MBD1, for example, couples CpG methylation to repressive methylation of H3 lysine 9 through its association with the histone methyltransferase, SetDB1, during replication. MBD2 is known to recruit the histone deacetylase (HDAC) 1 and 2 containing NuRD/Mi-2 complex, of which MBD3 is thought to be a component in some instances (Zhang et al. 1999) but not in others (Le Guezennec et al. 2006). Similarly, MeCP2 is thought to initiate local chromatin condensation through the HDAC containing Sin3a silencing complex (Figure 4).

The principal interactions of methyl binding proteins in mammalian cells, as described above, are consistently observed across tissues. However, it is becoming increasingly clear that these proteins are promiscuous with respect to their biochemical associations (Bogdanovic and Veenstra 2009). Each individual MCBP associates with many partners to accomplish its transcriptional function and these interactions can be tissue specific or locus specific within a given nucleus. This promiscuity is evident in the list of the MCBPs and their known biochemical interactions summarized in a review by Bogdanovic and Veenstra (given in Table 5 below). Once again, implicit in the widespread communication of MCBPs is the integrative and combinatorial nature of epigenetic signals. Through alternative arrangement of MCBPs and their partners, the

24

cell or organism as the case may be, can fine tune the hardwired transcriptional program to suit its particular requirements.

Figure 4. Methyl Binding Domain Protein Family and their Co-Repressors
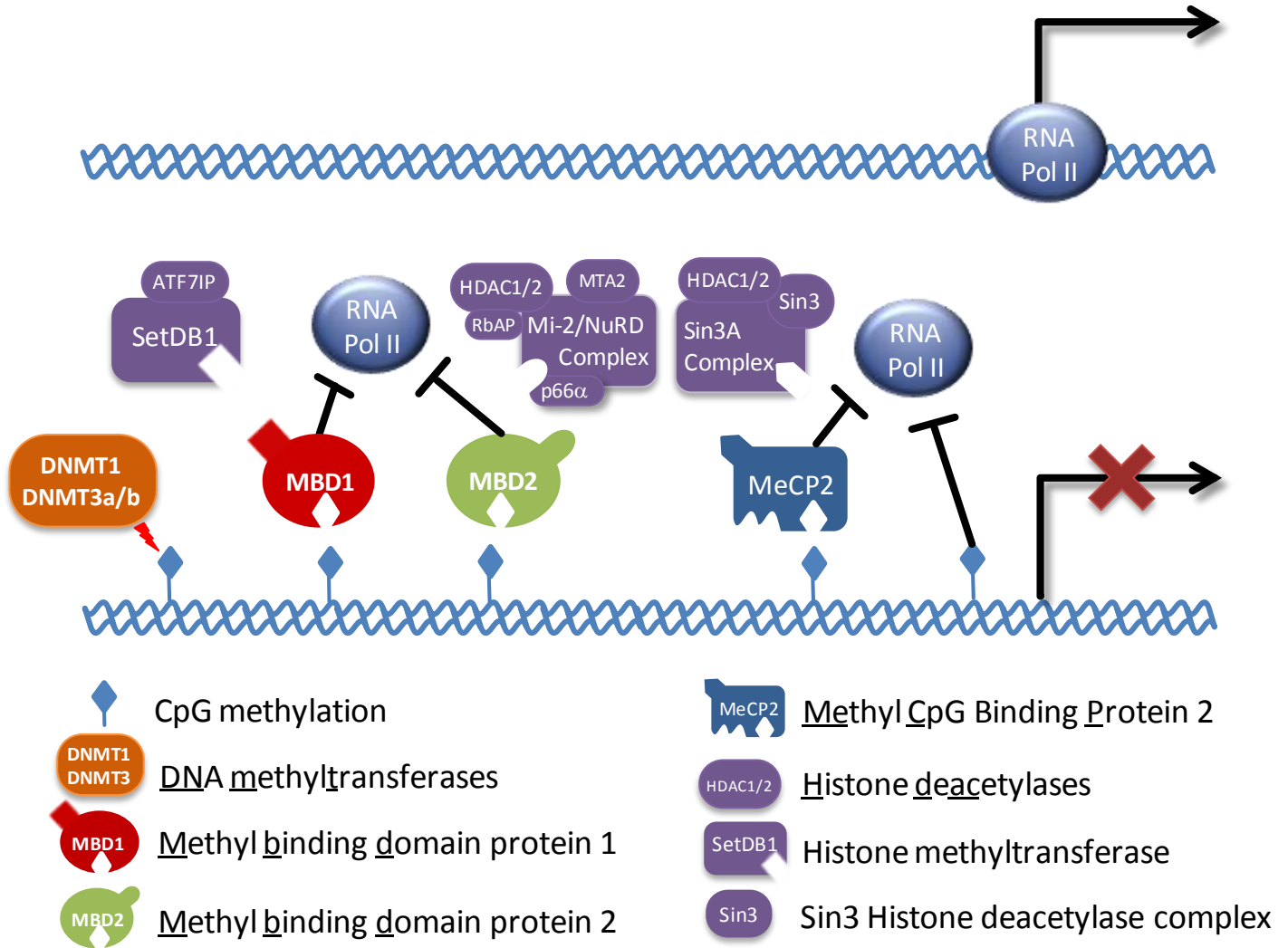
Figure 2: CpG methylation leads to transcriptional silencing through MCBPs: Cytosine (in the context of a CpG di-nucleotide) is methylated by DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) leading to methyl-binding domain protein association (e.g., MBD1, MBD2, MeCP2). These MCBPs in turn recruit co-repressor complexes (e.g., SetDB1, NuRD/Mi-2, and Sin3a) which modify the local chromatin environment leading to stable transcriptional silencing.

www.manaraa.com

Table 5. Methyl DNA Binding Proteins and their Interactions

Protein	Interacting partner	Effects of the interaction	Reference
MeCP2	Sin3A, HDACs	Transcriptional repression	Jones et al. (1998), Nan et al. (1998)
	c-ski, N-CoR	Transcriptional repression	Kokura et al. (2001)
	HMGB1	Unknown	Dintilhac and Bernues (2002)
	Sin3B, HDAC2	Transcriptional repression	Rietveld et al. (2002)
	Dnmt1	Targeting of maintenance DNA methylation	Kimura and Shiota (2003)
	H3K9 methyltransferase	Transcriptional repression	Fuks et al. (2003b)
	CoREST complex	Repression of neural genes	Ballas et al. (2005), Lunyak et al. (2002)
	Brm (SWI/SNF complex)	Transcriptional repression	Harikrishnan et al. (2005), Hu et al. (2006)
	YB-1	Alternative splicing	Young et al. (2005)
	ATRX	Epigenetic regulation of neural development	Nan et al. (2007)
	HP1	Transcriptional repression during myogenic differentiation	Agarwal et al. (2007)
	CREB1	Transcriptional activation	Chahrour et al. (2008)
MBD1	MPG	DNA repair	Watanabe et al. (2003)
	Suv39h1-HP1	Transcriptional repression	Fujita et al. (2003)
	MCAF1, MCAF2, SETDB1, CAF-1 p150	Transcriptional repression, inheritance of epigenetic states	Ichimura et al. (2005), Reese et al. (2003), Sarraf and Stancheva (2004)
	PML-RARα, HDAC3	PML-RARα-mediated silencing	Villa et al. (2006)
MBD2	Mi-2, MTA1-3, P66α/β, HDAC1/2, RbAp46/48, DOC-1, PRMT5, MEP50 (NuRD complex)	Transcriptional repression	Brackertz et al. (2002), Le Guezennec et al. (2006), Zhang et al. (1999)
	Sin3A	Transcriptional repression	Boeke et al. (2000)
	Tax	Transcriptional activation	Ego et al. (2005)
	TACC3, HATs, pCAF	Transcriptional activation	Angrisano et al. (2006)
	GCNF	Oct-4 silencing	Gu et al. (2006)
	Dnmt1	Targeting of maintenance DNA methylation?	Tatematsu et al. (2000)
	RFP	Transcriptional repression	Fukushige et al. (2006)
MBD3	Mi-2, MTA1-3, P66α/β, HDAC1/2, RbAp46/48, DOC-1 (NuRD complex)	Transcriptional repression	Le Guezennec et al. (2006), Wade et al. (1999), Zhang et al.

26

			(1999)
	Dnmt1	Targeting of maintenance DNA methylation	Tatematsu et al. (2000)
	CDK2AP1, GCNF	Oct-4 silencing	Deshpande et al. (2009), Gu et al. (2006)
MBD4	Sin3A, HDAC1	Transcriptional repression	Kondo et al. (2005)
	FADD	Genome surveillance/apoptosis?	Screaton et al. (2003)
	MLH1	DNA repair	Bellacosa et al. (1999)
	RFP	Enhancement of transcriptional repression	Fukushige et al. (2006)
Kaiso	Tcf3	Suppression of Wnt signaling	Ruzov et al. (2009)
	p120	Wnt signaling?	Daniel and Reynolds (1999), Prokhortchouk et al. (2001)
	N-CoR	Transcriptional repression	Yoon et al. (2003)

Table 5. Methyl DNA Binding proteins are listed along with the proteins and protein complexes with which an association has been demonstrated. The function associated with each biochemical interaction is also given as are the relevant references. The data for this table was compiled from: (Bogdanovic and Veenstra 2009)

The precise role that MCBPs play in cancer is not well understood. One could make a convincing case that their presence promotes the repression of protective genes and therefore drives cancer progression; the opposite argument could be made as well by considering the beneficial repression of tissue restricted oncogenes and parasitic elements. To date, a few studies have demonstrated a functional role for MCBPs in promoting extra-mammary tumor progression (Berger and Bird 2005a; Sansom et al. 2003a; Pulukuri and Rao 2006). In addition, isolated gene expression changes in breast cancer cell lines mediated by MCBPs have been demonstrated (Lin and Nelson 2003). However, functional studies examining the role of MCBPs in promoting the pathogenesis of breast cancer are needed.

The Special Role of MBD2 in Development and in Cancer

Substantial evidence now exists that supports a role for MBDs in the transcriptional silencing of genes in tumors (Esteller 2008; Ballestar and Esteller 2005). Among methyl-binding proteins, MBD2 is unique in that it binds the largest known proportion of genes in human tumors. Moreover, MBD2 is commonly the only MBD found at tumor suppressor gene promoters; important exceptions do exist where other MBDs are simultaneously present (Lopez-Serra et al. 2008a).

MBD2 null mice develop normally and are completely viable and fertile, with the exception of a mild maternal behavioral phenotype (Hendrich et al. 2001). A recent study cast suspicion on the idea that MBDs play redundant roles during development; a triple knockout mouse with loss of function of MBD2, MeCP2 and Kaiso displayed only the MeCP2 dependent Rett syndrome phenotype (Martin Caballero et al. 2009). This

28

study essentially rules out compensation by other MCBPs as an explanation for the lack of developmental lethality in MBD2 knockout animals.

Several studies have shown a role for MBD2 in gene expression patterning and differentiation in post developmental mice. For example, MBD2 is required for the complete silencing of fetal hemoglobin in adult human betaYAC transgenic mice (Rupon et al. 2006). In addition, MBD2 contributes to the suppression of the Xist gene in fibroblasts from male mice, in which this gene is not required for X-inactivation (Barr et al. 2007). Interestingly, this repression was exclusive to MBD2; MeCP2, MBD1, and Kaiso were not able to mediate a similar repression of Xist. MBD2 is also required for the proper repression of IL-4 in the Th-1 subset of mouse helper T-cells (Hutchins et al. 2002; Hutchins et al. 2005). In MBD2 null animals, GATA-3 is no longer required for activation of IL-4 expression and it is ectopically expressed in a subset of the MBD2 null animal's T-cells. Similarly, pancreatic genes are ectopically expressed in inappropriate segments of the MBD2 null mouse gut (Berger et al. 2007). These examples further illustrate the integrative nature of activating (e.g., GATA-3) and repressive (e.g., MBD2) signals in maintaining appropriate temporal and spacial gene expression in mammals. It is interesting to note despite these MBD2 dependant effects there is not overt phenotypic effect on animal viability.

Of particular relevance to the current work is the evidence for the exacerbating role of MBD2 in tumorigenesis. Anti-sense RNA mediated knockdown of MBD2 in human lung and colo-rectal cancer cells diminished their ability to form tumors in nude mice (Campbell, Bovenzi, and Szyf 2004). Furthermore, MBD2 null animals showed decreased adenoma formation and enhanced survival when bred with an Apc^{Min} mouse

29

model of spontaneous colo-rectal cancer, shown below in a figure from the relevant work (Figure 5, (Sansom et al. 2003a). By contrast, MBD4 null animals demonstrated increased adenoma formation in the same model, consistent with the DNA repair function associated with this MBD protein (Millar et al. 2002).

Given the known exacerbating role of MBD2 in tumor models, we sought to examine the role MBD2 was playing in breast cancer, where abnormal methylation is an early event and predicts a poor prognosis (Corn 2009; Lustberg and Ramaswamy 2009; Dumont et al. 2009; Martens et al. 2009).

Figure 5. Loss of MBD2 reduces adenoma formation and promotes survival in a mouse model of colon cancer.
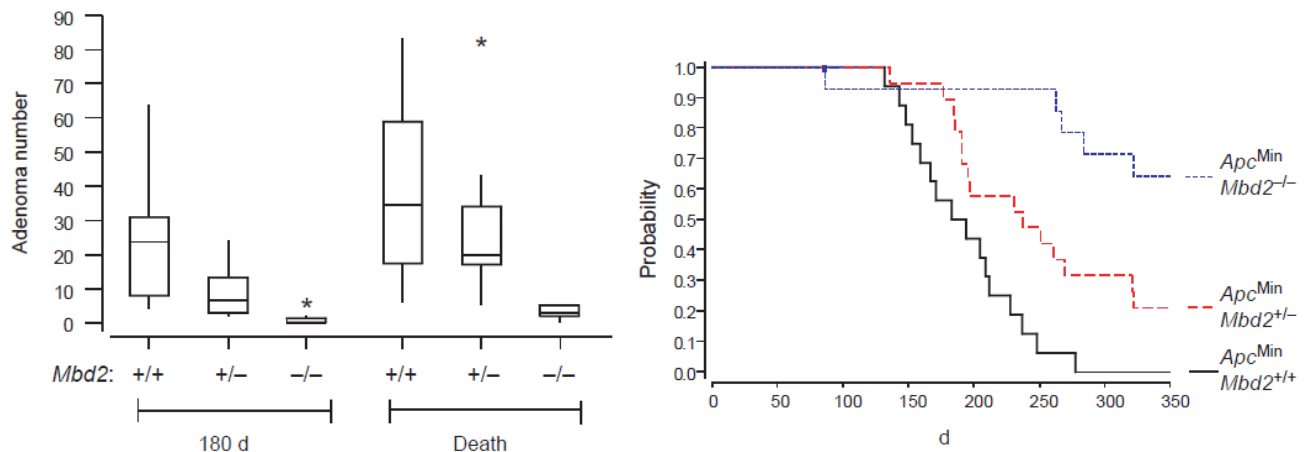
Sanson, OJ *et al*, Nature Genetics, 2003

Figure 5. In breeding experiments conducted by others, MBD2 heterozygote and null mice showed a reduction in the occurrence and size of neoplastic lesions in the Apc[Min] mouse model of colon cancer. In addition, MBD2 +/- and -/- animals demonstrated increased survival. On the left is charted the progressively decreased tumor number and increased survival in MBD2 +/+, +/- and -/- mice. On the right a survival curve is shown. The effects on tumor burden and survival were MBD2 dose dependent, that is heterozygotes demonstrated an intermediate phenotype with respect to adenoma formation and survival. Image: (Sansom et al. 2003)

<center>**Materials and Methods:**</center>

Cell culture

   MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468 cells (ATCC, Manassas, VA) were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% v/v FBS and penicillin/streptomycin. SK-BR-3 cells were cultured in McCoy's 5A medium (Mediatech Inc, Manassas, VA) supplemented with 10% v/v FBS. MCF-10a cells were cultured in DMEM F12 Hams (Gibco, Carlsbad, CA) media supplemented with 5% v/v horse serum, penicillin/streptomycin, 20ng/ml recombinant human EGF (Invitrogen, Carlsbad, CA), 10 ng/ml of insulin, and 20 ng/ml of Dexamethasone (Sigma-Aldrich, St. Louis, MO). All cells were held at 37 degrees C under 95% air and 5% carbon dioxide and sub-cultured before complete confluence. DNA methylation patterns are unstable over time in culture conditions; these cell lines were maintained in limited passage states by returning to frozen stocks regularly. Low passage frozen stocks were stored in a mixture of 10% DMSO, 20% FBS, and 70% DMEM. Cells were frozen in cryo-storage tubes initially placed in controlled cooling vessels at -80 deg C (to maximize post-thaw viability) and subsequently kept under liquid nitrogen.

Reverse Transcriptase PCR (cDNA synthesis)

   Total RNA was purified from cultured breast cancer cells by disruption in 1mL of Trizol^{TM} reagent (Invitrogen, Carlsbad, CA). RNA purification was performed according to the product directions, briefly by chloroform based (0.2mL) aqueous phase extraction, isopropanol precipitation, rehydration in DEPC treated water and storage at -80 deg C.. One microliter of purified RNA was quantified by measuring absorbance at 260nm using

<center>31</center>

a Nanodrop spectrophotometer (NanoDrop Products, Wilmington, DE). Complementary

DNA (cDNA) was generated from mRNA by reverse transcriptase PCR (iScript Kit, Bio-

Rad Labs, Hercules, CA) using 1ug of total RNA. A negative control with no reverse

transcriptase enzyme (-RT control) was always included to verify amplification of cDNA

only (and not residual co-purified DNA) in subsequent PCR assays. Reactions were

performed by incubation at 42 deg C for 45 min, after which cDNA was diluted 1:10 and

stored at -20 deg C or used for subsequent qPCR reactions.

SYBR Green fluorescence based quantitative real-time PCR

Gene specific mRNA levels and enrichment by ChIP were both measured using

quantitative PCR essentially as described previously (Rupon et al. 2006; Barrett et al.

2004).   Gene quantification, primers were designed either manually, using the

PrimerQuest tool (Integrated DNA Technologies), or auto-generated using the

qPrimerDepot tool (http://primerdepot.nci.nih.gov/) (Cui, Taub, and Gardner 2007). All

primers were verified through a 4 log range using serial dilutions of reference cDNA or

gDNA followed by standard curve analysis. A list of qPCR primers is given in Appendix

B of this dissertation. All amplifications were performed using 40 cycles of 'standard'

two step PCR plus dissociation curve program on either and ABI 7300 or ABI 7900 HT

instrument (Applied Biosystems). All reactions were performed in either 96 or 384 well

microtiter plates using SYBR green chemistry. A commercially available 2X master mix

was used containing enzyme and PCR additives. (Applied Biosystems, CA) To this mix

was added molecular biology grade water, approximately 5ng of RNA (cDNA) per

32

reaction, and oligonucleotide primers at a final concentration of 100nM (from a 1 pM/ul working stock).

Analysis of qPCR data

Quantitative PCR data was analyzed in MS Excel by relative quantification using the $2^{-\Delta CT}$ method as described previously (Schmittgen and Livak 2008). Endogenous control primers were included from at least one reference gene (Cylcophillin A, Glyceraldehyde Phosphate Dehydrogenase [GAPDH], or beta-actin) on every plate. Relative quantification results are reported as a ratio of means for each sample, e.g., MBD2/GAPD of at least two replicate reactions per gene per plate. Error is reported as standard deviation adjusted for the ratio of means operation according to the following formula where z_σ represents the cumulative error, $\frac{\bar{x}}{\bar{y}}$ is the ratio of means and x_σ and y_σ are the individual standard deviations of the mean for each set of qPCR replicates:

$$z_\sigma = \frac{\bar{x}}{\bar{y}} \sqrt{\left(\frac{x_\sigma}{\bar{x}}\right)^2 + \left(\frac{y_\sigma}{\bar{y}}\right)^2}$$

A simple approximation of the cumulative error given by this formula is the sum of the individual errors for each set of qPCR replicates ($\approx x_\sigma + y_\sigma$), for example the standard deviation of all MBD2 replicates plus the standard deviation of all Cylcophillin A replicates for a given biological sample.

Western Blots:

Protein immunoblots were performed following SDS polyacrylamide gel electrophoresis of Bradford/Lowry normalized protein lysates in 4% SDS plus protease

inhibitors essentially as described previously (Kransdorf et al. 2006a). Antibodies for MBD2 were obtained from Upstate and Santa Cruz Biologicals (SC-D15). Antibodies for MeCP2 and Beta-Actin, were obtained from Abcam Inc. Antibodies for DNMT1 were a generous gift from Shirley Taylor, PhD.

3DlrECM Culture

Human mammary adenocarcinoma cells were grown in 3 dimensional culture media composed of laminin rich extracelluar matrix (3D-lrECM, Cultrex ®, Trevigen, Gaithersburg, MD) as previously described (Genee Y. Lee et al.). This basement membrane substitute functions to provide a scaffold for formation of higher order epithelial structures characteristic of *in situ* epithelial tissues (Nelson and Bissell 2005). Briefly, basement membrane extract (BME) was thawed overnight at 4 deg C and kept on ice at all times as it irreversibly solidifies into a gel at 15 deg C or above. Culture vessels were pre-coated with a thin layer of BME and incubated at 37 deg C for 5 min to insure that cultured cells are not were not in direct contact with plastic. Cells were trypsinized, pelleted and resuspended in Matrigel by slowly pipetting to prevent bubble formation, all cultures were started at a cellular concentration between $5x10^5$ and $1x10^6$ cells per ml. Following a5 minute incubation at 37 deg C, the BME gel was overlaid with complete growth medium and returned to the 37 deg C incubator. Overlaid media was changed every 2 days. 3D structures were visualized by collecting whole BME aspirates in chamber slides, complete dehydration of matrigel and a 10 minute fixation in 50% Methanol 50% Acetone solution at -20 deg C. Fixed BME specimens were visualized

34

with light microscopy or stained with 1X Hoechst nuclear stain (Sigma-Aldrich, St. Louis, MO) in PBS and visualized by fluorescence microscopy.

Lentivirus cloning and infection

Lentiviral vectors and packaging was accomplished essentially as previously described (Wiznerowicz and Trono 2003). Briefly, the lentivirus system was used to mediate the efficient delivery, integration and stable expression of shRNA that was rapidly FACS selectable. Rapid selection was essential to avoid the loss of populations of interest, i.e., those cells whose growth or survival was negatively affected. Infectious lentiviral particles were generated by co-expressing the virion packaging elements and the vector genome containing engineered components (e.g. shRNAs and transgenes) into packaging cells (in this case the 293T human embryonic kidney cell expressing a temperature-sensitive version of SV40 Large T antigen). The core and enzymatic components of the virion were originally derived from HIV-1, while the envelope was derived from vesicular stomatitis virus (VSV) due to the high stability and broad tropism of its G protein. By convention, the former elements are designates as the LV packaging system the latter as the envelope. Currently, three generations of HIV-based LV packaging systems have been successively developed for production of lentivirus by transient transfection. The first generation LV packaging system encompasses all HIV-1 genes besides the envelope. The second generation LV packaging system is additionally deleted in all viral auxilliary genes, i.e. vpr, vif, vpu and nef. The third generation LV packaging system comprises only gag, coding for the virion main structural proteins, pol, responsible forthe retrovirus-specific enzymes, and rev, which encodes a post-

35

transcriptional regulator necessary for efficient gag and pol expression. The third generation packaging system offers maximal biosafety but is more cumbersome, involving the transfection of four different plasmids in the producer cells. We used the second generation LV packaging system in our studies (see Appendix C for plasmid maps). For this system, the vector itself is the only genetic material transferred to the target cells. It typically comprises the shRNA expression cassette flanked by cis-acting elements necessary for its encapsidation, reverse transcription and integration. In this system, the reverse transcription rearrangement is used to generate self-inactivating (SIN) HIV-1-derived vectors, which lose the transcriptional capacity of the viral long terminal repeat (LTR) once transferred to target cells (Wiznerowicz and Trono 2003; Choi et al. 2001). This minimizes the risk of emergence of replication competent recombinants (RCR) and avoids problems linked to promoter interference. The bicistronic pLV-THM vector allows for the simultaneous expression of a transgene and GFP marker to facilitate tracking of transduced cells.

In order to insert shRNA hairpin forming oligos the pLVTHM vector was digested with MluI and ClaI and the siRNA oligonucleotide was designed with the appropriate complementary ends. Otherwise annealing and ligation was performed as described above for pSuperior cloning. Oligos and target sequences used to generate the pLV-THM clones are given in Table 1.

36

Table 6. Lentivirus shRNA Oligos and Sequencing Primers

MBD2 B Target Sequence	GGGTAAACCAGACTTGAAT
MeCP2 Target Sequence	AAGCCTTTCGCTCTAAAGTGG
Maspin Target Sequence	GCCGTTGATCTGTTCAAACAA
DAPK1 Target Sequence	AAGCATGTAATGTTAATGTTA
KLK10 Target Sequence	AACACAGTGGGTGGTGAGTTC
Scramble Target Sequence	ACGCGTAACGCGGGAATTT
shMBD2-B-Aa	CGCGCCCCGGGTAAACCAGACTTGAATTTCAA
shMBD2-B-Aas	TTCTCTTGAAATTCAAGTCTGGTTTACCCGGGG
shMBD2-B-Bs	GAGAATTCAAGTCTGGTTTACCCTTTTTAT
shMBD2-B-Bas	CGATAAAAAGGGTAAACCAGACTTGAA
shMeCP2-Aa	CGCGCCCCAAGCCTTTCGCTCTAAAGTGGTTC
shMeCP2-Aas	CTCTTGAACCACTTTAGAGCGAAAGGCTTGGGG
shMeCP2-Bs	AAGAGACCACTTTAGAGCGAAAGGCTTTTTTTAT
shMeCP2-Bas	CGATAAAAAAAGCCTTTCGCTCTAAAGTGGT
shMaspin-Aa	CGCGCCCGCCGTTGATCTGTTCAAACAATTCA
shMaspin-Aas	TCTCTTGAATTGTTTGAACAGATCAACGGCGGG
shMaspin-Bs	AGAGATTGTTTGAACAGATCAACGGCTTTTTAT
shMaspin-Bas	CGATAAAAAGCCGTTGATCTGTTCAAACAA
shDAPK1-Aa	CGCGCCCAAGCATGTAATGTTAATGTTATTCA
shDAPK1-Aas	TCTCTTGAATAACATTAACATTACATGCTTGGG
shDAPK1-Bs	AGAGATAACATTAACATTACATGCTTTTTTTAT
shDAPK1-Bas	CGATAAAAAAAGCATGTAATGTTAATGTTA
shKLK10-Aa	CGCGCCCAACACAGTGGGTGGTGAGTTCTTCA
shKLK10-Aas	TCTCTTGAAGAACTCACCACCCACTGTGTTGGG
shKLK10-Bs	AGAGAGAACTCACCACCCACTGTGTTTTTTTAT
shKLK10-Bas	CGATAAAAAAACACAGTGGGTGGTGAGTTC
shScramble-Aas	CGCGCCCCACGCGTAACGCGGGAATTTTTTCAA
shScramble-As	TTCTCTTGAAAAAATTCCCGCGTTACGCGTGGGG
shScramble-Bas	GAGAAAATTCCCGCGTTACGCGTTTTTTTAT
shScramble-Bs	CGATAAAAAACGCGTAACGCGGGAATT
H1 Forward Sequencing Primer	GAATTCGAACGCTGACGTC

Table 1: Lentivirus shRNA cloning oligonucleotides and sequencing primers. Sequences are given in the 5' to 3' direction for all oligonucleotides. Four complementary fragments were annealed for each shRNA targeting construct in the following orientation: Vector 3' – A oligo pair – B oligo pair – 5' Vector. The "A" and "B", sense ("s") and antisense ("as") oligonucleotides are marked accordingly. Oligonucleotide ends are MluI and ClaI compatible.

37

MTT Assay

The MTT Assay was performed as described previously (Scudiero et al. 1988). Briefly, cells were trypsinized and diluted to between 50,000 and 100,000 cells/ml. 100ul of cells were plated in each well of a 96 well culture dish (5000 to 10000 cells). For drug sensitivity assays, all cells were treated after 24 hours keeping the final culture volumes at 100ul per well. A series of two-fold serial dilutions of untreated cells was included in each assay as a standard reference. At the time of assay analysis, 20ul of a 5mg/ml filtered solution of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma Aldrich) was added to each well. Dishes were incubated for 2-4 hours (depending on cell density) and media was removed. Cells and formazan crystals were solubilized in acid isopropanol, 150ul per well (4mM HCl, 0.1% NP-40 in isopropyl alcohol). Absorbance was read on a 96 well plate reader at 595nm with a reference read at 620nm. All absorbances were normalized to the standard dilution curve and reported as standardized relative cell densities.

Xenograft growth, hematogenous seeding of the lung, and *in vivo* bioimaging of nude mice

Human tumor xenograft growth and *in vivo* hematogenous metastases to the lung was assayed essentially as described previously (Welch 1997; Klerk et al. 2007). For xenografts, 6 week old female Balb/C nu/nu mice were injected with $5x10^5$ or $1x10^6$ cells (GFP positive, SC and KD lentivirally transduced and sorted MDA-MB-231 and MDA-MB-435). Cells were suspended in a 1:1 DMEM:lrBME mixture (Gibco, Carlsbad, CA and Cultrex Inc) and 50ul was injected with a 25½ gauge needle and 1cc syringe

38

symmetrically in the subcutaneous space overlying the dorsolateral aspects of both hind flanks. Each mouse acted as its own control, receiving scramble (left) and knockdown (right) cells in opposite flanks. Mice were observed 2-3 times per week until the appearance of a palpable mass. Upon the detection of a palpable tumor, caliper measurements and bioimaging using GFP fluorescence filters. (IVIS 200, Xenogen Corp. Almeda, CA) was performed every second day. Standard IVIS 200 fluorescence acquisition settings were used including an exposure time between 4-10 seconds, normal background subtraction, medium binning, and a 1.5cm focal plane height.

Lung tumors were seeded in 6 week old female BalbC nu/nu mice by tail vein canulization and injection of $5x10^5$ or $1x10^6$ cells in 500ul of PBS. Mice were observed for signs of respiratory distress and for hemodynamic stabilization at the injection site. Mice were observed daily following injection for signs of distress. Mice were sacrificed at 4 weeks post injection. Lungs and heart were perfused with PBS and the airspaces were dilated with saline prior to extraction of the thoracic block and immediate fixation in formalin or flash freezing in optimum cutting temperature (OCT) media. Sections were stained in hematoxylin and eosin as described elsewhere and tumors appeared as basophilic (cellular) regions in the otherwise cell poor lung parenchyma upon gross macroscopic and microscopic examination. Approval for all animal work was obtained from the VCU Institutional Animal Care and Use Committee and all procedures were conducted according to VCU DAR guidelines.

39

MBD2 knockdown leads to growth inhibition of breast cancer cells

Our initial objective was to identify whether loss or attenuation of MBD2 leads to a detectable change in the growth or behavior of breast cancer cells. To this end, we designed a drug selectable MBD2 shRNA delivery system (pSuperior, described in detail in Chapter 2) and attempted to select stable MBD2 knockdown breast cancer cells. Despite significant effort, these early attempts to select stable MBD2 shRNA expressing cell lines met with limited success. We surmised that a growth or survival penalty was indeed being imposed in breast carcinoma cells lacking MBD2 and we began searching for alternate methods. We settled on two alternatives: transient siRNA transfection and lentiviral transduction.

We used targeted siRNA double stranded oligonucleotides (Qiagen) to knockdown both MBD2 and MeCP2 in MCF-7, MDA-MB-231 and SK-BR-3 human breast cancer cell lines. We confirmed knockdown of these genes by quantitative PCR, using a siNEG control siRNA as a reference for nonspecific gene expression changes. Immediately there were several problems with this approach. We found that the mRNA knockdown using siRNA peaked at between 48hrs and 96hrs post transfection and then gene levels began to rise again (time course not shown). Furthermore, non-specific toxicity from the Lipofectamine transfection reagent was a significant confounding factor, with maximal toxicity occurring at the precise timepoints corresponding to peak mRNA knockdown. Nevertheless, we were able to detect an increase in apoptosis by Annexin V staining and flow cytometry analysis (Vybrant Assay, Invitrogen, Carlsbad, CA). The overall effect

40

was small in magnitude (see Chapter III results), however, and no persistent change in growth was apparent.

It became clear that we needed a more rapid and persistent method of knocking down MBD2 in order to test its full spectrum of anti-cancer effects on our breast cancer lines. We chose to try an anti-MBD2 lentiviral shRNA transduction system which allowed rapid FACS based selection of stable knockdown populations. This system had several advantages over transient siRNA and antibiotic selection methods. The transduction was immediately stable and required no selection yet was highly efficient in terms of the percentage of cells infected. Moreover, we could eliminate noise from the uninfected cells because we were able to sort by GFP co-expression. We were left with an essentially pure population of stable knockdown breast cancer cells and corresponding controls within 1 week (or slightly less) from the date of viral infection (as compared to 3-6 weeks with antibiotic selection). In addition, there was very little apparent toxicity from the virus or the low serum infection conditions.

The pLV-THM viral genome vector was cotransfected into HEK-293T packaging cells along with two helper plasmids (lacking any regions of homology capable of generating recombinant virions). One of the helper plasmids contained the lentiviral structural proteins and another encoded the vesicular stomatitis virus envelope protein to give the virus enhanced epithelial tropism. The resulting virus was shed into the supernatant of the HEK-293T cell supernatant; this supernatant was collected and filtered to eliminate any cellular carryover, complexed with 1 ug/mL of polybrene (Sigma Aldrich) to improve the viral transduction, and was used to infect subconfluent breast cancer cells (24 hour incubation with gentle rocking at 37 deg C in a CO_2 incubator). As

41

mentioned above, GFP fluorescence was used to trace successful transduction in the breast cancer cells, appearing 24 to 48 hours following the overnight infection and gradually peaking in intensity over the next 48 hours (Figure 6). MBD2 knockdown was confirmed in sorted populations by qRT-PCR and western blot (Figure 7).

As we hypothesized, we found that MBD2 knockdown using the lentivirus method led to significant growth inhibition when we compared MBD2 shRNA transduced cells against scramble control virus transduced cells (Figures 8 and 9). Surprisingly, however, the growth suppression was not immediate. In fact the cells were essentially indistinguishable in terms of growth for the first two weeks following infection (1 week following FACS). At that point the MBD2 knockdown cells began to proliferate at a significantly reduced rate compared to scramble controls in every breast cancer cell line tested.

We found that MCF-7 cells were least sensitive to MBD2 knockdown, with a maximum of 49% growth inhibition as measured by MTT assay. Other breast cancer cell lines were somewhat more sensitive to MBD2 deficiency, with anti proliferative effects ranging from 77% for MDA-MB-231; 92% inhibition of SK-BR-3; and 94% inhibition of MDA-MB-435 cells. Importantly, anti-proliferative effects required between 5-7 days of sustained MBD2 protein deficiency to manifest, and the peak effect took several weeks to develop. To determine the mechanism of death or growth inhibition, we performed apoptosis assays (PARP cleavage) and measured senescence associated beta-galactosidase levels and found no significant increase in either PARP cleavage or senescence (not shown).

42

Figure 6. GFP positivity in shRNA lentivirus transduced breast cancer cells
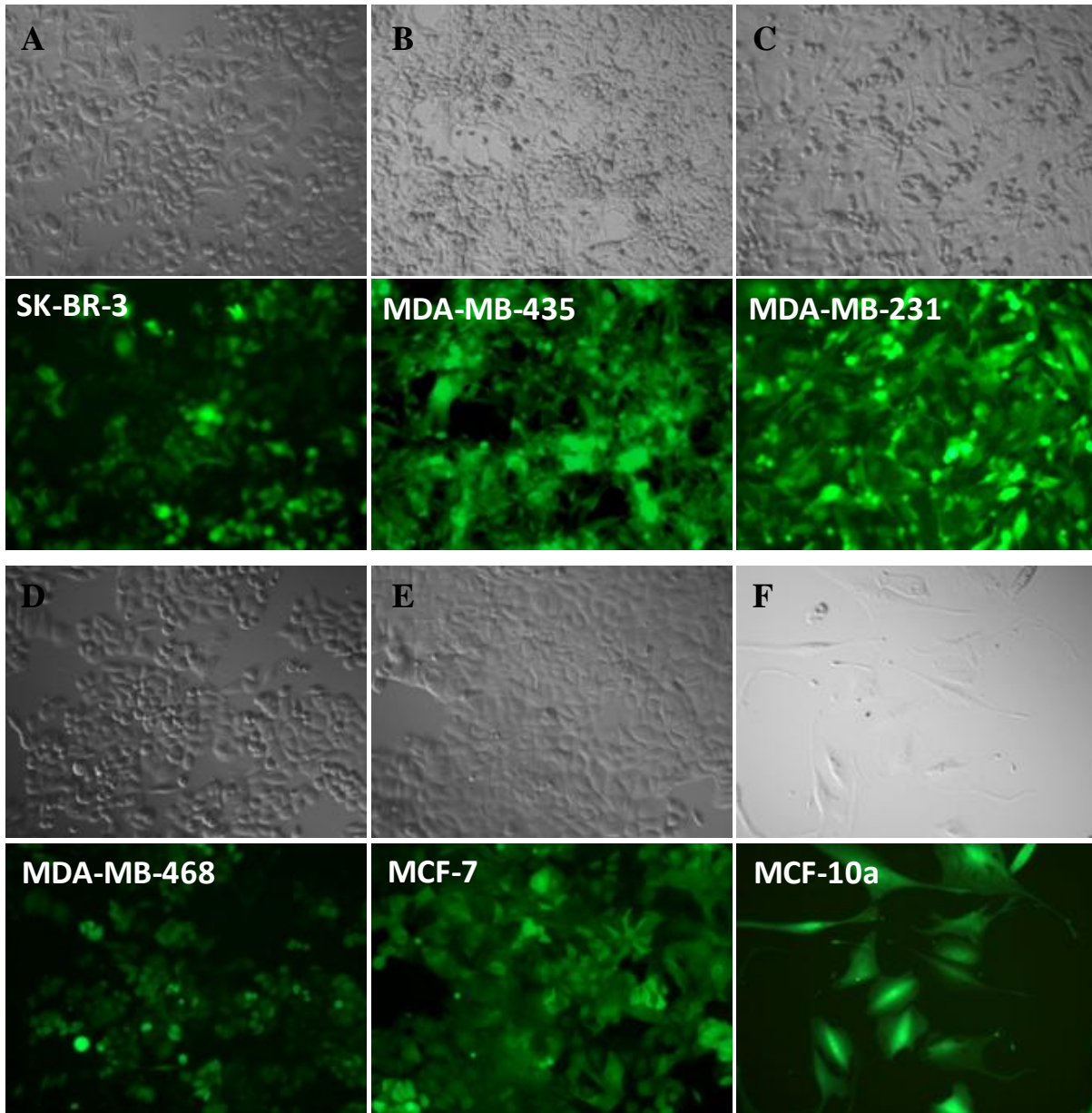
Figure 6. Verification of lentivirus transduction and expression by GFP fluorescent microscopy of breast cancer cell lines prior to FACS segregation of positive cells. Cells were infected with filtered supernatant from 293T helper (packaging) cells. Shown are representative phase contrast and GFP fluorescent microscope images from SK-BR-3 (A), MDA-MB-435 (B), MDA-MB-231 (C), MDA-MB-468 (D), MCF-7 (E), and MCF-10a (F) breast cancer cells. These images were obtained prior to sorting at 5 days post infection. GFP positivity confirmed by microcopy and subsequently by FACS ranged from 85% to 98% indicating sufficient lentiviral titers in crude supernatant to produce high infection efficiencies in all lines.

43

Figure 7. shRNA knockdown lines: qPCR and Immunoblots

SKBR3

100%
75%
50%
25%
0%

shScramble   shMBD2

MDA-MB-435

100%
75%
50%
25%
0%

shScramble   shMBD2

MDA-MB-231

100%
75%
50%
25%
0%

shScramble   shMBD2

SK-BR-3

Vector shSCR shMBD2

MDA-MB-435

Vector shSCR shMBD2

MDA-MB-231

Vector shSCR shMBD2

αMBD2

Coomasie
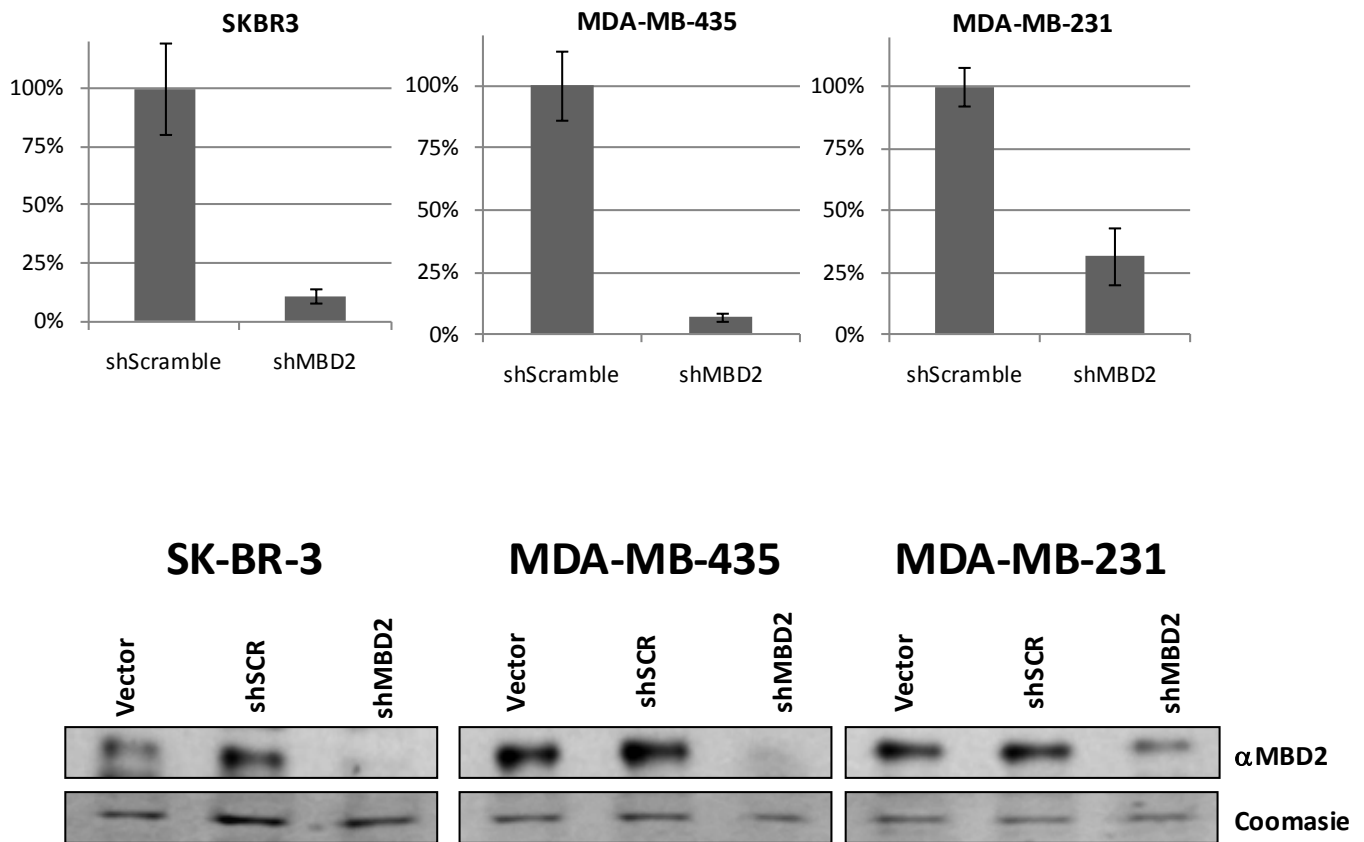
Figure 7. Quantitative RT-PCR (above) and anti-MBD2 immunoblots (below, 25 ug total protein) were used to confirm knockdown of MBD2 mRNA and protein. A pLV-THM Vector and Scramble control virus were used as references for comparison of relative MBD2 levels. qRT-PCR data is normalized to an internal control gene (Cyclophilin A) to account for differences in total RNA levels between samples. Error bars represent the standard error of the ratio of the mean calculated as described in qPCR methods from three qPCR replicates per sample. A Coomasie stained gel is shown as a loading control for the immunoblots.

Figure 8. Cell proliferation Assay Demonstrates Growth Arrest in MBD2

Knockdown Breast Cancer Lines
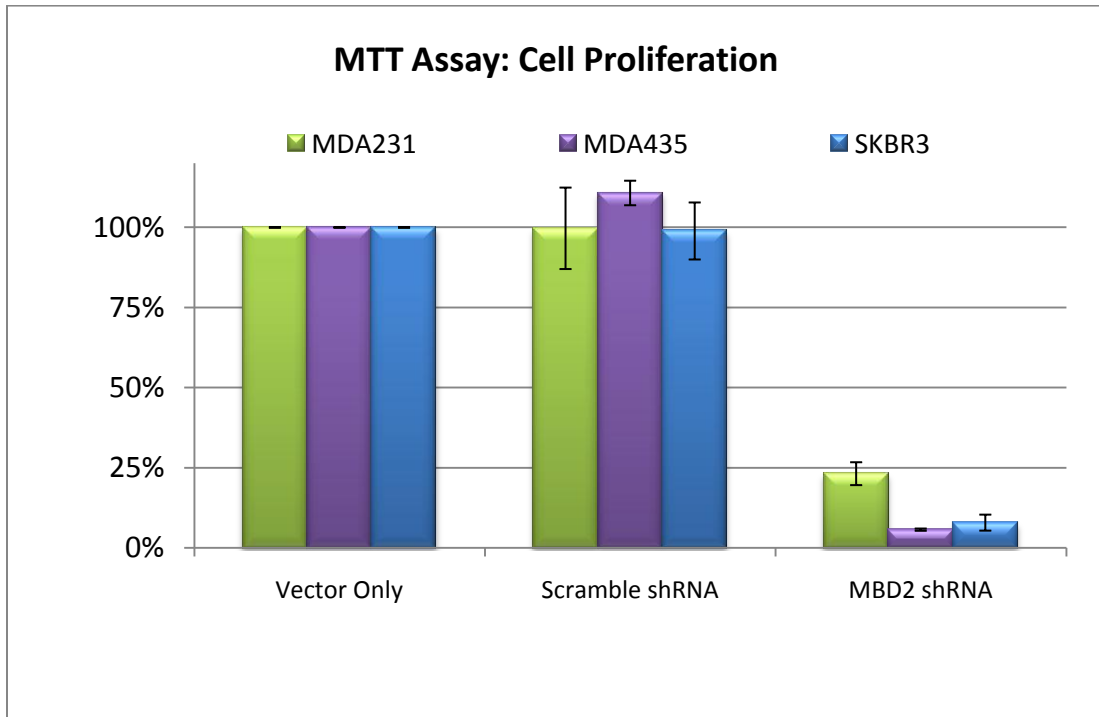
Figure 8. Cell proliferation was measured by MTT assay for MDA-MB-231, MDA-MB-435, and SK-BR-3 breast cancer cells. Each bar represents the mean and error bars are 95% CI for the 595 nm absorbance normalized to vector controls from 3 replicates at 72 hours following plating of equal cell numbers in a 96 well dish. This assay was performed approximately 14 days following lentivirus infection and subsequent FACS sorting. Three cell lines showing significant growth inhibition are shown (left to right, MDA-MB-231, MDA-MB-435, and SK-B3-3). Little difference was observed between vector only and scramble controls, however MBD2 knockdown virus (Right) was significantly growth inhibited.

Figure 9. Breast Cancer Cell Line Growth Curves
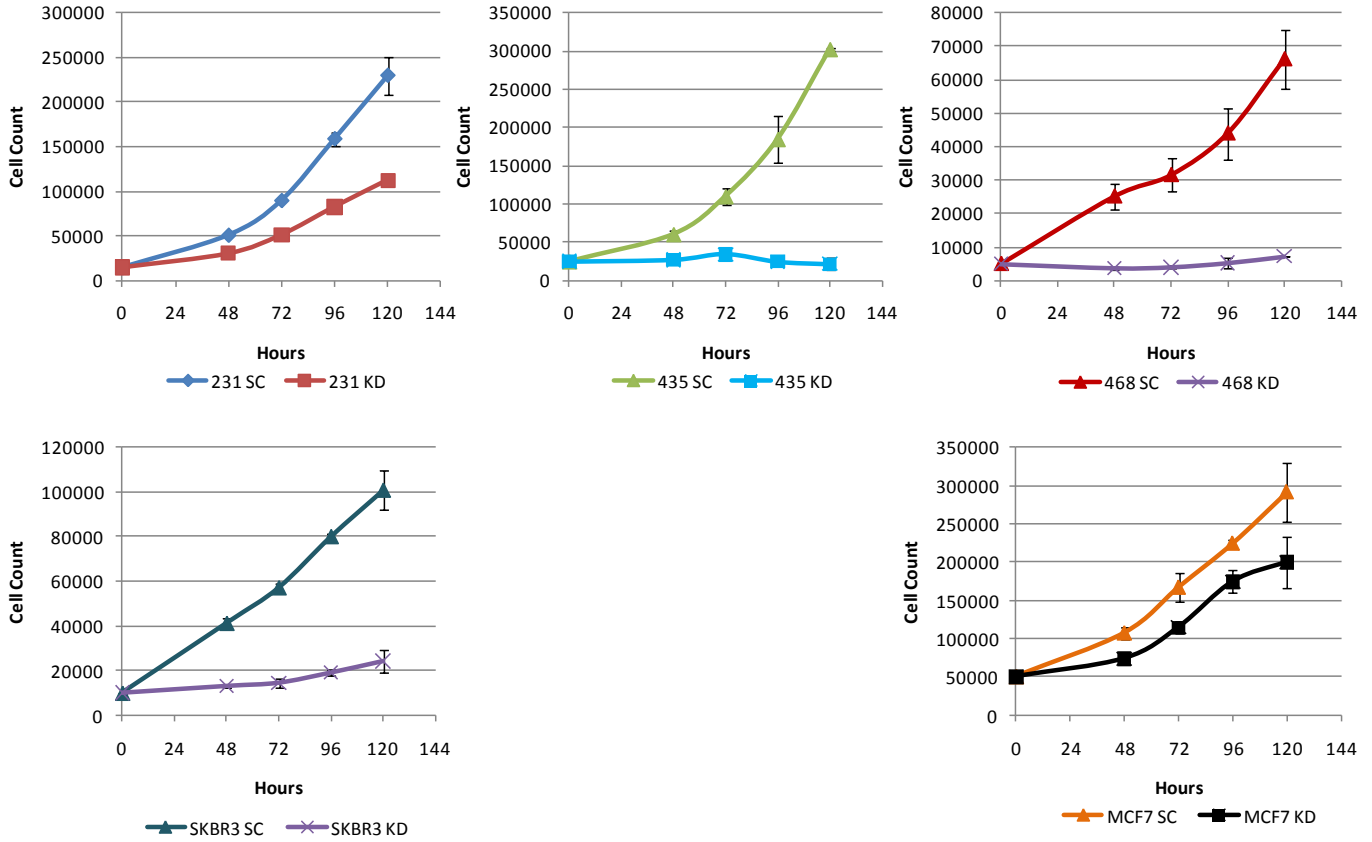
Figure 9. Breast cancer cell line growth curve analysis. Cells were counted (Coulter counter) every 24 hours for 5 days (beginning 14 days post virus infection and 7 days post FACS sorting. The starting point of the 5 day growth assay is indicated here as time 0 hours). Each point represents a mean count from three wells of a 24 well culture plate and error bars are the corresponding 95% confidence intervals. Error bars are plotted for all time points, though small variations are obscured at some data points. Top row, left to right: MDA-MB-231, MDA-MB-435, and MDA-MB-468. Bottom Row, left to right: SK-BR-3 and MCF-7.

MBD2 knockdown leads to increased tumor cell death but not sensitization to anti-neoplastic agents

A significant problem in the treatment of certain breast cancers is the lack of an additive biological modifier; for example in advanced and triple negative tumors where estrogen receptor or Her2/neu targeted therapies provide no benefit. Even when available therapies initially provide benefit, the appearance of resistance following an initial period of tumor regression is common. This is likely due to two factors: the presence of a small subpopulation of resistant cells within a heterogeneous tumor which are enriched during therapy and the dosing limitations imposed by generalized toxicity which thwart complete eradication of the tumor. We were therefore interested in the response of our MBD2 knockdown lines to chemotherapeutic agents. We hypothesized that, if we were able to identify those compounds capable of acting synergistically with MBD2 knockdown, we would find substantially increased killing of the knockdown cells compared to scramble controls using standard doses of these anti-tumor agents. We chose several chemotherapeutic agents that are used as first line therapy in breast cancer and several others which we thought might act in concert with MBD2 knockdown to sensitize tumor cells. Endocrine therapy (hormone therapy, e.g., Tamoxifen) and biologic therapies (e.g., humanized anti-Her2, Herceptin) were not used for these studies for reasons discussed later. The DNMT inhibitor 5-aza-2-deoxy cytidine (Decitabine) was likewise not tested because we knew from our methylation studies (described later) that knockdown cells were not significantly more sensitive to this compound. The drug classes commonly used as first line chemotherapy include nitrogen mustard derivatives (cyclophosphamide, Cytoxan), anthracyclines (Doxorubicin) and taxanes (Paclitaxel).

47

We also tested the activity of two additional classes of therapy: the HDAC inhibitor, Vorinostat, and a platinum compound, Cisplatin based on our suspicion and that of others (Steele et al. 2009) that these compounds may be effective in combination with other epigenetic strategies. To isolate any altered sensitivity, we tested the compounds individually rather than in combinations. We found that the principal predictor of survival of the breast cancer lines we examined was MBD2 knockdown alone, as shown in Figure 10. All the compounds tested exhibited dose dependent toxicity in control and MBD2 knockdown lines, albeit variable in magnitude. However, when normalized for the effect of MBD2 knockdown, none of the drug/cell line combinations tested exhibited significantly enhanced sensitivity to therapy. Nonetheless, the dose normalized death of breast cancer cells was uniformly higher in MBD2 knockdown lines. The percent survival was determined by MTT assay at 96 hours following a single doses of 0.2, 1.0, or 5.0 uM Vorinostat, Cytoxan, Doxorubicin, and Cisplatin and 0.01, 0.1, or 1.0 uM Paclitaxel. The mean and standard deviation of the percent survival from three experimental samples was normalized to untreated scramble controls and is plotted individually for each compound in Figures 11-15. Dose response curves were also plotted for each compound (Figures 16 and 17) and a representative MTT assay plate is shown (Figure 18).

48

Figure 10. Cell lines used for drug assays and their baseline sensitivity to MBD2 Knockdown
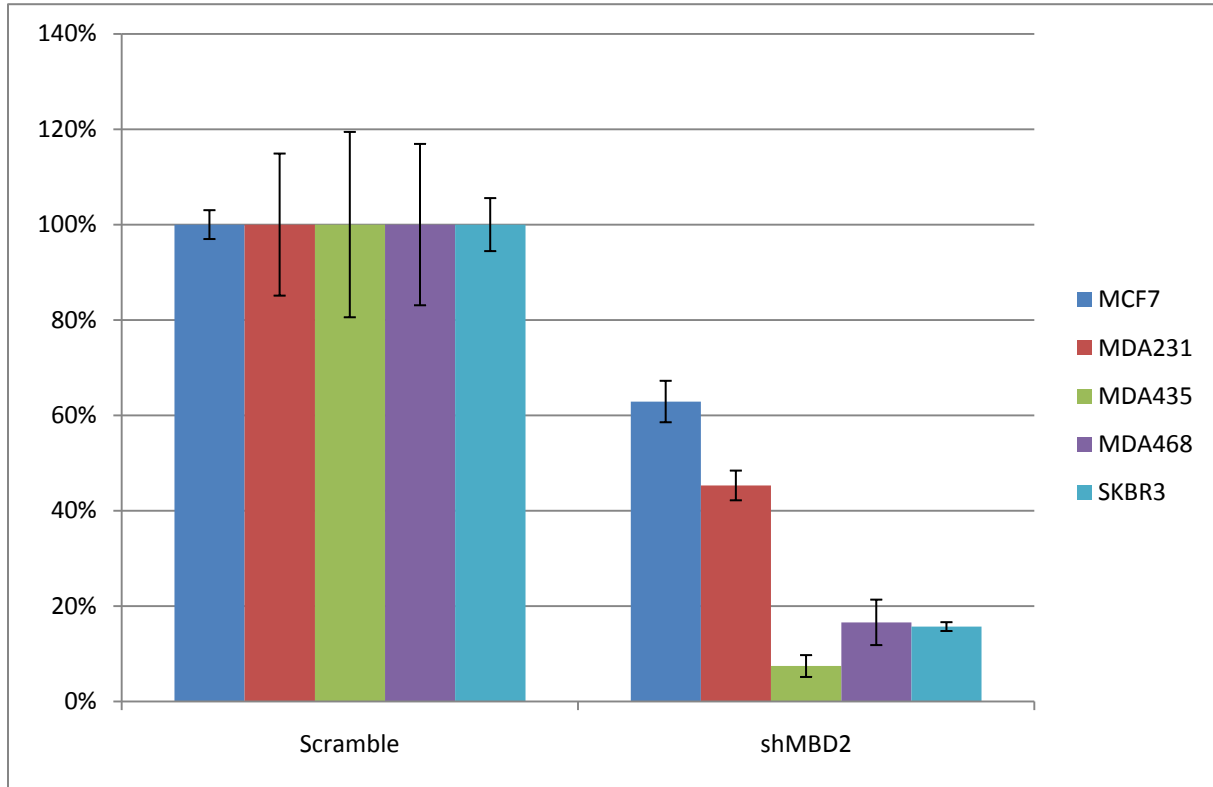
Figure 10. Relative proportion of metabolically active cells after 96 hours (assay time, 14 days post virus infection and 7 days post FACS sorting) measured by MTT assay as described above. These lines were infected with lentivirus (SC or KD) and cultured in parallel with the drug treatment experimental samples, although the lines shown here were not treated with any chemotherapeutic compound. The baseline sensitivity of these lines to MBD2 knockdown varied from 63% residual viability for MCF-7 cells to 7% viability for MDA-MB-435 cells. Each well of a 96 well culture plate was seeded with $1x10^4$ cells at 0 hours. Vertical bars represent the mean and standard error of the mean for three assay replicates per cell line normalized to Scramble controls. This assay was repeated with on each 96 well assay plate (shown in subsequent figures) with no significant variation in baseline sensitivity to MBD2 knockdwon.

Figure 11. MBD2 Knockdown and Scramble Control Cell Percent Viability Following Cytoxan Treatment
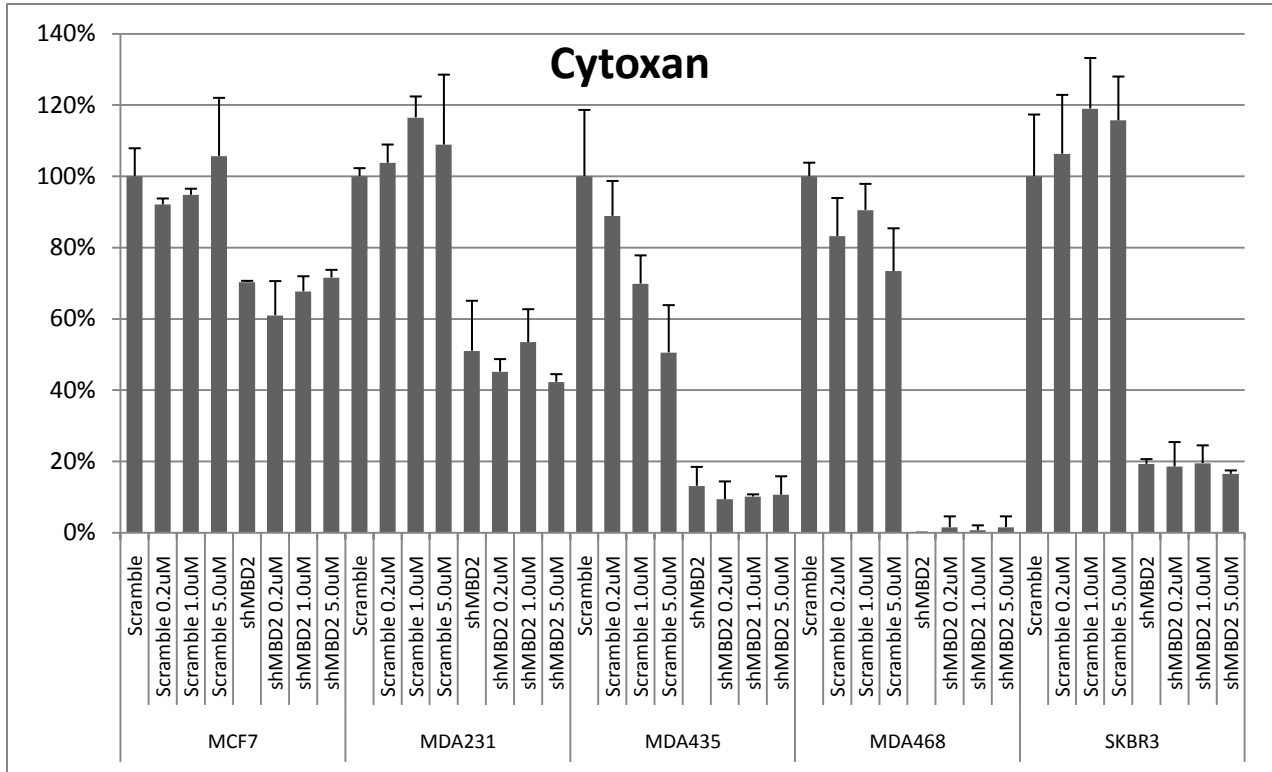
Figure 11. Metabolically active cells following 96 hours of drug treatment measured by MTT assay as described above. Breast cancer cell lines were transduced with Scramble or shMBD2 lentivirus, sorted by FACS, and duplicate 96 well plates were seeded with $1x10^4$ cells (0 hours). Cells were treated with 0.2uM, 1.0uM, or 5uM Cytoxan at 24 hours. Vertical bars represent the mean and standard error for three replicates from a single assay plate. Samples were normalized to untreated Scramble controls.

Figure 12. MBD2 Knockdown and Scramble Control Cell Percent Viability

Following Doxorubicin Treatment
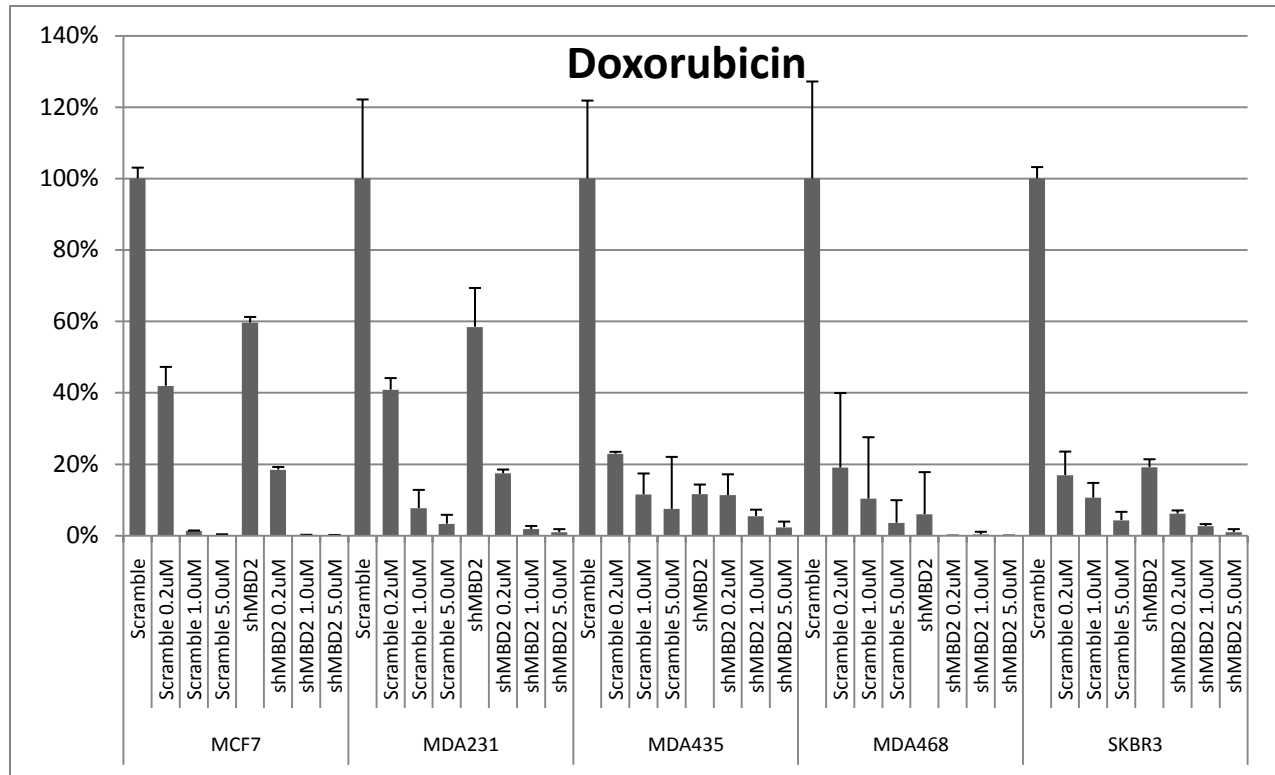
Figure 12. Metabolically active cells following 96 hours of drug treatment measured by MTT assay as described above. Breast cancer cell lines were transduced with Scramble or shMBD2 lentivirus, sorted by FACS, and duplicate 96 well plates were seeded with $1x10^4$ cells (0 hours). Cells were treated with 0.2uM, 1.0uM, or 5uM Doxorubicin at 24 hours. Vertical bars represent the mean and standard error for three replicates from a single assay plate. Samples were normalized to untreated Scramble controls.

Figure 13. MBD2 Knockdown and Scramble Control Cell Percent Viability Following Paclitaxel Treatment
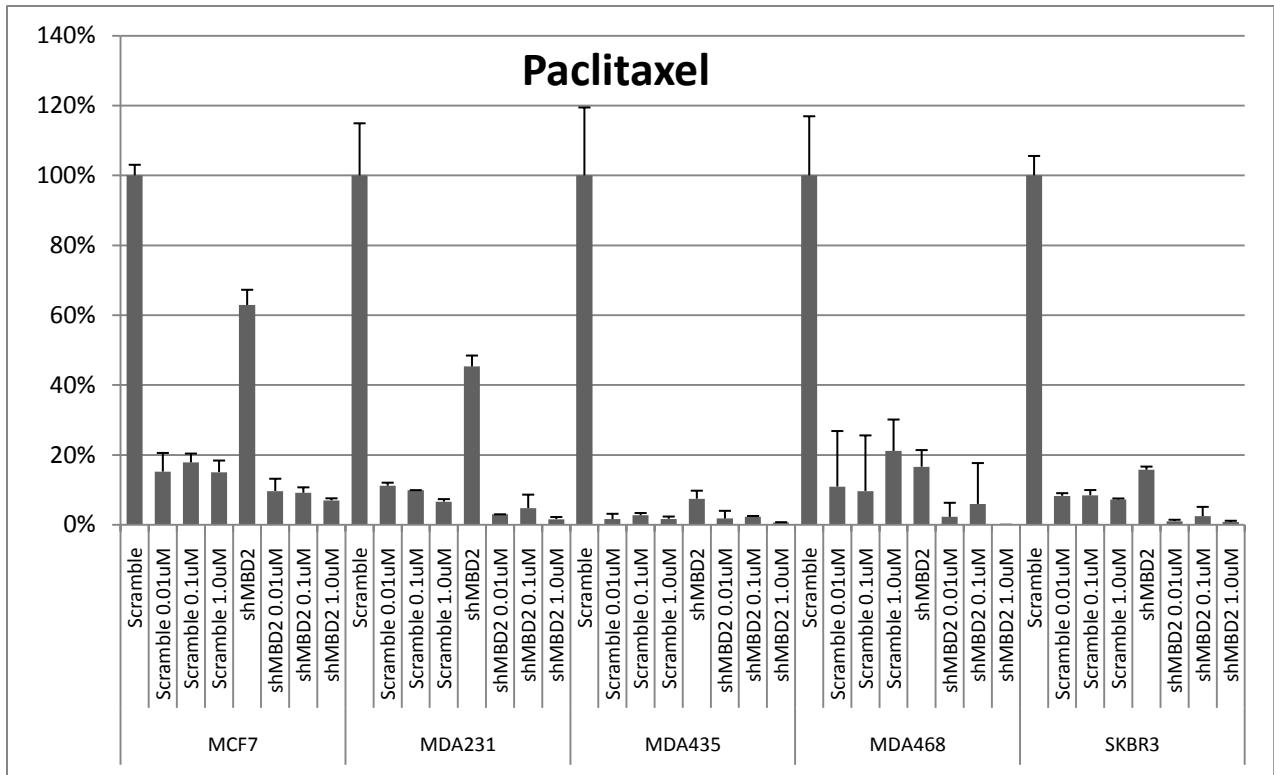
Figure 13. Metabolically active cells following 96 hours of drug treatment measured by MTT assay as described above. Breast cancer cell lines were transduced with Scramble or shMBD2 lentivirus, sorted by FACS, and duplicate 96 well plates were seeded with $1x10^4$ cells (0 hours). Cells were treated with 0.01uM, 0.1uM, or 1.0uM Paclitaxel at 24 hours. Vertical bars represent the mean and standard error for three replicates from a single assay plate. Samples were normalized to untreated Scramble controls.

Figure 14. MBD2 Knockdown and Scramble Control Cell Percent Viability Following Vorinostat Treatment
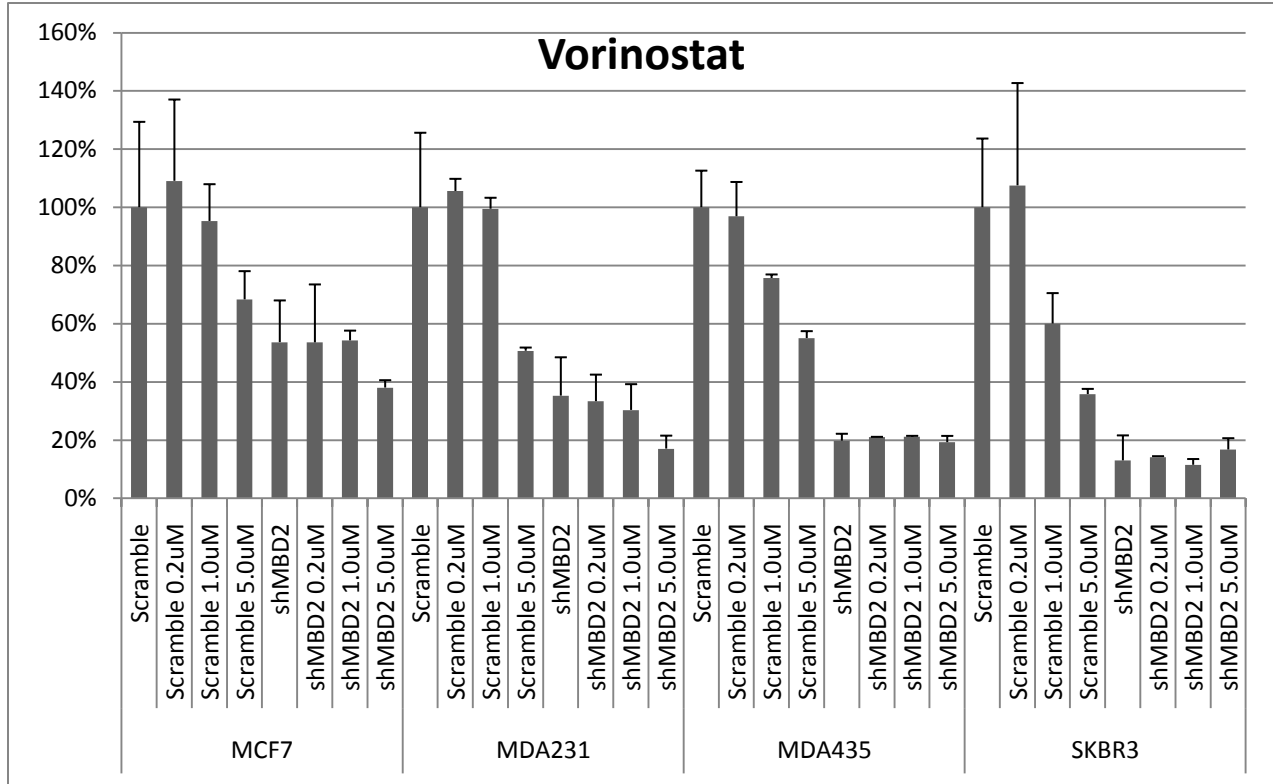
Figure 14. Metabolically active cells following 96 hours of drug treatment measured by MTT assay as described above. Breast cancer cell lines were transduced with Scramble or shMBD2 lentivirus, sorted by FACS, and duplicate 96 well plates were seeded with $1x10^4$ cells (0 hours). Cells were treated with 0.2uM, 1.0uM, or 5uM Vorinostat at 24 hours. Vertical bars represent the mean and standard error for three replicates from a single assay plate. Samples were normalized to untreated Scramble controls.

Figure 15. MBD2 Knockdown and Scramble Control Cell Percent Viability Following Cisplatin Treatment
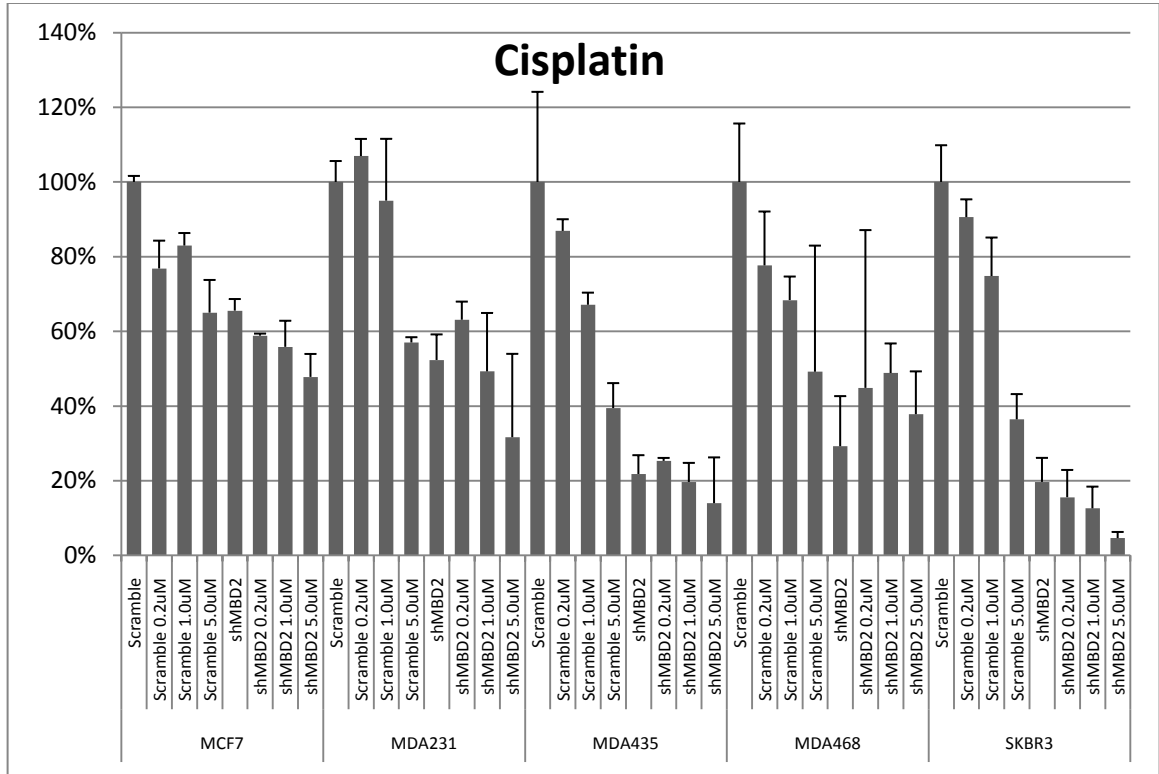
Figure 15. Metabolically active cells following 96 hours of drug treatment measured by MTT assay as described above. Breast cancer cell lines were transduced with Scramble or shMBD2 lentivirus, sorted by FACS, and duplicate 96 well plates were seeded with $1x10^4$ cells (0 hours). Cells were treated with 0.2uM, 1.0uM, or 5uM Cisplatin at 24 hours. Vertical bars represent the mean and standard error for three replicates from a single assay plate. Samples were normalized to untreated Scramble controls.

Figure 16. Dose Response Curves for Paclitaxel, Cytoxan, and Doxorubicin
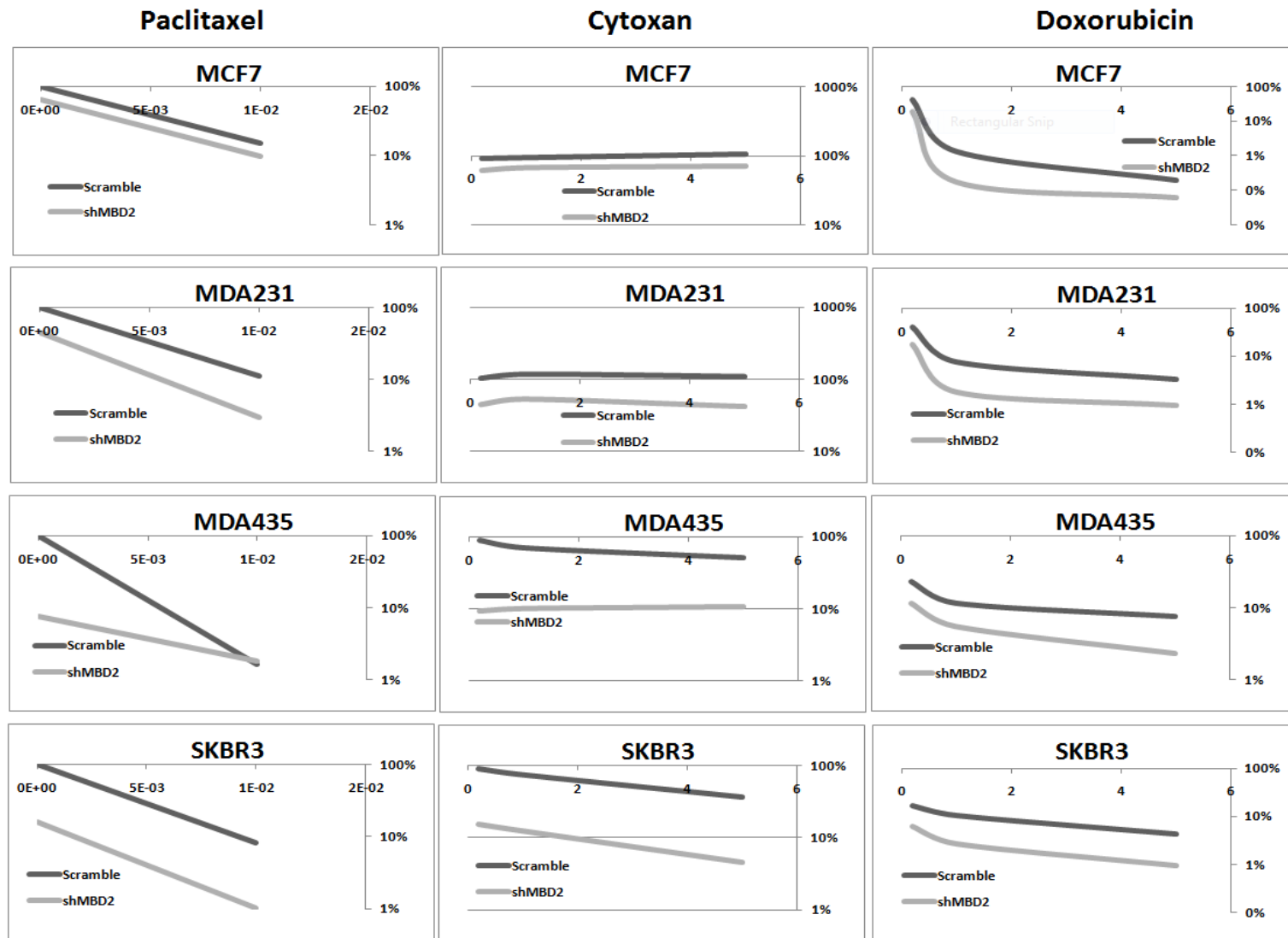
Figure 16. Chemotherapy sensitization data plotted as dose response curves. Drug dose (uM) is given on the x-axis and the y-axis represents percent cell viability (note the y-axis is logarithmic). Scramble control and knockdown curves are plotted as separate lines (KD-dark gray, SC-light gray).

Figure 17. Dose Response Curves for Paclitaxel, Cytoxan, and Doxorubicin
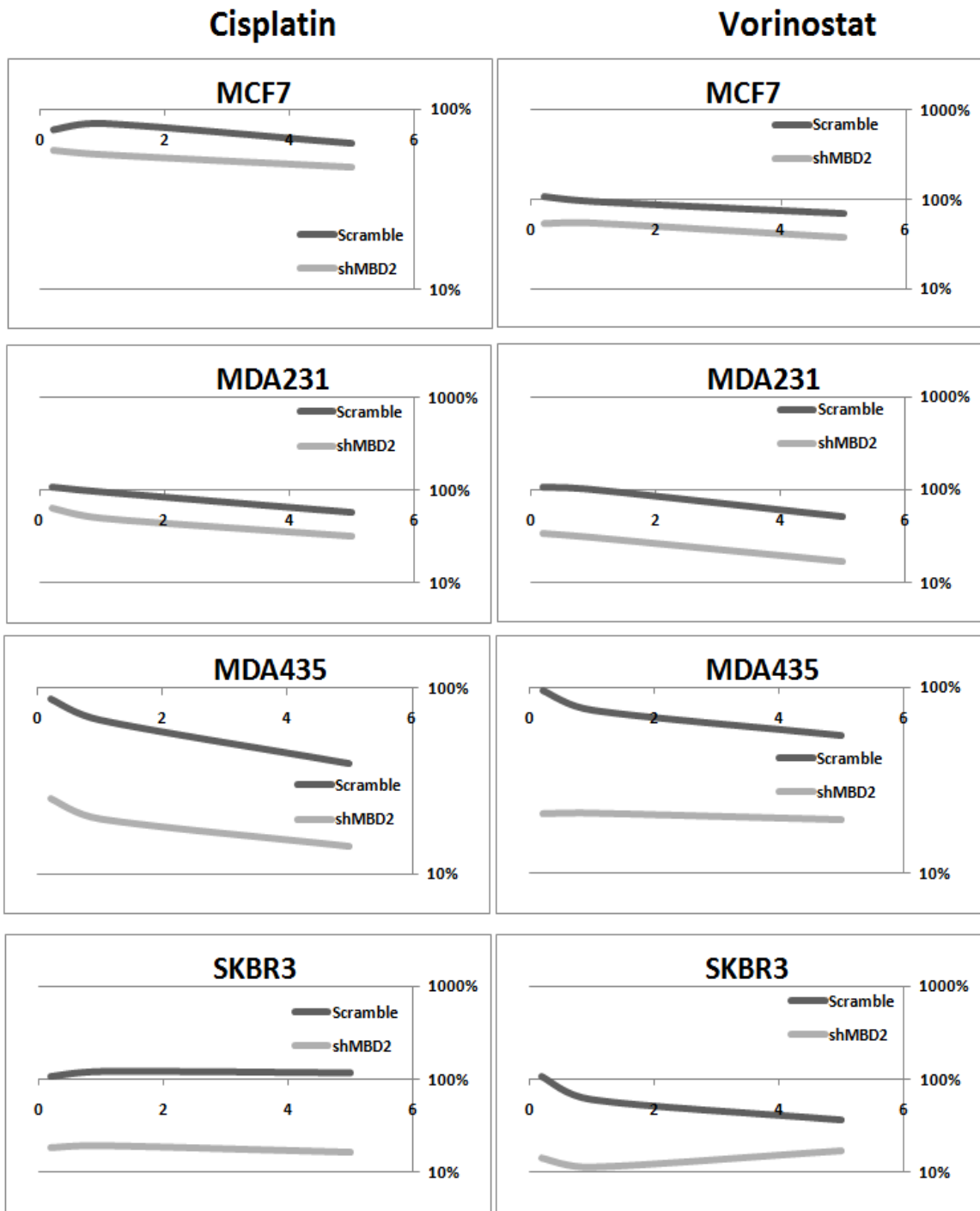
Figure 17. Chemotherapy sensitization data plotted as dose response curves. Drug dose (uM) is given on the x-axis and the y-axis represents percent cell viability (note the y-axis is logarithmic). Scramble control and knockdown curves are plotted as separate lines (KD-dark gray, SC-light gray).

Figure 18. MTT Drug Sensitivity Assay (Vorinostat Plate)
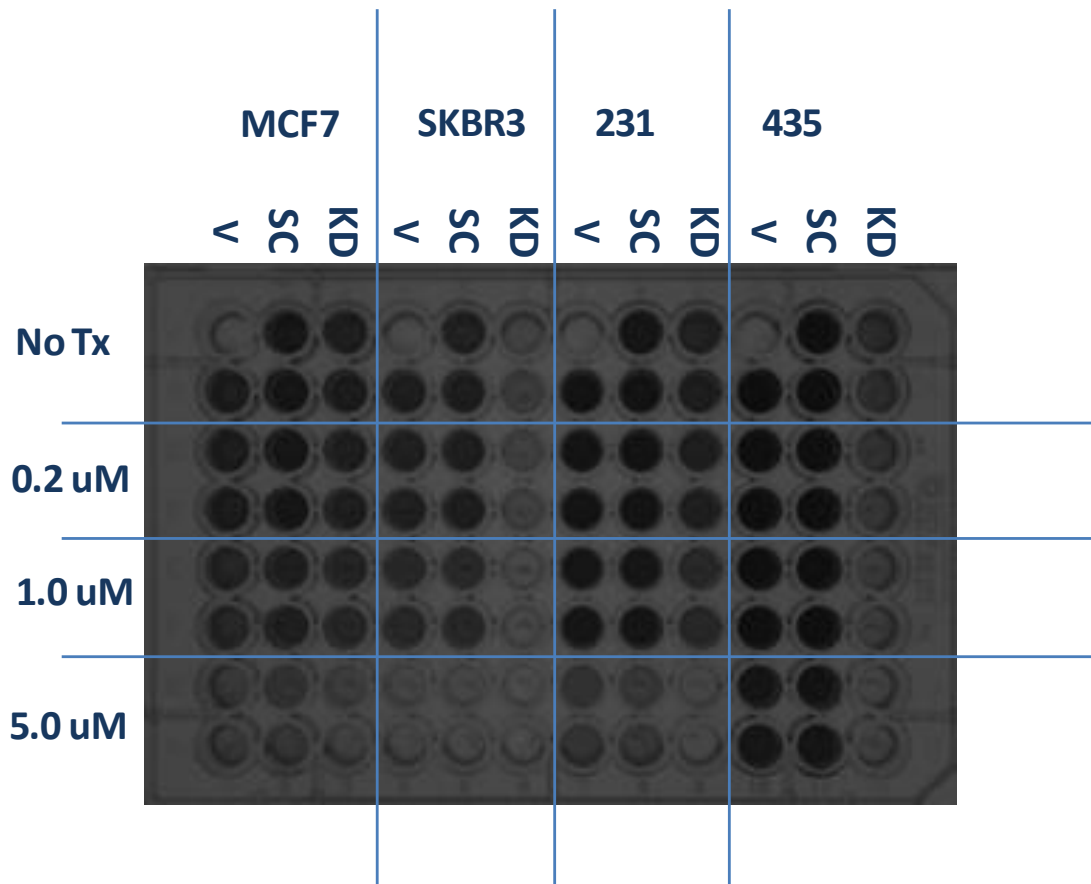
Figure 18. A representative image of the 96 well format MTT assay used for drug sensitivity screening. V: Vector only, SC: Scramble, KD: MBD2 Knockdown. One blank well is present in the top left well for each of the four cell lines; this blank was used to baseline the 96-well plate reader. The doses (Vorinostat plate is shown) varied from untreated (No Tx) to 5uM as labeled on the left.

MBD2 knockdown inhibits xenograft tumor growth in BALB/c nu/nu mice.

We next explored whether MBD2 knockdown had any effect on the ability of aggressive breast cancer lines to form tumors in animals. This experiment was a logical progression from our observation that MBD2 knockdown significantly impaired the growth of our breast cancer cell lines in culture. The rationale behind this progression is the widely held idea that measuring the growth of human xenograft tumors in a mouse is a more physiologically relevant system in for studying the *in vivo* biology of cancer (Welch 1997). Using the same method, stable lentiviral transduction of MBD2 targeted shRNA, we infected and FACS purified sufficient cells to establish xenograft tumors in the subcutaneous space of immunocompromised mice (we determined empirically that 5×10^5 to 1×10^6 cells per injection were required as fewer cells were incapable establishing tumors). We hypothesized that MBD2 knockdown would diminish the ability of breast cancer cells to grow in mice, paralleling the effect on their growth *in vitro*.

We implanted the MBD2 knockdown and scrambled control transformed lines in opposite flanks of 8 week old female BALB/c nu/nu nude mice as described above and schematically below (Figure 19). We assayed subsequent tumor growth using two independent methods. We followed the mice daily until a palpable mass appeared at the site of injection and then made regular caliper measurements of the mass, from which a tumor volume was calculated. In addition to caliper measurements we took advantage of the GFP positivity of our lentivirally transduced cells using *in vivo* bio-fluorescent imaging to non-invasively follow tumor growth in live anesthetized mice. We found that MBD2 knockdown lead to a substantial decrease in the ability of aggressively

58

tumorigenic lines (MDA-MB-231 and MDA-MB-435) to form tumors in mice. Mean GFP fluorescence and median tumor volume calculated from caliper measurements was significantly greater in control lines compared to MBD2 knockdown tumors for all time points following the initial appearance of a palpable tumor (Figures 20-24). Knockdown tumors demonstrated steady, unrelenting growth and mice were eventually sacrificed due to the large unilateral masses which resulted. Knockdown derived tumors, on the other hand, were slow to grow. In the case of MDA-MB-435, the initial fluorescent density at the site of implantation was cleared within 1 week (Figure 20). A single recurrent tumor appeared late at the MDA-MB-435 knockdown injection site. In order to verify persistent knockdown, this tumor was resected and examined by qPCR and immunohistochemistry. We found that MBD2 protein and mRNA remained at low levels in this tumor (Figure 24). Interestingly, the recurrent (resistant) tumor no longer expressed the MBD2 dependent tumor suppressor gene, Maspin (discussed in greater detail in Chapter II). We conclude that MBD2 knockdown imposes a significant impediment to tumor growth in nude mice, however, at least one mechanism of escape/resistance exists which can lead to late recurrence of Knockdown tumors.

Figure 19. GFP expressing tumor Xenografts
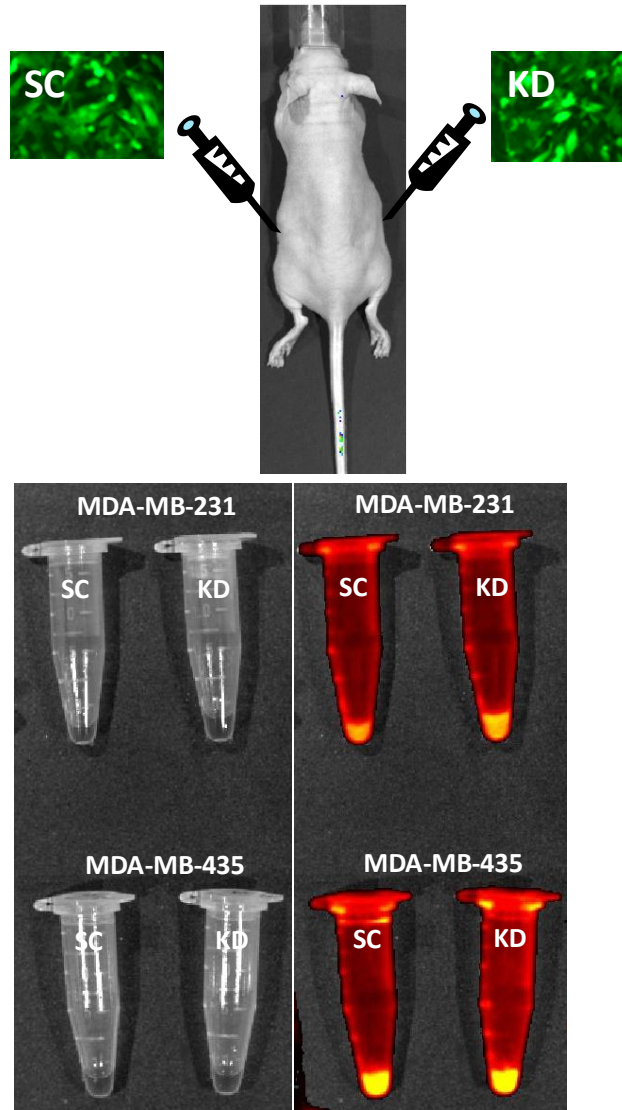
Figure 19. Shown above is a schematic representation of xenograft experimental design. (Top) The subcutaneous space of nude mice was injected with GFP expressing Scramble (SC) control cells on the left and MBD2 Knockdown (KD) cells on the right dorsolateral aspects of the flanks. FACS sorted cells were injected 14-16 days following lentivirus infection. Following injection, residual cells in their respective microtubes were imaged with the fluorescent bioimager to insure adequate signal and consistent fluorescence intensity between samples (bottom, left: B&W photograph; bottom, right: overlayed GFP fluorescence.)

60

Figure 20. *In vivo* biofluorescent imaging shows clearance of MBD2 knockdown xenograft tumors in nude mice.
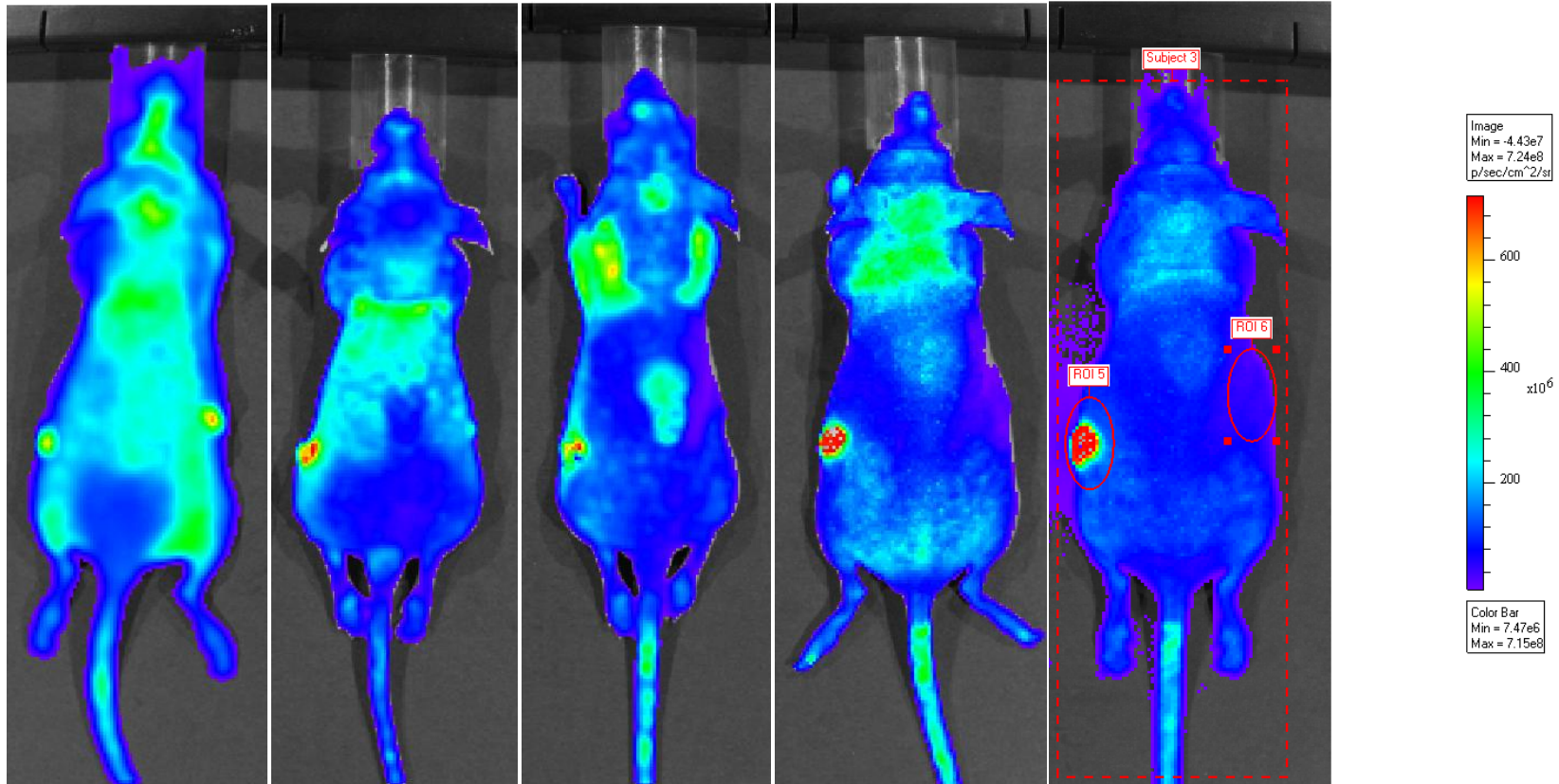
Figure 20. *In vivo* biofluorescent images captured with the IVIS Xenogen 200 imager show initial bilateral fluorescence (mouse on the left) from MDA-MB-435 Scramble (left flank) and Knockdown (right flank) tumor injections. Images 2-5 were taken at 5 day intervals following the appearance of a palpable tumor in nude mice. This progression demonstrates clearance of the initial Knockdown tumor and unrelenting growth of the Scramble control tumor. Mice were given a single challenge of 5×10^5 MDA-MB-435 cells.

Figure 21. *In vivo* biofluorescent imaging of MDA-MB-231 tumors in nude mice.
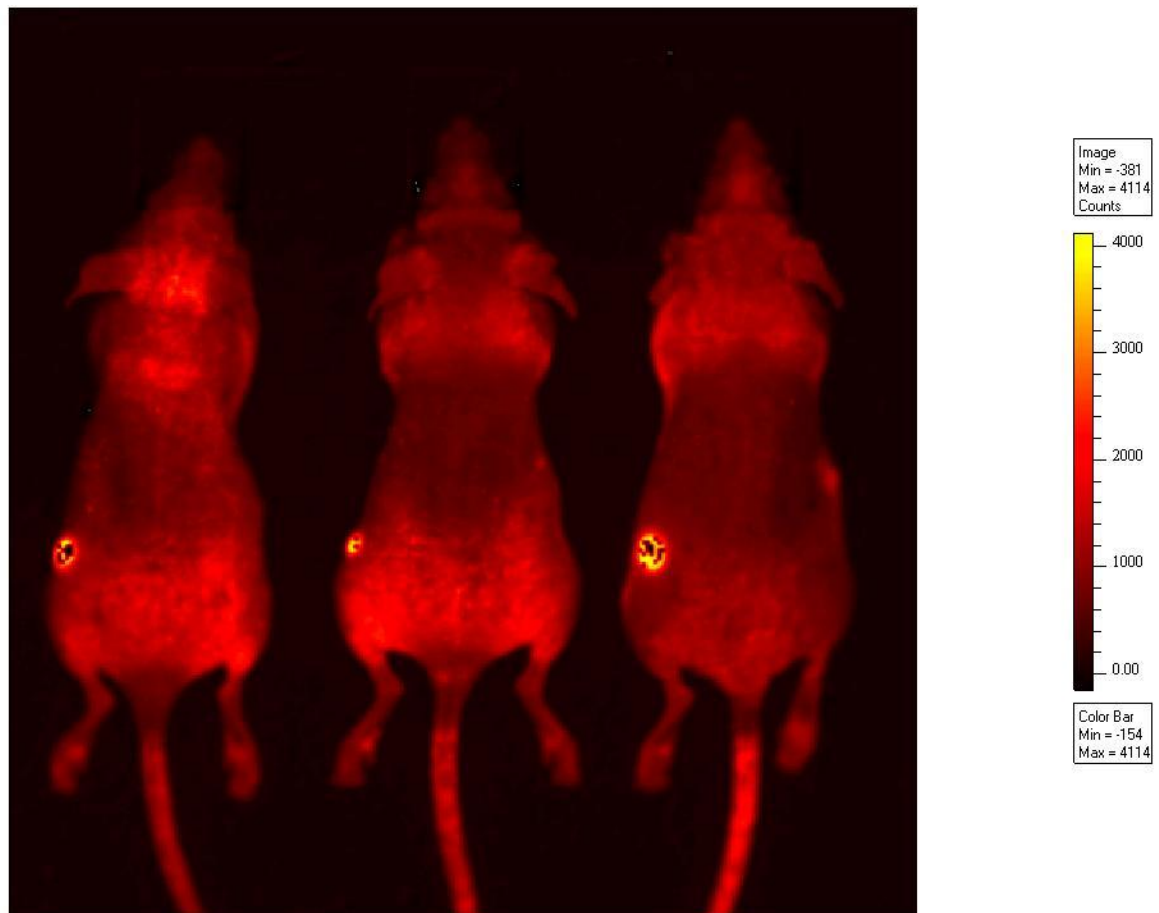
Figure 21. *In vivo* biofluorescent images captured with the IVIS Xenogen 200 imager show large fluorescent densities corresponding to growing GFP expressing tumors on the left flanks of three nude mice (Scramble controls) and no or relatively small fluorescent signal on the right (MBD2 Knockdown).   Images were taken 14 days after xenograft injection.

Figure 22. *In vivo* **biofluorescent imaging of MDA-MB-435 tumors in nude mice.**
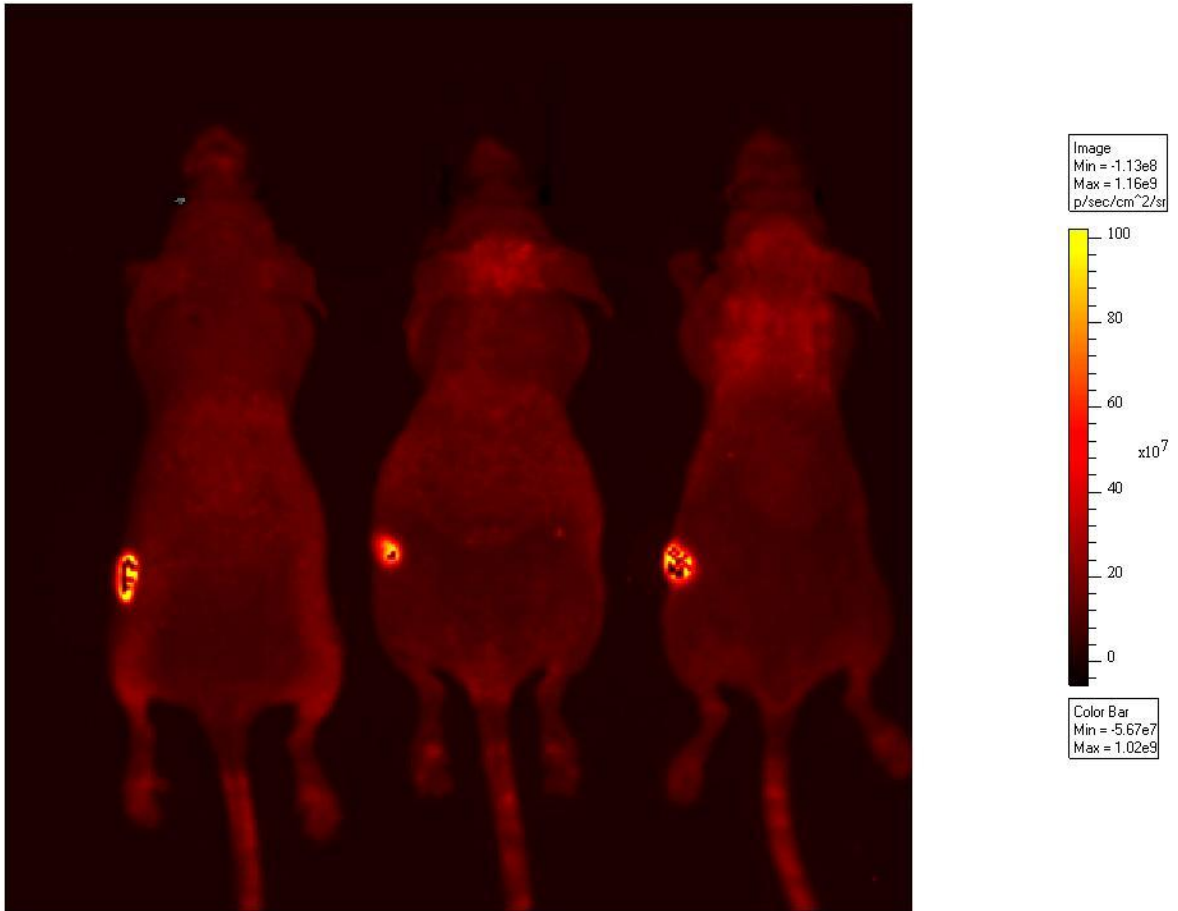
Figure 22. *In vivo* biofluorescent images captured with the IVIS Xenogen 200 imager show large fluorescent densities corresponding to growing GFP expressing tumors on the left flanks of three nude mice (Scramble controls) and no fluorescent signal on the right (MBD2 Knockdown). Images were taken 14 days after xenograft injection.

63

Figure 23. *In vivo* growth of MDA-MB-231 and MDA-MB-435 xenograft tumors in nude mice.
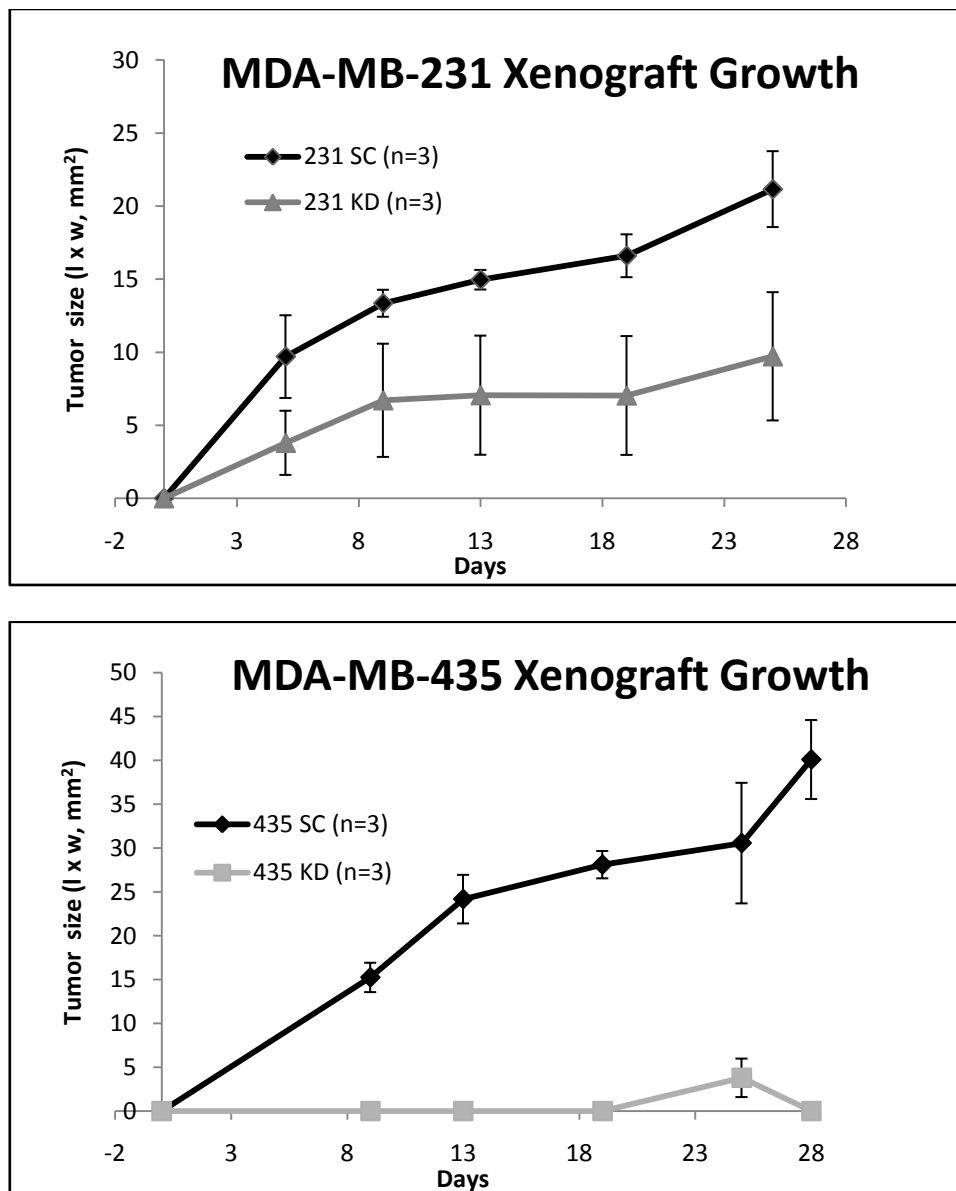
Figure 23. Caliper measurements were made of palpable tumors and used to calculate the mean and standard error (SEM) of tumor area (determined by multiplying length x width with length held constant as the longest tumor diameter and the corresponding orthogonal axis as width) of Scramble and Knockdown tumors from 3 nude mice per cell line. Measurements were taken at approximately 5 day intervals.

Figure 24. Recurrence of an MBD2 negative Maspin Positive Tumor in a Nude Mouse
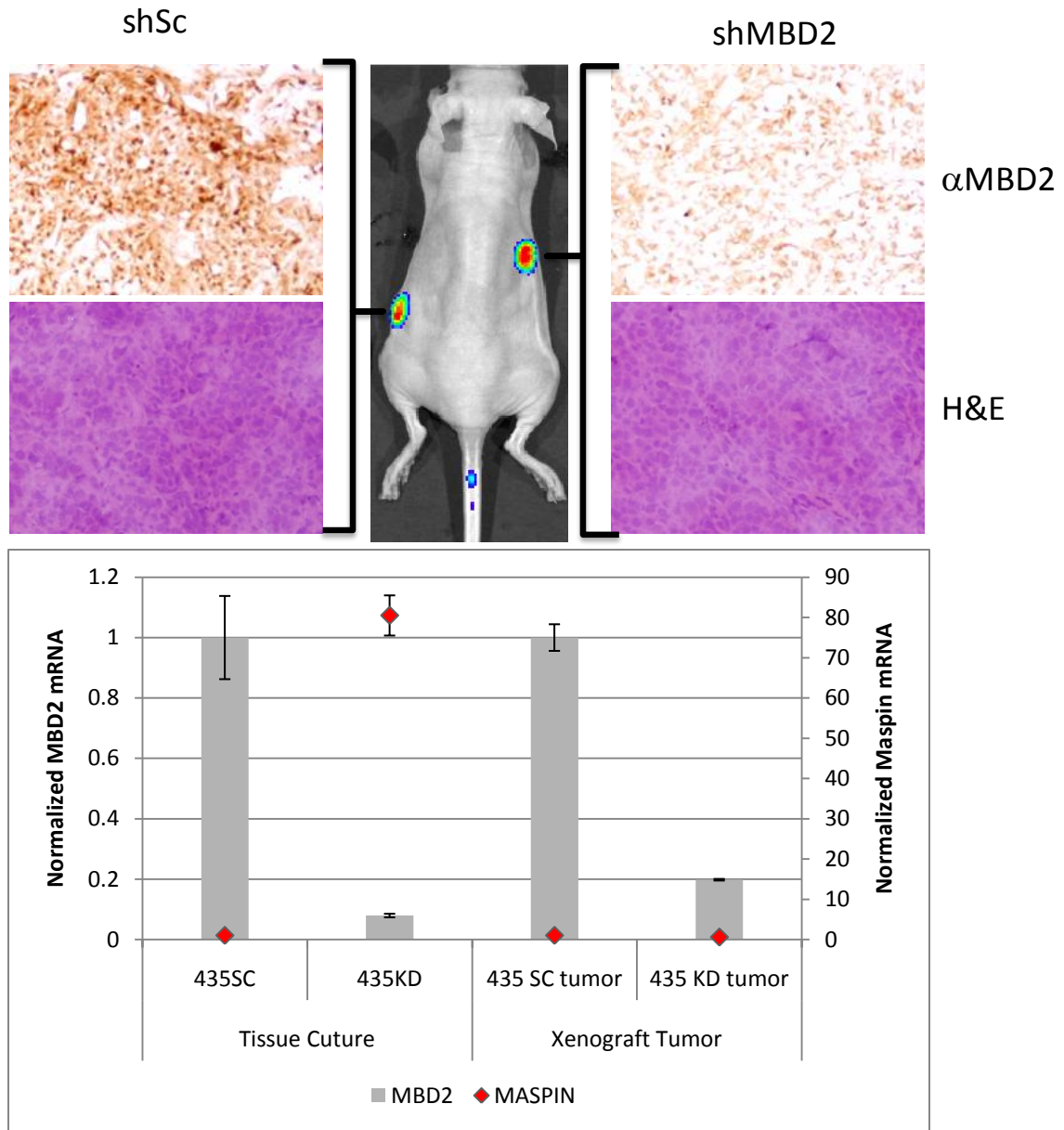
Figure 24. A single large and aggressive tumor developed in an MDA-MB-435 MBD2 knockdown injected mouse (above). We examined this tumor by immunohistochemistry and found MBD2 expression was still low (anti-MBD2 IHC and H&E stained sections of resected tumor). Upon further examination, we determined this tumor expressed very low levels of the MBD2 knockdown induced gene, Maspin (qRT-PCR graph, below). Note MBD2 and Maspin expression is plotted on separate Y-axes. We took this to mean there was a compensatory mechanism capable of resilencing MBD2 induced genes and providing an escape mechanism from MBD2 knockdown mediated growth suppression.

MBD2 knockdown restores normal epithelial morphology in 3D culture

We noticed that MBD2 knockdown cells exhibited distinctive characteristics upon microscopic examination of cultured cells. Knockdown (KD) cells were less fusiform in shape compared to untreated and scramble control transfected cells. In addition, the knockdowns grew more diffusely on tissue culture treated plastic, without forming the tight overlapping colonies observed in controls (Figure 25). Though the effect was variable between cell lines, in general the dense 'cobble stone' morphology of control cells was replaced by a more diffuse, 'fried egg' appearance, which more closely resembled the relatively normal morphology of the immortalized non-transformed MCF-10a line (Soule et al. 1990) (shown for comparison, bottom panel, Figure 25).

We hypothesized that MBD2 may be required for tumor related epithelial de-differentiation and to test this we examined the knockdown cell growth in 3 dimensional laminin-rich extracellular matrix extracts. In these basement membrane-like extracts normal epithelial cells and differentiated (low grade) epithelial populations form structures which recapitulate *in situ* morphology (i.e., acini, ducts, terminal lobules etc.) while poorly differentiated (high grade) populations do not (Xiang and Muthuswamy 2006). We found that knocking down MBD2 was sufficient to induce an increase in the number of differentiated epithelial structures formed in 3D cultures. Stable MBD2 knockdown MCF-7 cells formed normal epithelial structures 70% of the time, [CI=0.55-0.83], while untransfected and scrambled shRNA transfected controls formed these structures with reduced frequency, 46% (CI=0.39-0.53, p≤0.038) and 37% (CI=0.29-0.45, p≤0.012) respectively (Figures 26 and 27). Furthermore, MBD2 knockdown cells were able to form higher order structures in 3D lrECM cultures which grossly

66

recapitulated *in situ* epithelial multicellular morphology, e.g., ducts and terminal lobules (Figure 28).

These observations suggested to us that the overall effect MBD2 had on epithelial tumor cell differentiation was to promote and maintain a state of de-differentiation to a precursor state or trans-differentiation to a more mesenchymal-like population. The former is associated with a subpopulation of stem like cells thought to contribute to tumor formation, adaptation, and resistance (Visvader 2009). The latter trans-differentiation (epithelial to mesenchymal transition or EMT) has been associated with the acquisition of invasive characteristics which are the hallmark of pathological progression in carcinomas, i.e., invasion and metastasis (Thiery et al. 2009).

Figure 25. 2D Morphology of MBD2 knockdown cells
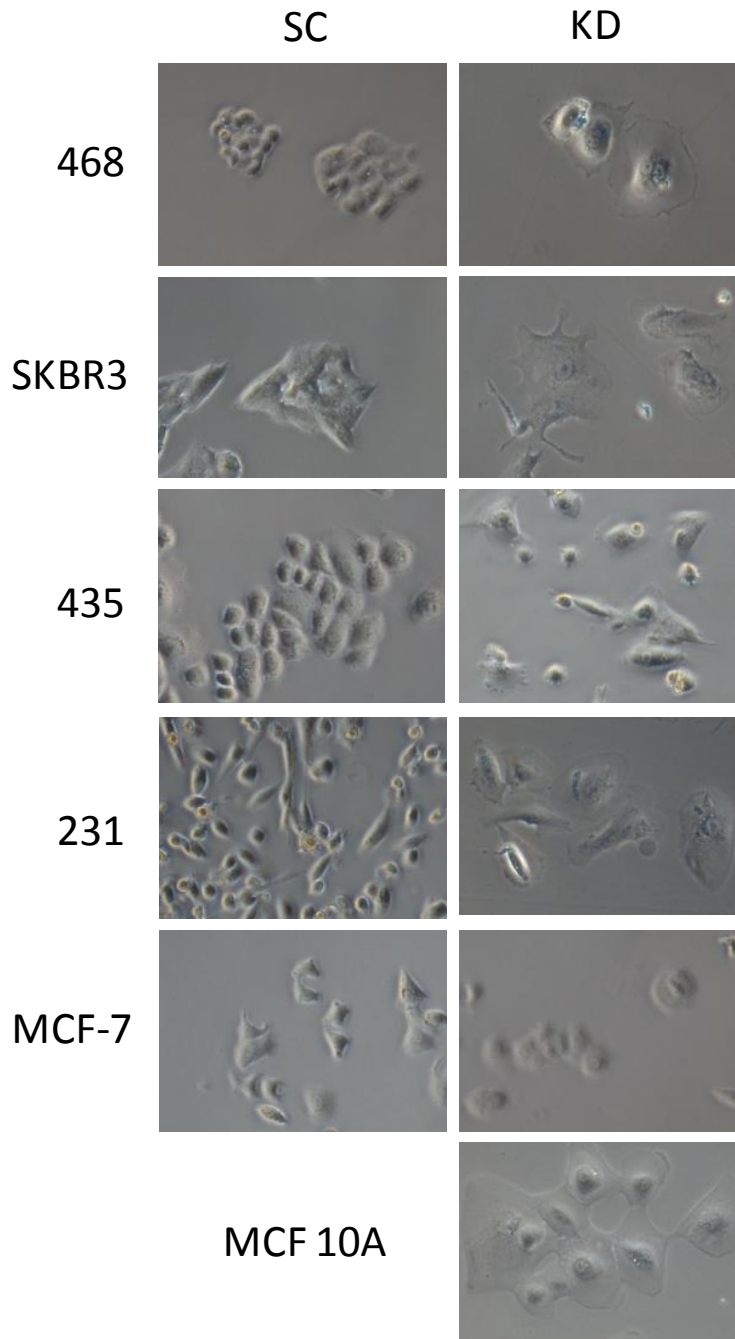
Figure 25. MBD2 knockdown cells change their morphology in culture. Knockdowns (KD) cells are less fusiform in shape and colonies grow more diffusely on the plate. The effect is variable between cell lines but in general the dense 'cobble stone' morphology is replaced by a more diffuse, 'fried egg' appearance, which more closely resembles the relatively normal morphology of the MCF-10a line (shown for comparison, bottom). All images were captured at the same magnification using a 40X phase contrast objective.

68

Figure 26. 3D lrECM Culture

MCF7

MCF7 Scrambled shRNA

MCF7 MBD2 shRNA
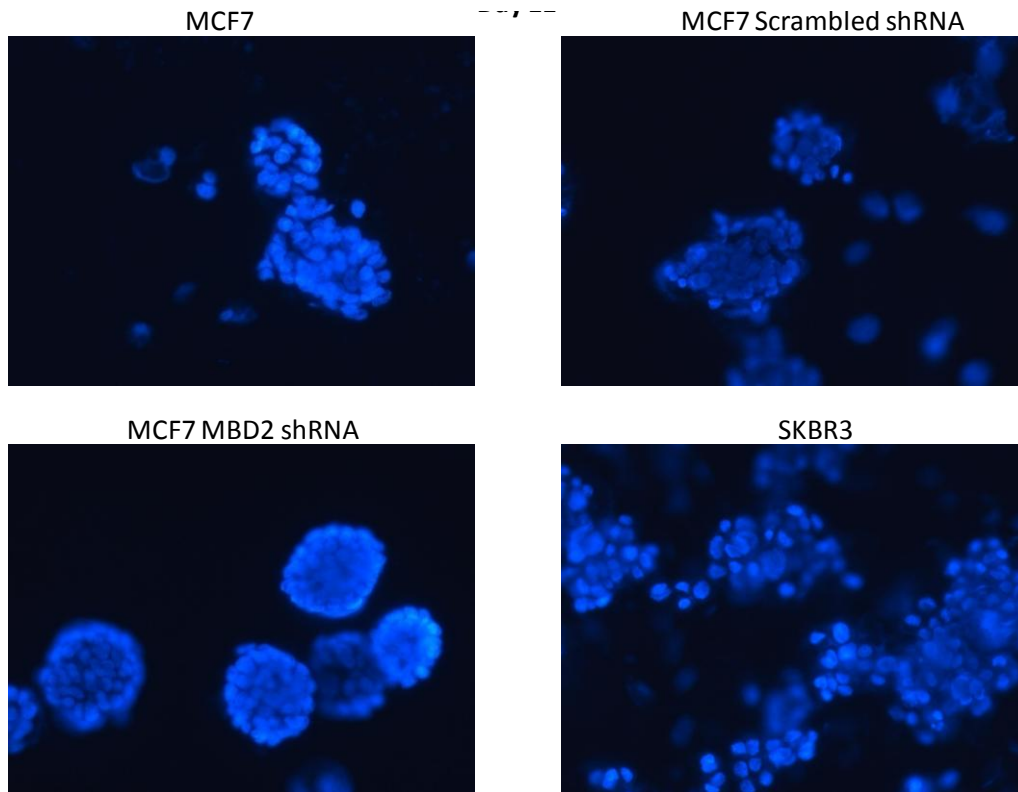
SKBR3

Figure 26. MCF-7, MCF-7 Scramble, MCF7 Knockdown and SK-BR-3 cells grown in 3D lrECM for 10 days. Representative images taken of control and MCF-7 Knockdown cells show knockdowns form significantly more organized 'mammospheres' (acini) than controls or than the disorganized SK-BR-3 cells. All cells were stained with 1X Hoechst in PBS for 10 minutes and imaged with a 40X objective, DAPI filter. Fluorescent nuclear staining allows visualization of individual cells within 3D colonies; these cells are difficult to distinguish by light microscopy.

Figure 27. Mammosphere Formation Assay
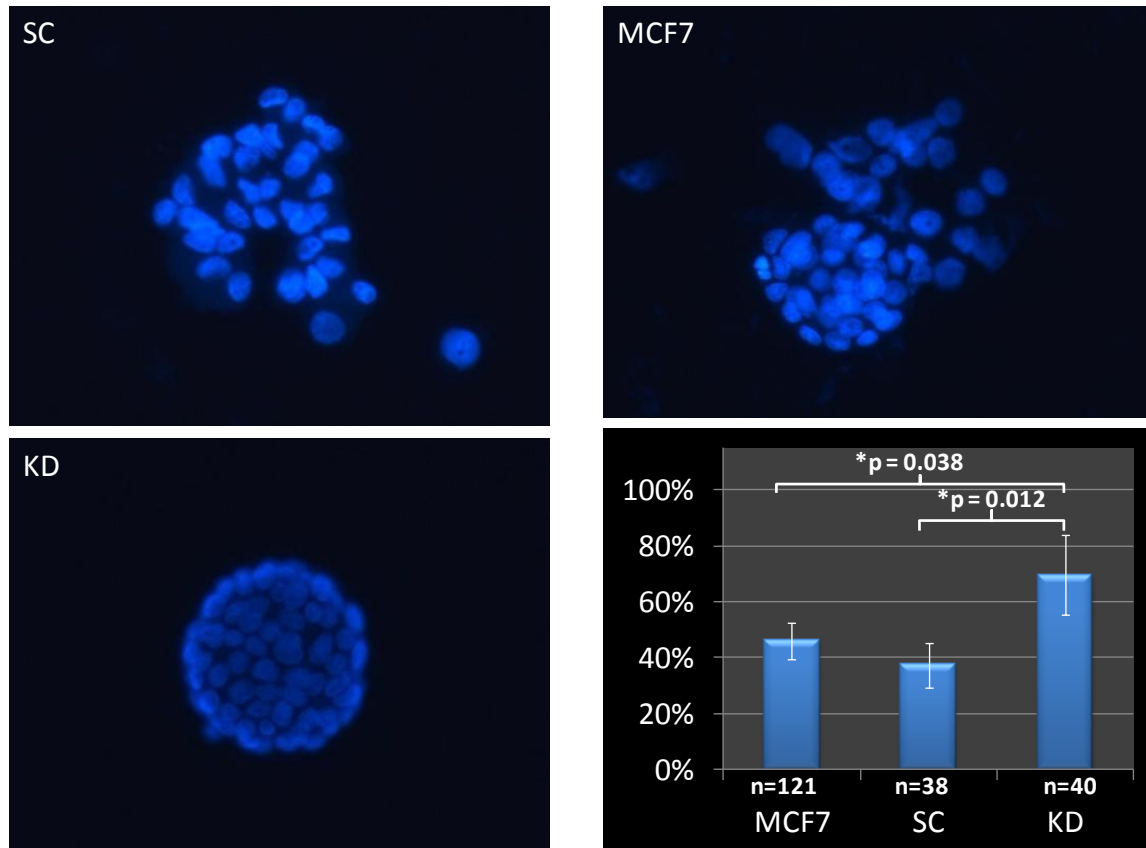
Figure 27. MCF-7 shMBD2 knockdown cells form significantly more organized mammosphere structures in 3D lrECM cultures when compared to MCF-7 and MCF-7 Scramble control lines. Cells stained with 1X Hoechst in PBS, 40X objective, DAPI filter. Mammospheres were only counted if they contained greater than 15 cells and organized and disorganized colonies were manually distinguished based on spherical morphology and evidence of polarization such as a clearly defined lumen. Counts are charted in the lower right hand panel. Total counts for each category are indicated below vertical bars and p values (*) were derived by students T-test. (n = organized + disorganized colonies). In contrast to control cells (MCF-7 and MCF-7 SC), knockdowns (KD) formed a significantly increased proportion of organized structures.

Figure 28. MBD2 knockdown promotes higher order structure formation
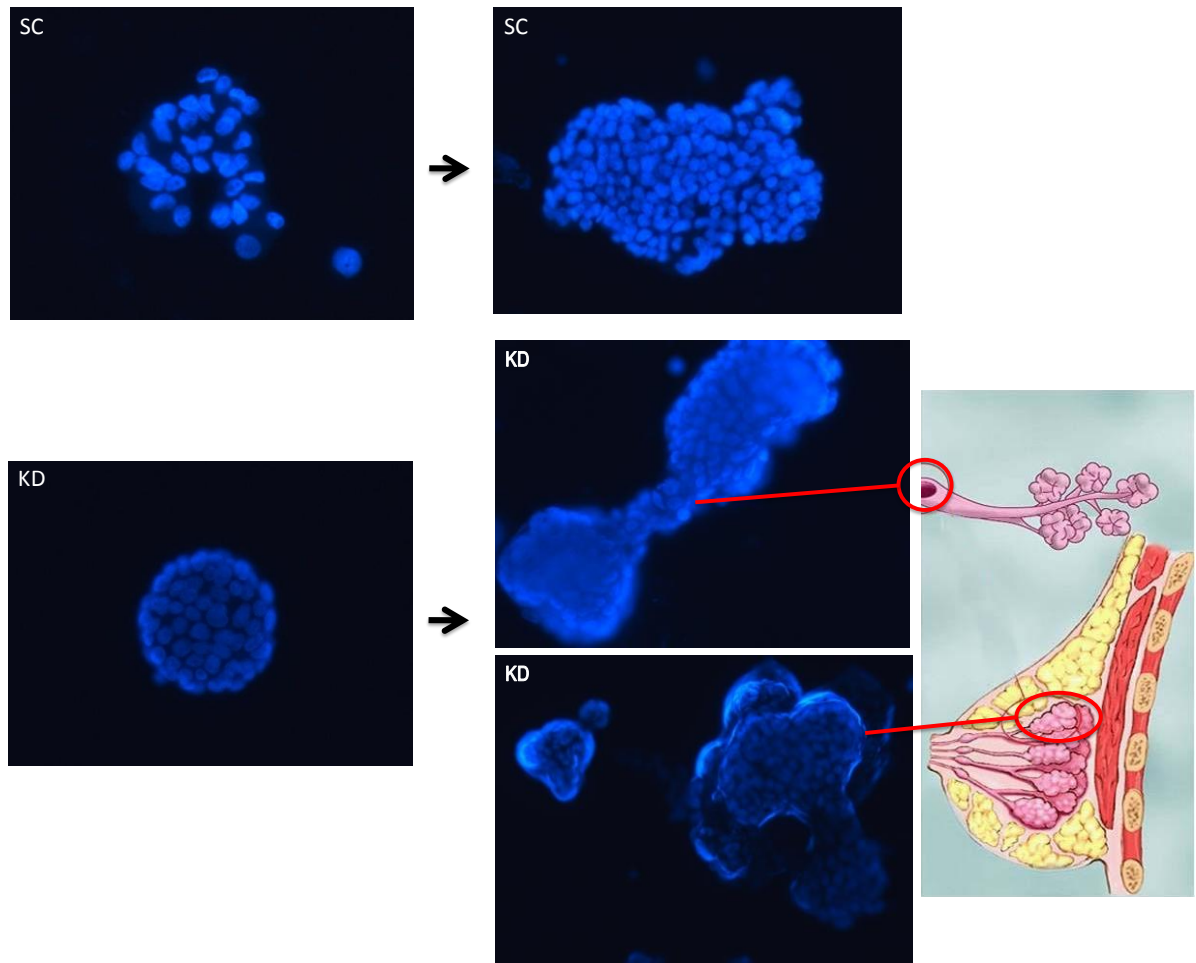
Figure 28. MCF-7 shMBD2 cells form organized structures that recapitulate *in situ* epithelial morphology, e.g., ducts and terminal lobules. Higher order structures were imaged between day 15 and 20 in lrECM culture as described in methods and in the legend for Figure 26.

<center>**Discussion**</center>

MBD2 is required for the rapid growth of breast cancer cells.

Normal mammalian epithelial cells do not require MBD2 for survival. In order to live through malignant transformation, tumor cells must inactivate the sentinel genes which protect against cancer by promoting death and clearance of neoplastic cells. Because many of these sentinel genes are silenced by abnormal DNA methylation, we asked whether breast cancer cells develop a dependence on MBD2. If this was the case, then we would expect a survival penalty to be paid if MBD2 protein was removed. As summarized below, we observed precisely such a penalty in human breast cancer cell lines in which double stranded RNA interference was used to reduce MBD2 protein levels. We took this observation as a confirmation of our hypothesis: human breast cancer cells become dependent on the Methyl DNA Binding Domain Protein 2 for growth, albeit to variable degrees in different lines. In the process, we made some interesting and unexpected observations regarding the specifics of the role of MBD2 in promoting growth; these are discussed further below.

We found that MBD2 knockdown by stably integrated, lentivirally delivered shRNA led to nearly complete growth inhibition in certain human breast cancer cell lines (MDA-MB-435, MDA-MB-468, and SK-BR-3) and significant but somewhat less growth inhibition in several others (MCF-7 and MDA-MB-231), Figures 8 and 9. Several factors presumably underlie the variable response to MBD2 knockdown. First, as discussed briefly above, the pattern of abnormal methylation is highly inconsistent between tumors and between divergent populations within a single tumor. As such, a variable response to a strategy that targets abnormally methylated and silenced genes is not all that

<center>72</center>

unexpected. Second there is the inherent inconsistency in our experimental knockdown approach. It is clear from our studies that a high degree of knockdown, approximately 80% reduction of mRNA or greater, is necessary to produce a phenotype. This degree of change in message, in our experience, corresponds to an even greater (90% or higher) reduction in MBD2 protein as measured by immunoblot, Figure 7. We make several inferences here. First, some amplification occurs from transcription to translation of MBD2; this is an indirect sign that regulation of MBD2 occurs largely at the transcriptional level. In addition, MBD2 protein is present in sufficient excess in breast cancer cells that a considerable reduction is necessary to reach the functional threshold required for growth inhibition. The implication here is that any pharmacologic inhibition of MBD2 will need to be potent enough to achieve similar reduction in MBD2 function to induce growth inhibition.

Our observations are largely consistent with the two inferences made above, i.e., variability in response is due to variable methylation and that significant knockdown is required to produce a phenotype. MCF-7 cells are somewhat less aggressive and slower growing than MDA-MB-435 cells to begin with. Their blunted response to MBD2 inhibition can be taken to mean that the degree and character of abnormal methylation in MCF-7 cells is less developed than that in MDA-MB-435 cells. Furthermore, we found efficient MBD2 knockdown was difficult to achieve in MDA-MB-231 cells compared to, for example MDA-MB-435 and SK-BR-3, as illustrated in Figure 7. The relatively high proliferative rate observed for MDA-MB-231 (Figure 8 and 9) may in fact be due to residual MBD2 rather than inherent insensitivity to MBD2 knockdown.

73

The timing of MBD2 knockdown is important

An important difference between our methodology and previous reports of MBD2 knockdown in tumor cells was the use of a lentiviral shRNA system. This method allowed us to avoid what we believe were the twin pitfalls of previous studies: short lived transient knockdowns and long selection times for stable transformants. Transient knockdowns do not persist for the two to three weeks we found to be required for growth inhibition to fully manifest. Conversely, the long periods (3-6 weeks) required for antibiotic selection and regrowth of stable clones by the standard method allow for copurification of cells that have developed resistance to MBD2 imposed growth inhibition. Lentivirus transduction and FACS purification circumvent these pitfalls and provide the best approach we have found for observing the delayed growth inhibition imposed by MBD2 knockdown.

The temporal relationship between MBD2 knockdown and growth inhibition was itself quite surprising. Following lentivirus infection on day 1, cells were passaged and recovered for 5-7 days in culture. Next, GFP expression was confirmed by fluorescent microscopy before the cells were sorted by FACS. The purified knockdown cells were divided for qPCR, western blot, and growth analysis after sorting. The cells were grown for another week to expand the sorted populations. They were subsequently harvested for qPCR and western blot analysis or counted and replated for growth analysis in 96 well plates (MTT assay, drug sensitivity) or 24 well plates (growth curves). Therefore, the temporal window used for *in vitro* growth analysis occurred somewhere between 14 and 20 days post transduction. It appears somewhat serendipitous that the processing delay

74

which preceded the analysis window allowed the growth inhibitory effects of MBD2 knockdown to fully develop.

The reason for the delay in growth suppression is not clear. We know from transient MBD2 knockdown studies conducted in our lab that 3-4 days is sufficient time for MBD2 mRNA and protein levels to be significantly reduced (see Chapter III for details). We also know that 3-4 days following MBD2 knockdown is sufficient time for secondary, MBD2 induced, expression changes. For example, increased expression of gamma globin in erythroid populations is observed at 72 and 96 hours after knockdown. It is possible that tumor suppressor expression is being induced with similar timing in our breast cancer cell lines although the maximal effect on growth takes longer to evolve.

On the other hand, it is possible that stable re-expression of MBD2 regulated tumor suppressor proteins in breast cancer takes longer; complete target gene derepression may take several weeks, corresponding to 10-20 cell divisions. Moreover, an extended period may be required for passive epigenetic reprogramming following MBD2 knockdown, not unlike the time required for induced pluripotent stem (iPS) cell reprogramming. A time course of exogenous inducible factor expression in the generation of iPS cells showed that at least two weeks of expression were required before pluripotent iPS cells were formed. Moreover, iPS formation was enhanced by longer periods of expression (Brambrink et al. 2008).

Our analysis of gene expression changes in MBD2 knockdown breast cancer cells is reported in the following chapter; however, the analyses described in Chapter II were conducted at timepoints corresponding to peak growth suppression. Therefore, a time

75

course study to fully interrogate the temporal relationship between MBD2, target gene induction, and resulting phenotype remains to be performed.

Combining MBD2 inhibition with chemotherapy

An important goal of our study from the outset was to determine whether MBD2 knockdown would sensitize breast cancer cells to chemotherapy. In many ways, this was our most promising hypothesis; it seemed plausible that MBD2 would restore expression of tumor suppressors (DNA damage response genes, for example) that would sensitize to compounds targeting the corresponding pathways (e.g., DNA damaging agents like anthracyclines, cyclophosphamide, platinum compounds, etc.) Surprisingly, MBD2 knockdown had a greater direct effect on growth than as sensitizing agent for any of the compounds we tested.

Using stable MBD2 knockdown and control lines, we tested the dose dependent toxicity of several compounds, corresponding to classes of chemotherapy commonly used as first line treatment in breast cancer: a nitrogen mustard derivative (cyclophosphamide, Cytoxan), an anthracycline (Doxorubicin), and a taxane compound (Paclitaxel). We also tested the activity of two additional classes of therapy: the HDAC inhibitor, Vorinostat, and a platinum compound, Cisplatin. Several endocrine and biologic therapies are now used commonly in breast cancer. These include Selective Estrogen Receptor Modulators (SERMs), aromatase inhibitors, and monoclonal antibodies against Her2/neu. These were not used in for these studies because either their clinical utility depends on preclassification of tumors and/or their mechanism of action was not amenable to an *in vitro* study (i.e., aromatase inhibitors function at sites away from the tumor and Herceptin depends largely on the immune system and type 2 antibody dependent cell mediated

76

cytotoxicity, ADCC). However, in the case of SERMs, for example Tamoxifen, there may be some combined effect of estrogen receptor inhibition and an anti-MBD2 strategy; this compound merits testing in future studies.

We found that the effect of MBD2 knockdown was observed in treated and untreated populations. However, at the doses we tested, there was no conclusive evidence of any synergistic (supra-additive) effect of MBD2 knockdown plus another compound. Alternatives to synergy include additive effects, non-additive (mutually exclusive) effects and protective or negative effects (i.e., where two compounds used together are less effective than either alone). We found there was an additive effect which was particularly evident at low doses of Paclitaxel, Doxorubicin, and SAHA (Figures 16 and 17). This was important to establish because we hypothesize that the consequence of MBD2 inhibition will remain largely restricted to tumor cells. In other words, a specific inhibitor of MBD2 will likely be very well tolerated and therefore can have significant clinical utility in lowering effective doses of more toxic therapies when used in combinations.

Two important points need to be made here. First, any successful combined therapy strategy depends of course on choosing the right combination. We have tested only a small subset of available therapies and have therefore covered only a small fraction of the potentially synergistic compounds that might act through pathways crippled by epigenetic dysregulation in breast cancer. Second, the doses we tested corresponded to therapeutic levels often used in the clinical setting. These doses led to significant toxicity in our *in vitro* studies. It would be informative to repeat the treatments, doxorubicin and paclitaxel in particular, at lower doses to determine if any synergy is unmasked at lower ranges.

77

Functional inhibition of MBD2 significantly impairs the growth of tumors in mice.

Immunocompromised mice provide a useful experimental model for studying the biology of human tumors in an environment that more closely resembles their *in situ* physiology. These mice offer the ability to explore tumor-host interactions including endocrine/paracrine regulation, innate immune response, and direct tumor-stromal interactions. In contrast to *in vitro* growth, a xenograft tumor must be able to extract nutrients from its interstitial environment, recruit a blood supply as it grows, and evade clearance by any residual elements of the mammalian innate and adaptive immune system. The athymic nude mice we used have a leaky immunocompromised phenotype (as opposed to SCID mice which completely lack B and T cell function). Therefore, these mice retain the ability to clear certain tumors and are a slightly more stringent xenograft model than fully immunodeficient animals. From a practical standpoint, this affected our study in several ways. First, we were unsuccessful in grafting less aggressive tumor cell lines in these mice (MCF-7, MDA-MB-468, and SK-BR-3), despite repeated attempts with large starting cell numbers. Even highly tumorigenic lines, MDA-MB-231 and MDA-MB-435, required relatively high starting tumor cell numbers to establish successful xenografts. However, once successfully established, Scramble control tumors rapidly outgrew their Knockdown counterparts in our studies (Figures 20-23). The effect of MBD2 knockdown on *in vivo* tumor growth was somewhat more dramatic than the one observed for tumor growth *in vitro*. MDA-MB-435 knockdown cells were completely cleared in 5 out of 6 mice and though MDA-MB-231 knockdown

78

cells were able to grow, they formed significantly smaller and slower growing tumors than control cells (Figures 21 and 22).

Out of 6 mice implanted with MBD2 knockdown MDA-MB-435 cells, a single exceptional tumor was able to escape the MBD2 knockdown imposed growth inhibition in our xenograft study. We resected this tumor and examined the expression of MBD2 protein (Figure 24). MBD2 was still significantly suppressed in this rapidly growing and invasive mass. We further examined the expression of Maspin, an MBD2 induced gene and found it to be resuppressed to pre-MBD2 knockdown levels. This observation suggests that at least one mechanism for evading MBD2 knockdown imposed growth inhibition *in vivo* exists. As this tumor developed after 40 days, and given a doubling time of 24 hours for this cell line, it is possible that it arose from a single clone within the original population ($2^{40} = 1.1 \times 10^{12}$). Further studies are necessary to determine the precise mechanism of resistance in this clonally derived population. Based on the assumption of clonal origin, we concluded that this mechanism of phenotypic conversion was highly improbable for any given MBD2 knockdown cell. A further discussion of resistance mechanisms can be found in the summary section of Chapter IV.

Our *in vivo* studies illustrate an important point: a cytostatic effect can be a highly effective anti-tumor strategy. By significantly impairing the growth of breast cancer cells, even without outright killing, the balance between growth and clearance was tipped towards clearance.

Several other mechanisms of *in vivo* growth inhibition may be at work as well. By restoring the expression of epigenetically silenced genes, it is quite possible that we have blunted the ability of MBD2 knockdown cells to perform any of a number of acquired

79

functions necessary for *in vivo* growth. Among these functions is the ability to survive and proliferate in a relatively oxygen poor environment, in stark contrast to their environment in a culture vessel. In other words, MBD2 knockdown cells may be sensitized to hypoxia induced cell death, a feature of normal epithelial cells which are heavily dependent on oxidative metabolism. Tumor cells circumvent this obstacle in several ways, including the upregulation of the anaerobic metabolic machinery and the expression of angiogenic factors that aggressively recruit a de novo blood supply.

Furthermore, it is known that the expression of carcinoembryonic antigens is a red flag for the immune system during cancer surveillance. These antigenic factors are inappropriately expressed by neoplastic cells and subsequently detected by the innate and adaptive immune system. Not surprisingly, these antigens are often silenced in tumors which must evade the immune system to survive. It is conceivable that these silenced auto-antigens are reactivated by MBD2 knockdown in breast cancer cells, particularly given the likelihood that such antigenic elements are hypermethylated during tumor onset and/or progression. It is also possible that they are being recognized by conserved and intact elements of the nude mouse's innate immune system (e.g., macrophages in the subcutaneous space or circulating NK cells). In order to fully evaluate the mechanism of clearance of MBD2 knockdown derived tumors, further studies are needed.

Epithelial morphology is restored in MBD2 knockdown MCF-7 cells

A common distinguishing feature of cancer cells in contrast to their normal counterparts is a variable degree of dedifferentiation. In fact, certain high grade tumors bear little morphologic resemblance to their tissue of origin; in fact molecular markers

80

are required to identify and classify these tumors for the purposes of treatment. Importantly, high grade (poorly differentiated) tumors are correlated strongly with poor prognoses.

One of the first significant observations made during the course of the studies described in this dissertation was that MBD2 knockdown cells developed a strikingly different cellular morphology and pattern of growth. Upon microscopic examination it became clear that stable populations of MBD2 knockdown cells were less fusiform in shape compared to untreated and scramble control transfected cells. In addition, the pattern of growth of knockdown cells was more dispersed. Instead of forming the overlapping colonies observed in controls (Figure 25), MBD2 knockdown cells seem to form a more uniform monolayer that is evenly distributed in the culture vessels. In addition, the dense 'cobble stone' morphology of control cells became a more diffuse, 'fried egg' shape, with a decreased relative profile of nucleus to cytoplasm. MBD2 knockdown morphology more closely resembled the relatively normal morphology of the immortalized non-transformed MCF-10a cell line (Figure 25).

In order to better study the morphological differences between knockdown and control cells, we cultured these populations in collagen and laminin based extracellular matrix extracts which allowed the formation of three dimensional structures capable of recapitulating *in situ* morphology. We observed a significant difference in the formation of structures resembling epithelial rests, acini, terminal lobules and ducts in MBD2 knockdown MCF-7 cells compared to scramble control and untransfected cells (Figures 26-28). Similar observations were not made in other cell lines, which remained disorganized in 3D cultures, in contrast to their morphology differences in standard 2D

81

plastic culture plates. MCF-7 cells were unique in that they formed acini (mammospheres) at a lower frequency even in control populations (~40%). However, the percentage of mammospheres compared to disorganized multicellular colonies was significantly enhanced in highly knocked down MBD2 clones (~70%).

We are able to garner from these observations that MBD2 is not the only factor involved in maintaining the epithelial disorganization in breast cancer cells, but that it is playing an exacerbating role in the disorganized growth of certain populations. The utility of this observation in isolation is limited; however, taken together with growth inhibition and a decrease in the *in vivo* turmorigenic potential, it suggests that MBD2 plays a pleotropic role in the pathologic dedifferentiation of breast cancer.

In addition, the changes in morphology apparently maintained by MBD2 in breast cancer cells are reminiscent of a phenomenon termed the epithelial to mesenchymal transition (EMT). In pathological EMT, epithelial cells transdifferentiate to resemble fusiform mesenchymal cells which acquire the ability to migrate, invade locally, and metastasize to distant sites (Thiery et al. 2009). Since only a subset of cells within a heterogeneous tumor are capable of this transition (a population thought to have stem cell like characteristics) it is noteworthy that our observation is not an all-or-nothing effect but rather a shift in the probability of organized growth. Similarly it is not altogether surprising that only one of the cell lines we examined was capable of producing a change of the magnitude necessary for detection in a our mammosphere formation assay, which offers rather limited sensitivity and is suitable for distinguishing relatively dramatic differences only.

82

In summary, more sensitive assays to characterize the differentiation state of these cells are required to provide a less ambiguous picture of the mechanism(s) underlying the changes described in this Chapter. We have undertaken several studies with the express purpose of shedding light on MBD2 dependent changes in breast cancer. In the Chapters that follow, we attempt to characterize the gene expression differences, CpG methylation status, and compensatory adaptations that underlie the phenotypes described above and, more generally, the mechanistic basis for the pathologic role of MBD2 in breast cancer.

Chapter II: MBD2 is required for transcriptional inhibition of a subset of methylated tumor suppressor genes in breast cancer

Introduction:

DNA Methylation, CpG Islands and Transcription

Most mammalian genomes have high levels of DNA methylation and are depleted of CpGs due to the frequent deamination of methyl-cytosine to thymidine. Deamination results in methylCpG:TpG mismatches which are later repaired during DNA replication causing the genome to have more A/T than G/C content. The remaining CpGs are unevenly distributed throughout the genome. Approximately 60% gene promoters in mammals are imbedded in unmethylated stretches of DNA with high CpG density, known as CpG islands (Klose and Bird 2006). It is not clear how the CpG islands are maintained in an unmethylated state and what protects them from the action of DNA methyltransferases. It is clear that these sequences are not intrinsically immune to methylation since some of them acquire DNA methylation during normal development (Suzuki and Bird 2008) or are found to be aberrantly methylated in cancers (Jones and Baylin 2007). Given that most of the gene promoters are methylation-free, the question of whether DNA methylation is essential for regulation of gene expression on a global scale has been a subject of debate. One model of how DNA methylation exerts its repressive effect on transcription involves CpG methylation which alters binding sites of transcription factors and directly interferes with gene activation (Clouaire and Stancheva 2008). In the second and significantly more frequently cited model, methylated cytosines are thought to serve as docking sites for proteins that specifically recognize and bind to

84

methylated CpGs and repress transcription indirectly via recruitment of corepressors that modify chromatin.

It is widely accepted that aberrant methylation is involved in cancer development (Jones and Baylin 2007). In particular, the importance of promoter cytosine methylation in tumor suppressor gene silencing has been established repeatedly and is discussed throughout the present work. In general, it is thought that methylated CpG islands in tumors are not a permissive setting for the initiation of transcription unless the methylation signal can be overridden by alterations in factors that modulate chromatin, a notable example being the removal of methylated cytosine binding proteins (Pulukuri and Rao 2006; Bakker, Lin, and Nelson 2002; Martin et al. 2008). The study of CpG island promoter methylation has been a driving force in DNA methylation research, particularly as it relates to cancer. Presumably, the reason for this focus on CpG islands has been the demonstrable ability of CpG-island associated hyper-methylation to permanently silence genes both physiologically during mammalian development and pathologically in cancer cells (Jones and Baylin 2007; Suzuki and Bird 2008; Gronbaek, Hother, and Jones 2007). This exists in stark contrast to the role of methylation at non-CpG island promoters, which have been largely overlooked because the correlation between methylation and transcription is far weaker (Jones and Takai 2001; Takai and Jones 2002).

Because approximately 40% of human genes are non CpG island genes, methylation dependent regulation at these loci cannot be ignored. Recent work has shown strong correlations between tissue-specific expression and methylation of non-CpG island associatied genes, for example Maspin (Futscher et al. 2002b). Maspin has a CpG rich promoter that does not meet established criteria for a CpG island. The MAGE gene

85

family is another example of genes that are commonly upregulated by epigenetic therapy even though their promoters do not fall within canonical CpG islands (Wischnewski et al. 2007). At least one report suggests non-CpG island containing genes are not responsive to 5-aza-dC and that observed transcriptional reactivation at these sites might be occurring through indirect mechanisms (Wozniak et al. 2007). The work in this chapter and the next deals to some extent with the role of MBD2 in the transcriptional silencing of Maspin, and the nature of MBD2's effects on CpG sparse genes.

Epigenetic dysregulation of transcription in cancer

Mammalian DNA methylation has been implicated in a diverse range of cellular functions and pathologies, including tissue-specific gene expression, cell differentiation, genomic imprinting, X chromosome inactivation, regulation of chromatin structure, carcinogenesis, and aging (Bird 2002b). DNA methylation is essential for normal development and remains indispensable for the survival of differentiated cells (Okano et al. 1999; Jackson-Grusby et al. 2001). Mechanistically, a methylated cytosine base can function to promote or preclude recruitment of regulatory proteins. The methyl mark is read through the MBD family of methyl-CpG binding proteins including MBD2 which are thought to mediate transcriptional repression through interactions with histone modifying complexes (Klose and Bird 2006; Hendrich and Bird 1998b). Alternatively, the methyl mark can exclude DNA binding proteins from their target sites, as has been shown for CTCF binding at the H19 locus (Hark et al. 2000). An important property of DNA methylation is that the patterns are dynamic and change throughout stages of development and disease. Importantly, the DNA methylome undergoes characteristic

86

changes during the pathogenesis of cancer. These include a genome wide loss of methylation which occurs concurrently with an aberrant local gain of methylation marks at certain loci. In particular, tumor suppressor gene promoters are targets of hypermethylation, which typically results in their transcriptional silencing (Jones and Baylin 2007; Jones and Baylin 2002). Moreover, recent models suggests that cancer may evolve from a population of nonneoplastic, polyclonal, epigenetically disrupted stem/progenitor cells, potentially with additional genetic lesions (Feinberg, Ohlsson, and Henikoff 2006).

Contrasting the pathologic role of DNA methylation and histone modifications

While the present work deals primarily with DNA methylation and the related function of MBD2, it is useful to remember that these factors exist in equilibrium with local chromatin states. Histone modifications have been implicated in a number of cancer related epigenetic phenomena. The use of the term ''heritable'' is often dropped from discussions of the epigenetic nature of histone modifications; in this context 'epigenetic' has taken to mean information carried by the genome (e.g., on chromatin) that is not coded by DNA. Despite this loosening of definitions, histone modification must be distinguished from DNA methylation which provides non-genetic memory of function that is reliably transmitted from generation to generation (consider imprinting, X chromosome inactivation, heterochromatin organization, and gene silencing). In addition there are environmentally induced changes, which are passed on from generation to generation, without the need for the original stimulus (best studied in plants) (Martienssen et al. 2008). However studies suggest genomic methylation is the basis for

87

transgenerational epigenetic stability in plants (Mathieu et al. 2007). There is no disputing that histone modifications are involved in epigenetic processes. If epigenetic memory is mediated by one or more of the histone modifications, then there should be a mechanism for the transmission of such modifications onto the chromatin of the replicating DNA. Such a mechanism has been proposed for H3K9 methylation in the transmission of heterochromatin (Hublitz, Albert, and Peters 2009). It is thought that lysine methylation of nucleosomes on the daughter strand occurs through a mechanism involving recruitment of HP1 and secondary targeted H3K9-methylation. This process ensures the transmission of the H3K9 methylation mark. This mechanism of transmission, along with the observation that patterns persist, have given histone lysine methylation an epigenetic status. The issue that remains, however, is whether the modification pattern inherited by the daughter chromatin is sufficient to impose the correct chromatin structure originating from the mother cell. In other words, a question remains as to whether lysine methylation is dictating memory of chromatin structure. In contrast, the epigenetic function of CpG methylation in this regard is more firmly established (Suzuki and Bird 2008).

The position-effect variegation exhibited during gene expression in Drosophila demonstrates a key property of epigenetic silencing; its ability to spread over genomic regions in a progressive way. In Drosophila, it seems to involve the cooperation of multiple processes, including noncoding RNAs, covalent modifications of chromatin, physical alterations in nucleosomal positioning, and DNA methylation. The high degree of mitotic stability of DNA dependent gene silencing coupled with the progressive nature

by which it is achieved makes pathological silencing of growth controlling and other genes an important factor in the development of cancer (Jones and Baylin 2007).

The central role of MBDs in epigenetic dysregulation

While the importance of DNA methylation in cancer has been well established, the focus in the field continues to shift towards related mechanisms such as chromatin modifications, which can also play a role in cancer development. It is widely acknowledged that covalent modifications of histones can control gene activity. Histone deacetylation and methylation of specific lysine residues such as lysine 9 in histone H3 or lysine 27 in histone H3 clearly participate in transcriptional silencing (Jenuwein 2006). However, as discussed above, the primacy of these marks is a matter of controversy. A key link between these covalent histone modifications and the stable epigenetic mark, DNA methylation, was established by pioneering experiments that showed that cytosine methylation could attract methyl DNA binding proteins (Nan et al. 1998a; Denslow and Wade 2007; Feng and Zhang 2001a; Fujita et al. 2003; Sarraf and Stancheva 2004). In a related example, a recent study showed that Brahma (Brm), which is a catalytic component of the SWI/SNF chromatin-remodeling complex, was recruited by the methylated DNA binding protein MeCP2 (Harikrishnan et al. 2005). Such experiments provide the most plausible mechanism for long term stability and transmission of histone modifications and architecture, specifically by exploiting the stability of CpG methylation. In addition they highlight the central role of MBDs in tying together the principal modalities associated with epigenetic effects on transcription.

89

As discussed in Chapter I, significant crosstalk is likely to exist between different epigenetic modalities. Heritable gene silencing involves, among other processes, the interplay between DNA methylation, histone covalent modifications, and nucleosomal remodeling. Some of the enzymes that contribute to these modifications include DNA methyltransferase (DNMTs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and complex nucleosomal remodeling factors (NURFs). While DNA methylation remains the most stable long term modification, it is clear that once established histone modifications and chromatin architecture are capable of recapitulating one another through the activity of multiple biochemical mediators within the nucleus.

A review of transcriptional dysregulation in cancer suggests that those factors which bind the epigenetic machinery into macromolecular repressive machines thereby maintaining persistent silencing of tumor suppressor genes at multiple levels were critical targets. We reasoned that one such target was MBD2, which (as discussed in Chapter I) links the two best characterized processes involved in stable long term transcriptional inactivity, namely CpG methylation and chromatin modifications. In the previous chapter we found that MBD2 knockdown cells were significantly altered from their control counterparts, consistent with our hypothesis that MBD2 was an important target in breast cancer. Here we sought to better define the specific influence of MBD2 on the maintenance of epigenetic dysregulation in tumors and thereby gain insight into the transcriptional basis for MBD2 dependent phenotypes in breast cancer cells.

90

<center>**Materials and Methods:**</center>

Design and cloning of shRNA vectors

We used the pSuperior Vector system (OligoEngine, Seattle, WA) for stable delivery of short harpin RNA (shRNA). This system allows rapid selection of a stable population by G418 resistance. This system also incorporates an inducible H1 promoter, which we thought may provide future utility for applications requiring tight regulation of shRNA expression:

Inducible H1 promoter (from 35nt upstream of BglII / HindIII cloning site AGATCTaagctt):
5'…GAATCT**TATA**AGTTCCCTATCAGTGATAGAGATCTaagctt…3'

We designed and ordered sense and anti-sense oligonucleotides that included the following features: terminal BglII and HindIII overhang complementary sites, a region corresponding to a 19bp target sequence in the gene being silenced, in this case MBD2, and a non-complementary loop region. The mechanism of RNAi involves loading the antisense strand of the siRNA duplex into a silencing protein complex which targets the corresponding messenger RNA molecule for cleavage. Using universal RNA design features, Shou Zhen Wang in our lab generated two targeted sequences and a non-targeting scrambled sequence to be used as a negative control. This control is necessary to account for any confounding effects of a non-sequence dependent response to double stranded RNA, for example a type one (alpha/beta) interferon response. It is worthwhile to note that the scramble control doesn't account for sequence specific induction of the interferon response. Four oligonucleotides were ordered for each target to reduce the cost of synthesis (sequences are given in Table 1). These oligonucleotides were annealed by

<center>91</center>

slow cooling from 95 deg C, and ends were phosphorylated to increase the efficiency of ligation into the vector with T4 polynucleotide kinase (NEB, Ipswich, MA) at 37 deg C for one hour. The pSuperior vector was cut with the appropriate restriction enzymes and a 12kb band was resolved and cut from a 1% agarose gel and purified using a Qia-quick Gel purification kit (Qiagen, Valencia, CA). The annealed and phosphorylated oligos were cloned between the unique BglII and HindIII or between the HindIII and XhoI sites and ligated overnight at 16 deg C in the presence of T4 DNA ligase (NEB). This positions the forward oligo at the correct position downstream from the H1 promoter's TATA box to generate the desired siRNA duplex. 4ul of the ligation was transformed by heat shock at 42 deg C for 20 sec into DH5-alpha chemically competent cells (Invitrogen, Carlsbad, CA) and positive clones were selected on ampicillin containing LB agar plates. Individual colonies were selected and grown overnight at 37 deg C with agitation in LB/amp media. Plamids were purified from the overnight cultures using column purification kits (Qiagen) and were subsequently validated by fluorescent automated sequencing (VCU Molecular Biology Core). The shRNA target sequences, shRNA cloning oligos and H1 Forward sequencing primer sequences are given in Table 7 below.

92

Table 7. shRNA Targets, Cloning and Sequencing Primers

MBD2 B Target Sequence	GGGTAAACCAGACTTGAAT
MBD2 C Target Sequence	GAACAGCCACGTCAGCTTT
Scramble Target Sequence	ACGCGTAACGCGGGAATTT
shMBD2-B-Aas	CTCTTGAAATTCAAGTCTGGTTTACCCGGG
shMBD2-B-As	GATCCCCGGGTAAACCAGACTTGAATT
shMBD2-B-Bas	AGCTTAAAAAGGGTAAACCAGACTTGAATT
shMBD2-B-Bs	TCAAGAGAATTCAAGTCTGGTTTACCCTTTTTA
shMBD2-C-Aas	CTCTTGAAAAAGCTGACGTGGCTGTTCGGG
shMBD2-C-As	GATCCCCGAACAGCCACGTCAGCTTTT
shMBD2-C-Bas	AGCTTAAAAAGAACAGCCACGTCAGCTTTT
shMBD2-C-Bs	TCAAGAGAAAAGCTGACGTGGCTGTTCTTTTTA
shScramble-Aas	CTCTTGAAAAATTCCCGCGTTACGCGTGGG
shScramble-As	GATCCCCACGCGTAACGCGGGAATTTT
shScramble-Bas	AGCTTAAAAAACGCGTAACGCGGGAATTTT
shScramble-Bs	TCAAGAGAAAATTCCCGCGTTACGCGTTTTTTA
H1 Forward Sequencing Primer	GAATTCGAACGCTGACGTC

Table 7: Table shows shRNA target sequences along with cloning and sequencing primers used to generate and verify shRNA clones. Sequences are given in the 5' to 3' direction for all oligonucleotides. Four complementary fragments were annealed for each shRNA targeting construct in the following orientation: Vector 3' – A oligo pair – B oligo pair – 5' Vector. The "A" and "B", sense ("s") and antisense ("as") oligonucleotides are marked accordingly. Oligonucleotide ends are BglII and HindIII compatible.

Transfection of breast cancer cell lines

All cell lines were transfected in 6cm culture vessels between 80 and 95% confluency. Transfections were performed using the Lipofectamine ™ 2000 reagent (Invitrogen) using maxi scale purified plasmid DNA essentially as detailed in the product insert/protocol with optimization of DNA concentrations when required to maximize transfection efficiency and minimize toxicity.

Selection and isolation of knockdown Clones

Transfected populations were subcultured at a 1 to 10 dilution 24-48 hours post transfection by disruption in the presence of a 0.25% trypsin/EDTA solution (Gibco, Carlsbad, CA). Cultures were placed in antibiotic selection media (Geneticin or Zeocin, Invitrogen, Carlsbad, CA) at a cell line dependent concentration predetermined by killing curve analysis. Antibiotic media was changed every 72 hours for 2 to 4 weeks until the appearance of single colonies of resistant clones. Single clones were isolated and replated in 6 well tissue culture plates and allowed to grow in selection antibiotic. Clonal populations were subsequently screened by SYBR green based real-time quantitative PCR as well as immunoblot analysis for gene knockdown.

Cluster Analysis of qPCR Data:

Cluster analysis was performed and heat map figures were generated from tabular gene expression data essentially as described previously (Eisen et al. 1998). The Cluster analysis tools are designed primarily for manipulation and display of microarray data so some minor reformatting of quantitative PCR results was necessary prior to import. A

94

detailed explanation of formatting input data is available in the software help files and therefore will not be provide here. The analysis operations performed on raw data were limited to log transformation (to provide consistency in fold change intensity) and gene normalization; array normalization was never performed to preserve quantitative relationships between biological samples. The preferred algorithm for clustering small data sets is self organizing map (SOM) analysis and therefore this algorithm was used almost exclusively. A modified Java treeview platform with an improved and user-friendly graphical interface was used to generate heat map figures (Saldanha 2004). All analysis software is available online from the developer's websites at no cost.

(http://rana.lbl.gov/EisenSoftware.htm, and http://jtreeview.sourceforge.net/).

Gene expression profiling of MBD2 Knockdown Breast Cancer Cells

To determine the extent to which MBD2 was required for tumor suppressor gene silencing in breast cancer and to determine which genes were mediating the effects presented in the previous chapter, we conducted a series of gene expression analyses. Since many of the effects we observed in MBD2 knockdown cells are not known to be induced by the DNA methylation inhibitor, Decitabine (5-aza-dC), we also sought to determine how MBD2 knockdown gene expression changes differed from those induce by 5-aza-dC. We chose to use quantitative PCR for our analysis for reasons discussed below. Changes in the mRNA levels of 48 tumor suppressor genes were probed in our cell lines. The list of genes we chose to screen was compiled from independent reports indicating methylation dependent silencing specifically in breast cancer. The complete names and function of each gene is given in Appendix A along with NCBI accession numbers and primer sequences in Appendix B. We validated all primers generated for our array of tumor suppressor genes using SYBR Green based fluorescent quantitative PCR. Figure 30 shows the results of this validation; the template used was serially diluted cDNA from MCF-7 cells. As expected, several genes were undetectable at higher dilutions, most likely due to the low starting mRNA copy number of these epigenetically silenced genes in breast cancer. Only primer sets that were quantitative over at least an order of magnitude were considered valid and used for subsequent tests.

In order to minimize transcriptional noise from a heterogeneous pool, we next selected stable clones expressing shRNA against MBD2 in the human mammary epithelial carcinoma line, MCF-7 (Figure 29). These clones were compared against

96

identically derived control shRNA expressing lines using high throughput quantitative PCR. In our comparison we included additional samples to ascertain how MBD2 dependent changes differed from those induced by the DNA methyltransferase inhibitor, 5-aza-2-deoxy-cytidine. Prior to extraction of RNA for analysis, these samples were treated for 96 hours with 4 uM 5-aza-dC. RNA was purified by Trizol extraction and reverse transcribed into cDNA; a 1:10 dilution of this cDNA was used for subsequent SYBR Green based qPCR analysis. Quantitative PCR results were clustered into groups using self-organizing map analysis (Figure 31). Though the starting RNA amount was held constant for all samples, several housekeeping genes were included in order to correct for small differences in starting material or changes in gene expression not related to our experimental variables. The normalized levels of housekeeping genes (GAPDH, b-Actin, CyclophilinA) did not vary appreciably between samples (Green arrows, Figure 31). In contrast, the levels of a number of tumor suppressor genes we examined were markedly induced by MBD2 knockdown. Representative genes were selected for subsequent analysis (bisulfite sequencing and ChIP) described in Chapter III; these genes are indicated on the heat map by red arrows in Figure 31.

Cluster analysis allowed us to distinguish several categories of gene response in our panel. The largest category of genes were those induced primarily by 5-aza-dC (4uM, number of genes: n=30, 67%). Another group of genes was simultaneously induced by MBD2 knockdown and 5-aza-dC in additive fashion (n=11, 24%). Finally a smaller subset of genes appeared to respond to MBD2 knockdown alone (n=7, 15%) in our stable cells. Genes in this subset were designated MBD2 responsive target genes and were of particular interest to us as candidates for mediating the growth inhibition and morphology

97

phenotypes elicited by MBD2 knockdown; these target genes were used for several subsequent analysis experiments as discussed further below.

We inferred from the clustering pattern that MBD2 knockdown acts on a specific subset of genes in breast cancer independently of global methylation changes, such as those induced by 5-aza-dC and that perhaps these two perturbations had unrelated and locus specific mechanisms of action.

Figure 29. Stable shRNA mediated knockdown of MBD2 in MCF-7 clones
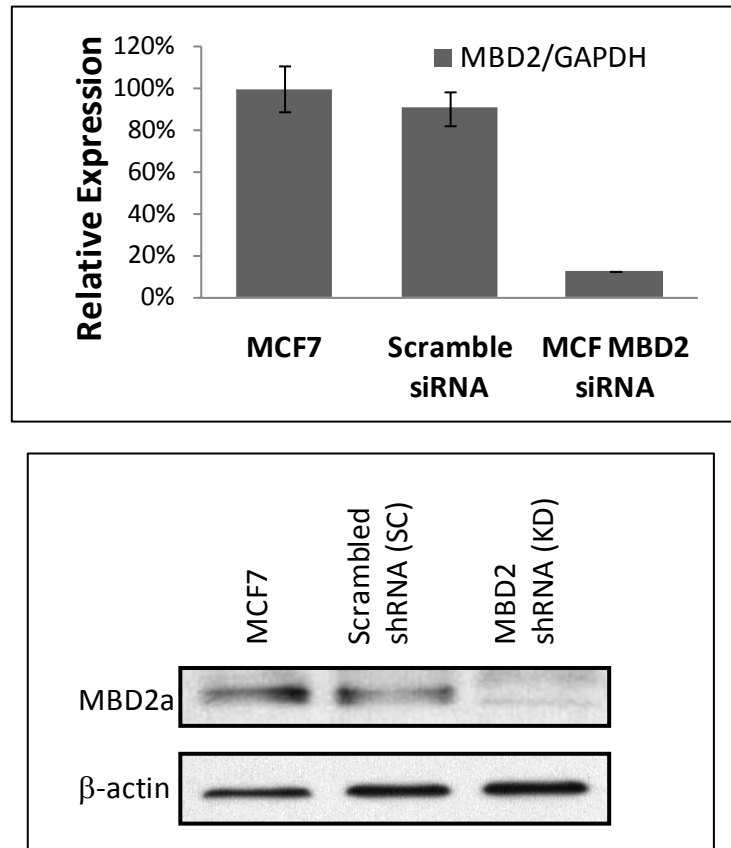
Figure 29. We selected and screened MCF-7 clones for high level knockdown of MBD2. A representative clone is shown, mRNA levels were measured by qPCR and immunobloting for MBD2 was used to confirm mRNA (above) and protein levels (below) in untransfected, scramble control transfected and MBD2 shRNA transfected cells.

98

Figure 30. Testing and standardization of qPCR array primers

cDNA Dillution

10^{-1} 10^{-2} 10^{-3} 10^{-4}

CLCA2
ADAM23
DIRAS3
KLK10
HIC1
PAX5
ESR2
ACTB
TWIST1
RASSF1
RARB
PLAGL1
MASPIN
GSTP1
GPC3
ATM
BCSG1
DAPK1
PGR
HOXA5
TGFBR2
LAMA5
APC
FHIT
TP53
CDH13
DAB2IP
TFAP2a
SYK
PRDM2
p16/CDKN2A
THRB
THBS1
RB1
CCND2
BRCA1
BAX
CDH1
ESR1
PYCARD
PTEN
PRSS8
TNFSF10
NME1
TIMP3
SFN
SCGB3A1
SOCS1
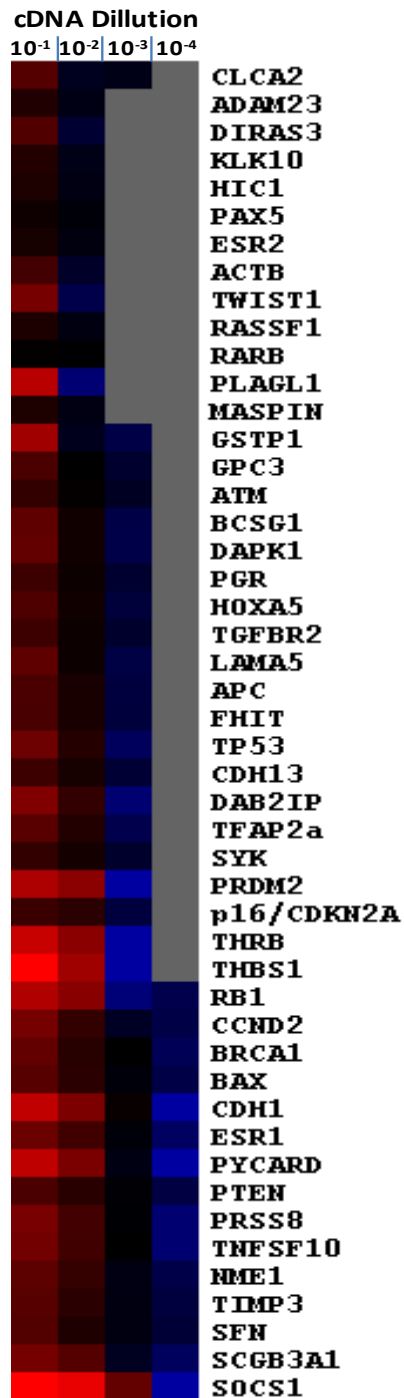
Figure 30. Quantitative PCR primers for epigenetically silenced breast cancer specific genes were validated using serial dilutions of MCF-7 cDNA. This figure shows the results of quantitative PCR gene expression analysis as a color coded heat map. Heat maps illustrate relative expression changes for each gene (rows) using a continuum of color, in this case blue (low expression) to red (high expression). Undetectable expression is shown in gray. Not surprisingly, many of these genes are expressed at low levels in wild type MCF-7 breast cancer cells. Only primers quantitative through at least an order of magnitude were used for subsequent tests.

99

Figure 31. Gene expression changes following MBD2 knockdown and 5-deoxy-Azacytidine treatment.
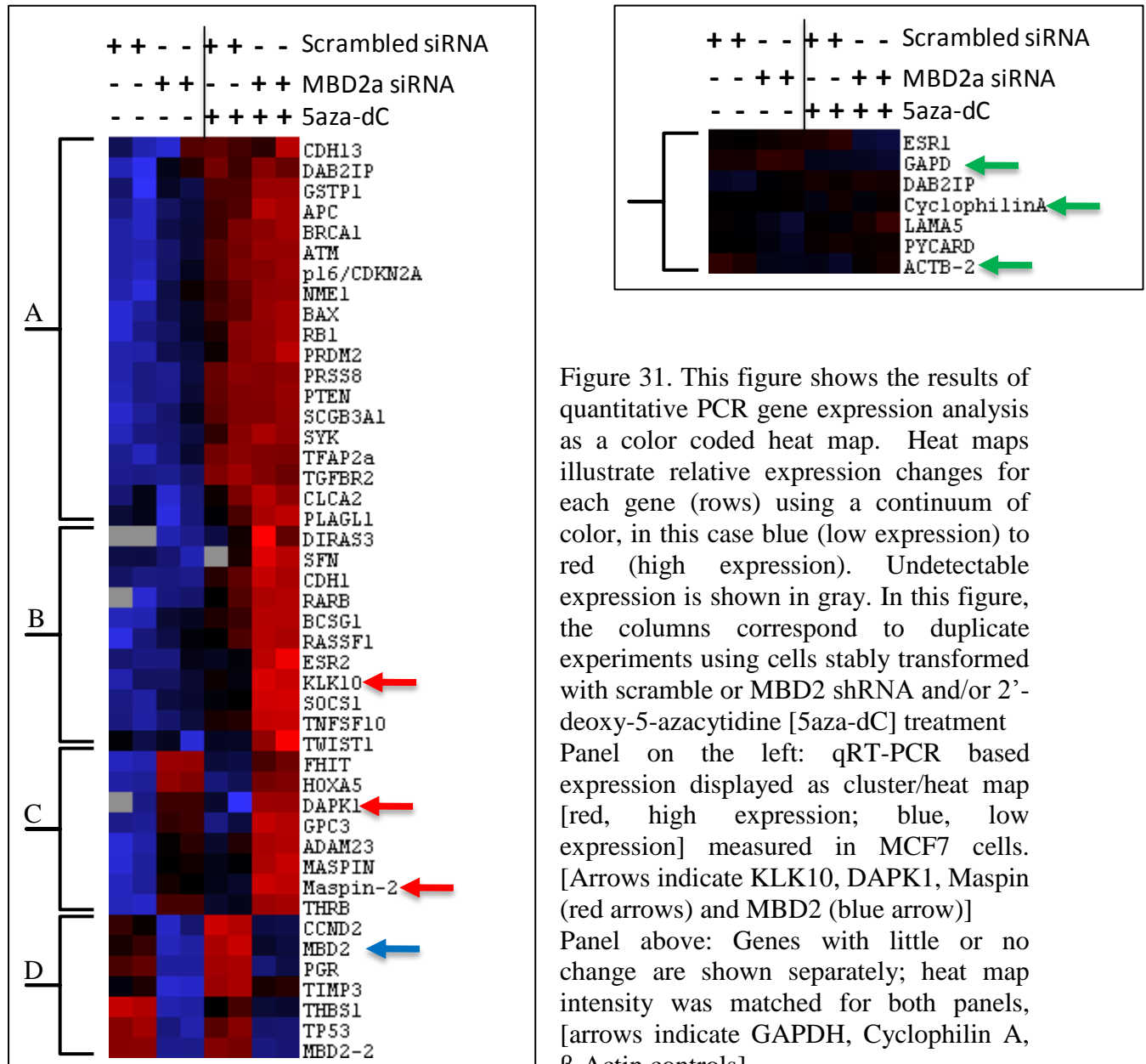
Figure 31. This figure shows the results of quantitative PCR gene expression analysis as a color coded heat map. Heat maps illustrate relative expression changes for each gene (rows) using a continuum of color, in this case blue (low expression) to red (high expression). Undetectable expression is shown in gray. In this figure, the columns correspond to duplicate experiments using cells stably transformed with scramble or MBD2 shRNA and/or 2'-deoxy-5-azacytidine [5aza-dC] treatment

Panel on the left: qRT-PCR based expression displayed as cluster/heat map [red, high expression; blue, low expression] measured in MCF7 cells. [Arrows indicate KLK10, DAPK1, Maspin (red arrows) and MBD2 (blue arrow)]

Panel above: Genes with little or no change are shown separately; heat map intensity was matched for both panels, [arrows indicate GAPDH, Cyclophilin A, β-Actin controls].

On the left, the gene clusters labeled A, B, C, and D correspond to differentially regulated groups of genes. Cluster A contains genes predominantly induced by 5aza-dC and relatively unaffected by MBD2 knockdown. Cluster B contains genes induced by 5aza-dC and MBD2 knockdown in additive fashion. Cluster C contains MBD2 dependant genes which were not induced by 5aza-dC. Finally, cluster D contains those genes which were downregulated by MBD2 shRNA, including MBD2 itself [Blue Arrow].

100

Tumor suppressor gene expression is altered in growth restricted lentivirally transduced breast cancer cell lines.

We used high throughput quantitative PCR as described above to examine the expression of epigenetically silenced genes in lentivirally transduced MBD2 knockdown and scramble control SK-BR-3, MDA-MB-231, and MDA-MB-435 cells. These cells were of particular interest because of the changes in *in vitro* and *in vivo* growth described in Chapter I. We found that, in similar fashion to our previous gene expression analyses, specific subsets of tumor suppressor genes from the panel of genes tested were reexpressed in MBD2 knockdown cells (Figure 31). Not surprisingly, there was some degree of cell line specificity with respect to MBD2 induced changes in gene expression, for instance DAPK1 was induced in MDA-MB-231 and MDA-MB-435 cells but not in SK-BR-3 cells. Other MBD2 responsive genes identified in our initial examination of MCF-7 cells were responsive in all three of the lentivirally transfected cell lines (e.g., Maspin, KLK10, GPC3 and THRB). Of all the genes examined, Maspin, DAPK1, and KLK10 were the most uniformly and/or robustly induced tumor suppressors, raising the possibility that these genes may play a role in mediating MBD2 dependent growth inhibition of breast cancer cells. Once again control genes (Cyclophilin A and GAPD) were used for normalization and as before the expression level of these genes remained relatively constant across all samples.

101

Figure 32. Tumor Suppressor Gene Expression Changes in Lentivirus Transduced Breast Cancer Cell Lines.
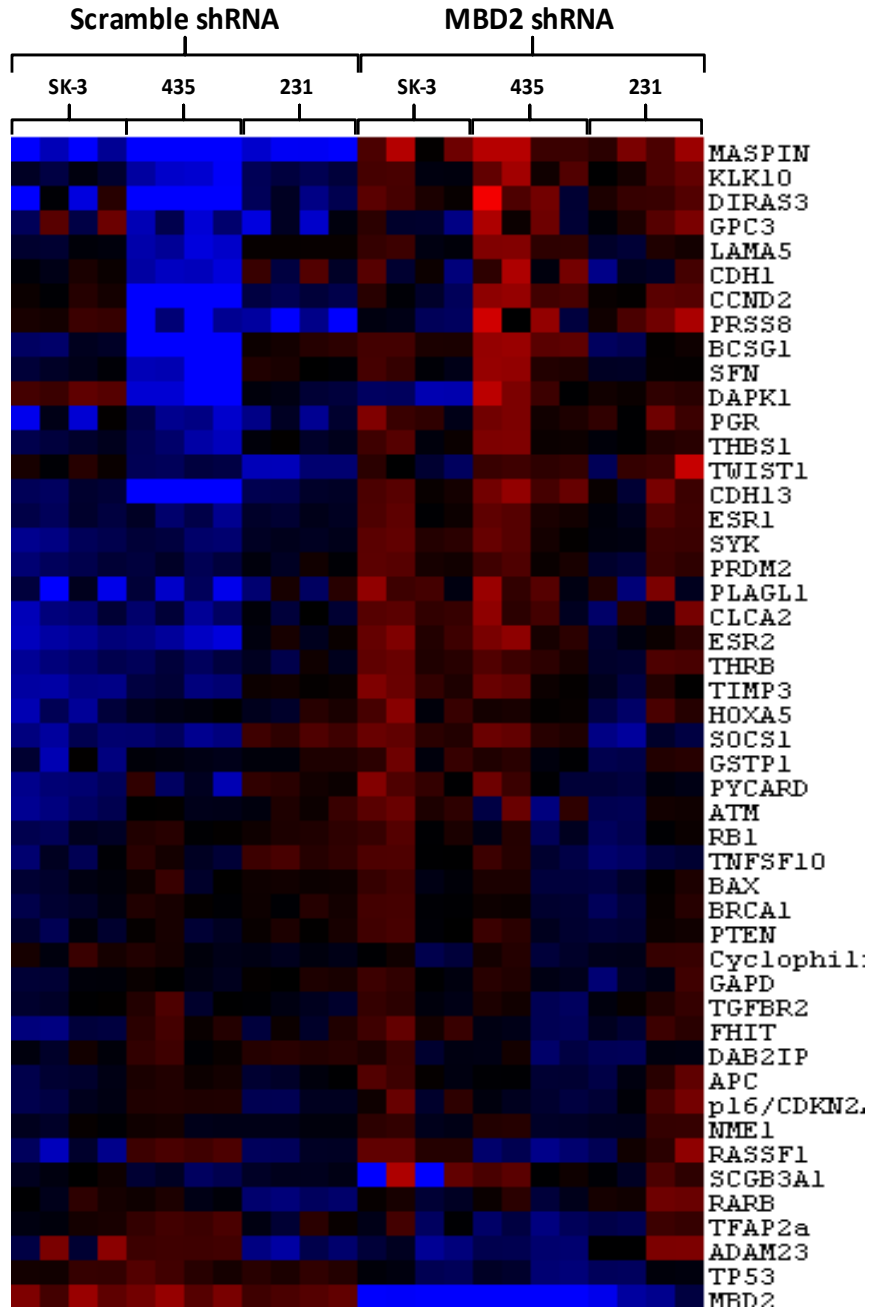
Figure 32. Quantitative RT-PCR was used to measure the gene expression levels of known hypermethylated and transcriptionally silenced genes in breast cancer. Three cell lines which demonstrated significant growth inhibition were analyzed, MDA-MB-231, MDA-MB-435, and SK-BR-3. Scramble controls are shown in the left half of the figure and knockdowns are on the right. Red is used to show relatively high expression and blue for relatively low expression for each gene. GAPD and Cyclophilin A control genes were included and cluster near the lower middle region, corresponding to relatively little change across samples. MBD2 levels (confirming knockdown) are shown in the last row.

102

MBD2 target genes vary independently of each other but knocking them down individually does not rescue MBD2 dependent morphological differences.

We attempted to identify the MBD2 target gene responsible for mediating the epithelial morphology changes by using siRNA to knockdown each gene in the MBD2 dependent cluster individually. We transfected MBD2 knockdown MCF-7 cells with double stranded siRNA oligonucleotides targeted against each gene in the MBD2 dependent cluster and verified the magnitude and specificity of the resulting gene knockdowns by quantitative RT-PCR. The genes in the MBD 2 dependent cluster are listed in Table 8 along with the siRNA target sequences used to knock them down individually. We found that the siRNAs were both effective and specific for each gene (Figure 33). It was clear from our analysis that MBD2 target genes varied independently of each other; only one target gene was affected in each case. The significance of this observation was to confirm that these genes were indeed downstream targets of MBD2 rather than indirect targets of one another. We next sought to determine if any of the MBD2 responsive genes were mediating the changes in the morphology of these MBD2 knockdown MCF-7 cells by carrying out 3D culture assays on each siRNA transfected population. We found that while we were able to specifically knock down the individual genes, no single tumor suppressor target we tested was able to reverse the organized growth of MBD2 knockdown cells (Figure 34). This finding suggested to us that the morphology changes we observed in these cells were being driven by another MBD2 dependent effector, or more likely, multiple downstream effectors.

103

Table 8. siRNA oligonucleotide sequences

Gene	Description	Accession	siRNA Sequence
ADAM23	ADAM metallopeptidase domain 23	NM_003812	CGCGGGTGACATTTCACTATA
DAPK1	death-associated protein kinase 1	NM_004938	AAGCATGTAATGTTAATGTTA
FHIT	fragile histidine triad gene	NM_002012	TTCGCTCTTGTGAATAGGAAA
GPC3	glypican 3	NM_004484	CAGCCGAAGAAGGGAACTAAT
HOXA5	homeobox A5	NM_019102	CCTCTCCGAGAGACAAATTAA
SERPINB5	serpin peptidase inhibitor, clade B (ovalbumin), member 5	NM_002639	GCCGTTGATCTGTTCAAACAA
THRB	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)	NM_000461	TCCAGAATGATTACTAACCTA

Table 8. Listed in this table are the seven MBD2 dependant genes found in cluster C of Figure 31. MBD2 target gene names, description are shown on the left and the siRNA sequences used to knock these genes down individually are shown on the right.

104

Figure 33. siRNA mediated knockdown of individual target genes
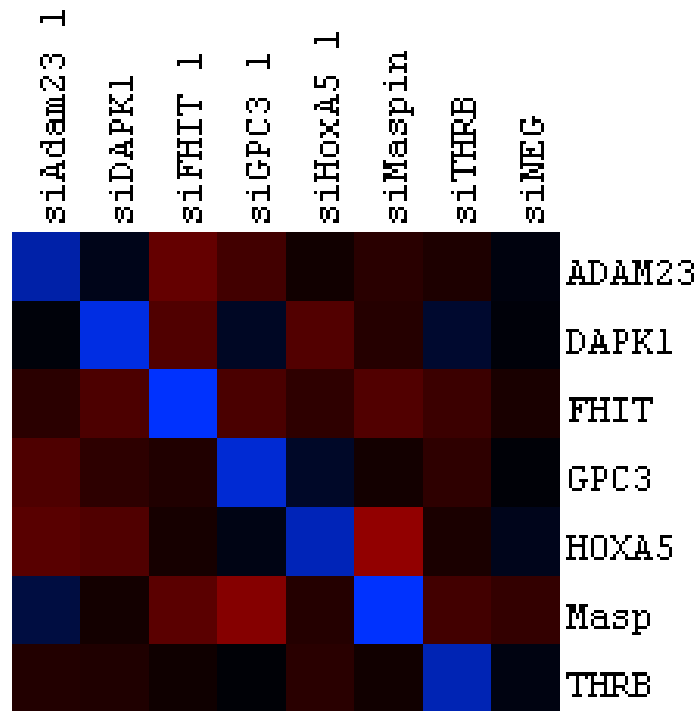
Figure 33. Transfection of stable MBD2 knockdown lines with short double stranded RNA oligonucleotides (siRNA) led to specific silencing of downstream target genes. A negative (non-targeted) siRNA oligonucleotide was used as a control (siNEG). All siRNA oligo based knockdowns were performed using MBD2 KD MCF-7 cells at approximately 80% confluence in a 24 well tissue culture plate. Each transfection was performed using 20 pico moles of siRNA (Qiagen) and 1 ul of Lipofectamine 2000 transfection reagent (Invitrogen) diluted in Opti-MEM serum free media (Gibco).

Figure 34. Acini formation is not affected by individual target gene knockdown.
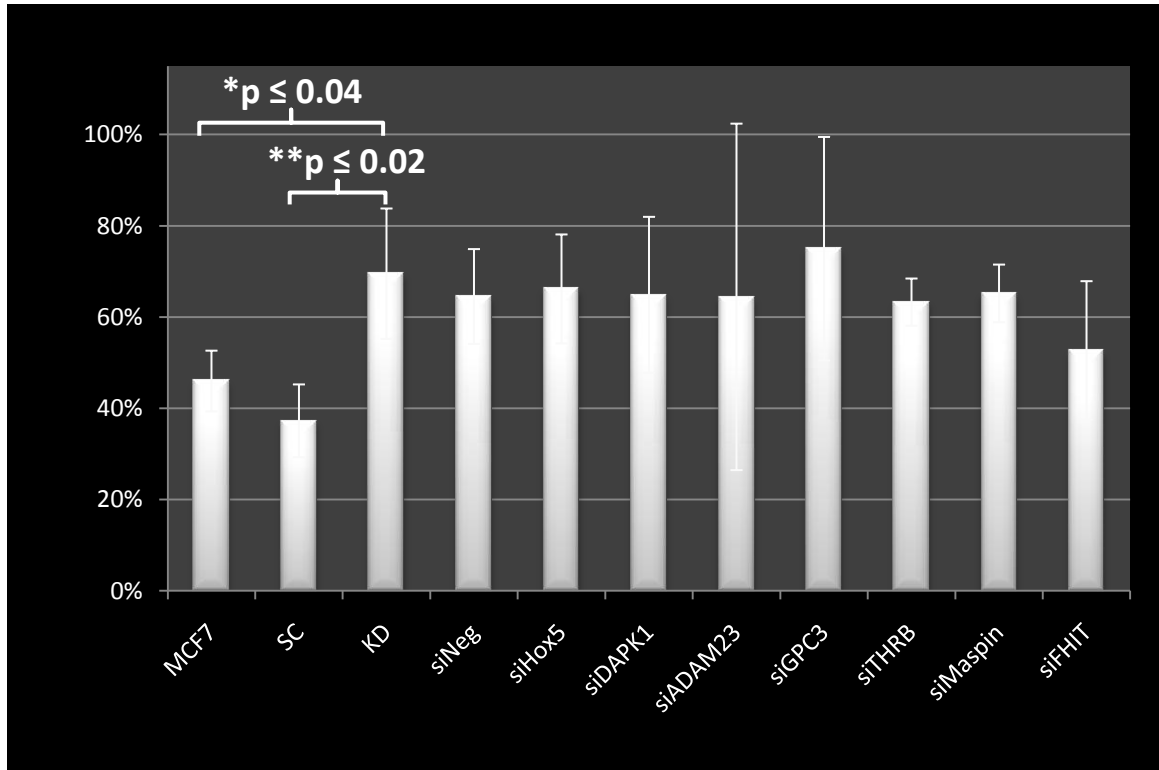
Figure 34. Mammosphere formation assay using MBD2 target gene knockdown cells. As described above, we measured percent mammosphere formation in our target gene siRNA treated lines in order to determine whether any specific gene was responsible for the increased proportion of mammospheres in knockdowns lines (Chapter I). No significant reversion to disorganized growth was observed in any of the MBD2 target gene knockdown populations.

106

<div align="center">

Discussion

</div>

MBD2 dependent gene repression in breast cancer

The only recognized function of MBD2 in humans is its role in binding to genomic cytosine methylation and initiating transcriptional repression through downstream changes in chromatin. As discussed throughout the current work, the obvious mechanism of action of MBD2 in aggravating breast cancer is the inhibition of tumor suppressor gene expression. Until now, however, a link between MBD2 function and breast cancer pathogenesis has not been made. With this in mind, we sought to prove that MBD2 knockdown restored the expression of tumor suppressor genes in breast cancer and determine whether these genes were mediating any MBD2 dependent phenotypes. We went about this by probing the mRNA levels of putative MBD2 target genes in breast cancer cell lines, both MBD2 knockdown and control. We found that the expression of specific tumor suppressors known to be silenced in breast cancer was increased with MBD2 knockdown. Moreover, we found that many of the genes induced by MBD2 knockdown were different from those induced by 5-aza-dC. Given the known differences in cytosine methylation between individual tumors, it was not surprising to learn that the gene expression changes induced by MBD2 varied somewhat between cell lines. Though we currently lack sufficient evidence to support a central role for any single downstream gene in mediating MBD2 dependent phenotypes, it was interesting that several genes were consistently re-expressed in all cell lines which demonstrated growth inhibition. As discussed further below, our observations leave some doubt as to whether a single critical target is responsible for mediating the full spectrum of MBD2 activity in breast cancer.

107

A quantitative PCR assay for epigenetic silencing in breast cancer

The gene expression analyses described in this chapter was performed using SYBR green based quantitative PCR. This methodology was chosen over other global gene expression profiling approaches (e.g., including microarrays, SAGE, or massive multi-parallel sequencing) for several reasons. The first reason was one of practicality; we have all the equipment and reagents to perform high-throughput 384 well quantitative PCR in our laboratory (ABI Prism 7900 HT, Applied Biosystems). In addition, this technique requires the lowest initial investment while providing the highest quantitative reliability. Case in point: microarray results, even of the highest significance, must be subsequently verified by quantitative PCR in order to be considered valid; as such we went straight to the current gold standard for quantitative gene expression analysis. Most importantly, previous experience with microarrays in our laboratory (Rupon et al. 2006) demonstrated a significant limitation of this approach. The strength of microarrays is that they provide parallel gene expression data for large set of genes in contrast to PCR based assays which are primer dependent. However, we were interested specifically in the direct effects of MBD2 on hypermethylated tumor suppressor genes. We found previously that microarrays include a considerable amount of noise from secondary and tertiary genes which are not directly regulated by MBD2. Sifting through this data to identify candidate genes can be time consuming and ultimately fruitless.

Importantly, the list of genes reported to be transcriptionally inactivated by promoter hypermethylation in breast cancer remains relatively limited (Hoque et al. 2009; Esteller 2005). We discovered that we could adequately cover this set of genes, the targets of highest interest to us, using high throughput quantitative PCR. The list of genes we

108

chose for our analysis is given in Appendix A along with their putative functions and the pathways in which they have been shown to interact.

We must concede an obvious limitation of our methodology: analyzing a restricted set of genes will, by definition, generate an *a priori* biased data set. In contrast to unbiased genome wide approaches (e.g., ESTseq, Affymetrix Human Genome Arrays), we are unable to comprehensively evaluate transcriptome level changes. Moreover, it is quite likely we have omitted interesting primary target and secondary effector genes from our analysis. However, this analysis is more than adequate considering our stated purpose: to evaluate whether MBD2 maintains transcriptional silence of a specific set of tumor suppressor genes in breast cancer. Despite its limitations, our gene expression analysis has successfully met this objective and in the process provided interesting and novel information regarding the unique function of MBD2 in breast cancer.

MBD2 and 5-aza-deoxy-cytidine activate different genes in breast cancer

The DNMT inhibitor, 5-aza-dC, is a nucleotide analog that is incorporated into genomic DNA and acts to irreversibly inhibit methylation by forming covalent adducts with catalytic residues in the active site of DNA methyltransferase enzymes. While there remains some question as to whether the efficacy of 5-aza-dC in cancer depends on its effect on methylation or the non-specific effects of protein-DNA adduct formation, it is accepted that this compound leads to passive, replication dependent demethylation and changes in gene expression (Patra and Bettuzzi 2009). Because of the toxicity and carcinogenicity associated with 5-aza-dC, the present work is geared toward the development and testing of an alternate strategy for reactivating methylated tumor

109

suppressor genes in breast cancer. Consequently we were interested in comparing the gene expression changes induced by MBD2 knockdown to those induced by 5-aza-dC treatment. We found that MBD2 and 5-aza-dC did indeed act on different subsets of genes in MCF-7 cells. As expected, the widely acting 5-aza-dC induced the transcription of a larger number of genes than MBD2 knockdown, in fact more than twice as many out of those tested (85% vs 35%). From the panel of 48 genes only 10 (21%) were additively induced by MBD2 knockdown and 5-aza-dC. Only 7 genes (15%) were induced by MBD2 knockdown alone (Table 8). Finally, while none of the genes tested were found to be repressed by 5-aza-dC, 5 genes (11%) were repressed in MBD2 knockdown cells. We presume this last group of genes are indirect targets; though highly unlikely given what is known about the function of MBD2, we cannot completely rule out the possibility that MBD2 may act as an activator of transcription at certain loci. Taken as a whole, these data support our initial hypothesis that MBD2 knockdown is a more targeted method for reactivating methylated tumor suppressor genes than treatment with 5-aza-dC. Implicit in these results is the idea that these two strategies can have independent, gene specific mechanisms of action and that MBD2 binds and represses a specific subset of methylated tumor suppressor genes in breast cancer leading to more targeted gene disruption in tumors.

MBD2 maintains transcriptional silence of a specific subset of tumor suppressor genes

As discussed above, MBD2 was found to act specifically at a subset of tumor suppressor genes which accounted for only 35% of those tested. If we assume that all MBD2 and 5-aza-dC induced genes are methylated in MCF-7, and that all the MBD2 induced genes are direct targets of MBD2, then the percentage of methylated genes bound by MBD2 is 47%. Extrapolating further from this assumption, approximately half of the tumor suppressor genes methylated in breast cancer are targets of MBD2 mediated repression. This extrapolation is in line with previous reports of the high affinity of MBD2 for CpG rich tumor suppressor promoters (which represent approximately half of all promoters) in cancer (Lopez-Serra et al. 2008a; Fraga et al. 2003; Ballestar 2003) and is a provocative indicator of the fundamental role of MBD2 in breast cancer pathogenesis.

An important point needs to be stressed here regarding the results presented in this section and their implications. It is clear that MBD2 inhibition is able to restore tumor suppressor expression in a targeted way. This targeted gene restoration is both effective in limiting the growth of breast cancer (Chapter I) but equally important is the inherent source of selectivity built into the mechanism of action of MBD2. By reactivating a subset of tumor suppressor from a pool which is pathologically hypermethylated only in cancer, MBD2 holds promise as a target for selective killing of tumor cells without affecting the viability of normal tissues.

111

MBD2 regulated genes vary between breast cancer cell lines

In addition to a detailed analysis of gene expression changes in the MCF-7 cell line, we conducted parallel gene expression studies in 3 other human mammary epithelial lines: MDA-MB-231, MDA-MB-435 and SK-BR-3. These cell lines were of interest because in contrast to MCF-7 they demonstrate substantial growth inhibition. It is worthwhile to note that all three of these lines are considered somewhat more aggressive in terms of their growth and tumorigenic potential in animal models (Perou et al. 2000; Sorlie et al. 2001). It can be inferred here that increased tumorigenicity correlates with a greater degree of genetic and epigenetic dysregulation. Based on this assumption, the fact that these lines are more sensitive to MBD2 knockdown is intriguing and supports the selectivity hypothesis we have put forward: targeting epigenetic silencing of tumor suppressors will have the greatest destabilizing effect in those cells with the greatest degree of abnormal methylation, i.e., aggressive breast cancer cells.

We found that the pattern of gene expression changes varied to some degree between all the breast cancer cell lines we compared following MBD2 knockdown. This observation is consistent with the idea that hypermethylation events occur following a stochastic model that will vary between tumors. Another possible explanation for the variability observed is the differential expression of transcription factors and epigenetic modifiers in cell lines of distinct origin. Recall that MBD2 knockdown in and of itself is not an activator of transcription; this perturbation is more accurately thought of as the removal of a component of the repressive machinery. In order for this removal to lead to the robust transcriptional activation of a particular hypermethylated target, the relevant activating machinery must be intact in a given cell. This activating machinery consists of

112

sequence specific transcription factors, targeting components of the transcriptional machinery, and epigenetic modifiers that act in opposition to MBD2, for example histone acetyl transferases (HATs). As a result, variability in these factors is likely to be influencing the pattern of gene expression in MBD2 knockdown cells.

Do MBD2 dependent gene expression changes underlie phenotype?

An interesting phenomenon we currently have no explanation for was the repression of several tumor suppressor genes by MBD2 knockdown. It is likely that this repression is indirect, i.e., through the action of an intermediary MBD2 induced genes. In this group of genes, i.e., MBD2 induced genes, one stood out to us and bears mentioning here, p53. This tumor suppressor is mutated in nearly half of all human cancers and is often referred to as the 'guardian of the genome' for its role as a critical checkpoint protein in halting growth and promoting apoptosis of transformed cells. In our analysis, we found that p53 was uniformly down regulated in MBD2 knockdown breast cancer cells. The significance of this finding is not entirely clear, particularly given the fact all the lines we examined with the notable exception of MCF-7 bear well described mutations in p53 (Petitjean et al. 2007). Though opposite in character from the expression change one might assume would be protective, this observation is in fact consistent with growth inhibition in the setting of mutant p53. Bearing in mind that mutant p53 can acquire a gain of function whereby it can exacerbate pathological phenotypes, it is interesting to speculate whether MBD2 knockdown may in fact be acting somehow indirectly through the downregulation of mutant p53 in breast cancer. This observation is further unique in

113

that upregulation of mutant p53 is typically due to increased protein stability, not increases at the transcriptional level.

Among the genes we examined, several stood out as uniformly induced by MBD2 knockdown in all cell lines examined. Among these genes were Maspin (Mammary Associated Serine Protease Inhibitor) and KLK10 (Kalikrein 10). Epigenetic silencing of of these genes has previously been shown to be pathologically involved in the progression of breast cancer (Khalkhali-Ellis 2006; Zhang et al. 2006). We became interested in the possibility that all or some of the phenotypic consequences of MBD2 knockdown were mediated through one of these targets. However, as discussed below, we have been unable to show that MBD2 acts through a single target and it is more likely that the growth inhibition and morphologic changes induced by MBD2 knockdown are the result of the combined effects of multiple downstream target genes.

MBD2 likely acts through multiple targets to affect the phenotype of breast cancer

We maintain that the effects of MBD2 knockdown on the behavior of breast cancer cells are mediated through target gene expression changes and we have shown that MBD2 is indeed acting as a modifier of methylated tumor suppressor gene induction. In order to evaluate the importance of individual MBD2 target genes, we used siRNA directed against the 7 exclusively MBD2 induced genes in MCF-7 knockdown cells and tested whether any of the resulting knockdown lines reverted to the abnormal morphology characteristic of control (MBD2 expressing) MCF-7 cells. We found that none of the single target gene knockdowns was effective in 'rescuing' MBD2 knockdown induced changes. This analysis was, however, limited to the morphologic changes in

114

MCF-7 cells and we have not yet addressed the growth inhibition in other breast cancer cell lines in a similar manner.

We cannot discount an important limitation inherent in our siRNA based knockdown strategy, namely the transient nature of the resulting gene repression. We know from our earlier studies that stable MBD2 knockdown, lasting weeks rather than days, is required to bring about the full magnitude of the growth inhibition we observed. It is quite possible that a similar, sustained knockdown of MBD2 induced genes is required to reverse these effects.

Taken at face value, we interpret the failure of MBD2 target gene knockdown to rescue phenotype to mean one of two things. First, it is possible that a gene not included in our panel (and therefore one which we remain unaware of) is responsible for mediating MBD2 knockdown induced morphologic changes. The second explanation and the one we consider to be more likely based on general probability and the pleotropy exhibited by cell lines, is that the combined effect of multiple downstream targets underlie MBD2 dependent changes. It is experimentally difficult to address multiple targets directly in a rescue experiment; we are currently conducting studies which involve the restoration of a shRNA immune MBD2 mutant to verify that the changes we are observing are indeed mediated solely by lack of MBD2 function in breast cancer.

We have shown that MBD2 is required for maximal growth of breast cancer cells, abnormal morphology of poorly differentiated populations, and for the transcriptional

115

repression of pathologically methylated tumor suppressor genes. Up to this point we have considered MBD2 largely in isolation from other aspects of epigenetic dysregulation in breast cancer. While a useful model for evaluating the specific function of MBD2, this reductionist approach artificially discounts the complex network of events that occur simultaneously to drive pathogenesis of breast cancer. Among these events are changes in CpG methylation and feedback regulation of and by other epigenetic modifiers, notably other methyl binding proteins and DNA methyltransferases. As detailed in the following chapter, an examination of these interconnected elements confirms important coregulatory phenomena are indeed occurring.

Chapter III: The interplay of transcription, methylation, epigenetic factors and MBD2 target genes

Introduction:

DNA Methylation in different species

Non-vertebrate species have very low levels of methylation. *Neurospora Crassa* for example, has low levels of methlylation which are thought to correspond to ancient transposition events (Selker et al. 2003). *Neurospora* utilizes DNA methylation which is dependent on the H3K9 DNA methyltransferase dim-5, perhaps establishing a functional convergence at the invertebrate level between histone and DNA methylation. (Tamaru and Selker 2001). It is interesting that *Drosophila Melanogaster* whose genome has very high levels of mutation from transposable elements, has very low levels of methylated DNA (Yoder, Walsh, and Bestor 1997). Despite the low levels of methylation, two potential methylase-like genes have been indentified in Drosophila. Of these two genes, deletion of Dnmt-2 in Drosophila resulted in complete abolishment of methylation. In contrast, the overexpression of Dnmt-2 resulted in hypermethylation at CpT and CpA dinucleotides (Kunert et al. 2003). Drosophila also contains a single homolog that corresponds to mammalian MBD2/3 (Roder et al. 2000). GST-fusions and two hybrid experiments have revealed that the Drosophila MBD2/3 interacts with Mi-2/NuRD complex via p55 and Mi-2 subunits (Marhold, Brehm, and Kramer 2004). Bandshift experiments using MBD2/3 and the mammalian homolog MBD2 demonstrated that the

117

fly MBD2/3 interacted with CpA and CpT methylated oligonucleotides, but could not bind CpG methylated probes. These experiments may indicate that Drosophila MBD2/3 is similar in function to human MBD2, however it is not fully understood whether MBD2/3 targets Mi-2/NuRD complexes to CpA/T methylation sites or if it facilities other protein-protein or protein-DNA interactions. In Drosophila, it is likely that DNA methylation plays a more important role during embryonic development. Liquid chromatography has demonstrated that the highest levels of DNA methylation occur during early stages of fly embryogenesis (Lyko, Ramsahoye, and Jaenisch 2000).

Compared to Drosophila, other insect species such as cabbage moth *Mamestra brassicae*, the peach potato aphid *Myzus persicae*, and the mealy bug *Planococcus citri* exhibit somewhat higher levels of DNA methylation (Field et al. 2004). In the honeybee *Apis Melifera*, DNA methylation plays an important role in the division of social structures. Honeybee larvae treated with small interfering RNA targeting a de novo DNA methyltransferase, Dnmt3, developed into queens with fully functional ovaries, while control RNAi treated larvae did not (Kucharski et al. 2008).

In stark contrast to invertebrates and fungi which are virtually methylation free, vertebrate genomes are highly methylated (Tweedie et al. 1997). *Xenopus laevis* and zebrafish, *Danio rerio*, have a high content of genomic DNA methylation as well as functional DNMTs and MBDs (McGowan and Martin 1997; Stancheva and Meehan 2000a; Veenstra and Wolffe 2001). Mammals use DNA methylation for parental imprinting; that is allele-specific regulation in which parental origin determines expression of a single allele (Reik, Dean, and Walter 2001b). Put another way, imprinting is able to affect the differential expression of maternal and/or paternal alleles

118

based on a DNA methylation pattern established during sex specific gametogenesis. Several severe diseases such as Beckwith–Wiedemann, Prader–Willi, and Angelman syndromes and even various types of cancer result from defects in imprinting (Bittel and Butler 2005; Lalande and Calciano 2007).

Dynamics of DNA methylation during development

During mammalian embryogenesis the de novo methyltransferases, DNMT3a and DNMT3b methylate previously unmethylated CpG sequences. A third methyltransferase called DNMT1 functions both as a maintenance methylase, copying the pre-existing methylation marks onto the new strand during replication and as a de novo DNA methyltransferase (Jeltsch 2006; Fatemi et al. 2002; Gowher et al. 2005). The precise mechanism of targeting of de novo methylation is not fully understood, however it is clear that the DNMT family of methyltransferases are critical for normal embryonic development.

Knock-out studies of DNMT1 in embryonic stem (ES) cells and in mouse embryos resulted in significantly reduced levels of DNA methylation. The loss of DNMT1 proved to be lethal with the majority of embryos not passing mid-gestation, although the ES cells remained viable and proliferative (Li, Bestor, and Jaenisch 1992b). Similar experiments involving DNMT1-knockouts in *X. laevis* embryos, resulted in a hypomethylated genome and the embryos displayed a premature expression pattern of several mesodermal markers (Stancheva and Meehan 2000b). The knockouts also exhibited p53-induced apoptosis and embryonic lethality (Stancheva, Hensey, and Meehan 2001). Similar observations have been made on cultured fibroblasts derived from conditional DNMT1

119

mouse knockouts that undergo p53-dependent programmed cell death (Jackson-Grusby et al. 2001). Theses DNMT1-depleted fibroblasts showed a reactivation of placental and germ line markers. These results suggest an important role for DNMT1, and therefore CpG methylation, in establishing a footprint of tissue-specific gene expression during and after embryonic development.

Knockdown studies of DNMT1 in zebrafish appeared to recapitulate the effects observed in mice and *Xenopus*. Approximately 40% of the embryos died as a result of DNMT1 depletion (Rai et al. 2006). An important finding of this study was that the response to DNMT1 deficiency is largely organ-specific. One of the most affected organs was the gut. The reduced intestinal differentiation also caused the loss of expression of *fabp2*, a marker of terminally differentiated epithelial cells. Markers of eye development such as *otx-2* and *otx-5* appeared to be expressed at similar levels in both control embryos and DNMT1 knockdowns. Histological evidence did suggest a severe disorganization of retinal structures.

Collectively these studies have emphasized the importance of DNMT1, and indirectly CpG methylation, in establishing a blueprint for tissue-restricted gene expression during development. DNMT1 has been reported to interact with methyl-CpG binding proteins as well as with HDACs and histone methyltransferases to repress transcription (Kimura and Shiota 2003; Fuks et al. 2003; Tatematsu, Yamazaki, and Ishikawa 2000). In addition, DNMT1 has been found to interact with the Rb tumor suppressor protein to repress transcription from promoters containing E2F binding sites. This interaction serves to link DNMT1 to a growth regulatory pathway that frequently is

120

disrupted in cancer (Robertson and Wolffe 2000). To date however, no association between DNMT1 and MBD2 has been established in human malignancy.

Demethylation and remodeling of methylation patterns in development

DNA methylation patterns are both stable and heritable in somatic cells. There are two distinct periods during mammalian development when the normally stable DNA methylation patterns are dynamically remodeled. The first global remodeling of DNA methylation occurs during gametogenesis (Mann and Bartolomei 2002). During gametogenesis there is an erasure of DNA methylation marks, which serves to reset the existing imprinting in gametes. This demethylation event is followed by a wave of remethylation which is necessary to reestablish the parental imprint. The second global remodeling of DNA demethylation occurs following fertilization during the preimplantation period of the embryonic development. This demethylation does not appear to affect imprinted regions established during gametogenesis. In contrast to the DNA methylation patterns in the oocyte, sperm DNA is highly methylated (Morgan et al. 2005). The high level of methylation correlates with its inactive chromatin state and compact structure. Immunohistochemistry and bisulfite conversion experiments in mice showed that the male pronucleus gets rapidly demethylated shortly after fertilization, while the maternal genome displays a slow but progressive drop in DNA methylation levels consistent with passive demethylation (Oswald et al. 2000; Mayer et al. 2000). Later during implantation, the global DNA methylation levels of both the paternal and the maternal contributions to the genome steadily increase. Similar developmental changes

121

involving a wave of demethylation followed by increasing levels of DNA methylation post fertilization have also been described in other placental mammals (Dean et al. 2001).

Despite the general observation regarding changes in global methylation, any direct observations of active demethylation remain controversial. Moreover, it is not fully understood how DNA methylation and demethylation are targeted or regulated. The physiological role of DNA demethylation in vertebrate development is also speculative. One recent study in zebrafish embryos identified demethylase activity involving an AID deaminase, MBD4 glycosylase, and Gadd45a during the late gastrula to segmentation stage (Rai et al. 2008). The protein Gadd45a has also been implicated with other demethylation events associated with DNA repair (Barreto et al. 2007). It is interesting to note that Gadd45a knockout mice did not exhibit any global or site specific hypermethylation which leaves the role of Gadd45a in demethylation in dispute (Engel et al. 2009). Similarly, a controversial DNA demethylase function has been proposed for MBD2 and MBD3 (Bhattacharya et al. 1999; Brown et al. 2008; Hamm et al. 2008). The biochemical energetics of direct demethylation of 5-methyl-cytosine (i.e., the direct enzymatic cleavage of the sigma carbon-carbon bond of the 5- methyl group without partial or complete reclamation of the heterocyclic ring) are unfavorable casting doubt on the possibility that MBD2 or MBD3, or any other single protein for that matter functions in this capacity (Klose and Bird 2006; Berger and Bird 2005a; Hendrich et al. 2001; Ginder, Gnanapragasam, and Mian 2008). An active process of demethylation likely does exist, given the rapid waves of global demethylation during development. A more plausible mechanism than direct demethylation is likely to involve a cooperative

deaminase/glycosylase (i.e., AID/MBD4) step followed by resolution of the apyrimidinic site by the nucleotide excision repair machinery (Rai et al. 2008).

Another look at the MCBPs and their specific roles in gene repression

MBDs and Kaiso-like proteins are the two major families of methyl CpG binding proteins in mammals. MeCP2 was the first methyl-CpG binding protein discovered during a screen to identify factors that bind to unmethylated DNA. Instead, protein factors, initially named MeCP1 and MeCP2, that bind specifically to methylated DNA were isolated (Meehan et al. 1989). MeCP2 is a 53-kDa protein containing a N-terminal methyl-CpG binding domain (MBD) and a C-terminal transcriptional repression domain (TRD) (Nan et al. 1998a). Later screens based on mammalian expressed sequence tag (EST) database homology searches for sequences encoding a conserved MBD domain led to the identification of four additional proteins currently known as MBD1, MBD2, MBD3 and MBD4 (Hendrich and Bird 1998b). The MBD family proteins, including MeCP2, are highly conserved in all vertebrates (Clouaire and Stancheva 2008). MBD2 and MBD3 are closely related to each other and share 77% identity outside the MBD domain (Hendrich et al. 2001). It is likely MBD2 and MBD3 represent the ancestral MBD family founders since a homologous MBD2/3-like protein is present in invertebrates, including Drosophila, where low levels of DNA methylation are detectable only in early development (Marhold, Brehm, and Kramer 2004). All MBD proteins, except MBD3, specifically recognize and bind to methylated DNA in vitro and in vivo (Hendrich and Bird 1998a). Mammalian MBD3, contains a critical mutation in the MBD domain and therefore does not bind preferentially to methylated DNA.

123

Figure 35. The Methyl Binding Proteins
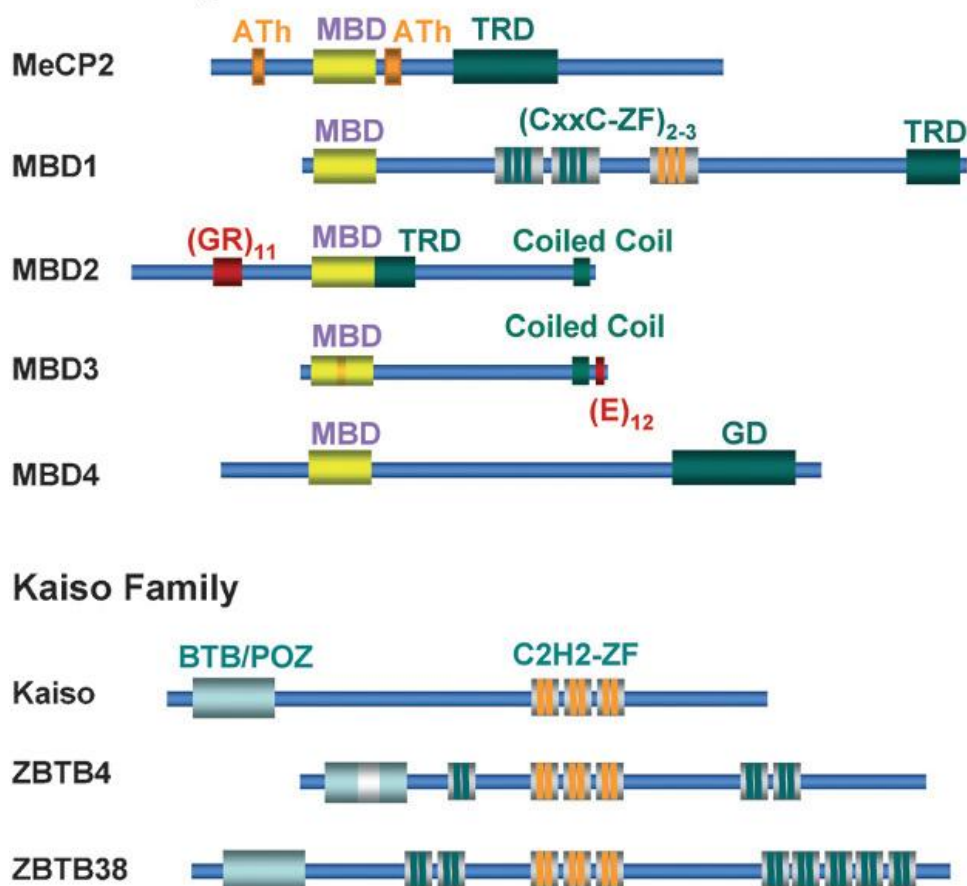
Figure 35. Families of methyl-CpG binding proteins. MBD family proteins share a conserved MBD domain, which is required for binding to methylated DNA. MBD3 carries a mutation (shown in orange) in the MBD domain and does not bind to methylated CpGs. MeCP2 has two AT-hook motifs (ATh) which potentially could bind AT-rich DNA. These motifs are not required for highaffinity binding to sequences containing a methylated CpG followed by an [A/T]4 run. MBD1 is characterized by two (or three in some isoforms) CxxC-type zinc fingers. The third CxxC motif (orange) binds unmethylated CpGs. TRD indicates transcriptional repression domains mapped by functional and deletion analyses. GD indicates the glycosylase domain of MBD4, which is involved in excision of CG:TG mismatches. The (GR)11 motif of MBD2 is a stretch of glycine and arginine residues that can be methylated by PRMT5 protein methylase [98]. (E)12 is a glutamate rich domain. The Kaiso family of proteins is characterized by three homologous C2H2 zinc finger motifs that are required for binding to methylated and in some instances unmethylated DNA. In addition, all proteins of this family carry a BTB/POZ domain likely to be involved in either homo- or heterodimerization or protein-protein interactions. ZBTB4 and ZBTB38 have additional three and seven, respectively, zinc finger motifs. (Figure from T Clouaire and I Stancheva. Cell. Mol. Life Sci. (Clouaire and Stancheva 2008)

124

The individual MBD Proteins and their Function in Transcriptional Repression

MeCP2

MeCP2 is a potent transcriptional repressor and studies have suggested that MeCP2 might serve as a global transcriptional silencer (Nan et al. 1998a). In addition to binding methylated DNA, MeCP2 associates with various co-repressor complexes such as Sin3a, NCoR, and c-Ski at the sites of its occupancy (Jones et al. 1998; Kokura et al. 2001). Given its well described association with Rett Syndrome, it was surprising that transcriptional profiling of MeCP2-null mice brains displayed only subtle changes in gene expression (Tudor et al. 2002). Recent microarray studies have confirmed this finding using RNA isolated from the cerebellum of MeCP2 mutant mice (Jordan et al. 2007). The microarray study did not reveal any large changes in expression. The greatest change was in the Irak1 gene, which showed a twofold increase in expression. Special AT-rich sequence binding protein 1 (SATB1) was identified as one of the few genes upregulated in two MeCP2-null mouse models. SATB1 is known to specifically bind to nuclear matrix attachment regions (MARs) and mediate formation of chromatin loops (Horike et al. 2005). In a similar large scale expression study using cultured fibroblast cell lines from two RTT patients revealed only 49 upregulated and 21 downregulated potential MeCP2 targets, some of which were known to be expressed in brain tissues (Traynor et al. 2002). The most compelling result of this study was the striking differences observed in different clones obtained from the same RTT patients, which may be indicative of epigenetic pleomorphism between individual clonal populations, not unlike the variability between breast cancer cell lines in the current work.

125

MBD1

MBD1 is also a transcriptional repressor which acts through its repression domain like other family members. It is has been shown to function as a transcriptional repressor both in vivo and in vitro, and depending on the splicing isoform, it can bind methylated as well as unmethylated DNA (Ohki et al. 1999; Fujita et al. 2000). MBD1 is the largest protein in the MBD family, and Like the other family members, it associates with chromatin modifiers such as the Suv39h1–HP1 complex to enhance DNA methylation-mediated transcriptional repression (Fujita et al. 2003). Experiments using human HeLa cells demonstrated that MBD1 associated with the H3K9 methyltransferase SETDB1 (Sarraf and Stancheva 2004). During S phase the MBD1–SETDB1 complex is recruited to chromatin by the chromatin assembly factor CAF1 to establish new H3K9 methyl marks.bFunctional experiments showed that removal of DNA methylation disrupted the formation of MBD1–SETDB1–CAF1 interaction on the p53BP2 promoter, which lead to the loss of H3K9 methylation. An MBD1 mouse knockout has been obtained, but no severe developmental defects were found. MBD1-null mice had a normal morphology and appeared healthy, although they carried a number of minor neural defects like reduced hippocampal neurogenesis and had problems with spatial learning. Another interesting feature of this knockout was reduced genomic stability and an increase in expression of the Intracisternal-A particle retrotransposon (Zhao et al. 2003). This is the likely resulting from the lack of proper H3K9 methylation . Because H3K9 methylation is involved in the silencing of genomic repetitive elements, a reactivation of retrotransposon sequences in the MBD1-null mice was observed.

126

MBD2

MBD2, is a 44-kDa protein which shares extensive sequence homology with MBD3 (Hendrich and Tweedie 2003). MBD2 is been shown to bind methylated CpGs in vitro and in vivo; like the other MBD family members, it confers DNA methylation mediated transcriptional silencing through its repression domain (Wade 2001). MBD2 associate with HDAC1 and HDAC2 in the Mi-2/NuRD chromatin remodeling complex (Zhang et al. 1999).   For a more detailed review of the function of MBD2 in development and cancer, see the introductions for Chapter I and Chapter II.

MBD3

MBD3 unlike the other MBD family members does not preferentially bind methylated DNA. In contrast, MBD3 in lower vertebrates such as amphibians does preferentially bind methylated DNA. It is an essential subunit of at least some or most of the Mi-2/NuRD chromatin remodeling complexes (Zhang et al. 1999; Sakai et al. 2002). MBD2 and MBD3 both associate with Mi-2/ NuRD. However the association appears to be mutually exclusive, forming two distinct complexes (Le Guezennec et al. 2006; Denslow and Wade 2007; Bowen et al. 2004; Feng and Zhang 2001b). In spite of the striking sequence similarity between MBD2 and MBD3, the two proteins do not carry out redundant functions during early development. The MBD3 knockout is lethal at embryonic day 8.5. This is in stark contrast to MBD2-null mice which only displayed a mild maternal phenotype as discussed above. MBD3-null ES were seriously compromised in their ability to differentiate as they failed to shut down the expression of undifferentiated ES cell markers such as Oct4, Nanog, and Rex1 (Kaji et al. 2006).

127

Aberrant gene expression in MBD3-null cells is the most probable reason why cells of the inner cell mass fail to develop into late epiblasts after implantation (Kaji, Nichols, and Hendrich 2007).

MBD4

The last member of the MBD family, MBD4, has a very distinct functional role. MBD4 is a thymine glycosylase and serves as a DNA repair protein at targeted sites of cytosine deamination (Hendrich et al. 1999). The CpG dinucleotide is under-represented in methylated genomes because of spontaneous hydrolytic deamination of methylated cytosine which causes mCpGTpG transitions (Bird 1980). Because of the spontaneous deamination, any non-methylated CpG can mutate to UpG. MBD4 has been shown to excise and repair both mutated nucleotides. Because of this important repair function, it is not surprising that mutations in MBD4 have been found in various human carcinomas associated with microsatellite instability (Riccio et al. 1999). MBD4-null mice had a two to three times higher number of mCpG-TpG transitions showing that MBD4 acts to reduce the mCpG-TpG mutation rate (Millar et al. 2002). MBD4-null mice exhibited mild phenotypic abnormalities. Importantly, when they were crossed with mice carrying a germline mutation in the Apc (adenomatous polyposis coli) gene MBD4-null animals show an accelerated tumor formation and accelerated tumor progression (Millar et al. 2002).

Kaiso (ZBTB33), ZBTB4, and ZBTB38

128

Kaiso is a zinc finger domain protein that like the MBD family, can preferentially bind methylated DNA (Prokhortchouk et al. 2001a; Filion et al. 2006). Kaiso appears to have several important functions in lower vertebrates. Kaiso antisense knockdown in X. laevis caused a premature activation of zygotic transcription which eventually led to apoptosis and developmental arrest (Ruzov et al. 2004). This phenotype closely resembled the one induced by the DNMT1 antisense depletion, suggesting that DNA methylation-mediated repression mechanisms are partly responsible for repression of embryonic transcription before the mid-blastula transition (Stancheva and Meehan 2000a). Unlike the experiments in X. laevis, Kaiso knockout in mice resulted in no apparent abnormalities; however, when Kaiso-null mice were crossed with ApcMin/+ mice susceptible for intestinal tumorigenesis, it resulted in a delayed onset of tumor formation (Prokhortchouk et al. 2006).

Structural predictions and patterns of MBD Occupancy of Methylated DNA

The molecular functions of methyl-CpG binding proteins depend on their recognition and occupancy of methylated DNA. Differential binding properties are essential to their roles in vivo. Deletion analyses identified a minimal region of MeCP2 responsible for the interaction with methylated CpGs (Nan, Meehan, and Bird 1993). Further comparison with other MBD proteins defined the MBD domain to a protein motif of about 75 amino acids (Nan et al. 1998b). Since the classical MBD was described, proteins containing MBD-like domain, including ESET/SETDB1 and TIP5, have also been identified in different species (Clouaire and Stancheva 2008). However, these MBD-like domains are

129

not predicted to form specific interactions with methylated DNA and likely serve other functions.

Sequence comparison of all human MBD family proteins show the presence of 16 strictly conserved amino acids within the MBD domain. MBD3, which does not bind preferentially to methylated DNA, lacks four of these conserved residues (Hendrich and Bird 1998a). The structural information available so far does not explain why MBD4, whose MBD domain is related to MeCP2 more than any other, would display an altered DNA binding specificity (Hendrich et al. 1999).

About 70 – 80% of CpGs are methylated in mammalian genomes, creating a relatively high number of potential binding sites for MBD proteins. It is not clear what determines the pattern of occupancy at CpG sites, though several models have been proposed (Clouaire and Stancheva 2008). One possible model would be that each MBD protein randomly occupies any available methylated CpG site. In such a model, the relative abundance of each MBD protein within a cell together with the methylation density will dictate the occupancy of individual methylated sites. This random behavior supports the idea of functional redundancy between MCBPs and was, until recently, the principal argument to explain the relatively mild phenotypes of MBD1, MBD2 and Kaiso null mice. This, however, does not appear to be the case at least in mice (Martin Caballero et al. 2009).   In a second model, other factors may influence the distribution of MBD proteins within a cell nucleus, making it non-uniform and non-random, with each MBD protein occupying unique sites in the genome. This "solo" model would predict that a subset of genes might be affected by the loss of one MBD protein but not another. Examples of genes misexpressed in the absence of specific MBD proteins have been

130

identified, and the phenotypes of MBD-deficient mice, although subtle, are slightly different (Hendrich and Bird 1998c; Martin Caballero et al. 2009).

It remains unclear which type of binding is retained in cancer cells, which tend to accumulate aberrant DNA methylation patterns. If the various members of the MBD family display different DNA binding specificity, this would infer that they recognize and bind to more complex sequences than a single methylated CpG. In vitro experiments have shown that, unlike MBD2, MeCP2 requires a run of four or more A/T base pairs adjacent to methylated CpG for high affinity binding (Klose et al. 2005). These findings constitute the first example where the enhanced binding specificity towards a particular set of methylated sequences allows discriminative binding site occupancy of an MBD protein. Whether this is the case for other MBD proteins remains to be determined. As discussed earlier, our data support a slightly modified "overlapping + solo" model, where some genes are bound by MBD2 alone, while other methylated targets are either unbound or occupied by different methyl-binding proteins, as has been suggested by others (Lopez-Serra et al. 2006; Lopez-Serra et al. 2008b).

Materials and Methods:

Cloning of mutant MBD2 expression constructs

A full length MBD2 clone was obtained from the IMAGE Consortium and the coding sequence was subcloned into the pCDNA 4.0 plasmid using PCR amplification to introduce restriction complementary ends and followed by ligation into a TA cloning vector, amplification, BamH1-HindIII restriction product purification, and ligation. A V5 antigen coding oligonucleotide was cloned into the pCDNA 4.0 vector using an in frame restriction site on the 3' side of MBD2 to generate a C-terminus tagged MBD2 protein. Site directed mutagenesis was performed to generate shRNA a shRNA immune mutant using the QuickChange II kit (Stratagene, La Jolla, CA). Oligonucleotides used for PCR, V5 tag cloning and mutagenesis are given in Table 3.

Table 9. MBD2 Tagging, Cloning, and Mutagenesis Primers

5' MBD2 Cloning PCR (BamHI)	GGATCC**ATG**CGCGCGCACCCGGGGGGGAGGC
3' MBD2 Cloning PCR (EcoRI)	GAATTC**TTA**GGCTTCATCTCCACTGTCCATTTCAATATCCATC
V5 – Sense Strand (EcoR1-XhoI)	AATTCGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG**TAA**C
V5 – Anti-sense Strand (EcoR1-XhoI)	TCGAG**TTA**CGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCG
shRNA B Target Sequence	GGGTAAACCAGAC***T***TGAAT
Sense B-Mutagenesis Primer (T→C)	ATCAAAATAAGGGTAAACCAGAC***C***TGAATACAACATTGCCAATTAGA
Anti-Sense B-Mutagenesis Primer	TCTAATTGGCAATGTTGTATTCA***G***GTCTGGTTTACCCTTATTTTGAT
shRNA C Target Sequence	GAACAGCCACGTCAGCT***T***T
Sense C-Mutagenesis Primer (T →G)	ACAGCCACGTCAGCT***G***TTCTGGGAGAAGAGG
Anti-Sense C-Mutagenesis Primer	CCTCTTCTCCCAGAA***C***AGCTGACGTGGCTGT

Table 9: MBD2 Tagging, Cloning, and Mutagenesis Primers used to restore shRNA immune and tagged MBD2. Restriction complementary overhangs were included in all cloning oligonucleotides for vector acceptance and ligation. Mutagenesis primers were designed using Stratagene's QuickChange II web tools. Start and Stop codons (for frame reference) are indicated in bold, as are the mutagenized bases.

132

Bisulfite conversion

Bisulfite conversion reactions were performed as described previously by members of the Ginder Lab (Rupon et al. 2006; Singal et al. 2002). Briefly, purified genomic DNA was collected by stepwise detergent based lysis, alkaline protein precipitation, and isopropanol precipitation (Aquapure Genomic DNA Isolation Kit, Bio-Rad Labs, Hercules, CA). The 500ng to 1ug of gDNA was denatured and unmethylated cytosines were hydrolytically deaminated to uracil in the presence of a sodium bisulfite conversion reagent at 50 degrees C overnight (Methyl-Detector Kit, Active Motif, Carlsbad, CA). Converted DNA was purified following an on-column desulfonation step. Purified converted DNA was stored at -20 deg C.

Bisulfite sequencing primer design and data analysis

Regions of interest were PCR amplified using methylation insensitive primers. These primers were designed using the web based primer design tool, MethPrimer (http://www.urogene.org/methprimer/index1.html).(Li and Dahiya 2002) Primers were optimized to specifically amplify bisulfite converted DNA only. PCR thermal cycling program was a modified touchdown PCR with an initial 20 cycles of progressively lower annealing temperatures from 60 to 50 deg C (-0.5 deg C per cycle) and subsequently 25 more cycles of standard 3 step PCR with an annealing temperature of 45 deg C. PCR products were checked for appropriate size bands by gel electrophoresis and were subsequently cloned (pGEM T-Easy kit, Promega, Madison, WI). At least 10 clones from each converted sample were sequenced by fluorescent automated sequencing. The

133

resulting sequence traces were analyzed for complete conversion as well as methylation using the open source Java BiQ Analyzer software package (http://biq-analyzer.bioinf.mpi-sb.mpg.de/).(Bock et al. 2005) This software and the associated web tools were used also to generate all 'lollipop' style methylation diagrams.

Table 10. Bisulfite PCR Primers

DAPK1 Promoter Bis PCR F1	TAGTTTAGTAATGTGTTATAGGTGGGG
DAPK1 Promoter Bis PCR R1	AAAAAACAAAATCCCCRAC
DAPK1 Promoter Bis PCR F2	TTTTTATTTATTTTTTAGTTGTGTTTT
DAPK1 Promoter Bis PCR R2	CCTTAACCTTCCCAATTACTC
MASPIN Promoter CpG island Bis PCR F1	TTGTTAAGAGGTTTGAGTAGGAGAG
MASPIN Promoter CpG island Bis PCR R1	CCCACCTTACTTACCTAAAATCACA
MASPIN Exon2 CpG island Bis PCR F1	TTTTATTATGTGGTTAGGTTGGTTT
MASPIN Exon2 CpG island Bis PCR R1	TAATTACCCAACAAATCTCCAAATT
MASPIN Exon4 CpG island Bis PCR F1	ATTTTAGTATTTTGGGAGGTTAAGG
MASPIN Exon4 CpG island Bis PCR R1	TTTTTAAAACAAAATCTCACTCTATC
KLK10 Promoter Bis PCR F1	TTGAGAAAGAGGTTTTTATTGGTTT
KLK10 Promoter Bis PCR R1	CAAACACCCTTTTAACCTACAAC
KLK10 Promoter Bis PCR F2	ATATATTTTAGTAGATTTTGGTTATGAGAG
KLK10 Promoter Bis PCR R2	AAAAAAACCCAACTAACTTAAAAAC

Table 10: Bisulfite PCR primers designed using the MethPrimer tool. Sequences are given in the 5' to 3' direction for all oligonucleotides. Note the absence of "C" from any of the sense strand primers indicating specificity for bisulfite converted DNA.

Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation assays were performed to determine whether/where MBD2 binds to its target genes and to map patterns of histone acetylation essentially as described previously (Rupon et al. 2006; Kransdorf et al. 2006a). We used commercially available ChIP grade antibodies (Upstate, Temecula, CA; Abcam, Cambridge, MA) recognizing MBD2 and various post-translational histone modifications to map MBD2/co-repressor mediated changes in chromatin architecture. Briefly, we crosslinked

134

adherent cells in culture vessels (150mm dishes) using 1% formaldehyde in growth medium for 10 minutes at room temperature. Formaldehyde was quenched with 125mM glycine. We washed twice with 1X PBS and then collected cells by scraping into 1X lysis media containing 1mM PMSF and Complete^{TM} Mini - protease inhibitor cocktail tablets (Roche, Indianapolis, IN). Chromatin was subsequently sheared DNA (water bath type sonicator, Diagenode, Sparta, NJ) according conditions optimized to produce fragments between 200 and 500 base pairs. Precipitations were performed using sheared chromatin from approximately $1x10^6$ cells at 4 degrees C°, overnight using 5μg of affinity purified ChIP grade anti-bodies. Positive control (anti-acetyl H3K9,14) and negative (normal IgG) controls were included and probed with GAPDH primers. In our experience, background and non-specific contamination was reduced by using magnetic protein-G coated beads (Active Motif, Carlsbad, CA) to isolate immune-precipitates. We then performed SYBR green based qRT-PCR to measure target sequence enrichment (~200-500bp resolution promoter proximal CpG rich regions) in column purified, immunoprecipitated DNA (Active Motif, Carlsbad, CA). Enrichment is reported as a proportion of starting chromatin (input) under both specific (e.g., anti-MBD2, anti-acH3K9, etc.) and non-specific (normal IgG) conditions.

Results:

Tumor Suppressor CpG islands remain methylated after gene expression is restored.

Though it is now widely accepted that CpG methylation varies inversely with transcription, the primacy of either methylation or transcription at tumor suppressors in cancer remains a matter of controversy. We recognized that our system provided a model to investigate a temporal relationship between DNA methylation and changes in transcription, specifically at the hypermethylated promoters of tumor suppressor genes in breast cancer. We used bisulfite sequencing to examine CpG methylation at the promoters of the genes which became transcriptionally active following MBD2 knockdown both before and after transcription was restored. Based on conventional wisdom regarding passive demethylation at actively transcribed genes, we hypothesized methylation levels would be decreased following transcriptional activation. However, there were no formal grounds on which to rule out the possibility that methylation levels remained stable at genes induced by MBD2 knockdown. This experiment allowed us to distinguish between these two possibilities by establishing the methylation state of promoter proximal CpGs under both transcriptionally active and repressed conditions.

We found that methylation remained remarkably stable through large swings in expression following MBD2 knockdown (Figure 36). At the promoter KLK10 and DAPK1, which were heavily methylated in MCF-7 cells, there was virtually no change in methylation levels. Similarly at the Maspin promoter in MDA-MB-231 cells, which was relatively CpG sparse and significantly less methylated than the other genes examined,

136

overall methylation levels were stable between stable Scramble and Knockdown cells although methylation at single CpG sites varied.

MBD2 binds to Methylated CpG Island associated promoters and methylCpG-bound MBD2 is decreased in shRNA knockdown cells.

We confirmed that MBD2 protein was bound directly at the promoter CpG islands of methylated target genes, DAPK1 and KLK10, by Chromatin Immunoprecipitation in control MCF-7 cells. When we used chromatin from MBD2 knockdown cells for ChIP, we found significantly reduced enrichment of DAPK1 and KLK10 promoter DNA. Recall that the methylation levels at the gene promoters remained unchanged in knockdown cells; nonetheless there was significantly less MBD2 protein present in knockdown cells and ChIP assays confirmed there was proportionately less MBD2 bound at methylated loci (Figure 37). Taken together with the bisulfite sequencing and gene expression data, we concluded that by reducing MBD2 bound at promoter associated CpG islands in tumor cells we were essentially uncoupling CpG methylation from its repressive effect on transcription.

137

Figure 36. Bisulfite Sequencing Showed No Change in the Methylation of Activated Genes in MBD2 Knockdown Cells

Maspin Promoter
Scramble shRNA

Maspin Promoter
MBD2 shRNA

KLK10 Promoter
Scramble shRNA

KLK10 Promoter
MBD2 shRNA

DAPK1 Promoter
Scramble shRNA

DAPK1 Promoter
MBD2 shRNA
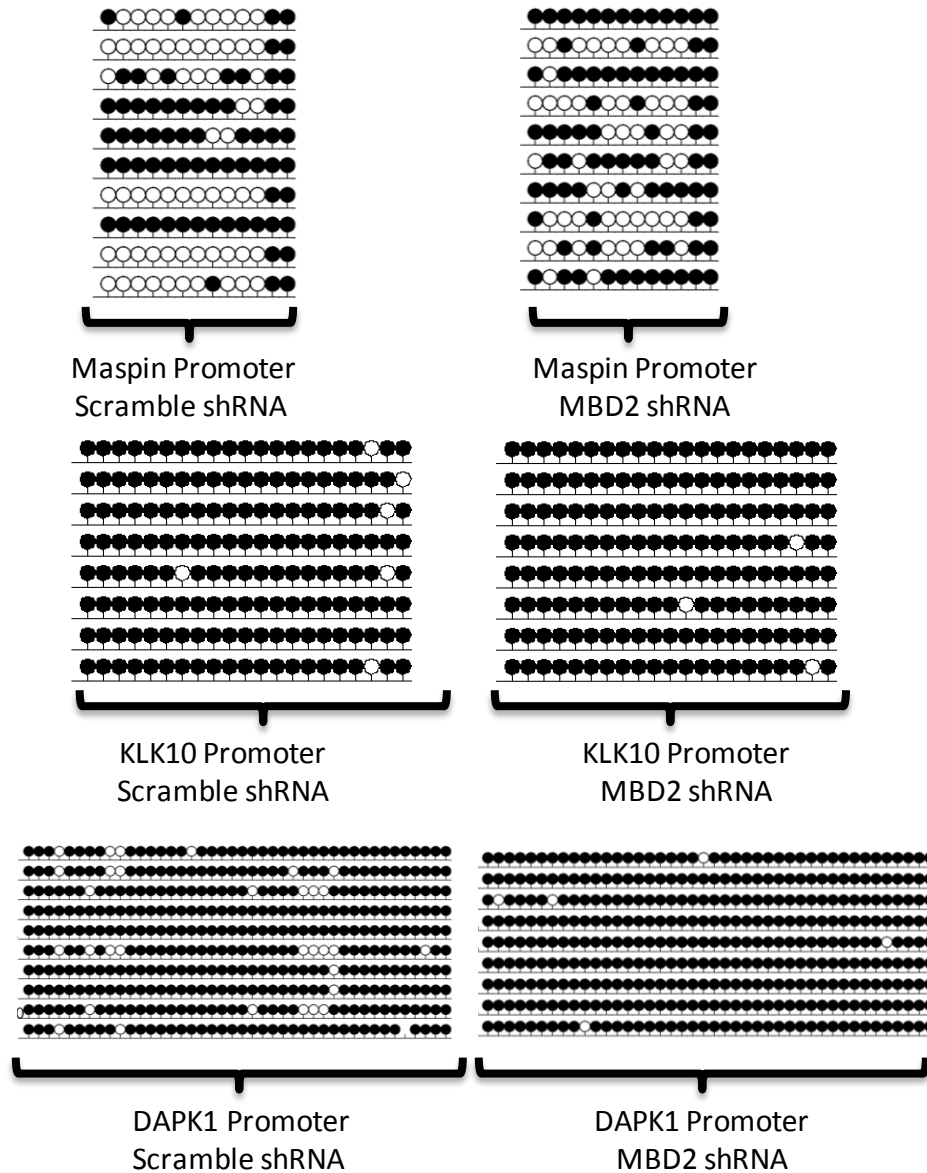
Figure 36. Bisulfite conversion and sequencing was performed to assess the degree of CpG methylation at the promoters of (top to bottom) Maspin, KLK10, and DAPK1 in breast cancer cell lines where these genes were found methylated at baseline and were transcriptionally activated following MBD2 knockdown. Sequencing data was decoded using the BiQ analyzer software package. Filled black circles represent methylated sites and empty (white) circles are unmethylated sites. The relative position of CpGs is not illustrated, see Appendix C for maps of promoter CpG distribution and bisulfite primer position. The bisulfite sequencing analysis shows that no appreciable decrease in methylation occurs at promoter associated CpGs in MBD2 knockdown cells.

Figure 37. Chromatin Immunoprecipitation shows MBD2 binds to target genes DAPK1 and KLK10 directly
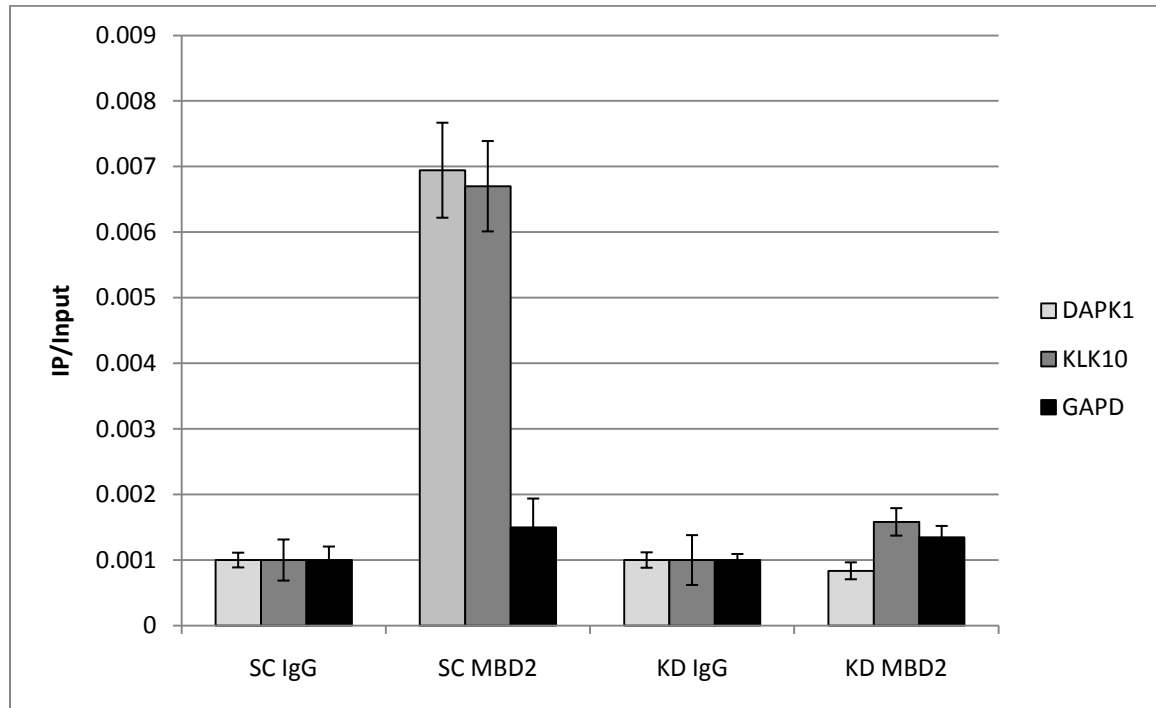
Figure 37. Quantitative RT-PCR was used to probe anti-MBD2 or normal IgG control immunoprecipitated chromatin (ChIP) from Scramble (SC) and shMBD2 knockdown (KD) MCF-7 cells. Binding of MBD2 to DAPK1 and KLK10 promoters is evident in SC cells (SC MBD2); this binding is reduced to background levels in KD cells (KD MBD2). There is no enrichment of the actively transcribed GAPD promoter DNA by anti-MBD2. This control gene was included to verify the absence of non-specific enrichment or genomic DNA contamination.

139

Restoration of MBD2 expression in knockdown cells leads to resilencing of MBD2 dependent genes

Up to this point, we had not addressed an important limitation of the shRNA driven knockdown methodology we were using. Though we attempted to control for the non-specific effects of shRNA expression in our tumor cells by using a scrambled shRNA which did not target any known gene, we could not account for the possibility that there were sequence specific effects mediating gene expression and phenotypic differences which did not depend on the loss of function of MBD2 protein. In other words, we had not formally excluded the possibility that the observations we attributed to a reduction in MBD2 protein were not the result of a non-specific response to anti-MBD2 shRNA (e.g., Interferon alpha/beta response). Furthermore, we could no longer entertain the notion that MBD2 dependent changes relied on transcription associated demethylation of MBD2 repressed genes. We therefore dismissed the assumption that MBD2 knockdown was initiating an irreversible 'hit-and-run' effect on target gene expression.

It was important to establish that restoration of MBD2 led to the reversal of MBD2 dependent changes. Given this realization, and in order to nail down MBD2 as the sole mediator of genotypic and phenotypic changes in breast cancer, we conducted a rescue experiment. We hypothesized that restoring MBD2 protein in knockdown cells would re-establish tumor suppressor gene silencing and reverse knockdown dependent phenotypic changes.

We obtained a full length MBD2 cDNA clone from the IMAGE consortium and generated a shRNA immune expression construct by site directed mutagenesis. Specifically, we introduced a single, silent (amino acid neutral) nucleotide substitution

140

within the shRNA binding site of the MBD2 coding sequence. This allowed us to express the normal MBD2 protein in our stable shRNA expressing breast cancer lines. We added an N-terminal V5 epitope tag to our mutant MBD2 expression construct in order to follow the *in situ* expression of the exogenously expressed protein. We confirmed expression of the mutant MBD2 protein and the specificity of the V5 epitope tag in knockdown breast cancer cells by Western Blot analysis (Figure 38). We further confirmed nuclear localization and gross DNA binding capacity of exogenous V5 tagged MBD2 protein by fluorescent immunocytochemistry (Figure 39). We found that the shRNA binding site mutant (pCMV-B mutant) was capable of restoring near endogenous levels of MBD2 protein; wild type MBD2 (pCMV MBD2) and a second expression construct with an off target mutation (pCMV-C mutant) were not expressed (Figure 38, top).

As hypothesized, we found that restored MBD2 expression in stable knockdown lines led to resuppression of target gene expression (Figure 38, bottom), consistent with our earlier observation that promoter methylation remained stable despite transcriptional activation.

Figure 38. Expression of shMBD2-B mutant cDNA is able to restore MBD2 expression in a stably knocked down cell line.
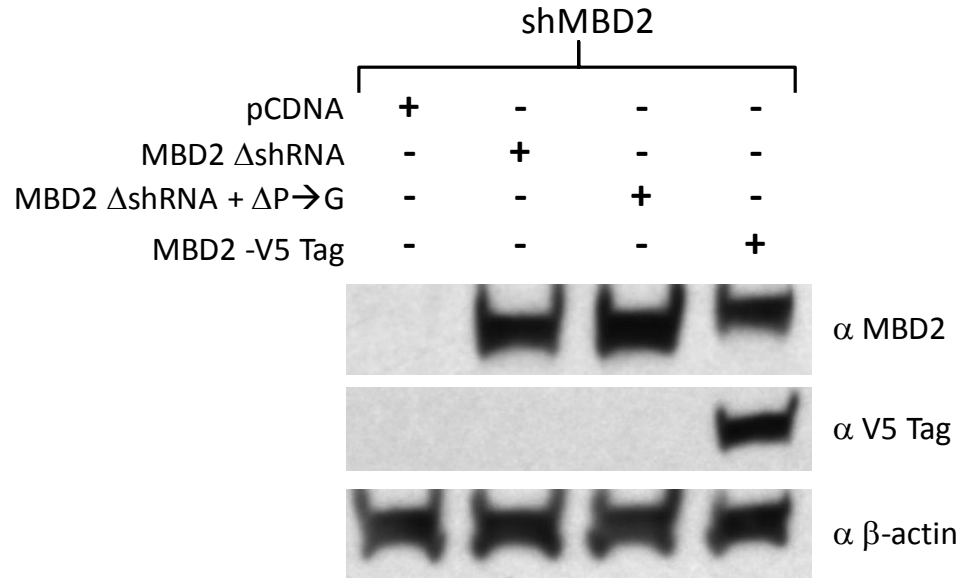
Figure 38. Western blot analysis shows expression of MBD2 shRNA (Δ-MBD2) binding site mutant in stably knocked down MCF-7 cells. Expression of a double mutant used for functional analysis and a V5 epitope tagged mutant MBD2 is shown as well. Blotting with an anti-V5 antibody demonstrates specificity of this antibody for the tagged MBD2 only (far right bands). Note the slightly decreased mobility in the tagged MBD2 indicating the small increase in molecular weight conferred by the V5 tag. Also note the absence of detectable MBD2 in this knockdown line with transfection of a pCDNA vector only control only (Far left, absent bands).

142

Figure 39. Immunocytochemsitry shows nuclear localization and DNA binding of Exogenous V5-Tagged MBD2 in MCF-7 Cells

Figure 39. Anti-V5-FITC fluorescent immunocytochemistry against untagged (3 images on the left) and V5 Tagged (right) MBD2 demonstrates the specificity of anti-V5 to recognize the tagged MBD2 only. Exogenous MBD2 localizes to the nucleus (DAPI) and retains the ability to bind DNA in mitotic forms (white arrowheads).

143
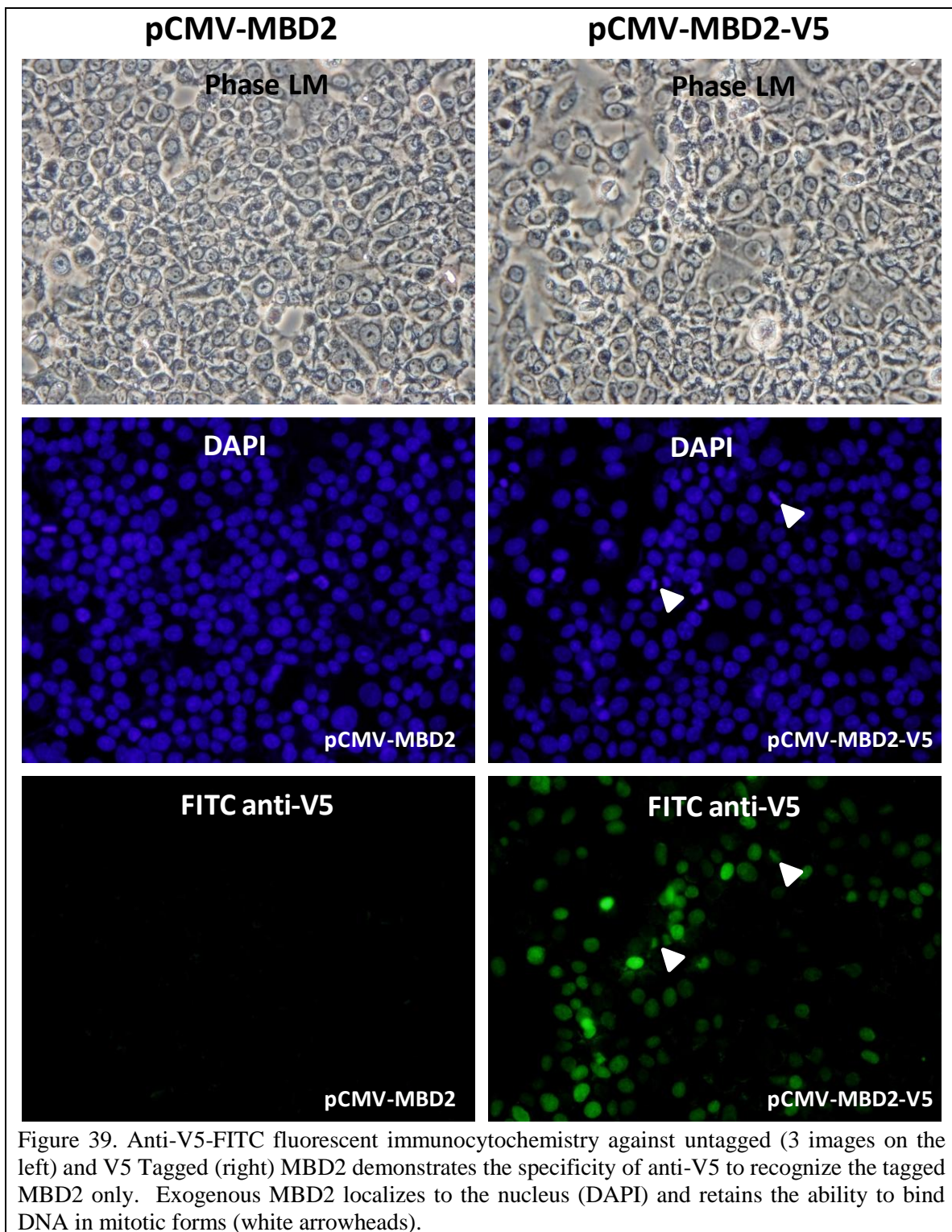
Figure 40. MBD2 Rescue with Exogenous shRNA Binding Site Mutant cDNA Expression Construct Leads to Resilencing of MBD2 target genes.
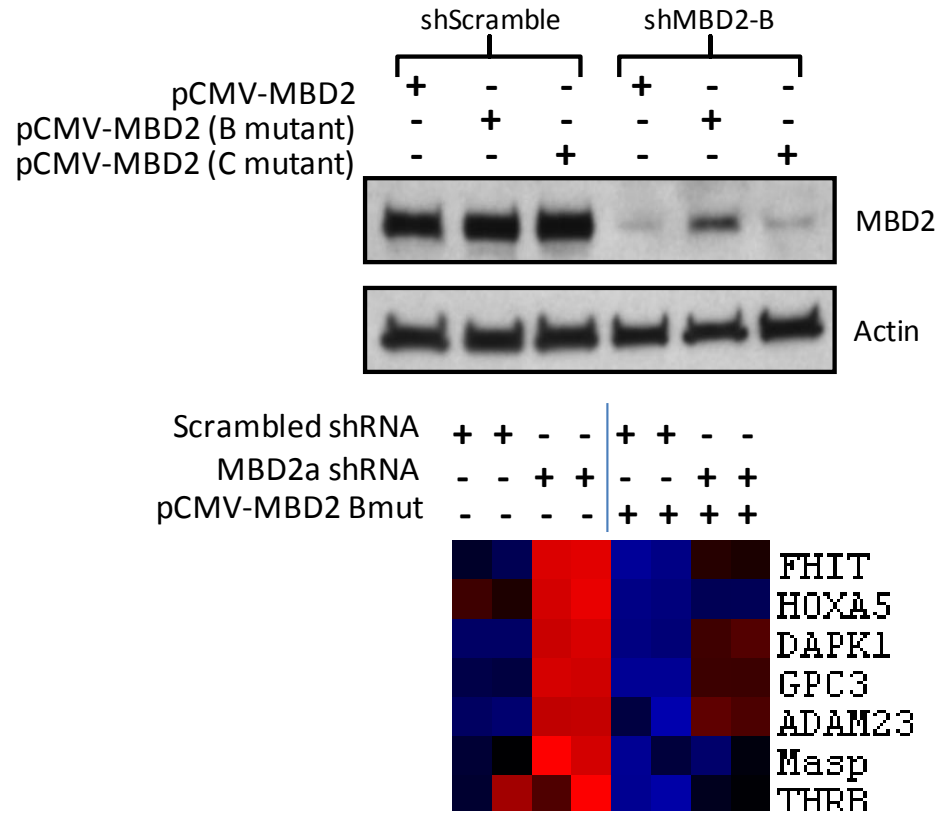
Figure 40. Westen blot analysis of MBD2-B shRNA expression in Scramble and Knockdown cells demonstrates the ability to restore endogenous levels of MBD2 in stably knocked down cells. Expression of wild type MBD2 and an MBD2-C mutant is not observed to the same degree demonstrating the specificity of stable shMBD2 B expression. Below, a heat map of gene expression measuring mRNA levels of all MBD2 target genes in MCF-7 demonstrates that rescue with an MBD-B expression construct leads to resuppression of MBD2 target gene, suggesting that these genes remain methylated and poised for resuppression in the presence of adequate levels of MBD2.

144

MBD2 down regulates DNMT1

We were interested in the relationship between the epigenetic factors which establish and maintain tumor suppressor silencing in breast cancer. CpG methylation in normal and neoplastic cells depends on the activity of the DNA Methyltransferase enzymes (Klose and Bird 2006). We hypothesized that breast cancer cells would attempt to maintain pathological silencing of tumor suppressor genes by promoter hypermethylation due to a survival advantage and might therefore implement or select for compensatory mechanisms such as upregulation of DNMT1. We examined the feedback regulation of the DNA methyltransferase, DNMT1, in MBD2 knockdown cells by western blot. We found that the levels of DNMT1 protein were increased in breast cancer cells that stably expressed anti MBD2 shRNA (Figure 41). A mechanistic link between MBD2 levels and DNMT1 remains to be established, however, we envisage a sensor mechanism exists in tumor cells which upregulates the DNA methyltransferase machinery in order to preserve the abnormal promoter hypermethylation on which these cells depend for unchecked proliferation. Such a sensor mechanism may be important for intrinsic or acquired resistance to anti MBD2 therapy in breast cancer.

145

Figure 41. MBD2 knockdown increases DNMT1 protein
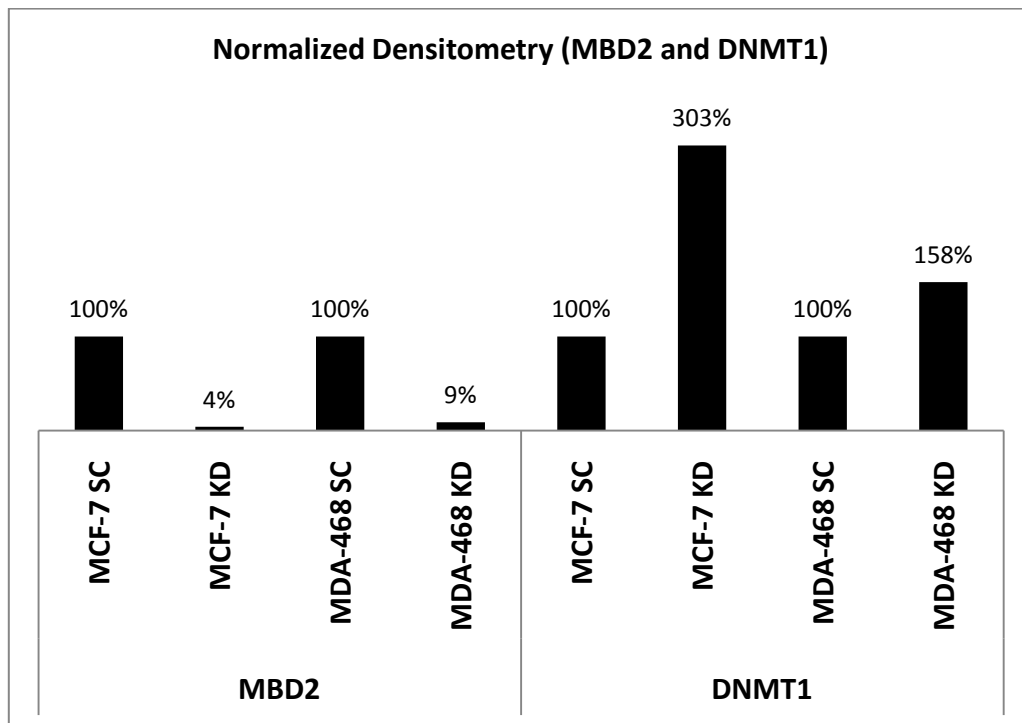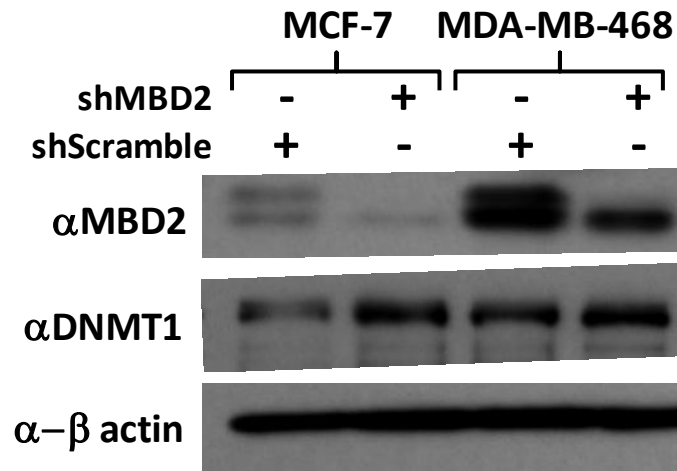
Figure 41. (Above) A preliminary Western blot with antibodies directed against MBD2 and DNMT1 protein and corresponding densitometry analysis shows an increase of approximately 3 fold in DNMT1 levels in MBD2 knockdown MCF-7 cells compared to scramble controls. This increase is present, though somewhat less pronounced, in MDA-MB-468 cells as well. (Below) Scramble control normalized densitometry of the western blot shown above demonstrates a >90% knockdown of MBD2 protein in both MCF-7 and MDA-MB-468. A corresponding increase of 3 fold in DNMT1 protein was observed in MCF-7 knockdown cells and 1.5 fold in MDA-MB-468.

146

MBD2 post transcriptionally down regulates MeCP2 and the two proteins cooperate in promoting tumor viability.

MBD2 has been shown to bind certain tumor suppressor promoters in combination with other MBD proteins. MeCP2 in particular is commonly found along with MBD2 bound to methylated tumor suppressors (Lopez-Serra et al. 2006). In addition, MeCP2 was found to be expressed at higher than normal levels in human breast cancers, suggesting this protein may be playing an exacerbating role (Billard et al. 2002; Muller et al. 2003). We were interested in determining whether MBD2 and MeCP2 were playing complementary roles in breast cancer. We considered the possibility that MeCP2 was compensating for MBD2 knockdown and perhaps there was a mechanism to increase MeCP2 levels in MBD2 knockdown cells. We also sought to determine whether knocking MeCP2 and MBD2 down simultaneously, would more effectively inhibit tumor cells than knocking either one down alone.

We evaluated changes in MeCP2 protein levels in stable MBD2 knockdown cells and MBD2 overexpressing cells by western blot analysis. We found that MeCP2 protein was increased by approximately 3 fold over baseline in MBD2 knockdown cells (Figure 42). Interestingly, MeCP2 levels remained relatively stable when we overexpressed MBD2. This suggested to us that the feedback inhibition of MBD2 protein was being mediated indirectly through an MBD2 repressed target. In this scenario, normal levels of MBD2 are sufficient to maintain complete silencing of the intermediate factor and overexpression therefore has minimal effect.

We were interested in determining whether a combined knockdown of MBD2 and MeCP2 would lead to an increased effect in breast cancer. We used transient transfection

147

of either MBD2, MeCP2, or both siRNAs into several breast cancer cell lines (MDA-MB-231, MDA-MB-468, and SK-BR-3 cells and confirmed individual and combined knockdown by quantitative real time PCR (Figure 43). We then examined the acute cytotoxicity of siRNA transformation by measuring cell death and apoptosis quantitatively using Annexin V/PI flow cytometry. MCF-7 cells were included in the apoptosis study as well but these cells showed very little baseline or induced annexin V positivity. We found that MBD2 or MeCP2 knockdown alone led to an increased proportion of apoptotic cells (Figure 44). Furthermore, we found that combined knockdown of both genes led to an additive increase in apoptotic cells in 2 out of 3 cell lines. SK-BR-3 cells did not show an additive effect, however this might be explained by the relatively poor MBD2 knockdown achieved in the double transfected cells. (Figure 43 and 44)

Figure 42. MBD2 Knockdown up regulates MeCP2 Protein
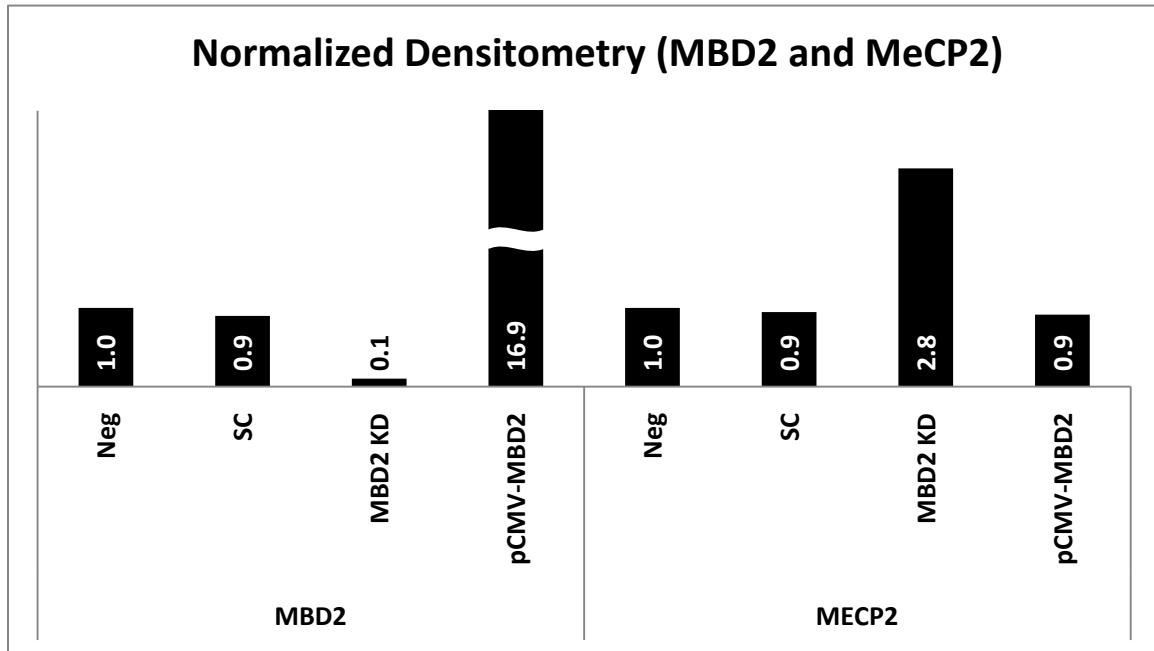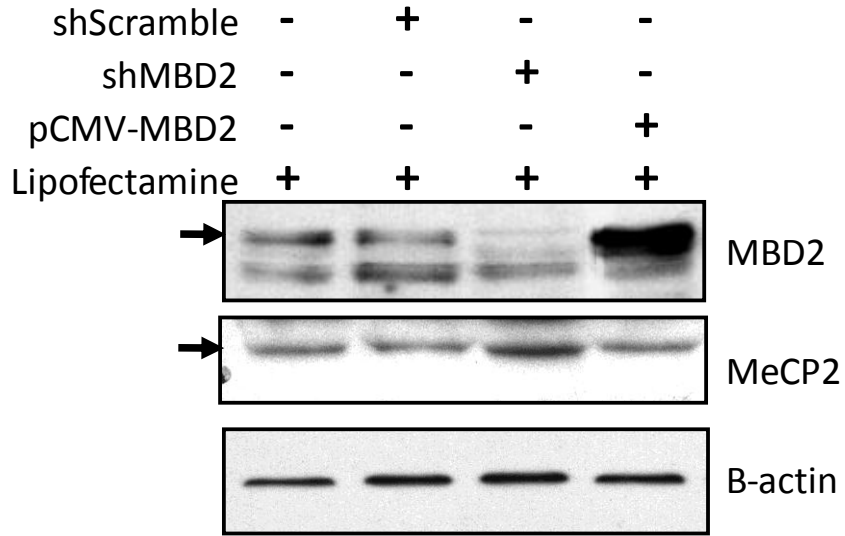
Figure 42. A preliminary Western Blot analysis of MBD2 and MeCP2 protein levels in MBD2 knockdown and overexpression demonstrates 2.8 fold increase in the level of MeCP2 protein in knockdown cells. MeCP2 levels remain relatively unaffected by overexpression of MBD2 suggesting an indirect mechanism of feedback through an unidentified MBD2 target gene which is fully silenced with normal levels (or overexpression) of MBD2.

149

Figure 43. Simultaneous siRNA mediated Knockdown of MBD2 and MeCP2
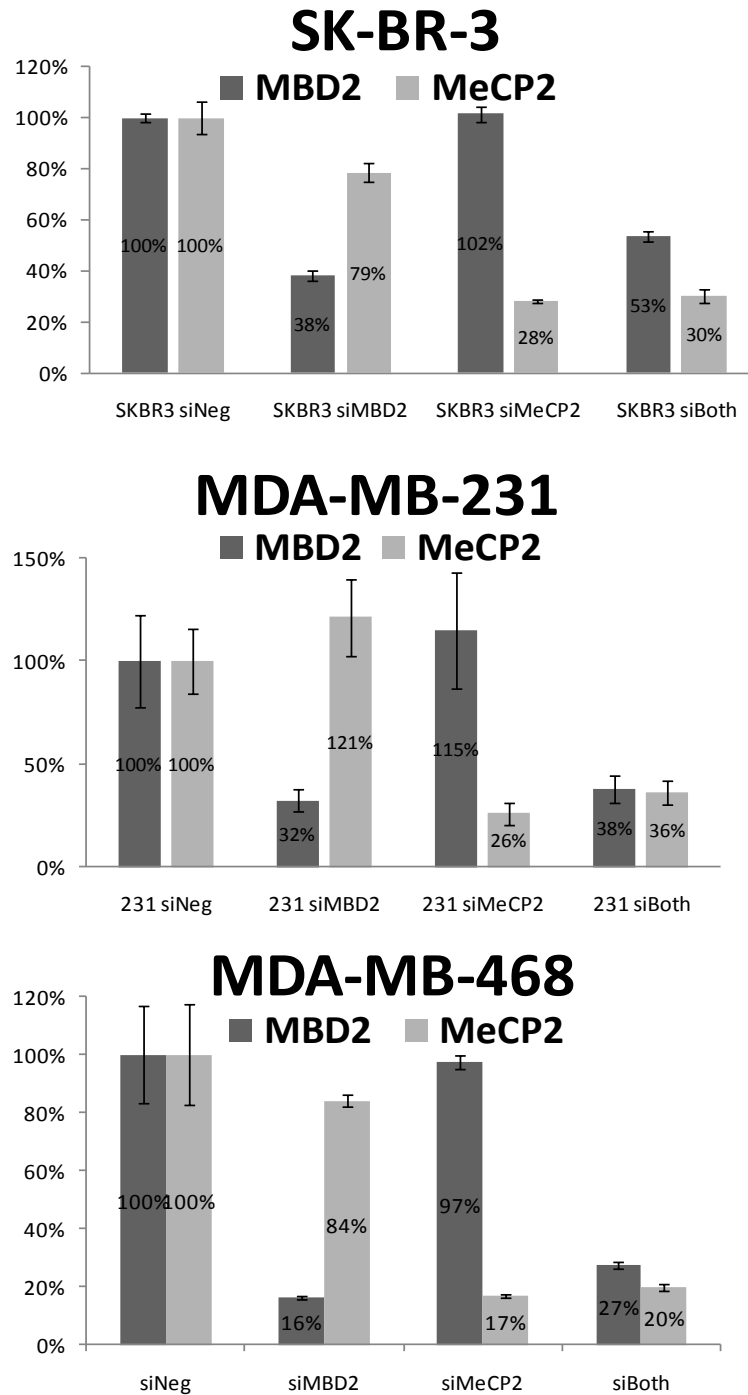
Figure 43. qRT-PCR to confirm knockdown of MBD2 and MeCP2 mRNA in siRNA transfected tumor cells lines at 72hrs post transfection. All siRNA knockdowns were performed using cells at approximately 80% confluence in a 24 well tissue culture plate. Each transfection was performed using 20 pico moles of total siRNA (Qiagen) and 1 ul of Lipofectamine 2000 transfection reagent (Invitrogen) diluted in Opti-MEM serum free media (Gibco). Bars represent cyclophilin nomalized mean and standard deviation from 3 qRT-PCR replicates for each transfection.

150

Figure 44. Annexin V PI Flow Cytometry for Measuring Apoptosis
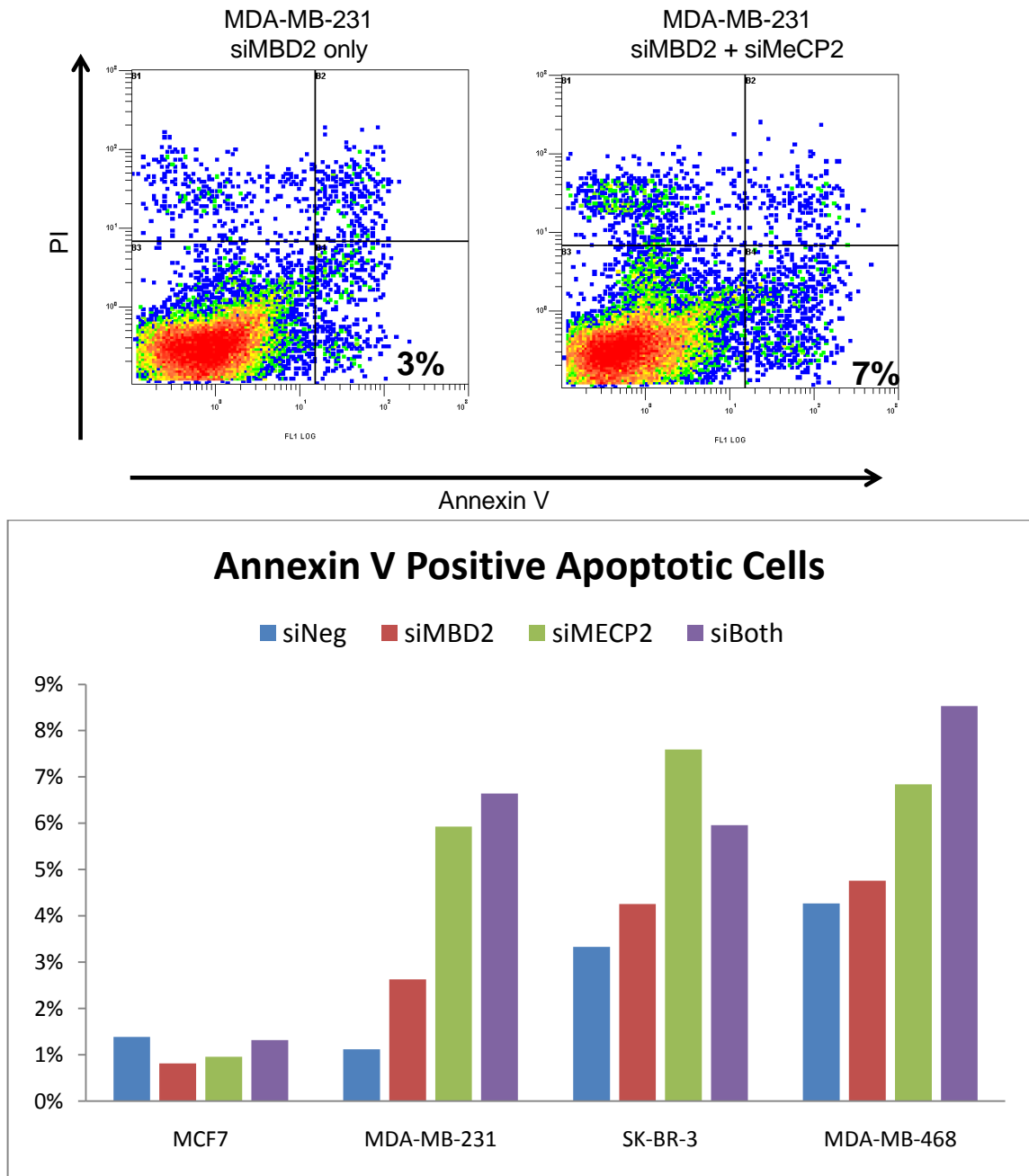
Figure 44. Annexin V-FITC and Propidium Iodide (PI) staining was performed to measure apoptotic and necrotic cells, respectively, in siNeg (control siRNA), siMBD2, siMeCP2 KD and both siMBD2 and siMBD2 combined. The populations were counted by flow cytometry (representative flow cytometer scatter plot, above) and results are reported as percent apoptotic cells (below). These results represent a single FACS experiment, therefore no error bars are included.

151

<center>**Discussion**</center>

DNA methylation remains stable through changes in transcription

It is widely accepted that an inverse correlation exists between DNA methylation at promoter proximal CpG sites and transcriptional activity. However recent genome wide studies cast this association in less absolute terms than previously believed. In particular, the high resolution bisulfite mapping of methylation and transcription across 3 human chromosomes has shown that certain promoters are differentially methylated in ontologically distinct populations; however, DNA methylation was found to be independent of transcriptional state at as many as two thirds of these genes (Eckhardt et al. 2006). With regard to the pathological methylation of CpG islands in cancer, an association between methylation and transcription is more firmly established; methylation in this particular circumstance is strongly correlated with transcriptional inactivity (Jones and Baylin 2007; Jones and Baylin 2002; Brock, Herman, and Baylin 2007). However the degree to which this methylation depends on transcription has yet to be characterized. In addition, the plasticity of promoter methylation at hypermethylated CpG islands in cancer remains a matter of some speculation.

We have identified a cluster of MBD2 dependent genes whose transcriptional state can be digitally switched by the presence or absence of MBD2. We recognized that these genes provide a model for studying the stability of CpG island hypermethylation and the temporal relationship between transcription and methylation. We began by examining the basal methylation state of several candidate MBD2 dependent genes: Maspin, KLK10, and DAPK1 in MCF-7 cells. We found that KLK10 and DAPK1, which have large CpG islands were heavily methylated. Maspin, which has a relatively smaller non-

<center>152</center>

canonical CpG island, surprisingly was unmethylated in MCF-7 cells where this gene is robustly induced by MBD2 knockdown. This raised our suspicion that the effect of MBD2 knockdown on Maspin expression might be indirect. In MDA-MB-231 cells the Maspin promoter was found to be methylated, in contrast to MCF-7 cells. Nevertheless, we noted that methylation at Maspin promoter's CpG rich region did not occur to the same degree as found at the canonical CpG islands of DAPK1 or KLK10. This observation is consistent with checkered reports regarding Maspin's somewhat atypical methylation dependent regulation in breast cancer (Futscher et al. 2002a; Wozniak et al. 2007; Domann et al. 2000).

In order to measure the covariation of methylation and transcription, we examined CpG methylation in stably knocked down MBD2 breast cancer cells where expression of corresponding genes was significantly induced. We found that, despite robust increases in transcription, there were no appreciable changes in the promoter methylation at every gene we examined. Several inferences can be drawn from these observations which deserve mention here. First, these data cast doubt on the idea that passive demethylation invariably occurs at transcribed loci due competition between the transcriptional machinery, DNA binding proteins, and methyltransferases (Hsieh 1999). Furthermore, it suggests that methylation in and of itself is not the prime impediment to transcription of hypermethylated tumor suppressor genes. It implies instead that, as we hypothesized, recruitment of MBD2 to particular hypermethylated promoters is essential for the maintenance of their transcriptional inactivity. Put another way, MBD2 is essential for coupling hypermethylation to transcriptional inhibition, presumably through its role in directing local changes in chromatin, e.g., histone modifications such as histone

153

deacetylation or other repressive mechanisms. As discussed below, we go on to reinforce this idea by verifying that MBD2 is bound directly to methylated promoters and showing that restoration of MBD2 in knockdown cells is sufficient to re-silence these and other MBD2 dependent genes.

MBD2 acts directly at methylated promoters to affect changes in tumor suppressor gene expression

We had found that MBD2 was required for target gene repression in breast cancer cells. However we had not experimentally shown that this gene repression was directly mediated by the binding of MBD2 to the promoter proximal methylated CpG islands of tumor suppressor genes. In order to confirm that MBD2 was acting through the accepted mechanism, i.e., binding to promoter proximal methylCpG, we performed chromatin immunoprecipitations with antibodies directed against MBD2 and used quantitative PCR to probe for enrichment of tumor suppressor gene associated chromatin from MCF-7 cells.

We found that MBD2 was indeed bound to the CpG rich promoters of DAPK1 and KLK10 in MCF-7. We did not see enrichment of Maspin promoter DNA by ChIP, once again suggesting the MBD2 was not acting directly, at least not at the promoter, to induce transcription of this gene. At least one other report indicates that the epigenetic silencing of Maspin, and its reactivation by 5-aza-dC, are not dependent on promoter associated methylation, leaving lingering questions as to the mechanism of epigenetic silencing of this tumor suppressor in breast cancer (Wozniak et al. 2007).

154

Our results indicate that MBD2 binding is dependent on the presence of extended stretches of methylated CpGs, such as those found at the promoters of DAPK1 and KLK10 in MCF-7 cells. The affinity of MBD2 for even a few methylated CpGs is well established and argues against a requirement for long stretches of methylation for binding (Fraga et al. 2003; Lopez-Serra et al. 2008b). However, it stands to reason that fewer MBD2 molecules are able to bind a region with fewer CpG sites. Moreover, an equilibrium between bound and unbound MBD2 likely exists and would be shifted depending on the relative proximity of methylation sites. Therefore we consider it possible that the relatively low sensitivity of ChIP precludes the detection of MBD2 resident at partially methylated, CpG sparse promoters such as the one found at the Maspin gene. The sensitivity of ChIP is largely determined by several factors which may be working against us: the affinity and specificity of the ChIP antibody and the accessibility of the epitope being recognized. Regarding the latter, it has been shown that MBD2 has multiple interaction partners in the Mi2/NuRD complex which may be limiting access to MBD2 and reducing the efficiency of immunoprecipitation. It is interesting to speculate whether the specific NuRD components in this modular complex vary from promoter to promoter in a given cell and perhaps lead to differential ChIP sensitivity. For instance, the MBD2 containing corepressor complex at CpG sparse promoters may in fact be fundamentally different from the one resident at CpG rich promoters leading to variation in antibody recognition.

Despite the notable exception of Maspin, taken as a whole our data suggests that MBD2 functions by directly binding to CpG rich and heavily methylated tumor suppressor promoters and maintains transcriptional silencing. Because we found that

155

transcription was restored while promoter methylation remained intact, we inferred that MBD2 was indeed an essential link between methylation and transcriptional suppression at specific tumor suppressor genes in breast cancer.

Transiently restored expression of MBD2 leads to resuppression of target genes

In order to establish that MBD2 is both necessary and sufficient to suppress transcription of methylated target genes, and given that methylation was stabley maintained, we reasoned that restoring MBD2 in knockdown cells would re-establish transcriptional silencing. In order to restore functional levels of MBD2 protein in cells constitutively expressing anti-MBD2 shRNA, we needed to introduce a silent point mutation within the shRNA binding site of the expressed mRNA. We found we were able to transiently express the MBD2 B mutant message and generate significant levels of MBD2 protein in stable knockdown MCF-7 cells. By incorporating a co-translated V5 affinity tag, we were able to trace the expression and localization of exogenous protein; we verified that it targeted to the nucleus and further confirmed tight colocalization with heterochromatic mitotic forms.

We examined the expression levels of MBD2 regulated genes in knockdown and scramble control cells following restoration of MBD2 protein and found that the expression of these genes was silenced within 72 hours of transfection with the shRNA immune MBD2 construct. This finding confirmed the primary importance of MBD2 in maintaining the transcriptional repression of specific target genes. It further indicates that all associated elements required for the epigenetic silencing of MBD2 dependent genes remain intact and poised to resuppress transcription in the presence of MBD2.

156

The observation that restored MBD2 leads to restored silencing has dual mechanistic and clinical implications. First, these data provide indirect confirmation that methylation remains stable at other tumor suppressor genes despite increases in transcription in MBD2 knockdown cells. In providing this confirmation, we can discount the possibility that MBD2 knockdown induced expression changes are due to a 'hit and run' phenomenon at these loci. In other words, MBD2 knockdown is probably not inducing a persistent and irreversible change in site specific patterns of gene expression. Nevertheless, we can not rule out the possibility that irreversible changes are occurring at other sites and whether or not MBD2 knockdown induces some form of epigenetic reprogramming in breast cancer remains to be determined.

Finally, it appears for the moment as though any future clinical strategy aimed at inhibition of MBD2 in breast cancer will have to follow a long term treatment model in order to prevent the reversal of MBD2 induced expression changes. However, an indefinite treatment strategy is not necessarily indicated, with the notable exception of a preventative approach for high risk patients. A potent short term MBD2 dependent cytostatic effect may be sufficient to shift the balance between tumor growth and immune clearance or combinatorial killing, thereby achieving the therapeutic goal of tumor cell eradication.

MBD2 knockdown upregulates DNMT1 protein

Upon observing that DNA methylation at MBD2 knockdown induced genes remained methylated, we were intrigued by the possibility that tumor cells may activate compensatory mechanisms to maintain methylation and methylation dependent gene silencing. Such compensatory mechanisms would obviously benefit cancer cells which

157

depend on the inactivation of tumor suppressor genes for survival and proliferation. However we considered the possibility that such compensation might originate as benign feedback regulation intended to maintain methylation directed patterns of expression, for example during normal development and differentiation. We grew interested in DNMT1 in particular because of several reports linking it to methyl binding domain proteins, including MBD2, and chromatin modifying enzymes (Kimura and Shiota 2003; Fuks et al. 2003; Tatematsu, Yamazaki, and Ishikawa 2000).

We examined the levels of DNMT1 protein in control and MBD2 knockdown breast cancer cells by immunoblot and found that DNMT1 protein was increased following depletion of MBD2. DNMT1 levels increased by approximately 3 fold and 1.5 fold in MCF-7 and MDA-MB-468 MBD2 knockdown cells, respectively. At the time of this writing, we do not know the mechanism of increased DNMT1 in MBD2 knockdowns; moreover, we currently do not know whether the observed increase in DNMT1 has functional significance. Nonetheless, the observation is intriguing and raises the possibility that a feedback mechanism exists to maintain methylation directed gene silencing. Such a mechanism might be present in breast cancer cells alone, in other types of cancer, or perhaps more generally in normal cells.

While there may be a direct interaction underlying the observed relationship between MBD2 and DNMT1, we consider it more likely that DNMT1 is being induced indirectly by a methylated MBD2 dependent gene. Such a gene might be acting as a sensor for methylation directed silencing; a sort of methylation sensitive thermostat to keep methylation levels within an ideal window. According to this model, changes in the levels of this MBD2 regulated 'thermostat' gene would signal the methyltransferase

158

machinery to make compensatory changes in global DNA methylation, either increases in maintenance methylation or passive decreases, as required. It stands to reason that such a regulatory mechanism would benefit cells by preventing the depletion or pathologic accumulation of DNA methylation, both of which are known to be detrimental (Li, Bestor, and Jaenisch 1992a; Roll et al. 2008; Schmidt et al. 2007).

MBD2 knockdown post transcriptionally upregulates MeCP2 protein

Having observed an increase in the levels of DNMT1 in MBD2 knockdown cells, we grew interested in the effect of MBD2 knockdown on other functionally related proteins. In particular we were interested in MeCP2, a protein known to bind numerous methylated tumor suppressors, often simultaneously with MBD2 (Lopez-Serra et al. 2008a; Lopez-Serra et al. 2006). Because MeCP2 shares significant tumor suppressor silencing function with MBD2, we were alert to the possibility that compensatory increases in MeCP2 may be sufficient to suppress the cytotoxicity and tumor suppressor gene de-repression brought on by MBD2 knockdown.

We measured the expression and protein levels of MeCP2 in MBD2 knockdown MCF-7 cells and found that transcription of MeCP2, which itself has a large promoter associated CpG island, was not being induced. However, MeCP2 protein levels were increased by approximately 3 fold over controls in MBD2 knockdown cells. In addition, MeCP2 protein levels remained at background levels with overexpression of MBD2.

Considered together, these observations imply that that MeCP2 protein is somehow post-transcriptionally stabilized by MBD2 knockdown. Because there is was no significant increase in MeCP2 mRNA in MBD2 knockdown cells, it seems unlikely that

159

MBD2 represses MeCP2 transcription, either directly or indirectly through a secondary target gene. We cannot entirely rule out the possibility that a small increase in MeCP2 mRNA, below the dynamic range of our qPCR assay (approximately 2 fold), could be mediating a 3 fold increase in MeCP2 protein levels.

An immediate implication of the finding that MeCP2 protein is increased in MBD2 knockdown cells is the possibility that MeCP2 is compensating for reduced levels of MBD2 and maintaining transcriptional silence of at least those target tumor suppressors that are jointly bound by both MBDs. Given this possibility, we decided to investigate whether simultaneous knockdown of both proteins would lead to an enhanced cytotoxic effect in breast cancer.

Simultaneous knockdown of MBD2 and MeCP2 leads to an increase in apoptosis

In order to investigate whether MBD2 and MeCP2 act cooperatively to maintain inappropriate survival of breast cancer cells, we needed to knock both proteins down simultaneously. The most efficient method for the simultaneous knockdown of both genes was the use of siRNA, which in our hands provided the highest co-transfection efficiencies (>95%) when compared to plasmid transfection or lentivirus infection methods. Furthermore, we did not have the ability to segregate coinfected cells with lentivirus; shMBD2 + shMeCP2 infected cells as well as single virus transduced cells are GFP positive and FACS based sorting would not distinguish between the two. In addition, we made the choice to avoid an extended selection strategy such as that required for double antibiotic based MBD2+MeCP2 knockdown plasmid selection. As discussed previously, this approach had the significant disadvantage of selective purification over time of a population resistant to growth restriction and was therefore not suitable for

160

studying acute cytotoxicity. We do however concede that siRNA has its own limitations including some acute non-specific transfection-associated toxicity (which we controlled for using negative control siRNA) as well as transient knockdown.

We screened four breast cancer cell lines transfected with either MBD2 or MeCP2 targeted siRNA, or both together. Importantly, all transfections including negative control siRNA were conducted with equal amounts of siRNA to account for differences in toxicity associated with absolute amounts of exogenous double stranded RNA. We verified that these strategies provided adequate knockdown of target mRNA by quantitative PCR. Because the effects of transient siRNA transfection are inherently short lived, we screened for changes in acute toxicity using a hightly quantitative FACS based apoptosis assay.

We found that either MBD2 or MeCP2 knockdown alone led to increased apoptosis in 3 out of 4 cell lines tested. Moreover, we found that knocking both out simultaneously led to increased apoptosis in 2 out of 4 cell lines, i.e., 2 out of 3 sensitive lines. MCF-7 cells showed very little basal apoptosis by Annexin V positivity and no increase with any siRNA. This is not altogether surprising given a well described apoptotic pathway defect in these cells, specifically the absence of caspase 3. Furthermore, we can rationalize the absence of increased apoptosis in siMBD+siMeCP2 transfected SK-BR-3 cells based on the poor double knockdown achieved in this particular cell line. Interestingly, we found that all three sensitive cell lines were more responsive to MeCP2 than to MBD2 knockdown with respect to acute apoptotic cell death. It is worthwhile to note that, unlike MBD2, loss of MeCP2 function is not benign with respect to human development

161

and disease, though a post developmental requirement for MeCP2 has not been demonstrated.

In summary, our results strongly suggest that MBD2 and MeCP2 act cooperatively to maintain the growth of breast cancer cells. It remains to be determined if long-term, stable knockdown of both proteins simultaneously is capable of producing a more significant anti-tumor effect. We conclude that a combination of MeCP2 induced acute toxicity with MBD2 induced long term cytostasis may provide the maximal benefit to future breast cancer patients who are treated with methyl binding domain protein targeting agents.

Chapter IV: Summary and Perspectives

MBD2 inhibition as an epigenetic therapy in breast cancer

Breast cancer, like many malignancies, collects epigenetic abnormalities during progression from normal epithelium to frank carcinoma. An important, common, and early change in breast cancer is hypermethylation of tumor suppressor gene associated CpG islands. The origin of abnormal methylation in cancer is not completely understood, but two principal mechanisms are thought to be involved. The first is targeted methylation of specific genes by intrinsic dysfunction or irregular recruitment of de novo DNA methyltransferases (Linhart et al. 2007). The second and probably more common mechanism is global dysregulation of the epigenetic machinery in malignant and premalignant cells. This dysregulation leads to widespread methylation changes which occur at random and are enriched over time for those adaptations which promote survival, growth, metastases, or resistance to therapy (Jones and Baylin 2007; Widschwendter and Jones 2002).

The stochastic appearance of abnormal methylation in malignancy has been an obstacle to the utility and reliability of epigenetic therapies. Not all methylation is undesirable and therapies incapable of discriminating between normal and pathologic changes are inherently unpredictable. A more targeted approach for reactivating transcription that is specific, effective, and safe in non-neoplastic tissues is necessary. Methyl DNA Binding Domain (MBD) proteins are recruited to specific sites of CpG methylation and lead to local transcriptional inhibition. Inhibiting MBD proteins may provide an alternate, targeted approach for reactivating epigenetically silenced tumor suppressor genes in cancer. However, the role of MBDs in tumorigenesis must be better

163

characterized to validate their potential as therapeutic targets. We recognized this as an opportunity and initiated a series of studies to examine the function of MBD2 in breast cancer.

An important factor in choosing to study MBD2 was the knowledge that this protein is not required for normal mammalian development or for the viability of normal differentiated epithelial tissue. In addition, previous studies indicated that MBD2 bound a large number of methylated tumor suppressor CpG islands in tumor cells. We hypothesized, therefore, that inhibition of MBD2 was a viable strategy for selectively targeting tumor cells. We used double stranded shRNA mediated knockdown of MBD2 to study the function of this protein and found that depletion of MBD2 from breast cancer cells led to changes consistent with the hypothesis that breast cancer cells grow dependent on this protein. An integrated overview of our findings, a discussion of their consequences, and opportunities for further study are presented below.

Breast cancer depends on MBD2 for maximal growth

We found that MBD2 knockdown produced a variable and at times profound growth inhibition in human breast cancer cell lines in culture. In addition, the ability of aggressive breast cancer cell lines to form tumors in nude mice was significantly impaired by MBD2 knockdown. We made several correlative observations regarding the nature of MBD2's effects on growth rate. First, the antiprolifereative effects required a significant depletion of MBD2 (~80%) before becoming apparent, suggesting MBD2 mediated growth is not strictly speaking a dose dependent type of response. This observation implied that MBD2 is present in sufficient excess to adequately perform its

164

functions even at less than half of its baseline concentration in breast cancer cells. While it explains why MBD2 is not commonly found overexpressed in primary breast cancers, this observation conflicts with the finding of Sansom, Bird and colleagues who showed that knocking out a single allele of MBD2 reduced adenoma burden in Apc^{Min} mice (Sansom et al. 2003a). We submit that one reason for this conflict may be somewhat higher basal levels of MBD2 in cultured breast cancer cell lines than in normal epithelial cells. In addition, the upregulation of DNMT1 and MeCP2 we have described implies that compensatory mechanisms must be overcome in order to produce observable changes. Further studies are required to determine the existence of a dose dependent relationship between MBD2 and the phenotype of breast cancer, for example in future pharmacokinetics and pharmacodynamics type studies of MBD2.

We also found that variation existed between cell lines with respect to the degree of growth suppression produced by MBD2 knockdown. These variations were substantiated by differences in patterns of CpG island methylation between cell lines as well as differences in the spectrum of gene expression changes observed in MBD2 knockdown lines. Undoubtedly the full spectrum of phenotypic changes in a given cell line depends not only on the function of MBD2 but on the variable methylation, differential expression of other epigenetic modifiers and on genetic lesions that fundamentally cripple certain pathways (e.g., cell cycle arrest and apoptosis). The influence of such factors can not be underestimated and taken together with variability in MBD2 dependent response we conclude that a certain subset of tumors will ultimately be more susceptible to MBD inhibitors than others. The dependence of MBD2 induced growth inhibition on patterns of methylation, particular biochemical pathways, or specific modifying factors in breast

165

cancer must be more thoroughly characterized so that we may rationally predict which tumors will respond best to MBD2 inhibition in patients.

Rapid and Sustained depletion of MBD2 is necessary to inhibit growth

A significant observation regarding the conditions required to reproducibly inhibit the growth of breast cancer was that a rapid and persistent MBD2 knockdown was required. When we used transient double stranded siRNA oligonucleotides we achieved a rapid knockdown, however the depletion of MBD2 was short lived. We found that MBD2 knockdown by this method induced a slight increased acute toxicity; a relatively minor in increase in apoptosis was found in MBD2 knockdown cells, which we later found was augmented by simultaneous knockdown of MeCP2. On the other hand, persistent knockdown by antibiotic selection similarly failed to demonstrate significant growth inhibition. In this latter case, we surmised that during the extended period of selection and regrowth, we were losing the growth inhibited population and enriching for cells which at some low frequency were escaping MBD2 knockdown induced cytostasis. Consistent with this theory was our observation that stable clones were either more difficult (MDA-MB-231) or not possible to isolate (MDA-MB-435 and SK-BR-3) while stable Scramble shRNA transformants regrew at higher frequencies.

A lentivirus based shRNA transduction proved to be the ideal method for measuring the growth restriction imposed by MBD2. While a robust MBD2 knockdown was found within days of infection the growth inhibition took somewhat longer to fully materialize. We found that the maximum effect on growth occurred only after FACS purification of transduced cells and took between 2 and 3 weeks to develop. We reasoned that

166

derepression of downstream effectors and their subsequent cytostatic effects might require this period to reach peak activity.

It is interesting to speculate whether a gradual epigenetic reprogramming is occurring in MBD2 knockdown tumor cells, following a delayed onset model analogous to the reprogramming of iPS cells. Our bisufite sequencing data suggest that if such a mechanism is in use, it does not rely on the rewrtiting of CpG methylation at tumor suppressor promoters. However, the availability and cost of global epigenetic profiling methods is becoming increasingly permissive; looking ahead it will be interesting to make use of such strategies to examine the broad spectrum epigenetic variation in MBD2 knockdown tumor cells.

We also considered the possibility that an autocrine/paracrine feedback system was being disrupted by MBD2 knockdown. Tumor cells often secrete growth factors into their environment that promote their own growth as well as the growth of adjacent cells. If such a pathway was disrupted, then FACS based purification of knockdown cells may be a strict requirement for growth disruption. Recall that maximal growth inhibition occurred shortly after FACS purification, as detailed in our methods and discussed above. Importantly, our animal studies suggest that if autocrine/paracrine factors are being eliminated from the media of MBD2 knockdown cells *in vitro*, a suitable replacement is not supplied by the stromal environment in animals. Future studies aimed at correlating population purity with growth and using control cell conditioned media in rescue experiments will demonstrate whether such a mechanism is in fact at work.

167

MBD2's role in disrupting epithelial morphology and differentiation

We found that MBD2 knockdown resulted in distinct changes in morphology of breast cancer cells, which began to more closely resemble their normal counterparts in culture. MCF-7 knockdown clones also demonstrated a change in 3D morphology more reminiscent of normal epithelial tissue architecture. Addressing this observation alongside the growth inhibition which occurs in the same breast cancer cells, we contend that changes in morphology and proliferation are likely to be fundamentally related. It is conceivable that the net function of MBD2 knockdown is to restore elements of normal epithelial differentiation in high grade mammary derived tumors. Put another way, MBD2 is functioning in tumors to maintain an abnormally dedifferentiated state, essentially as we hypothesized. Consistent with this assertion is the acknowledged role of DNA methylation in tissue specific gene expression patterning during development (Suzuki and Bird 2008; Eckhardt et al. 2006). This normal pattern of expression is fundamentally dysregulated in tumors in large part through epigenetic modifications. We conclude that MBD2 knockdown functions to reverse elements of this epigenetic dysregulation and leads to redifferentiation of breast cancer cells towards a more normal state.

The attention of the breast cancer research community of late has hovered around several themes which converge here. This convergence can be distilled to the following three specific topics: the role of cancer stem cells, the tumor stromal microenvironment, and the epithelial to mesenchymal transition; each of these topics has been extensively reviewed elsewhere (Thiery et al. 2009; Weigelt and Bissell 2008; Gupta, Chaffer, and Weinberg 2009; Polyak and Weinberg 2009). With respect to our work, the proportion of

168

mamospheres (i.e., ordered acinar forms in 3D culture) has been associated with the presence of so called cancer stem cells. Similarly, 2D and 3D morphology changes such as the ones we have described have been associated with altered tumor-stromal interactions and pathological epithelial to mesenchymal transition in breast cancer. These phenomena have significant clinical implications and our observations, while still rudimentary, suggest that MBD2 may be playing a central role in mediating pathologic shifts in tumor composition and environment. Further studies aimed at integrating MBD2's role in maintaining a population of cancer stem cells, in mediating tumor stromal interactions and in maintaining pathologic EMT in breast cancer are needed.

MBD2 as a component of a combinatorial treatment strategy

Our studies to date have not uncovered a combined treatment approach that demonstrates bona fide synergy with MBD2 knockdown. We have tested several compounds covering the drug classes commonly used as first line chemotherapy in breast cancer. They included a nitrogen mustard derivative (cyclophosphamide, Cytoxan), anthracyclines (Doxorubicin) and taxanes (Paclitaxel). We have also tested the HDAC inhibitor, Vorinostat, and a platinum compound, Cisplatin. We found that the effect of MBD2 knockdown and the individual chemotherapies remained largely independent of each other, i.e., they were neither supra-additive nor did they annul one another. The consequences of this observation are multiple including the prospect that MBD2 may not function as a sensitizer in the strictest sense but may be an effective adjunct treatment. Of pressing importance is the outstanding need to determine the effects of MBD2 on normal tissues, for example normal epithelium and bone marrow. Such normal

169

mitotically active tissues are the well known sites of off target toxicities that limit the effectiveness of most chemotherapy. There is good reason to believe MBD2 inhibition will not affect normal tissues, as discussed extensively throughout this dissertation and elsewhere (Sansom, Maddison, and Clarke 2007b; Klose and Bird 2006; Berger and Bird 2005a; Martin Caballero et al. 2009; Sansom et al. 2005). However, ongoing and future studies must experimentally demonstrate the innocuous nature of MBD2 targeting strategies in normal human tissues, including primary epithelial cells and the lymphoid/myeloid compartment. Furthermore, additional studies which seek to identify biochemical and biological anti-neoplastic agents which act synergistically with MBD2 knockdown are needed.

MBD2 dependent gene regulation in breast cancer

Our studies of the gene expression changes induced by MBD2 knockdown in cultured breast cancer lines reveal insight in to the function of this pleotropic protein and provide a mechanistic backdrop for our phenotypic observations. Recall our contention that MBD2 knockdown offers a more targeted approach to methylated tumor suppressor gene reactivation. In support of this hypothesis, we found that only a subset of the tumor suppressor genes we examined were transcriptionally reactivated in MBD2 knockdown breast cancer cells. Further supporting this idea was the observation that patterns of gene expression in MBD2 knockdown cells overlapped only partially with those induced by 5-aza-dC treatment. ChIP experiments suggest that both direct and indirect tumor suppressor gene reactivation occurs at methylated promoters, with large CpG island-associated genes being preferentially reactivated through direct depletion of promoter

170

bound MBD2. Both direct and indirect (secondary) gene expression changes are no doubt involved in bringing about the full spectrum of phenotypic changes.

Not unlike the variation we have described in the response to MBD2 knockdown, there were subtle differences in the pattern of gene restoration between different cell lines. We attribute these changes to several factors; chief among these is the likelihood of differential methylation in different cells. In addition, we have not yet determined whether any MBD2 associated corepressor components are aberrantly expressed in different cell lines. To put it concisely, we do not yet know enough about the spectrum of CpG methylation, epigenetic defects, or MBD2 occupancy in these various cell lines; outside of the few genes we have surveyed more thoroughly, we can not be certain where MBD2 is acting directly and how this changes between cell lines. As the epigenomes of primary tumors and established cell lines become better characterized, we can revisit our gene expression data and perhaps glean further insight into the mechanism of differential response to MBD2 knockdown.

Finally, we remain open to the possibility that MBD2 has regulatory functions in the cell which are unrelated to its role in transcriptional silencing of methylated genes. Though no such activity has yet been convincingly attributed to MBD2, the evolutionary divergence of the MBD family of proteins suggests such function(s) may well exist. In order to fully evaluate the importance of the methyl DNA binding properties of MBD2 in mediating phenotypes in breast cancer, further studies are needed. One such study might involve the restoration of MBD2 with mutant lacking the ability to bind methyl CpG in stable knockdown cells and determining whether this mutant is capaple of reversing MBD2 dependent changes.

171

MBD2 likely acts through multiple targets to affect the phenotype of breast cancer

A question remains as to the mechanism of the growth inhibition that follows MBD2 knockdown. We have discussed a minor increase in acute toxicity, measurable as crude cell death (propidium iodide uptake) as well as apoptosis (Annexin V positivity). By far the greatest effect of MBD2 knockdown appears to be a cytostatic one, which occurs following an initial refractory period. The phenomenon we observe closely resembles cellular senescence, including replicative cessation and characteristic changes in morphology (Adams 2009; Caino, Meshki, and Kazanietz 2009). However we failed to note any increase in beta galactosidase activity commonly associated with cellular senscense (not shown). Additional studies to determine if cellular senescence is being induced are warranted, including assaying telomerase levels and activitiy.

Based on our unsuccessful efforts to identify downstream mediators of MBD2 dependent phenotypes, we are left with the impression that a single downstream target (direct or indirect) is probably not responsible for the full spectrum of changes. It is certainly possible that we have not yet found the right gene; admittedly the subset we are examining is small. However, it seems intuitively more likely to us that MBD2 dependent gene repression, which to our knowledge is not restricted to any functional category of tumor suppressor, is holding multiple pathways in check. In other words, inhibition of MBD2 is initiating a divergent cascade of secondary effects which depend on more than one, and probably many, downstream targets.

172

Based on such a model (MBD2 works through multiple targets), we will revisit the idea of resistance to MBD2 knockdown imposed growth inhibition in the following section. For the moment we acknowledge that further studies are required to identify the precise mechanism of knockdown induced growth inhibition and the key genes which mediate MBD2 dependent phenotypes.

Considering resistance to MBD2 knockdown

At several points in this dissertation we have described a population of cells which emerge from MBD2 knockdown imposed growth restriction; specifically, late developing tumors in mouse xenograft models and stable MBD2 knockdown clones which survive extended antibiotic selection. The mechanism of this acquired resistance remains unknown. As mentioned in the previous section, we interpret our data as a whole to suggest that MBD2 mediated gene repression is stifling multiple pathways in breast cancer. However we cannot exclude the possibility that a small group or perhaps even a single key downstream mediator exists. These mutually exclusive models of MBD2 function suggest one of two processes is required for a given cell to acquire resistance to MBD2 knockdown.

If a small group or a single gene is mediating MBD2 knockdown imposed growth restriction, resistance can develop by multiple mechanisms, including high level compensation for MBD2 by proteins with similar function or low level mutations in key MBD2 target genes or pathway components. If this was the case, we would expect a relatively homogeneous population of resistant clones with relatively uniform growth properties that would be difficult to distinguish from control cells. On the other hand, if

173

multiple pathways are involved resistance might develop at various points, including high level compensation or disruptions in any one of the downstream pathways. In the latter case, we would expect the population of resistant clones to be more heterogeneous with varied growth characteristics depending on the particular point of disruption.

In theory, a careful study of resistant clones might glean some insight into the mechanism underlying MBD2 knockdown imposed growth restriction. We currently do not have sufficient data vis-à-vis resistance to reliably draw any conclusions and therefore future studies aimed at better characterizing resistant populations are necessary. Furthermore, a clearer understanding of the mechanism(s) of resistance will allow us to better predict the clinical course of future strategies aimed at inhibiting MBD2 in breast cancer patients.

Promoter CpG island methylation is independent of transcription

DNA methylation is often thought to vary inversely with transcription and to be the principle mark that nucleates the formation of heterochromatin. However recent reports have led to the realization that this view is overly simplistic. Trancriptionally silent heterochromatin persists even in the absence of significant DNA methylation and conversely high levels of methylation, for example in bodies of actively transcribed genes, do not appear to affect transcription to any measureable extent (Suzuki and Bird 2008). It is still widely accepted, however, that promoter CpG island-associated methylation is a strict barrier to transcription, both at developmentally regulated sites of differential promoter methylation and pathologically in cancer.

174

We found that promoter CpG island methylation remained remarkably stable despite significant levels of transcriptional induction in MBD2 knockdown cells. This observation casts doubt on the idea that transcriptional interference leads to passive demethylation over time. Granted these observations are made in the abnormal environment of a tumor cell and generalizing to normal tissues may be injudicious. We concede there may pathologic factors at work to maintain CpG island methylation in cancer cells, nevertheless this observation is provocative. Considering the absence of methylation from most CpG islands, even in tissues where the respective genes are not expressed, we suggest that the mechanisms directing DNA methylation are largely independent of transcription.

This is not to say that the two are unrelated, a great deal of credible evidence exists linking CpG island hypermethylation to transcriptional silencing in development and disease. We can safely draw the following conclusion from our data: methyl-binding proteins such as MBD2 seem to be the primary link between hyper-methylation and transcription at hyper-methylated tumor suppressors. Further support for this conclusion is offered by our observation that restoring MBD2 expression in stable knockdown cells led to the re-silencing of persistently methylated tumor suppressor genes. In order to evaluate whether persistent methylation of transcribed genes occurs at sites of tissue-specific promoter methylation in normal cells, corresponding studies in normal tissues are required.

175

MBD2 is a component of a dynamic epigenetic network in breast cancer

Our work has largely focused on the specific function of MBD2; however a survey of the effects of MBD2 knockdown on other elements of the epigenetic landscape has led to some notable findings. As discussed throughout the present work, MBD2 does not exist in isolation. While this protein is clearly performing an important pathologic role in breast cancer, we must consider it in its broader environmental context.

The epigenetic silencing of tumor suppressor genes involves the coordinated action of methyltransferases, methyl binding proteins, and corepressor complexes; it is therefore not surprising that we found an element of coregulation exists between these factors.

We suspected that MBD2 knockdown cells may be implementing compensatory mechanisms to overcome the deleterious effects of knockdown. We found that levels of DNMT1 and MeCP2 protein were increased in stable knockdown cells. Increased levels of DNMT1 may be acting to counteract passive demethylation, perhaps accounting for the stability of CpG methylation at transcriptionally active tumor suppressor genes. Similarly, increases in other methyl binding proteins such as MeCP2 may be compensating to some degree for the loss of MBD2. In support of a compensation model, we found that simultaneous knockdown of MBD2 and MeCP2 led to an increase in apoptosis in breast cancer cell lines. These knockdown studies were performed with transient siRNA; long term stable knockdown studies to fully evaluate the requirement for MeCP2 in breast cancer cells are needed.

Conclusion

In conclusion, the body of work presented here strongly supports a pathological role for MBD2 in the growth and *in vivo* survival of breast cancer. We find that MBD2 depletion by targeted shRNA leads to reexpression of methylated tumor suppressor genes without a corresponding change in DNA methylation. Restored gene expression in turn leads to growth inhibition, organized growth in 3D culture models, and tumor clearance in animals. While further studies are needed to evaluate the effects of MBD2 inhibition in normal tissues, based on the studies reported here we conclude that targeting MBD2 is a viable strategy for inhibiting the proliferation of human breast cancer. A model of the function of MBD2 and a summary of MBD2 dependent effects in breast cancer cells is given below in Figure 45.

Figure 45. Summary and Model: The effects of MBD2 knockdown on CpG methylation, tumor suppressor gene expression, growth and in vivo clearance of breast cancer.
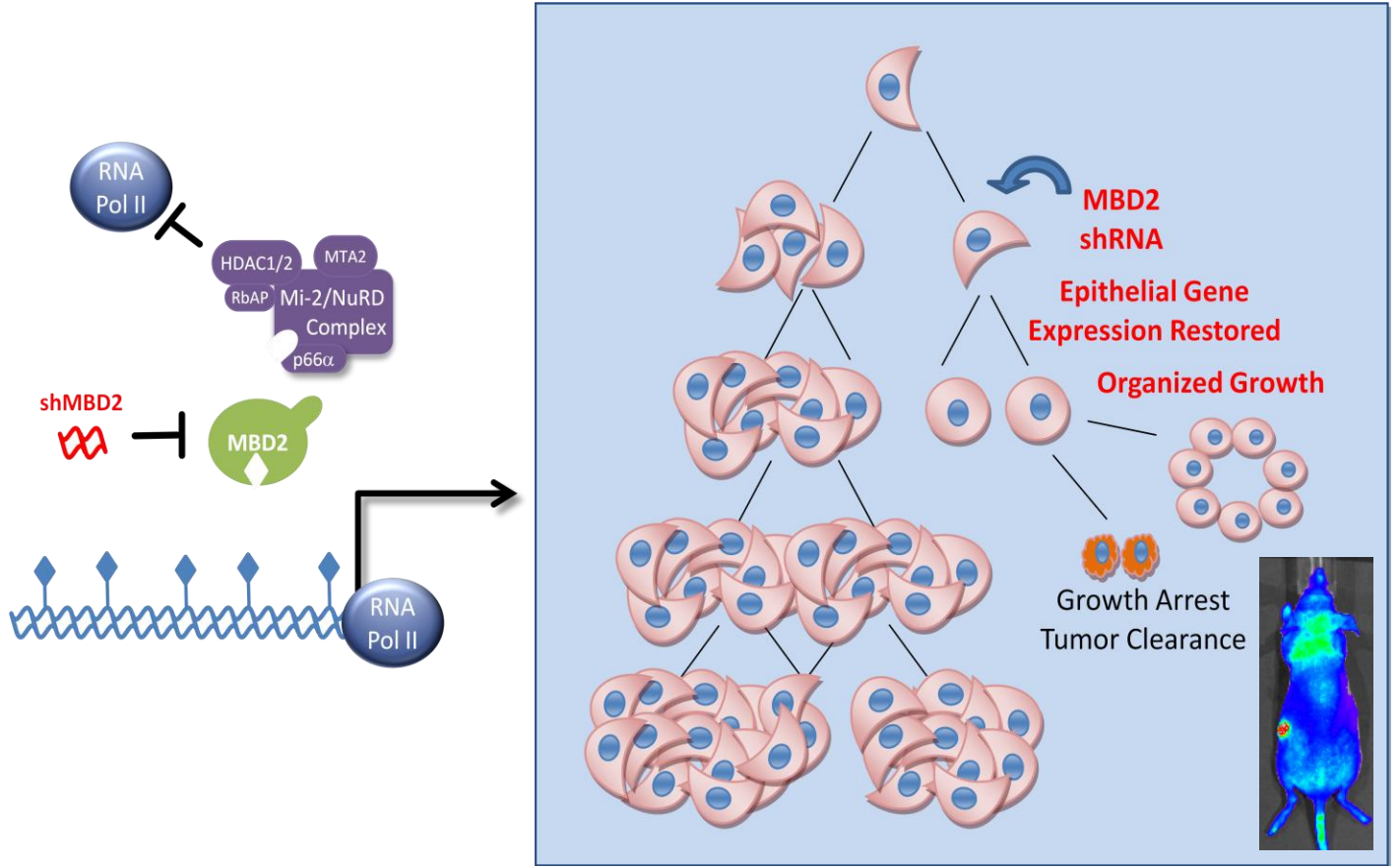
Figure 45. MBD2 depletion by targeted shRNA leads to reexpression of methylated tumor suppressor genes without a corresponding change in DNA methylation. Restored gene expression in turn leads to growth inhibition, organized growth in 3D culture models, and tumor clearance in animals.

178

Appendix A: Tumor Suppressor Genes Hyper-Methylated in Breast Cancer and their Function

Name	Extended Name	Function(s)	Biological Process(s)	Pathway(s)
ADAM23	ADAM metallopeptidase domain 23	Metalloprotease	Proteolysis; Cell adhesion-mediated signaling; Neuronal activities	
APC	adenomatosis polyposis coli	Non-motor microtubule binding protein	receptor mediated signaling pathway; intracellular signaling cascade; Cell adhesion; Apoptosis; Cell cycle control; Tumor suppressor	Angiogenesis->Adenomatous Polyposis of the Colon; Wnt signaling pathway->Adenomatous Polyposis Coli;
ATM	ataxia telangiectasia mutated (includes complementation groups A, C and D)	Nucleic acid binding; Non-receptor serine/threonine protein kinase; Nucleotide kinase	DNA repair; Protein phosphorylation; Stress response; Induction of apoptosis; Cell cycle control	p53 pathway feedback loops 2->ATM; EGF receptor signaling pathway->phosphatidylinositol 3-kinase; p53 pathway->Ataxia telangiectasia mutated (ATM) and Rad3-related (ATR);
BAX	BCL2-associated X protein	signaling molecule	Induction of apoptosis; Gametogenesis; Hematopoiesis; Cell cycle control; Cell proliferation and differentiation; Tumor suppressor	p53 pathway->BCL2-associated X protein; Apoptosis signaling pathway->Bcl-2 associated x protein; Huntington disease->Bcl-2-associated X protein;
BRCA1	breast cancer 1, early onset	Zinc finger transcription factor	DNA repair; mRNA transcription regulation; Cell cycle control; Tumor suppressor	
CCND2	cyclin D2	Kinase activator	Cell cycle control; Mitosis; Cell proliferation and differentiation	Wnt signaling pathway->Wnt Target Genes; Cell cycle->Cyclin D; PI3 kinase pathway->Cyclin d;

179

Name	Extended Name	Function(s)	Biological Process(s)	Pathway(s)
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	Cadherin	Cell adhesion-mediated signaling; Cell adhesion	Alzheimer disease-presenilin pathway->E-cadherin transmembrane fragment; Alzheimer disease-presenilin pathway->E-cadherin C-terminal fragment; Wnt signaling pathway->Cadherin; Alzheimer disease-presenilin pathway->E-cadherin N-terminal fragment; Alzheimer disease-presenilin pathway->E-cadherin; Cadherin signaling pathway->Cadherin; Alzheimer disease-presenilin pathway->E-cadherin intracellular fragment;
CDH13	cadherin 13, H-cadherin (heart)	Cadherin	Cell adhesion-mediated signaling; Cell adhesion; Oncogenesis	Wnt signaling pathway->Cadherin; Cadherin signaling pathway->Cadherin;
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	Kinase inhibitor	Cell cycle control; Tumor suppressor	Ras Pathway->p14/19 ARF; p53 pathway feedback loops 2->p14/19 ARF; p53 pathway->Alternative reading frame (tumor-suppresor protein); p53 pathway->ARF;
CLCA2	chloride channel, calcium activated, family member 2	Ion channel	Miscellaneous	
DAB2IP	DAB2 interacting protein	Molecular function unclassified	Biological process unclassified	EGF receptor signaling pathway->GAP;
DAPK1	death-associated protein kinase 1	Non-receptor serine/threonine protein kinase	Protein phosphorylation; Induction of apoptosis	
DIRAS3	DIRAS family, GTP-binding RAS-like 3	Molecular function unclassified	Biological process unclassified	

180

Name	Extended Name	Function(s)	Biological Process(s)	Pathway(s)
ESR1	estrogen receptor 1	Nuclear hormone receptor; Transcription factor; Nucleic acid binding	mRNA transcription regulation; Steroid hormone-mediated signaling; neuronal activity; Oogenesis; Cell cycle control; Mitosis; Cell proliferation and differentiation; Cell motility	
ESR2	estrogen receptor 2 (ER beta)	Nuclear hormone receptor; Transcription factor; Nucleic acid binding	mRNA transcription regulation; Steroid hormone-mediated signaling; neuronal activity; Oogenesis; Cell cycle control; Mitosis; Cell proliferation and differentiation; Cell motility	
FHIT	fragile histidine triad gene	Nucleotide phosphatase	Biological process unclassified	
GPC3	glypican 3	Cell adhesion molecule; Extracellular matrix glycoprotein	Cell adhesion	
GSTP1	glutathione S-transferase pi	transferase	Detoxification	
HIC1	hypermethylated in cancer 1	Zinc finger transcription factor; DNA-binding protein	mRNA transcription regulation	
HOXA5	homeobox A5	Homeobox transcription factor; Nucleic acid binding	mRNA transcription regulation; Segment specification; Neurogenesis	Wnt signaling pathway->Wnt Target Genes;
KLK10	kallikrein-related peptidase 10	Serine protease	Proteolysis	
LAMA5	laminin, alpha 5	Extracellular matrix linker protein	Cell adhesion-mediated signaling; Extracellular matrix protein-mediated signaling	Integrin signalling pathway->Laminin;

181

Name	Extended Name	Function(s)	Biological Process(s)	Pathway(s)
MGMT	O-6-methylguanine-DNA methyltransferase	DNA methyltransferase; DNA methyltransferase	DNA repair	
NME1	non-metastatic cells 1, protein (NM23A) expressed in	Nucleotide kinase	Pyrimidine metabolism	De novo purine biosynthesis->GDP kinase; De novo pyrimidine deoxyribonucleotide biosynthesis->dTDP kinase; De novo purine biosynthesis->dADP kinase; De novo pyrmidine ribonucleotides biosythesis->UDP kinase; De novo pyrimidine deoxyribonucleotide biosynthesis->dCDP kinase; De novo pyrmidine ribonucleotides biosythesis->CDP kinase; De novo pyrimidine deoxyribonucleotide biosynthesis->dUDP kinase; De novo purine biosynthesis->dGDP kinase;
PAX5	paired box gene 5 (B-cell lineage specific activator)	Homeobox transcription factor; DNA-binding protein	mRNA transcription regulation; Neurogenesis; Gut mesoderm development	
PGR	progesterone receptor	Nuclear hormone receptor; Transcription factor; Nucleic acid binding	mRNA transcription regulation; Steroid hormone-mediated signaling; neuronal activity; oncogenesis	
PLAGL1	pleiomorphic adenoma gene-like 1	zinc finger transcription factor	mRNA transcription regulation; developmental process	
PRDM2	PR domain containing 2, with ZNF domain	KRAB box transcription factor	Biological process unclassified	
PRSS8	protease, serine, 8 (prostasin)	Serine protease	Proteolysis; Immunity and defense; Spermatogenesis and motility	
PTCH1	patched homolog 1 (Drosophila)	Molecular function unclassified	Biological process unclassified	Hedgehog signaling pathway->Patched;

182

Name	Extended Name	Function(s)	Biological Process(es)	Pathway(s)
PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	Protein phosphatase; phosphatase	Phospholipid metabolism; Protein phosphorylation; Signal transduction; Cell adhesion; Immunity and defense; Induction of apoptosis; Cell cycle control; Cell proliferation and differentiation; Tumor suppressor	Insulin/IGF pathway-protein kinase B signaling cascade->Phosphatase and tensin homolog; Hypoxia response via HIF activation->phospatase and tensin homologue; PI3 kinase pathway->PTEN; p53 pathway->PTEN; Insulin/IGF pathway-protein kinase B signaling cascade->PTEN; Inflammation mediated by chemokine and cytokine signaling pathway->Phosphatase and tensin homologue; p53 pathway->Phosphatase and tensin homolog deleted on chromosome 10; p53 pathway feedback loops 2->PTEN; p53 pathway feedback loops 2->PTEN;
PYCARD	PYD and CARD domain containing	Protease inhibitor	Proteolysis; Apoptosis	
RARB	retinoic acid receptor, beta	Nuclear hormone receptor; Transcription factor; Nucleic acid binding	mRNA transcription regulation; Cell communication; Developmental processes; Oncogenesis	Vitamin D metabolism and pathway->Retinoid X Receptor, Alpha;
RASSF1	Ras association (RalGDS/AF-6) domain family 1	G-protein modulator	Signal transduction; Apoptosis	
RB1	retinoblastoma 1 (including osteosarcoma)	transcription factor; Nucleic acid binding	mRNA transcription regulation; Developmental processes; Cell cycle control; Tumor suppressor	p53 pathway feedback loops 2->Rb;
MASPIN SERPINB5	serpin peptidase inhibitor, clade B (ovalbumin), member 5	Serine protease inhibitor	Proteolysis	p53 pathway->Maspin;

183

Name	Extended Name	Function(s)	Biological Process(es)	Pathway(s)
SFN	stratifin	chaperones	Signal transduction; protein targeting and localization; Cell cycle	EGF receptor signaling pathway->14-3-3; FGF signaling pathway->14-3-3; p53 pathway->14-3-3 sigma; Parkinson disease->14-3-3; p53 pathway->14-3-3; PI3 kinase pathway->14-3-3;
SNCG	synuclein, gamma (breast cancer-specific protein 1)	signaling molecule; cytoskeletal proteins; miscellaneous function protein	MAPKKK cascade; apoptosis; oncogenesis	Parkinson disease->gamma-Synuclein;
SOCS1	suppressor of cytokine signaling 1	Kinase inhibitor	Cytokine and chemokine mediated signaling pathway	JAK/STAT signaling pathway->Suppressors of cytokine signaling;
SYK	spleen tyrosine kinase	Non-receptor tyrosine protein kinase	Protein phosphorylation; Intracellular signaling cascade; immune and defense; Embryogenesis; Cell proliferation and differentiation	B cell activation->Syk;
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	transcription factor	mRNA transcription regulation; Ectoderm development; Oncogenesis	
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	cytokine receptor; Serine/threonine protein kinase receptor; Protein kinase	Protein phosphorylation; Cytokine and chemokine mediated signaling pathway; Receptor protein serine/threonine kinase signaling pathway; developmental process	TGF-beta signaling pathway->TGFbetareceptor II; TGF-beta signaling pathway->TGFbetareceptors;
THBS1	thrombospondin 1	signaling molecule; enzyme regulator; extracellular matrix	Blood clotting; developmental process	p53 pathway->Thrombospondin-1;

184

Name	Extended Name	Function(s)	Biological Process(es)	Pathway(s)
THRB	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)	Nuclear hormone receptor; Transcription factor	mRNA transcription regulation; Steroid hormone-mediated signaling; Sensory perception; Developmental processes	
TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	Metalloprotease inhibitor	Proteolysis	
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	Tumor necrosis factor family member	Cytokine and chemokine mediated signaling pathway; Ligand-mediated signaling; Immunity and defense; Induction of apoptosis	Apoptosis signaling pathway->TNF-related apoptosis inducing ligand;
TP53	tumor protein p53 (Li-Fraumeni syndrome)	transcription factor	DNA repair; mRNA transcription regulation; Induction of apoptosis; Cell cycle control; Cell proliferation and differentiation; Tumor suppressor	P53 pathway feedback loops 1->P53; Huntington disease->P53 tumor suppressor phosphoprotein; Apoptosis signaling pathway->Tumor protein p53; p53 pathway by glucose deprivation->p53; p53 pathway->p53; p53 pathway->p53; p53 pathway feedback loops 2->p53; Wnt signaling pathway->Tumor suppressor p53;
TWIST1	twist homolog 1 (acrocephalosyndactyly 3, Saethre-Chotzen syndrome) (Drosophila)	Basic helix-loop-helix transcription factor; Nucleic acid binding	mRNA transcription regulation; Developmental processes; Cell proliferation and differentiation	

Appendix B: Quantitative RT-PCR Primers

Gene	refseq	Forward	Reverse
ACTB	NM_001101	CCTTGCACATGCCGGAG	GCACAGAGCCTCGCCTT
ADAM23	NM_003812	ACTGTGGTTTCCCATTTTCG	AATAAGGCTGTCCATCTGGC
APC	NM_000038	TCTCCATCTTCAGTGCCTCA	GGAGACAGAATGGAGGTGCT
ATM	NM_000051	TCATGGTCCAGTCAAAGAGTG	ACGGGTGTTGAAGGTGTCTT
BAX	NM_004324	CAGCCCATGATGGTTCTGAT	GACATGTTTTCTGACGGCAA
BCSG1	NM_003087	GATGGCCTCAAGTCCTCCTT	AGTGGCCGAGAAGACCAAG
BRCA1	NM_007294	GAAGGCCCTTTCTTCTGGTT	AGAGTGTCCCATCTGTCTGGA
CCND2	NM_001759	GCTTGGTCCAGTTCATCCTC	TGAGCTGCTGGCTAAGATCA
CDH1	NM_004360	GTCGAGGGAAAAATAGGCTG	GCCGAGAGCTACACGTTCAC
CDH13	NM_001257	CCTCAATGAATTCAGCTGGC	CTCGTTCTGTGCGTTCTCCT
CLCA2	NM_006536	CTGCGGCTTGTTGTAGTTGA	CCATGAATGGGACTGAGCTT
DAB2IP	NM_032552	GACTCGTGGGAGCGAGACT	GACCGCAACCACAGCTTC
DAPK1	NM_004938	CTCACGGCATTTCTTCACAA	TGGTGAGGCGTGACAGTTTA
DIRAS3	NM_004675	GCAGCTTCTGTTCCTTGGAG	CTTCTAGGCTGCTTGGTTCG
ESR1	NM_000125	AGGATCTCTAGCCAGGCACA	AAGCTTCGATGATGGGCTTA
ESR2	NM_001437	ATCACCCAAACCAAAGCATC	CCATGATCCTGCTCAATTCC
FHIT	NM_002012	ACCACTGTCCCGACTCTCTG	CTGTCCTTCGCTCTTGTGAA
GAPD	NM_002046	TTAAAAGCAGCCCTGGTGAC	CTCTGCTCCTCCTGTTCGAC
GPC3	NM_004484	CTTGAACATGGCATTGGTGT	ACAGCACGATTGAACATGGA
GSTP1	NM_000852	AATGAAGGTCTTGCCTCCCT	GACCTCCGCTGCAAATACAT
HIC1	NM_006497	ACAGGTTGTCACCGAAGCTC	CGACGACTACAAGAGCAGCA
HOXA5	NM_019102	TGGAACTCCTTCTCCAGCTC	AGATCTACCCCTGGATGCG
KLK10	NM_002776	CCGTTGAAGAGCGAGACCT	CTGCTGATGGCGCAACT
LAMA5	NM_005560	TTGGAGGAGGCAAAGAACTG	AACGTCACCCTGGACCTG
MASPIN	NM_002639	CCAGTGGCTCCTTTTCACAT	CTGTTCCTTTTCCACGCATT
MBD1	NM_015846	GCTGGATAGCACAAGATGCC	TGTGGACGCTCAGACACCTA
MBD2	NM_003927	AAGTGCTGGCAAGAGCGATGTCTA	TTTCCCAGGTACCTTGCCAACTGA
MBD3	NM_003926	CGGCTCTTGTTCATCTTGCT	GGCCACAGGGATGTCTTTTA

186

MeCP2	NM_004992	TGGACACGGAAGCTTAAGCAAAGG	CTCTCCCAGTTACCGTGAAGTCAA
NME1	NM_000269	GGCCCTGAGTGCATGTATTT	TTTGAGCAGAAAGGATTCCG
p16/CDKN2A	NM_058195	GTGAGAGTGGCGGGGTC	TCGTGCTGATGCTACTGAGG
PAX5	NM_016734	ACACTGCTCCCGATGTCAG	CATCTTCACCACCACAGAGC
PGR	NM_000926	CGATGCAGTCATTTCTTCCA	AATCTGTGGGGATGAAGCAT
PLAGL1	NM_002656	TTCCGGTTGAACGTCTTCTC	CAAACTTCTGGGAGGACTCG
PRDM2	NM_012231	CTTTGAAGAATGGTCCCTGG	CAAGCAGGAGCTGAAGGACT
PRSS8	NM_002773	ACGCCTTCATAGGTGATGCT	ATCTTGGATTACTCCGGTCG
PTCH1	NM_000264	TCTCCAATCTTCTGGCGAGT	CTCATATTTGGGGCCTTCG
PTEN	NM_000314	CGGTGTCATAATGTCTTTCAGC	TGAAGGCGTATACAGGAACAAT
PTEN	NM_002412	GAAATAGGCATTCAGCCAGG	GGGTCTGCACGAAATAAAGC
PYCARD	NM_013258	TGGTACTGCTCATCCGTCAG	CCCTCCTCAGTCGGCAG
RARB	NM_000965	ATCGTCCAACTCAGCTGTCA	TGCCAATACTGTCGACTCCA
RASSF1	NM_007182	AGGTGTCTCCCACTCCACAG	TGCAAGTTCACCTGCCACTA
RASSF1	NM_007182	GTCTCCCACTCCACAGGCT	AAGTTCACCTGCCACTACCG
RB1	NM_000321	CCTTCTCGGTCCTTTGATTG	CAGAAGGCAACTTGACAAGAGA
SCGB3A1	NM_052863	CTTCAGCGGGTTGAGGGT	CTCTGCGTGGCCCTGTC
SFN	NM_006142	GAGGAAACATGGTCACACCC	TGAGAACTGGACAGTGGCAG
SOCS1	NM_003745	AGAGGTGAGAAGGGGTCTGC	CTGGGATGCCGTGTTATTTT
SYK	NM_003177	AAAGAAGGGCAGGTGGTTG	GAGAGCGAGGAGGAGCG
TFAP2a	NM_003220	ATTGACCTACAGTGCCCAGC	TCCATGAAAATGCTTTGGAA
TGFBR2	NM_003242	CAGTTGCTCATGCAGGATTT	GCACGTTCAGAAGTCGGTTA
THBS1	NM_003246	TTGCCACAGCTCGTAGAACA	CAATGCCACAGTTCCTGATG
THRB	NM_000461	CAACCAGAAGGAAATCGCA	AAAGAGACCTCCTGCTCCG
TIMP3	NM_000362	GGTGAAGCCTCGGTACATCT	AGGACGCCTTCTGCAACTC
TNFSF10	NM_003810	CTCTCTTCGTCATTGGGGTC	TGCAGTCTCTCTGTGTGGCT
TP53	NM_000546	GCTCGACGCTAGGATCTGAC	CAGGTAGCTGCTGGGCTC
TWIST1	NM_000474	TCCATTTTCTCCTTCTCTGGAA	GGCTCAGCTACGCCTTCTC

Appendix C: Selected Vector and Gene Maps

shMBD2-B-pSuperior.Neo
4756 bp

Labels: f1(+) origin, M13(-20) Primer, T7 Primer, Amp, Neo, PGK Promoter, *Eco*RI (2231), H1 Promoter, Tet Repressor Site, *Bgl*II (2454), *Eco*RI (2489), *Hin*dIII (2515), *Xho*I (2536), T3 Primer, M13R Primer, shMBD2 B

pLVTHM-shMBD2
11135 bp

Labels: LTR, SD, psi, RRE, SA, EF1-alfa, cPPT, GFP, WPRE, LTR/SIN, tetO, H1, shMBD2, *Mlu* I (5612), *Cla* I (5679), LoxP, LTR/SIN, AmpR, ORI, SV40, gpt, pA

188

Fragment of hMaspin genomic
917 bp (molecule 37742 bp)

Fragment of hDAPK1 Genomic
1473 bp (molecule 235946 bp)

Fragment of hKLK10 Genomic
1756 bp (molecule 11147 bp)

190

List of References

Adams, P. D. 2009. Healing and hurting: Molecular mechanisms, functions, and pathologies of cellular senescence *Molecular Cell* 36, (1) (Oct 9): 2-14.

Amir, R. E., I. B. Van den Veyver, M. Wan, C. Q. Tran, U. Francke, and H. Y. Zoghbi. 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nature Genetics* 23, (2) (Oct): 185-8.

Appleton, K., H. J. Mackay, I. Judson, J. A. Plumb, C. McCormick, G. Strathdee, C. Lee, et al. 2007. Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 25, (29) (Oct 10): 4603-9.

Bakker, J., X. Lin, and W. G. Nelson. 2002. Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells. *The Journal of Biological Chemistry* 277, (25) (Jun 21): 22573-80.

Ballestar, E. 2003. Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer *The EMBO Journal* 22, (23): 6335 <last_page> 6345.

Ballestar, E., and M. Esteller. 2005. Methyl-CpG-binding proteins in cancer: Blaming the DNA methylation messenger. *Biochemistry and Cell Biology = Biochimie Et Biologie Cellulaire* 83, (3) (Jun): 374-84.

Barr, H., A. Hermann, J. Berger, H. H. Tsai, K. Adie, A. Prokhortchouk, B. Hendrich, and A. Bird. 2007. Mbd2 contributes to DNA methylation-directed repression of the xist gene *Molecular and Cellular Biology* 27, (10) (May): 3750-7.

Barreto, G., A. Schafer, J. Marhold, D. Stach, S. K. Swaminathan, V. Handa, G. Doderlein, et al. 2007. Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation *Nature* 445, (7128) (Feb 8): 671-5.

Barrett, D. M., K. S. Gustafson, J. Wang, S. Z. Wang, and G. D. Ginder. 2004. A GATA factor mediates cell type-restricted induction of HLA-E gene transcription by gamma interferon. *Molecular and Cellular Biology* 24, (14) (Jul): 6194-204.

Berger, J., and A. Bird. 2005. Role of MBD2 in gene regulation and tumorigenesis. *Biochemical Society Transactions* 33, (Pt 6) (Dec): 1537-40.

Berger, J., O. Sansom, A. Clarke, and A. Bird. 2007. MBD2 is required for correct spatial gene expression in the gut *Molecular and Cellular Biology* 27, (11) (Jun): 4049-57.

192

Berman, H., J. Zhang, Y. G. Crawford, M. L. Gauthier, C. A. Fordyce, K. M. McDermott, M. Sigaroudinia, K. Kozakiewicz, and T. D. Tlsty. 2005. Genetic and epigenetic changes in mammary epithelial cells identify a subpopulation of cells involved in early carcinogenesis *Cold Spring Harbor Symposia on Quantitative Biology* 70, : 317-27.

Bernstein, B. E., A. Meissner, and E. S. Lander. 2007. The mammalian epigenome. *Cell* 128, (4) (Feb 23): 669-81.

Bhattacharya, S. K., S. Ramchandani, N. Cervoni, and M. Szyf. 1999. A mammalian protein with specific demethylase activity for mCpG DNA *Nature* 397, (6720) (Feb 18): 579-83.

Billard, L. M., F. Magdinier, G. M. Lenoir, L. Frappart, and R. Dante. 2002. MeCP2 and MBD2 expression during normal and pathological growth of the human mammary gland *Oncogene* 21, (17) (Apr 18): 2704-12.

Bird, A. 2002. DNA methylation patterns and epigenetic memory. *Genes & Development* 16, (1) (Jan 1): 6-21.

Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation *Nature* 321, (6067) (May 15-21): 209-13.

Bird, A. P. 1980. DNA methylation and the frequency of CpG in animal DNA *Nucleic Acids Research* 8, (7) (Apr 11): 1499-504.

Bittel, D. C., and M. G. Butler. 2005. Prader-willi syndrome: Clinical genetics, cytogenetics and molecular biology *Expert Reviews in Molecular Medicine* 7, (14) (Jul 25): 1-20.

Bock, C., S. Reither, T. Mikeska, M. Paulsen, J. Walter, and T. Lengauer. 2005. BiQ analyzer: Visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics (Oxford, England)* 21, (21) (Nov 1): 4067-8.

Bogdanovic, O., and G. J. Veenstra. 2009. DNA methylation and methyl-CpG binding proteins: Developmental requirements and function *Chromosoma* 118, (5) (Oct): 549-65.

Boumil, R. M., and J. T. Lee. 2001. Forty years of decoding the silence in X-chromosome inactivation *Human Molecular Genetics* 10, (20) (Oct 1): 2225-32.

Bowen, N. J., N. Fujita, M. Kajita, and P. A. Wade. 2004. Mi-2/NuRD: Multiple complexes for many purposes. *Biochimica Et Biophysica Acta* 1677, (1-3) (Mar 15): 52-7.

193

Boyer, L. A., D. Mathur, and R. Jaenisch. 2006. Molecular control of pluripotency *Current Opinion in Genetics & Development* 16, (5) (Oct): 455-62.

Boyes, J., and A. Bird. 1991. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein *Cell* 64, (6) (Mar 22): 1123-34.

Brambrink, T., R. Foreman, G. G. Welstead, C. J. Lengner, M. Wernig, H. Suh, and R. Jaenisch. 2008. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells *Cell Stem Cell* 2, (2) (Feb 7): 151-9.

Brock, M. V., J. G. Herman, and S. B. Baylin. 2007. Cancer as a manifestation of aberrant chromatin structure. *Cancer Journal (Sudbury, Mass.)* 13, (1) (Jan-Feb): 3-8.

Brown, S. E., M. J. Suderman, M. Hallett, and M. Szyf. 2008. DNA demethylation induced by the methyl-CpG-binding domain protein MBD3 *Gene* 420, (2) (Sep 1): 99-106.

Caino, M. C., J. Meshki, and M. G. Kazanietz. 2009. Hallmarks for senescence in carcinogenesis: Novel signaling players *Apoptosis : An International Journal on Programmed Cell Death* 14, (4) (Apr): 392-408.

Campbell, P. M., V. Bovenzi, and M. Szyf. 2004. Methylated DNA-binding protein 2 antisense inhibitors suppress tumourigenesis of human cancer cell lines in vitro and in vivo. *Carcinogenesis* 25, (4) (Apr): 499-507.

Choi, J. K., N. Hoang, A. M. Vilardi, P. Conrad, S. G. Emerson, and A. M. Gewirtz. 2001. Hybrid HIV/MSCV LTR enhances transgene expression of lentiviral vectors in human CD34(+) hematopoietic cells *Stem Cells (Dayton, Ohio)* 19, (3): 236-46.

Clouaire, T., and I. Stancheva. 2008. Methyl-CpG binding proteins: Specialized transcriptional repressors or structural components of chromatin? *Cellular and Molecular Life Sciences : CMLS* 65, (10) (May): 1509-22.

Cooper, D. N., and M. Krawczak. 1989. Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes. *Human Genetics* 83, (2) (Sep): 181-8.

Corn, P. G. 2009. Methylation marks the path for biomarker development in breast cancer research *Cancer Biology & Therapy* 8, (23) (Dec 25).

Cortez, C. C., and P. A. Jones. 2008. Chromatin, cancer and drug therapies *Mutation Research* 647, (1-2) (Dec 1): 44-51.

Cross, S. H., and A. P. Bird. 1995. CpG islands and genes. *Current Opinion in Genetics & Development* 5, (3) (Jun): 309-14.

194

Cui, W., D. D. Taub, and K. Gardner. 2007. qPrimerDepot: A primer database for quantitative real time PCR. *Nucleic Acids Research* 35, (Database issue) (Jan): D805-9.

Dean, W., F. Santos, M. Stojkovic, V. Zakhartchenko, J. Walter, E. Wolf, and W. Reik. 2001. Conservation of methylation reprogramming in mammalian development: Aberrant reprogramming in cloned embryos *Proceedings of the National Academy of Sciences of the United States of America* 98, (24) (Nov 20): 13734-8.

Denslow, S. A., and P. A. Wade. 2007. The human mi-2/NuRD complex and gene regulation *Oncogene* 26, (37) (Aug 13): 5433-8.

Domann, F. E., J. C. Rice, M. J. Hendrix, and B. W. Futscher. 2000. Epigenetic silencing of maspin gene expression in human breast cancers. *International Journal of Cancer.Journal International Du Cancer* 85, (6) (Mar 15): 805-10.

Dumont, N., Y. G. Crawford, M. Sigaroudinia, S. S. Nagrani, M. B. Wilson, G. C. Buehring, G. Turashvili, et al. 2009. Human mammary cancer progression model recapitulates methylation events associated with breast premalignancy *Breast Cancer Research : BCR* 11, (6) (Dec 8): R87.

Eckhardt, F., J. Lewin, R. Cortese, V. K. Rakyan, J. Attwood, M. Burger, J. Burton, et al. 2006. DNA methylation profiling of human chromosomes 6, 20 and 22 *Nature Genetics* 38, (12) (Dec): 1378-85.

Egger, G., G. Liang, A. Aparicio, and P. A. Jones. 2004. Epigenetics in human disease and prospects for epigenetic therapy *Nature* 429, (6990) (May 27): 457-63.

Ehrlich, M. 2002. DNA methylation in cancer: Too much, but also too little *Oncogene* 21, (35) (Aug 12): 5400-13.

Ehrlich, M., M. A. Gama-Sosa, L. H. Huang, R. M. Midgett, K. C. Kuo, R. A. McCune, and C. Gehrke. 1982. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Research* 10, (8) (Apr 24): 2709-21.

Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns *Proceedings of the National Academy of Sciences of the United States of America* 95, (25) (Dec 8): 14863-8.

Engel, N., J. S. Tront, T. Erinle, N. Nguyen, K. E. Latham, C. Sapienza, B. Hoffman, and D. A. Liebermann. 2009. Conserved DNA methylation in Gadd45a(-/-) mice *Epigenetics : Official Journal of the DNA Methylation Society* 4, (2) (Feb): 98-9.

Esteller, M. 2005. DNA methylation, epigenetics and Metastasis
. In *Cancer metastasis - biology and treatment.* Vol. 7, 81-81-132Springer.

Esteller, M. 2008. Epigenetics in cancer *The New England Journal of Medicine* 358, (11) (Mar 13): 1148-59.

Evron, E., W. C. Dooley, C. B. Umbricht, D. Rosenthal, N. Sacchi, E. Gabrielson, A. B. Soito, et al. 2001. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. *Lancet* 357, (9265) (Apr 28): 1335-6.

Fatemi, M., A. Hermann, H. Gowher, and A. Jeltsch. 2002. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA *European Journal of Biochemistry / FEBS* 269, (20) (Oct): 4981-4.

Feinberg, A. P. 2007. Phenotypic plasticity and the epigenetics of human disease. *Nature* 447, (7143) (May 24): 433-40.

Feinberg, A. P., R. Ohlsson, and S. Henikoff. 2006. The epigenetic progenitor origin of human cancer *Nature Reviews.Genetics* 7, (1) (Jan): 21-33.

Feng, Q., and Y. Zhang. 2001a. The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. *Genes & Development* 15, (7) (Apr 1): 827-32.

Field, L. M., F. Lyko, M. Mandrioli, and G. Prantera. 2004. DNA methylation in insects *Insect Molecular Biology* 13, (2) (Apr): 109-15.

Filion, G. J., S. Zhenilo, S. Salozhin, D. Yamada, E. Prokhortchouk, and P. A. Defossez. 2006. A family of human zinc finger proteins that bind methylated DNA and repress transcription *Molecular and Cellular Biology* 26, (1) (Jan): 169-81.

Fraga, M. F., E. Ballestar, G. Montoya, P. Taysavang, P. A. Wade, and M. Esteller. 2003. The affinity of different MBD proteins for a specific methylated locus depends on their intrinsic binding properties *Nucleic Acids Research* 31, (6) (Mar 15): 1765-74.

Fujita, N., N. Shimotake, I. Ohki, T. Chiba, H. Saya, M. Shirakawa, and M. Nakao. 2000. Mechanism of transcriptional regulation by methyl-CpG binding protein MBD1 *Molecular and Cellular Biology* 20, (14) (Jul): 5107-18.

Fujita, N., S. Watanabe, T. Ichimura, S. Tsuruzoe, Y. Shinkai, M. Tachibana, T. Chiba, and M. Nakao. 2003. Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression *The Journal of Biological Chemistry* 278, (26) (Jun 27): 24132-8.

Fuks, F., P. J. Hurd, R. Deplus, and T. Kouzarides. 2003. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase *Nucleic Acids Research* 31, (9) (May 1): 2305-12.

196

Futscher, B. W., M. M. O'Meara, C. J. Kim, M. A. Rennels, D. Lu, L. M. Gruman, R. E. Seftor, M. J. Hendrix, and F. E. Domann. 2004. Aberrant methylation of the maspin promoter is an early event in human breast cancer. *Neoplasia (New York, N.Y.)* 6, (4) (Jul-Aug): 380-9.

Futscher, B. W., M. M. Oshiro, R. J. Wozniak, N. Holtan, C. L. Hanigan, H. Duan, and F. E. Domann. 2002a. Role for DNA methylation in the control of cell type specific maspin expression. *Nature Genetics* 31, (2) (Jun): 175-9.

Gamble, M. J., K. M. Frizzell, C. Yang, R. Krishnakumar, and W. L. Kraus. 2010. The histone variant macroH2A1 marks repressed autosomal chromatin, but protects a subset of its target genes from silencing *Genes & Development* 24, (1) (Jan 1): 21-32.

Genee Y. Lee, Paraic A. Kenny, Eva H. Lee, and Mina J. Bissell. 3D culture models of normal and malignant breast epithelial cells. http://escholarship.org/uc/item/1b2118cd., (accessed 12/8/2009).

Ginder, G. D., M. N. Gnanapragasam, and O. Y. Mian. 2008. The role of the epigenetic signal, DNA methylation, in gene regulation during erythroid development. *Current Topics in Developmental Biology* 82, : 85-116.

Goldberg, A. D., C. D. Allis, and E. Bernstein. 2007. Epigenetics: A landscape takes shape *Cell* 128, (4) (Feb 23): 635-8.

Gowher, H., C. J. Stockdale, R. Goyal, H. Ferreira, T. Owen-Hughes, and A. Jeltsch. 2005. De novo methylation of nucleosomal DNA by the mammalian Dnmt1 and Dnmt3A DNA methyltransferases *Biochemistry* 44, (29) (Jul 26): 9899-904.

Grant, S. 2009. Targeting histone demethylases in cancer therapy *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 15, (23) (Dec 1): 7111-3.

Gronbaek, K., C. Hother, and P. A. Jones. 2007. Epigenetic changes in cancer. *APMIS : Acta Pathologica, Microbiologica, Et Immunologica Scandinavica* 115, (10) (Oct): 1039-59.

Gupta, P. B., C. L. Chaffer, and R. A. Weinberg. 2009. Cancer stem cells: Mirage or reality? *Nature Medicine* 15, (9) (Sep): 1010-2.

Hamm, S., G. Just, N. Lacoste, N. Moitessier, M. Szyf, and O. Mamer. 2008. On the mechanism of demethylation of 5-methylcytosine in DNA *Bioorganic & Medicinal Chemistry Letters* 18, (3) (Feb 1): 1046-9.

Hanahan, D., and R. A. Weinberg. 2000. The hallmarks of cancer *Cell* 100, (1) (Jan 7): 57-70.

197

Harikrishnan, K. N., M. Z. Chow, E. K. Baker, S. Pal, S. Bassal, D. Brasacchio, L. Wang, et al. 2005. Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing *Nature Genetics* 37, (3) (Mar): 254-64.

Hark, A. T., C. J. Schoenherr, D. J. Katz, R. S. Ingram, J. M. Levorse, and S. M. Tilghman. 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus *Nature* 405, (6785) (May 25): 486-9.

Hendrich, B., and A. Bird. 1998a. Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Molecular and Cellular Biology* 18, (11) (Nov): 6538-47.

Hendrich, B., J. Guy, B. Ramsahoye, V. A. Wilson, and A. Bird. 2001. Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. *Genes & Development* 15, (6) (Mar 15): 710-23.

Hendrich, B., U. Hardeland, H. H. Ng, J. Jiricny, and A. Bird. 1999. The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. *Nature* 401, (6750) (Sep 16): 301-4.

Hendrich, B., and S. Tweedie. 2003. The methyl-CpG binding domain and the evolving role of DNA methylation in animals *Trends in Genetics : TIG* 19, (5) (May): 269-77.

Honorio, S., A. Agathanggelou, M. Schuermann, W. Pankow, P. Viacava, E. R. Maher, and F. Latif. 2003a. Detection of RASSF1A aberrant promoter hypermethylation in sputum from chronic smokers and ductal carcinoma in situ from breast cancer patients. *Oncogene* 22, (1) (Jan 9): 147-50.

Hoque, M. O., M. Prencipe, M. L. Poeta, R. Barbano, V. M. Valori, M. Copetti, A. P. Gallo, et al. 2009. Changes in CpG islands promoter methylation patterns during ductal breast carcinoma progression *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology* 18, (10) (Oct): 2694-700.

Horike, S., S. Cai, M. Miyano, J. F. Cheng, and T. Kohwi-Shigematsu. 2005. Loss of silent-chromatin looping and impaired imprinting of DLX5 in rett syndrome *Nature Genetics* 37, (1) (Jan): 31-40.

Horner, M. J., Ries, L. A., Krapcho, M., Neyman, N., Aminou, R., Howlader, N., Altekruse, S. F., et al. SEER cancer statistics review 1975-2006. 2009 [cited 12/13/2009 2009]. Available from http://seer.cancer.gov/csr/1975_2006/index.html (accessed 12/13/2009).

Hsieh, C. L. 1999. Evidence that protein binding specifies sites of DNA demethylation *Molecular and Cellular Biology* 19, (1) (Jan): 46-56.

198

Hublitz, P., M. Albert, and A. H. Peters. 2009. Mechanisms of transcriptional repression by histone lysine methylation *The International Journal of Developmental Biology* 53, (2-3): 335-54.

Hutchins, A. S., D. Artis, B. D. Hendrich, A. P. Bird, P. Scott, and S. L. Reiner. 2005. Cutting edge: A critical role for gene silencing in preventing excessive type 1 immunity *Journal of Immunology (Baltimore, Md.: 1950)* 175, (9) (Nov 1): 5606-10.

Hutchins, A. S., A. C. Mullen, H. W. Lee, K. J. Sykes, F. A. High, B. D. Hendrich, A. P. Bird, and S. L. Reiner. 2002. Gene silencing quantitatively controls the function of a developmental trans-activator *Molecular Cell* 10, (1) (Jul): 81-91.

Jabbour, E., J. P. Issa, G. Garcia-Manero, and H. Kantarjian. 2008. Evolution of decitabine development: Accomplishments, ongoing investigations, and future strategies. *Cancer* 112, (11) (Jun): 2341-51.

Jackson-Grusby, L., C. Beard, R. Possemato, M. Tudor, D. Fambrough, G. Csankovszki, J. Dausman, et al. 2001. Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation *Nature Genetics* 27, (1) (Jan): 31-9.

Jair, K. W., K. E. Bachman, H. Suzuki, A. H. Ting, I. Rhee, R. W. Yen, S. B. Baylin, and K. E. Schuebel. 2006. De novo CpG island methylation in human cancer cells. *Cancer Research* 66, (2) (Jan 15): 682-92.

Jeltsch, A. 2006. On the enzymatic properties of Dnmt1: Specificity, processivity, mechanism of linear diffusion and allosteric regulation of the enzyme *Epigenetics : Official Journal of the DNA Methylation Society* 1, (2) (Apr-Jun): 63-6.

Jenuwein, T. 2006. The epigenetic magic of histone lysine methylation *The FEBS Journal* 273, (14) (Jul): 3121-35.

Jones, P. A., and S. B. Baylin. 2007. The epigenomics of cancer *Cell* 128, (4) (Feb 23): 683-92.

Jones, P. A., and S. B. Baylin. 2002. The fundamental role of epigenetic events in cancer. *Nature Reviews.Genetics* 3, (6) (Jun): 415-28.

Jones, P. A., and P. W. Laird. 1999. Cancer epigenetics comes of age. *Nature Genetics* 21, (2) (Feb): 163-7.

Jones, P. A., and D. Takai. 2001. The role of DNA methylation in mammalian epigenetics *Science (New York, N.Y.)* 293, (5532) (Aug 10): 1068-70.

Jones, P. L., G. J. Veenstra, P. A. Wade, D. Vermaak, S. U. Kass, N. Landsberger, J. Strouboulis, and A. P. Wolffe. 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription *Nature Genetics* 19, (2) (Jun): 187-91.

199

Jordan, C., H. H. Li, H. C. Kwan, and U. Francke. 2007. Cerebellar gene expression profiles of mouse models for rett syndrome reveal novel MeCP2 targets *BMC Medical Genetics* 8, (Jun 20): 36.

Kaji, K., I. M. Caballero, R. MacLeod, J. Nichols, V. A. Wilson, and B. Hendrich. 2006. The NuRD component Mbd3 is required for pluripotency of embryonic stem cells *Nature Cell Biology* 8, (3) (Mar): 285-92.

Kaji, K., J. Nichols, and B. Hendrich. 2007. Mbd3, a component of the NuRD co-repressor complex, is required for development of pluripotent cells *Development (Cambridge, England)* 134, (6) (Mar): 1123-32.

Kamakaka, R. T., and S. Biggins. 2005. Histone variants: Deviants? *Genes & Development* 19, (3) (Feb 1): 295-310.

Karagiannis, T. C., and A. El-Osta. 2006. Clinical potential of histone deacetylase inhibitors as stand alone therapeutics and in combination with other chemotherapeutics or radiotherapy for cancer. *Epigenetics : Official Journal of the DNA Methylation Society* 1, (3) (Jul-Sep): 121-6.

Keenen, B., and I. L. de la Serna. 2009. Chromatin remodeling in embryonic stem cells: Regulating the balance between pluripotency and differentiation *Journal of Cellular Physiology* 219, (1) (Apr): 1-7.

Khalkhali-Ellis, Z. 2006. Maspin: The new frontier. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 12, (24) (Dec 15): 7279-83.

Kimura, H., and K. Shiota. 2003. Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1 *The Journal of Biological Chemistry* 278, (7) (Feb 14): 4806-12.

Klerk, C. P., R. M. Overmeer, T. M. Niers, H. H. Versteeg, D. J. Richel, T. Buckle, C. J. Van Noorden, and O. van Tellingen. 2007. Validity of bioluminescence measurements for noninvasive in vivo imaging of tumor load in small animals *BioTechniques* 43, (1 Suppl) (Jul): 7,13, 30.

Klose, R. J., and A. P. Bird. 2006. Genomic DNA methylation: The mark and its mediators. *Trends in Biochemical Sciences* 31, (2) (Feb): 89-97.

Klose, R. J., S. A. Sarraf, L. Schmiedeberg, S. M. McDermott, I. Stancheva, and A. P. Bird. 2005. DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. *Molecular Cell* 19, (5) (Sep 2): 667-78.

Kokura, K., S. C. Kaul, R. Wadhwa, T. Nomura, M. M. Khan, T. Shinagawa, T. Yasukawa, C. Colmenares, and S. Ishii. 2001. The ski protein family is required for

200

MeCP2-mediated transcriptional repression *The Journal of Biological Chemistry* 276, (36) (Sep 7): 34115-21.

Kouzarides, T. 2007. Chromatin modifications and their function *Cell* 128, (4) (Feb 23): 693-705.

Kransdorf, E. P., S. Z. Wang, S. Z. Zhu, T. B. Langston, J. W. Rupon, and G. D. Ginder. 2006a. MBD2 is a critical component of a methyl cytosine-binding protein complex isolated from primary erythroid cells. *Blood* 108, (8) (Oct 15): 2836-45.

Krassenstein, R., E. Sauter, E. Dulaimi, C. Battagli, H. Ehya, A. Klein-Szanto, and P. Cairns. 2004. Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 10, (1 Pt 1) (Jan 1): 28-32.

Kucharski, R., J. Maleszka, S. Foret, and R. Maleszka. 2008. Nutritional control of reproductive status in honeybees via DNA methylation *Science (New York, N.Y.)* 319, (5871) (Mar 28): 1827-30.

Kunert, N., J. Marhold, J. Stanke, D. Stach, and F. Lyko. 2003. A Dnmt2-like protein mediates DNA methylation in drosophila *Development (Cambridge, England)* 130, (21) (Nov): 5083-90.

Lalande, M., and M. A. Calciano. 2007. Molecular epigenetics of angelman syndrome *Cellular and Molecular Life Sciences : CMLS* 64, (7-8) (Apr): 947-60.

Le Guezennec, X., M. Vermeulen, A. B. Brinkman, W. A. Hoeijmakers, A. Cohen, E. Lasonder, and H. G. Stunnenberg. 2006. MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties. *Molecular and Cellular Biology* 26, (3) (Feb): 843-51.

Leonhardt, H., A. W. Page, H. U. Weier, and T. H. Bestor. 1992. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* 71, (5) (Nov 27): 865-73.

Li, B., M. Carey, and J. L. Workman. 2007. The role of chromatin during transcription *Cell* 128, (4) (Feb 23): 707-19.

Li, E., T. H. Bestor, and R. Jaenisch. 1992a. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69, (6) (Jun 12): 915-26.

Li, L. C., and R. Dahiya. 2002. MethPrimer: Designing primers for methylation PCRs. *Bioinformatics (Oxford, England)* 18, (11) (Nov): 1427-31.

Liang, G., M. F. Chan, Y. Tomigahara, Y. C. Tsai, F. A. Gonzales, E. Li, P. W. Laird, and P. A. Jones. 2002. Cooperativity between DNA methyltransferases in the

201

maintenance methylation of repetitive elements. *Molecular and Cellular Biology* 22, (2) (Jan): 480-91.

Lin, X., and W. G. Nelson. 2003. Methyl-CpG-binding domain protein-2 mediates transcriptional repression associated with hypermethylated GSTP1 CpG islands in MCF-7 breast cancer cells. *Cancer Research* 63, (2) (Jan 15): 498-504.

Linhart, H. G., H. Lin, Y. Yamada, E. Moran, E. J. Steine, S. Gokhale, G. Lo, et al. 2007. Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes & Development* 21, (23) (Dec 1): 3110-22.

Lopez-Serra, L., E. Ballestar, M. F. Fraga, M. Alaminos, F. Setien, and M. Esteller. 2006. A profile of methyl-CpG binding domain protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer *Cancer Research* 66, (17) (Sep 1): 8342-6.

Lopez-Serra, L., E. Ballestar, S. Ropero, F. Setien, L. M. Billard, M. F. Fraga, P. Lopez-Nieva, et al. 2008a. Unmasking of epigenetically silenced candidate tumor suppressor genes by removal of methyl-CpG-binding domain proteins. *Oncogene* (Jan 28).

Lustberg, M. B., and B. Ramaswamy. 2009. Epigenetic targeting in breast cancer: Therapeutic impact and future direction *Drug News & Perspectives* 22, (7) (Sep): 369-81.

Lyko, F., B. H. Ramsahoye, and R. Jaenisch. 2000. DNA methylation in drosophila melanogaster *Nature* 408, (6812) (Nov 30): 538-40.

Mann, M. R., and M. S. Bartolomei. 2002. Epigenetic reprogramming in the mammalian embryo: Struggle of the clones *Genome Biology* 3, (2): REVIEWS1003.

Marhold, J., A. Brehm, and K. Kramer. 2004. The drosophila methyl-DNA binding protein MBD2/3 interacts with the NuRD complex via p55 and MI-2 *BMC Molecular Biology* 5, (1) (Oct 29): 20.

Martens, J. W., A. L. Margossian, M. Schmitt, J. Foekens, and N. Harbeck. 2009. DNA methylation as a biomarker in breast cancer *Future Oncology (London, England)* 5, (8) (Oct): 1245-56.

Martienssen, R. A., A. Kloc, R. K. Slotkin, and M. Tanurdzic. 2008. Epigenetic inheritance and reprogramming in plants and fission yeast *Cold Spring Harbor Symposia on Quantitative Biology* 73, : 265-71.

Martin Caballero, I., J. Hansen, D. Leaford, S. Pollard, and B. D. Hendrich. 2009. The methyl-CpG binding proteins Mecp2, Mbd2 and kaiso are dispensable for mouse

202

embryogenesis, but play a redundant function in neural differentiation *PloS One* 4, (1): e4315.

Martin, V., H. F. Jorgensen, A. S. Chaubert, J. Berger, H. Barr, P. Shaw, A. Bird, and P. Chaubert. 2008. MBD2-mediated transcriptional repression of the p14ARF tumor suppressor gene in human colon cancer cells *Pathobiology : Journal of Immunopathology, Molecular and Cellular Biology* 75, (5): 281-7.

Mathieu, O., J. Reinders, M. Caikovski, C. Smathajitt, and J. Paszkowski. 2007. Transgenerational stability of the arabidopsis epigenome is coordinated by CG methylation *Cell* 130, (5) (Sep 7): 851-62.

Mayer, W., A. Smith, R. Fundele, and T. Haaf. 2000. Spatial separation of parental genomes in preimplantation mouse embryos *The Journal of Cell Biology* 148, (4) (Feb 21): 629-34.

McGhee, J. D., and G. D. Ginder. 1979. Specific DNA methylation sites in the vicinity of the chicken beta-globin genes. *Nature* 280, (5721) (Aug 2): 419-20.

McGowan, R. A., and C. C. Martin. 1997. DNA methylation and genome imprinting in the zebrafish, danio rerio: Some evolutionary ramifications *Biochemistry and Cell Biology = Biochimie Et Biologie Cellulaire* 75, (5): 499-506.

Meehan, R. R., J. D. Lewis, S. McKay, E. L. Kleiner, and A. P. Bird. 1989. Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. *Cell* 58, (3) (Aug 11): 499-507.

Millar, C. B., J. Guy, O. J. Sansom, J. Selfridge, E. MacDougall, B. Hendrich, P. D. Keightley, S. M. Bishop, A. R. Clarke, and A. Bird. 2002. Enhanced CpG mutability and tumorigenesis in MBD4-deficient mice. *Science (New York, N.Y.)* 297, (5580) (Jul 19): 403-5.

Mohammad, F., T. Mondal, and C. Kanduri. 2009. Epigenetics of imprinted long noncoding RNAs *Epigenetics : Official Journal of the DNA Methylation Society* 4, (5) (Jul): 277-86.

Morgan, H. D., F. Santos, K. Green, W. Dean, and W. Reik. 2005. Epigenetic reprogramming in mammals *Human Molecular Genetics* 14 Spec No 1, (Apr 15): R47-58.

Muggerud, A. A., J. A. Ronneberg, F. Warnberg, J. Botling, F. Busato, J. Jovanovic, H. Solvang, et al. 2010. Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer *Breast Cancer Research : BCR* 12, (1) (Jan 7): R3.

Muller, H. M., H. Fiegl, G. Goebel, M. M. Hubalek, A. Widschwendter, E. Muller-Holzner, C. Marth, and M. Widschwendter. 2003. MeCP2 and MBD2 expression in human neoplastic and non-neoplastic breast tissue and its association with oestrogen receptor status *British Journal of Cancer* 89, (10) (Nov 17): 1934-9.

Nan, X., R. R. Meehan, and A. Bird. 1993. Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2 *Nucleic Acids Research* 21, (21) (Oct 25): 4886-92.

Nan, X., H. H. Ng, C. A. Johnson, C. D. Laherty, B. M. Turner, R. N. Eisenman, and A. Bird. 1998a. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393, (6683) (May 28): 386-9.

Nelson, C. M., and M. J. Bissell. 2005. Modeling dynamic reciprocity: Engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation *Seminars in Cancer Biology* 15, (5) (Oct): 342-52.

Nishigaki, M., K. Aoyagi, I. Danjoh, M. Fukaya, K. Yanagihara, H. Sakamoto, T. Yoshida, and H. Sasaki. 2005. Discovery of aberrant expression of R-RAS by cancer-linked DNA hypomethylation in gastric cancer using microarrays *Cancer Research* 65, (6) (Mar 15): 2115-24.

Ohki, I., N. Shimotake, N. Fujita, M. Nakao, and M. Shirakawa. 1999. Solution structure of the methyl-CpG-binding domain of the methylation-dependent transcriptional repressor MBD1. *The EMBO Journal* 18, (23) (Dec 1): 6653-61.

Okano, M., D. W. Bell, D. A. Haber, and E. Li. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, (3) (Oct 29): 247-57.

Oki, Y., and J. P. Issa. 2006. Review: Recent clinical trials in epigenetic therapy. *Reviews on Recent Clinical Trials* 1, (2) (May): 169-82.

Old, R. W., and H. R. Woodland. 1984. Histone genes: Not so simple after all. *Cell* 38, (3) (Oct): 624-6.

Oswald, J., S. Engemann, N. Lane, W. Mayer, A. Olek, R. Fundele, W. Dean, W. Reik, and J. Walter. 2000. Active demethylation of the paternal genome in the mouse zygote *Current Biology : CB* 10, (8) (Apr 20): 475-8.

Patra, S. K., and S. Bettuzzi. 2009. Epigenetic DNA-(cytosine-5-carbon) modifications: 5-aza-2'-deoxycytidine and DNA-demethylation *Biochemistry.Biokhimiia* 74, (6) (Jun): 613-9.

Payer, B., and J. T. Lee. 2008. X chromosome dosage compensation: How mammals keep the balance *Annual Review of Genetics* 42, : 733-72.

Perou, C. M., T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, et al. 2000. Molecular portraits of human breast tumours. *Nature* 406, (6797) (Aug 17): 747-52.

Petitjean, A., E. Mathe, S. Kato, C. Ishioka, S. V. Tavtigian, P. Hainaut, and M. Olivier. 2007. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: Lessons from recent developments in the IARC TP53 database *Human Mutation* 28, (6) (Jun): 622-9.

Polyak, K., and R. A. Weinberg. 2009. Transitions between epithelial and mesenchymal states: Acquisition of malignant and stem cell traits *Nature Reviews.Cancer* 9, (4) (Apr): 265-73.

Prokhortchouk, A., B. Hendrich, H. Jorgensen, A. Ruzov, M. Wilm, G. Georgiev, A. Bird, and E. Prokhortchouk. 2001a. The p120 catenin partner kaiso is a DNA methylation-dependent transcriptional repressor. *Genes & Development* 15, (13) (Jul 1): 1613-8.

Prokhortchouk, A., O. Sansom, J. Selfridge, I. M. Caballero, S. Salozhin, D. Aithozhina, L. Cerchietti, et al. 2006. Kaiso-deficient mice show resistance to intestinal cancer *Molecular and Cellular Biology* 26, (1) (Jan): 199-208.

Pulukuri, S. M., and J. S. Rao. 2006. CpG island promoter methylation and silencing of 14-3-3sigma gene expression in LNCaP and tramp-C1 prostate cancer cell lines is associated with methyl-CpG-binding protein MBD2. *Oncogene* 25, (33) (Aug 3): 4559-72.

Rai, K., I. J. Huggins, S. R. James, A. R. Karpf, D. A. Jones, and B. R. Cairns. 2008. DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and gadd45 *Cell* 135, (7) (Dec 26): 1201-12.

Rai, K., L. D. Nadauld, S. Chidester, E. J. Manos, S. R. James, A. R. Karpf, B. R. Cairns, and D. A. Jones. 2006. Zebra fish Dnmt1 and Suv39h1 regulate organ-specific terminal differentiation during development *Molecular and Cellular Biology* 26, (19) (Oct): 7077-85.

Rassoulzadegan, M., V. Grandjean, P. Gounon, S. Vincent, I. Gillot, and F. Cuzin. 2006. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse *Nature* 441, (7092) (May 25): 469-74.

Reik, W., W. Dean, and J. Walter. 2001a. Epigenetic reprogramming in mammalian development *Science (New York, N.Y.)* 293, (5532) (Aug 10): 1089-93.

Riccio, A., L. A. Aaltonen, A. K. Godwin, A. Loukola, A. Percesepe, R. Salovaara, V. Masciullo, et al. 1999. The DNA repair gene MBD4 (MED1) is mutated in human carcinomas with microsatellite instability *Nature Genetics* 23, (3) (Nov): 266-8.

205

Riggs, A. D., and P. A. Jones. 1983. 5-methylcytosine, gene regulation, and cancer. *Advances in Cancer Research* 40, : 1-30.

Robertson, K. D., and A. P. Wolffe. 2000. DNA methylation in health and disease. *Nature Reviews.Genetics* 1, (1) (Oct): 11-9.

Roder, K., M. S. Hung, T. L. Lee, T. Y. Lin, H. Xiao, K. I. Isobe, J. L. Juang, and C. J. Shen. 2000. Transcriptional repression by drosophila methyl-CpG-binding proteins *Molecular and Cellular Biology* 20, (19) (Oct): 7401-9.

Roll, J. D., A. G. Rivenbark, W. D. Jones, and W. B. Coleman. 2008. DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. *Molecular Cancer* 7, (Jan 25): 15.

Rupon, J. W., S. Z. Wang, K. Gaensler, J. Lloyd, and G. D. Ginder. 2006. Methyl binding domain protein 2 mediates gamma-globin gene silencing in adult human betaYAC transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* 103, (17) (Apr 25): 6617-22.

Ruzov, A., D. S. Dunican, A. Prokhortchouk, S. Pennings, I. Stancheva, E. Prokhortchouk, and R. R. Meehan. 2004. Kaiso is a genome-wide repressor of transcription that is essential for amphibian development *Development (Cambridge, England)* 131, (24) (Dec): 6185-94.

Saha, A., J. Wittmeyer, and B. R. Cairns. 2006. Chromatin remodelling: The industrial revolution of DNA around histones. *Nature Reviews.Molecular Cell Biology* 7, (6) (Jun): 437-47.

Saito, M., and F. Ishikawa. 2002. The mCpG-binding domain of human MBD3 does not bind to mCpG but interacts with NuRD/Mi2 components HDAC1 and MTA2 *The Journal of Biological Chemistry* 277, (38) (Sep 20): 35434-9.

Sakai, H., T. Urano, K. Ookata, M. H. Kim, Y. Hirai, M. Saito, Y. Nojima, and F. Ishikawa. 2002. MBD3 and HDAC1, two components of the NuRD complex, are localized at aurora-A-positive centrosomes in M phase *The Journal of Biological Chemistry* 277, (50) (Dec 13): 48714-23.

Saldanha, A. J. 2004. Java treeview--extensible visualization of microarray data *Bioinformatics (Oxford, England)* 20, (17) (Nov 22): 3246-8.

Sansom, O. J., J. Berger, S. M. Bishop, B. Hendrich, A. Bird, and A. R. Clarke. 2003a. Deficiency of Mbd2 suppresses intestinal tumorigenesis. *Nature Genetics* 34, (2) (Jun): 145-7.

Sansom, O. J., S. M. Bishop, A. Bird, and A. R. Clarke. 2005. MBD2 deficiency does not accelerate p53 mediated lymphomagenesis *Oncogene* 24, (14) (Mar 31): 2430-2.

206

Sansom, O. J., K. Maddison, and A. R. Clarke. 2007a. Mechanisms of disease: Methyl-binding domain proteins as potential therapeutic targets in cancer. *Nature Clinical Practice.Oncology* 4, (5) (May): 305-15.

Sarraf, S. A., and I. Stancheva. 2004. Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. *Molecular Cell* 15, (4) (Aug 27): 595-605.

Schmidt, W. M., R. Sedivy, B. Forstner, G. G. Steger, S. Zochbauer-Muller, and R. M. Mader. 2007. Progressive up-regulation of genes encoding DNA methyltransferases in the colorectal adenoma-carcinoma sequence *Molecular Carcinogenesis* 46, (9) (Sep): 766-72.

Schmittgen, T. D., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols* 3, (6): 1101-8.

Schrump, D. S., M. R. Fischette, D. M. Nguyen, M. Zhao, X. Li, T. F. Kunst, A. Hancox, et al. 2006. Phase I study of decitabine-mediated gene expression in patients with cancers involving the lungs, esophagus, or pleura. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 12, (19) (Oct 1): 5777-85.

Scudiero, D. A., R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, and M. R. Boyd. 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines *Cancer Research* 48, (17) (Sep 1): 4827-33.

Selker, E. U., N. A. Tountas, S. H. Cross, B. S. Margolin, J. G. Murphy, A. P. Bird, and M. Freitag. 2003. The methylated component of the neurospora crassa genome *Nature* 422, (6934) (Apr 24): 893-7.

Singal, R., and G. D. Ginder. 1999. DNA methylation. *Blood* 93, (12) (Jun 15): 4059-70.

Singal, R., S. Z. Wang, T. Sargent, S. Z. Zhu, and G. D. Ginder. 2002. Methylation of promoter proximal-transcribed sequences of an embryonic globin gene inhibits transcription in primary erythroid cells and promotes formation of a cell type-specific methyl cytosine binding complex. The Journal of Biological Chemistry 277, (3) (Jan 18): 1897-905.

Slack, A., V. Bovenzi, P. Bigey, M. A. Ivanov, S. Ramchandani, S. Bhattacharya, B. tenOever, B. Lamrihi, D. Scherman, and M. Szyf. 2002. Antisense MBD2 gene therapy inhibits tumorigenesis. *The Journal of Gene Medicine* 4, (4) (Jul-Aug): 381-9.

Sorlie, T., C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, et al. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses

207

with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America* 98, (19) (Sep 11): 10869-74.

Soule, H. D., T. M. Maloney, S. R. Wolman, W. D. Peterson Jr, R. Brenz, C. M. McGrath, J. Russo, R. J. Pauley, R. F. Jones, and S. C. Brooks. 1990. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10 *Cancer Research* 50, (18) (Sep 15): 6075-86.

Stancheva, I., C. Hensey, and R. R. Meehan. 2001. Loss of the maintenance methyltransferase, xDnmt1, induces apoptosis in xenopus embryos *The EMBO Journal* 20, (8) (Apr 17): 1963-73.

Stancheva, I., and R. R. Meehan. 2000a. Transient depletion of xDnmt1 leads to premature gene activation in xenopus embryos *Genes & Development* 14, (3) (Feb 1): 313-27.

Steele, N., P. Finn, R. Brown, and J. A. Plumb. 2009. Combined inhibition of DNA methylation and histone acetylation enhances gene re-expression and drug sensitivity in vivo *British Journal of Cancer* 100, (5) (Mar 10): 758-63.

Surani, M. A., K. Hayashi, and P. Hajkova. 2007. Genetic and epigenetic regulators of pluripotency *Cell* 128, (4) (Feb 23): 747-62.

Suzuki, M. M., and A. Bird. 2008. DNA methylation landscapes: Provocative insights from epigenomics *Nature Reviews.Genetics* 9, (6) (Jun): 465-76.

Takai, D., and P. A. Jones. 2002. Comprehensive analysis of CpG islands in human chromosomes 21 and 22 *Proceedings of the National Academy of Sciences of the United States of America* 99, (6) (Mar 19): 3740-5.

Tamaru, H., and E. U. Selker. 2001. A histone H3 methyltransferase controls DNA methylation in neurospora crassa *Nature* 414, (6861) (Nov 15): 277-83.

Tatematsu, K. I., T. Yamazaki, and F. Ishikawa. 2000. MBD2-MBD3 complex binds to hemi-methylated DNA and forms a complex containing DNMT1 at the replication foci in late S phase *Genes to Cells : Devoted to Molecular & Cellular Mechanisms* 5, (8) (Aug): 677-88.

Taylor, S. M., and P. A. Jones. 1982. Mechanism of action of eukaryotic DNA methyltransferase. use of 5-azacytosine-containing DNA *Journal of Molecular Biology* 162, (3) (Dec 15): 679-92.

Taylor, S. M., and P. A. Jones. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* 17, (4) (Aug): 771-9.

Thiery, J. P., H. Acloque, R. Y. Huang, and M. A. Nieto. 2009. Epithelial-mesenchymal transitions in development and disease *Cell* 139, (5) (Nov 25): 871-90.

Ting, A. H., K. M. McGarvey, and S. B. Baylin. 2006. The cancer epigenome--components and functional correlates. *Genes & Development* 20, (23) (Dec 1): 3215-31.

Traynor, J., P. Agarwal, L. Lazzeroni, and U. Francke. 2002. Gene expression patterns vary in clonal cell cultures from rett syndrome females with eight different MECP2 mutations *BMC Medical Genetics* 3, (Nov 5): 12.

Tudor, M., S. Akbarian, R. Z. Chen, and R. Jaenisch. 2002. Transcriptional profiling of a mouse model for rett syndrome reveals subtle transcriptional changes in the brain *Proceedings of the National Academy of Sciences of the United States of America* 99, (24) (Nov 26): 15536-41.

Tweedie, S., J. Charlton, V. Clark, and A. Bird. 1997. Methylation of genomes and genes at the invertebrate-vertebrate boundary *Molecular and Cellular Biology* 17, (3) (Mar): 1469-75.

Umbricht, C. B., E. Evron, E. Gabrielson, A. Ferguson, J. Marks, and S. Sukumar. 2001. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. *Oncogene* 20, (26) (Jun 7): 3348-53.

Veenstra, G. J., and A. P. Wolffe. 2001. Constitutive genomic methylation during embryonic development of xenopus *Biochimica Et Biophysica Acta* 1521, (1-3) (Oct 31): 39-44.

Visvader, J. E. 2009. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis *Genes & Development* 23, (22) (Nov 15): 2563-77.

Waddington, C. H. 1957. *The strategy of genes; A discussion of some aspects of theoretical biology*. London: George Allen & Unwin.

Wade, P. A. 2001. Methyl CpG-binding proteins and transcriptional repression *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 23, (12) (Dec): 1131-7.

Watson, J. D., and F. H. Crick. 1953. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* 171, (4356) (Apr 25): 737-8.

Weigelt, B., and M. J. Bissell. 2008. Unraveling the microenvironmental influences on the normal mammary gland and breast cancer *Seminars in Cancer Biology* 18, (5) (Oct): 311-21.

Welch, D. R. 1997. Technical considerations for studying cancer metastasis in vivo *Clinical & Experimental Metastasis* 15, (3) (May): 272-306.

Widschwendter, M., and P. A. Jones. 2002. DNA methylation and breast carcinogenesis *Oncogene* 21, (35) (Aug 12): 5462-82.

Wischnewski, F., O. Friese, K. Pantel, and H. Schwarzenbach. 2007. Methyl-CpG binding domain proteins and their involvement in the regulation of the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 gene promoters *Molecular Cancer Research : MCR* 5, (7) (Jul): 749-59.

Wiznerowicz, M., and D. Trono. 2003; 2003. Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible RNA interference *Journal of Virology* 77, (16): 8957 <last_page> 8951.

Workman, J. L. 2006. Nucleosome displacement in transcription *Genes & Development* 20, (15) (Aug 1): 2009-17.

Wozniak, R. J., W. T. Klimecki, S. S. Lau, Y. Feinstein, and B. W. Futscher. 2007. 5-aza-2'-deoxycytidine-mediated reductions in G9A histone methyltransferase and histone H3 K9 di-methylation levels are linked to tumor suppressor gene reactivation *Oncogene* 26, (1) (Jan 4): 77-90.

Wutz, A., and J. Gribnau. 2007. X inactivation xplained. *Current Opinion in Genetics & Development* 17, (5) (Oct): 387-93.

Xiang, B., and S. K. Muthuswamy. 2006. Using three-dimensional acinar structures for molecular and cell biological assays. *Methods in Enzymology* 406, : 692-701.

Yoder, J. A., C. P. Walsh, and T. H. Bestor. 1997. Cytosine methylation and the ecology of intragenomic parasites *Trends in Genetics : TIG* 13, (8) (Aug): 335-40.

Zhang, Y., I. Bhat, M. Zeng, G. Jayal, D. E. Wazer, H. Band, and V. Band. 2006. Human kallikrein 10, a predictive marker for breast cancer *Biological Chemistry* 387, (6) (Jun): 715-21.

Zhang, Y., H. H. Ng, H. Erdjument-Bromage, P. Tempst, A. Bird, and D. Reinberg. 1999. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes & Development* 13, (15) (Aug 1): 1924-35.

Zhao, X., T. Ueba, B. R. Christie, B. Barkho, M. J. McConnell, K. Nakashima, E. S. Lein, et al. 2003. Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function *Proceedings of the National Academy of Sciences of the United States of America* 100, (11) (May 27): 6777-82.

Vita

Omar Y. Mian was born in Nairobi, Kenya on June 23, 1975. He came to the United States in 1984, where he now resides and holds citizenship. Before entering the MD/PhD program at Virginia Commonwealth University in 2004, he received a B.S. degree in Biology from James Madison University, an M.S. in Biotechnology from Johns Hopkins University. As a student at Virginia Commonwealth University, he was inducted into the Alpha Omega Alpha Medical Honor Society and the Association of Pathology Chairs Honor Society as well as the Phi Kappa Phi and Golden Key Academic Honor Societies. He received the C.C. Clayton Award as a second year student from the Microbiology and Immunology Dept. of the VCU Graduate School. He also received the American Medical Association's Seed Grant in 2008 and the Phi Kappa Phi Love of Learning Award in 2009. He received a Graduate Student Association Travel Award to attend the Keystone Conference on Epigenetics in Development and Disease in 2009. He presented his work at the 2008 ASCI/AAP/APSA joint meeting and received first place poster awards at the American College of Physicians Virginia Chapter Meeting in 2009, the VCU ICAMS retreat in 2008, and the Massey Cancer Center Retreat in 2008.

Omar currently lives in Richmond, VA with his wife, Lisa Gangi, and their children, Joseph and Olivia. He plans to resume medical school in the spring of 2010 and complete his MD degree in 2011.