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Mary Lucas Date

Methylation-Sensitive Regulation of TMS1 Expression and Chromatin Structure by the Ets Factor GABP

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Abstract

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Epigenetic silencing involving the aberrant DNA methylation of promoter-associated CpG islands is one mechanism leading to the inactivation of tumor suppressor genes in human cancers. However, the molecular mechanisms underlying this event remain poorly understood. TMS1/ASC is a novel proapoptotic signaling factor that is subject to epigenetic silencing in human breast and other cancers. The TMS1 promoter is embedded within a CpG island that is unmethylated in normal cells and is spanned by three DNaseI hypersensitive sites (HS). Silencing of TMS1 in cancer cells is accompanied by local alterations in histone modification, remodeling of the HS and hypermethylation of DNA.

In this study, we identified two roles for the GA-Binding Protein complex (GABP) in the regulation of transcription from the TMS1 locus. Initially, GABP was identified as a methylation-sensitive complex that bound a 55bp intronic element within HS2 *in vitro* and *in vivo* and functioned as an activator of transcription. In reporter assays the HS2 element conferred a 3-fold enhancement in TMS1 promoter activity, which was dependent on both intact GABPα binding sites and the presence of GABP proteins (GABPα and GABPβ1) in trans. Accordingly, downregulation of GABPα led to a concomitant decrease in TMS1 expression.

In addition, we identified a role for GABP in the maintenance of transcriptional competency at the TMS1 locus through the regulation of histone acetylation. Inhibition of GABP binding at the TMS1 locus through deletion of GABP binding sites correlated with a decrease in histone H3 acetylation at lysine 9 and 14. These data indicate that the

intronic HS2 element acts in cis to maintain transcriptional competency and hyperacetylated histones at the TMS1 locus and that this activity is mediated by the ets transcription factor, GABP.

In conclusion, this project demonstrates GABP is necessary for the activation of transcription from the TMS1 locus and the maintenance of a hyperacetylated chromatin state. Therefore, the loss of GABP binding at the TMS1 locus could be one mechanism leading to the loss of acetylated histones, the propagation of heterochromatic histone modifications and silencing of TMS1 expression in cancer.

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Chapter 1

Introduction

Epigenetics

Epigenetics is defined as a heritable change in the pattern of gene expression that is mediated by mechanisms other than alterations in the primary DNA sequence (1). The development and differentiation of various cell types are normal cellular processes regulated by epigenetics. During development, cells start in a pluripotent state in which they have the ability to differentiate into many cell types. Throughout differentiation, gene expression patterns become more limited and consequently stabilized for their restricted lineage. In addition to the combinatorial work of transcription factors, epigenetic events can contribute to the cell-type and time dependent expression of genes during the above mentioned processes (2-4). Epigenetic molecular mechanisms include methylation of DNA at CpG sequences (in vertebrates) (2, 3) and modification of histone tails (5).

An example of epigenetic regulation of gene expression that has been widely studied is the normal cellular process of X-chromosome inactivation (6). In female mammals, during early embryogenesis, one of the two X-chromosomes is silenced in order to achieve dosage compensation. X-inactivation is regulated by several factors, including non-coding RNAs, DNA methylation, histone acetylation, and deacetylation, as well as histone methylation. X-inactivation occurs soon after implantation and is accompanied by an increase in the expression and accumulation of Xist RNA; which coats the inactive X chromosome (6). Methylation of histone H3 at lysine 9 (H3K9me) is an early event in the initiation of X-inactivation. The histone methyltransferase Suv39h (7) is thought to be recruited to the locus by Xist, while methylation of H3 at lysine 4

(H3K4me) is associated with the active X (8, 9). DNA methylation and histone deacetylation are known events that occur at the inactive X chromosome in order for the active euchromatin to be converted into a transcriptionally silent state associated with highly condensed heterochromatin (10). However, the identification of factors that bring about DNA methylation and histone deacetylation in the spreading of X-chromosome inactivation remains to be determined. Nevertheless, summation of known information regarding X-chromosome inactivation demonstrates the regulatory connection between epigenetic mechanisms of gene regulation (e.g. DNA methylation and modification of histone tails).

Post-Translational Modification of Histones and Chromatin Structure

In eukaryotes, genomic DNA is packaged and organized into chromatin. Based on microscopic observations, chromatin is mainly divided into two distinct forms, euchromatin and heterochromatin. Euchromatin is defined as de-condensed chromatin during interphase, while heterochromatin is condensed and densely stained throughout the cell cycle (11) . Euchromatin replicates relatively early in the cell cycle and is known to be gene-rich and transcriptionally competent. Conversely, heterochromatin is relatively gene poor, transcriptionally inactive, late-replicating and enriched in repetitive sequences found at pericentric and telomeric regions.

Research has shown that chromatin plays an important role in the regulation of gene expression and has characteristic changes in cancer cells (12). Chromatin structure regulates the access of factors that control DNA-mediated reactions like transcription,

DNA replication and DNA repair, to specific region of the DNA. The basic unit of chromatin is the nucleosome which consists of 146 base pairs of DNA wrapped around a histone octamer that contains two molecules of each histone H2A, H2B, H3 and H4. Post-translational modifications of histones such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and poly-ADP-ribosylation play an important role in the regulation of chromatin structure and function (13-15) (Figure 1). The combinatorial patterns of different post-translational histone modifications and the resulting effect on gene expression have been termed the "histone code". Modification of lysine residues located in the N-terminal region of histones, by acetylation or deacetylation, changes the configuration of nucleosomes. Acetylation of lysines by histone acetylases (HAT) is associated with an open chromatin structure, which facilitates gene transcription and can be found at active genes within euchromatin. Acetylation of lysine residues 9 and 14 at histone H3 can be detected at the TMS1 and $p16^{INK4A}$ locus (16, 17) when these gene are actively expressed. Conversely, heterochromatic region of the genome, that maintain a transcriptionally silent state, are enriched for deacetylated histones (12). Histone methylation can be associated with both gene activation and repression depending on the residues modified and the extent of methylation. Trimethylation of lysine 4 at histone H3 (H3K4me3) is typically associated with the 5' ends of active genes, whereas trimethylation of lysine 9 at histone H3 (H3K9me3) is enriched at compact pericentromeric heterochromatin, which is transcriptionally inert (12). In addition, like methylation, ubiquitination is associated with both gene activation and repression. Ubiquitination of lysine 123 and 120 at histone H2B is associated with active genes, whereas, ubiquitination of lysine 119 at histone H2A is found at inactive

Table 1: Chromatin Modifications

Histone Post-Translational Modifications		
Mark	*Transcriptionally relevant sites*	*Transcriptional roles*
Acetylated lysine	H3 (9,14,18,56), H4 (5,8,13,16), H2A, H2B	Activation
Phosphorylated serine/ threonine	H3 (3,10,28), H2A, H2B	Activation
Methylated arginine	H3 (17,23), H4 (3)	Activation
Methylated lysine	H3 (4,36,79) H3 (9,27), H4 (20)	Activation Repression
Ubiquitylated lysine	H2B (123*/120) H2A (119)	Activation Repression
Sumoylated lysine	H2B (6/7), H2A (126)	Repression
Isomerized proline	H3 (30-38)	Activation/ Repression

Figure 1: *Activating and repressive chromatin modifications*. Specific amino acid residues in histones can be subjected to multiple modifications which elicit opposing roles in transcription . Each modification is inhibitory to subsequent modification of the same residue. * Yeast (*Saccharomyces cerevisiae*) Adapted from Berger *et. al.* 2007: The complex language of chromatin regulation during transcription.

genes (18). It is noteworthy to mention that the addition of one modification to a residue can chemically block additional modifications of that amino acid or others nearby. For example, lysine methylation and acetylation can not co-exist at the same lysine residue. Methylation of lysine 9 at histone H3 blocks potential acetylation, and vice versa.

DNA Methylation

Within the mammalian genome DNA methylation occurs at cytosine residues within the dinucleotide 5'-CpG-3' (Figure 2). Early during embryonic development, DNA methylation patterns undergo dramatic reprogramming after fertilization. Demethylation of DNA is the first event which occurs in the male pronucleus and is independent of DNA replication (19, 20). After zygote formation, both maternal and paternal chromosomes undergo progressive demethylation. By the blastocyst stage, most, but not all, of the methylation marks that are inherited from the gametes are erased (21-23). The methylation marks on imprinted genes are protected from demethylation. As a result, parental imprints are preserved. Embryonic DNA methylation patterns are established after implantation through *de novo* methylation which begins in the inner cell mass (undifferentiated cells in the blastocyst which gives rise to the entire fetus) of a blastocyst (21, 22). Genetic studies of the function of DNA methyltransferases in embryonic development show that the establishment of methylation patterns requires both *de novo* and maintenance methyltransferases activities, and the maintenance of genomic methylation above a certain level (24, 25).

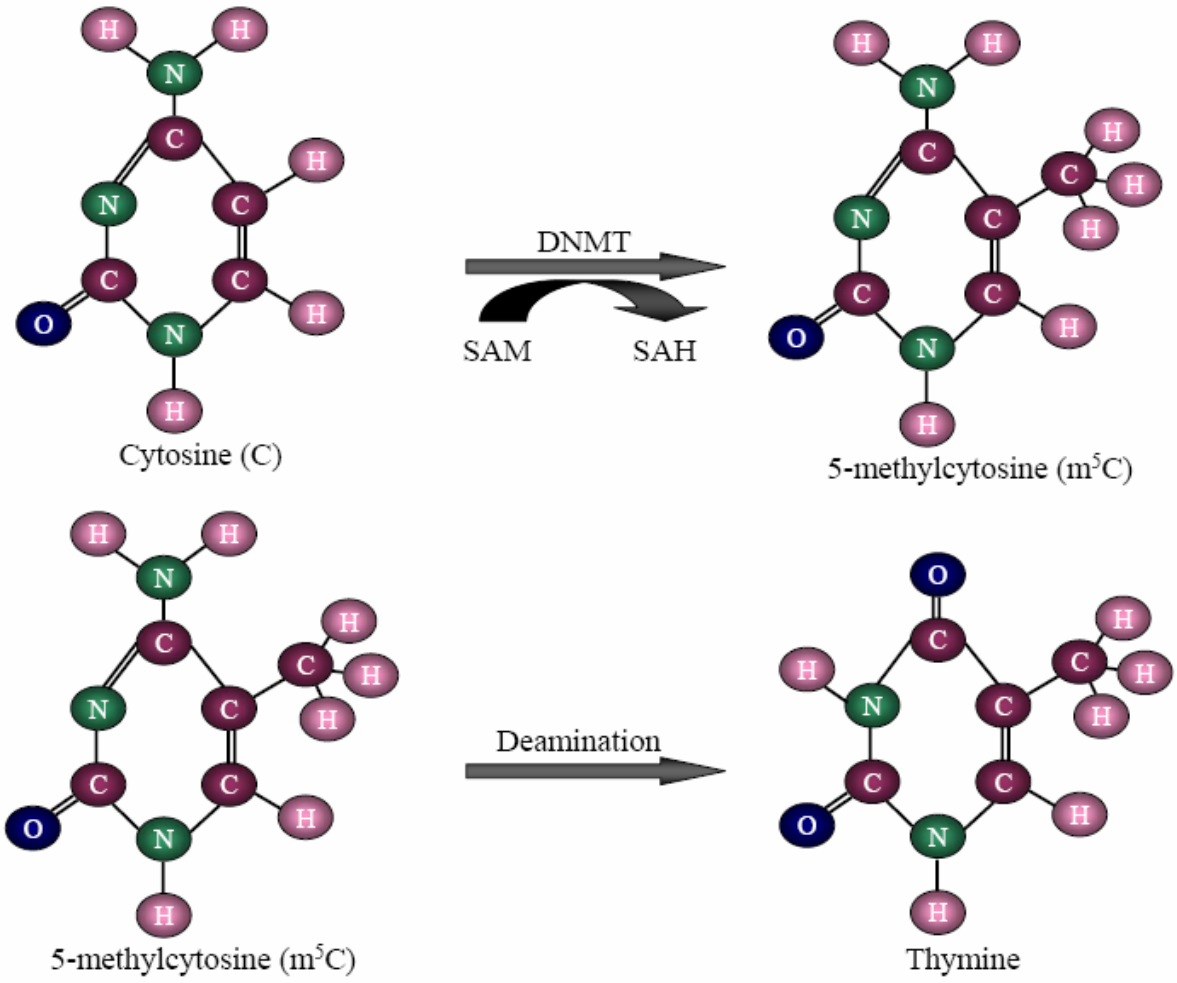

Figure 2: *Methylation of cytosine residues within the dinucleotide CpG.* DNMTs catalyze the transfer of a methyl group from S-adenosyl-methionine (SAM) to the carbon-5 position of cytosine in the dinucleotide 5'-CpG-3'. SAM is subsequently converted to S-adensoyl-homocysteine (SAH). Methylated cytosines are more sucseptible to deamination forming thymidine. Thus over evolution, methylated CpGs have deaminated to TpG. Adapted from Gronbaek *et. al.* 2007. Epigenetics changes in cancer. APMIS.

CpG Islands

In the context of DNA methylation, sequences within the genome can be classified into two different groups: CpG poor regions and CpG islands. As the name implies, CpG poor regions of the mammalian genomic DNA contain few CpG dinucleotides, and these are largely methylated (26). The depletion of CpG dinucleotides in these regions can best be explained by the fact that methylated CpG dinucleotides are prone to mutation leading to a decrease in CpG content during evolution.

CpG islands are approximately 200bp to several kb in length having a GC content greater that 55% and an observed CpG/expected CpG ratio of 0.65 (27). CpG islands are usually found in the promoter region of genes and usually extend from the promoter region into the first exon and sometimes into intron 1 (12). It has been estimated that there are ~29,000 CpG islands in the genome and that approximately 70% of all genes contain a promoter associated CpG island (28). Most of the CpG dinucleotides in the genome are heavily methylated while, in contrast, the CpG dinucleotides in CpG islands, especially those associated with gene promoters, are usually unmethylated. CpG islands probably have a high frequency of CpG dinucleotides due to the fact that methylated cytosines are more susceptible to deamination forming thymidine (29) (Figure 2). Thus, over evolution, methylated CpGs have deaminated to TpG while the unmethylated CpGs in the CpG island have been retained. The methylated state of CpG dinucleotides in the bulk of the genome helps to suppress unwanted transcription, whereas the unmethylated state of CpG islands in gene promoters permits active gene transcription (Figure 3). There are however some CpG islands that

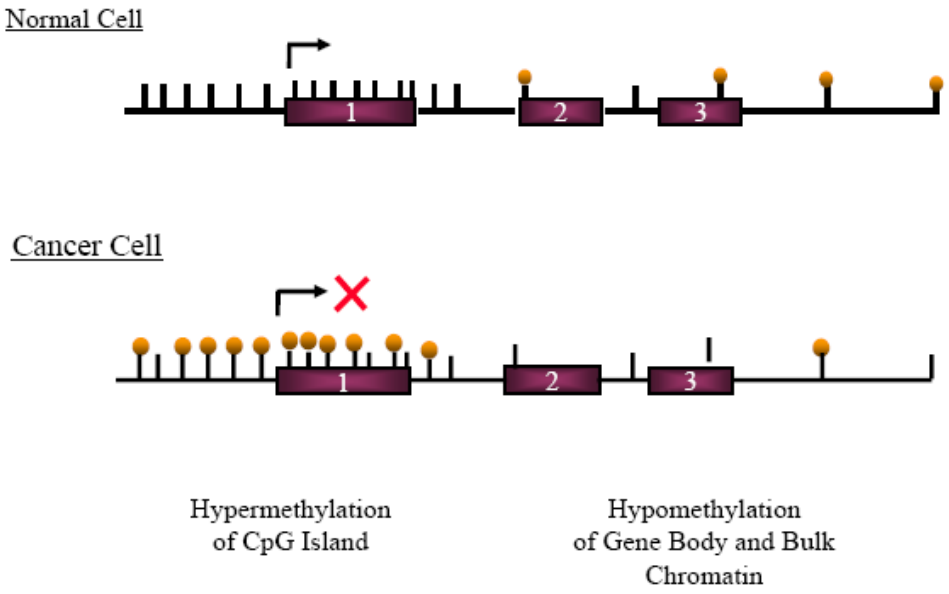

Figure 3: *Patterns of DNA methylation in normal and cancer cells.* In normal cells, most CpG sites (vertical ticks) in the genome are methylated (filled circles), except for those in CpG "islands"- CpG dense DNA that surrounds the promoters of ~70% of genes in the genome. In cancer cells, the DNA methylation pattern is shifted with hypomethylation of CpG sites within the gene body and bulk chromatin and hypermethylation of CpG-islands. This latter hypermethylation even is associated with gene silencing.

are normally methylated, such as the CpG islands associated with the transcriptionally silent genes on the inactive X-chromosome of females and the silent alleles of some "imprinted genes," which are programmed such that only one parental allele of the gene is expressed in normal tissues (30).

DNA Methyltransferases (DNMTs)

Methylation of DNA in the human genome is tightly controlled during development by the action of DNA methyltransferases (DNMTs). DNMTs catalyze the transfer of a methyl group from S-adenosyl-methionine to the carbon-5 position of cytosine in the dinucleotide 5'-CpG-3' (Figures 2 and 4). This epigenetic mark is copied after DNA synthesis, resulting in heritable changes in chromatin structure (12). Three active DNA methyltransferases have been discovered in mammals; DNMT1, DNMT3A and DNMT 3B (Figure 4).

Mammalian DNMTs are classified into two groups based upon their proposed activities, maintenance and *de novo*. "Maintenance" methylation activity, possessed by DNMT1, is responsible for the preservation of methylation patterns once established through subsequent cell divisions. *De novo* methylation, which is involved in the establishment and rearrangement of methylation patterns during embryogenesis or differentiation process in adult cells, is controlled by DNMT3A and 3B (31, 32). However, proteins have been identified that contain all or a subset of the conserved DNA methyltransferase motifs but exhibits no DNA methyltransferase activity *in vitro* (33), such as DNMT2 and DNMT3L. DNMT2 was later determined to be a tRNA

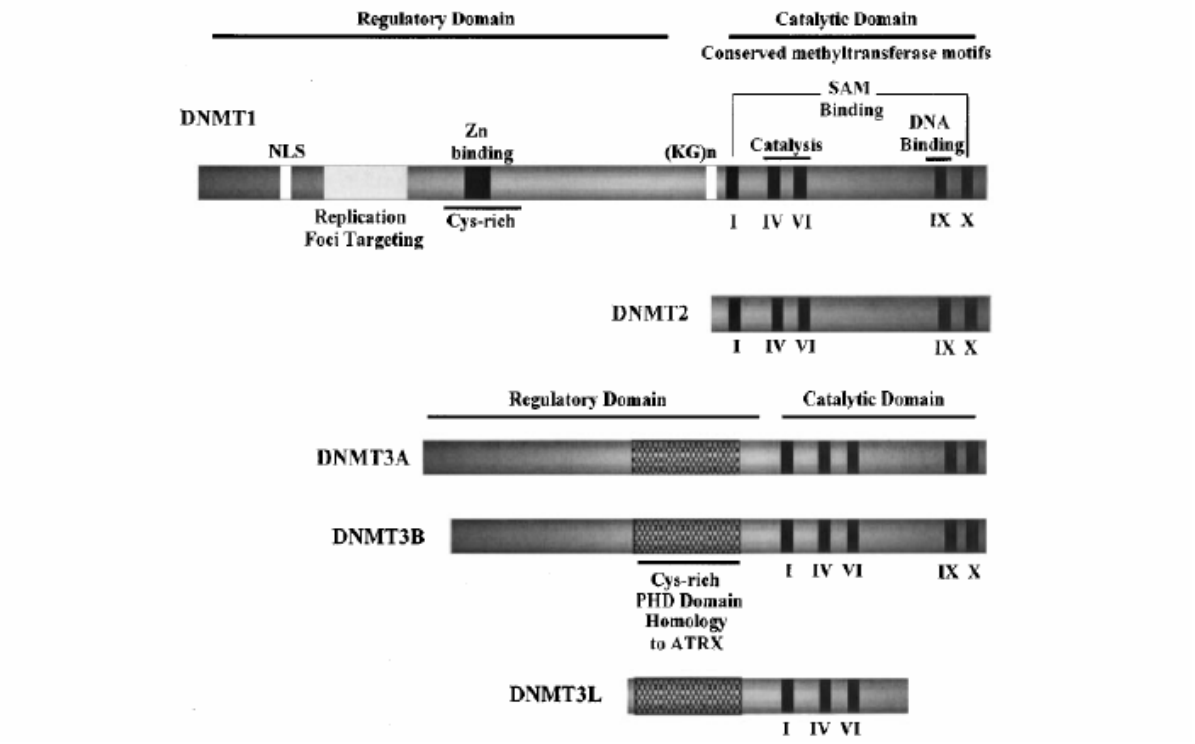

Figure 4: *Schematic of DNA methyltransferases and DNMT-like proteins.* DNMT1, 3A and 3B can be divided into two domains, regulatory and catalytic. Conserved motifs (roman numerals) involved in DNA methylation are indicated with black boxes. Of note: DNMT1- replication foci targeting domain and zinc binding domain; DNMT3A/B- cystein-rich PHD (plant homeodomain). DNMT2 and DNMT3L contain conserved motifs however, do not posses any known enzymatic activity involved in DNA methylation. DNMT2 is a tRNA methyltransferase, while DNMT3L stimulates the activity of DNMT3A and B. Adapted from Robertson *et al.* 2001: DNA methylation, methyltransferases and cancer.

methyltransferase (34) and is now sometimes referred to as TRDMT1 (tRNA aspartic acid methyltransferase 1) to reflect its biological function. DNMT3L (DNMT3-Like) is homologous to the DNMT3 family. DNMT3L lacks a functional catalytic domain but acts as a stimulator of *de novo* methylation by DNMT3A and B (35) (Figure 4).

DNMT1

DNMT1 is the most abundant methyltransferase in somatic cells (36). During DNA replication DNMT1 is located in the replication complex (via an association with the proliferating cell nuclear antigen (PCNA)) where it recognizes the normally methylated CpG sites in the parental strand and catalyzes the addition of the methyl group to the corresponding CpG site in the daughter strand (37). DNMT1 shows preference for hemimethylated DNA *in vitro*, demonstrating a 10-40 fold preference for hemimethylated DNA as compared to an unmethylated substrate (38, 39). DNMT1 is essential for proper embryonic development, X chromosome inactivation and imprinting as determined by the generation of Dnmt1 knockout mice (24, 40, 41). These mice show arrested development prior to the 8 somite stage and a nearly 70% reduction in the genomic 5-methylcytosine content (24).

DNMT3

Even though patterns of DNA methylation are generally accurately passed to daughter cells, these patterns can also be created or erased. Creation of methylation

patterns *de novo* is catalyzed by other cytosine methyltransferases, distinct from DNMT1. The DNMT3 family of DNA methyltransferases can methylate hemimethylated and unmethylated DNA at the same rate (42). DNMT3A and DNMT3B were identified through database searches for proteins containing conserved methyltransferase motifs (25).

Knockout of Dnmt3a and Dnmt3b in mice blocks *de novo* methylation in early post-implantation mouse embryos and causes embryonic lethality, but has little effect on the maintenance of pre-existing methylation (25). The mouse knockout for Dnmt3b revealed that it is required for the maintenance of DNA methylation of minor satellite repeats adjacent to centromeres suggesting that it may also be necessary for the maintenance of methylation. Studies using mouse germ cells in which Dnmt3a was disrupted but still preserved in somatic cells by conditional knockout technology, determined that Dnmt3a is required for methylation of most imprinted loci in germ cells (43). It is of note to mention that the phenotype of Dnmt3l knockout mice is indistinguishable from Dnmt3a conditional mutants, except for methylation at one locus (43). Subsequently, Dnmt3l was determined to have a role in the methylation of imprinted loci (44).

Impact of DNA Methylation on Gene Expression and Chromatin Structure

DNA methylation can interfere with gene expression by directly inhibiting the interaction of some transcription factors with their recognition sequence. The binding of transcription factors like E2F, CREB, AP2, cMyc/ Myn, NF-κB, and GABP to DNA is

blocked by methylation (45-50). In contrast, methyl-C-binding proteins (MBD) preferentially bind methylated CpG sites. The MBD family of proteins consists of five members: MBD1, MBD2, MBD3, MBD4 and MeCP2, which contain a homologous methyl-CpG binding domain. There is also non-homologous protein Kaiso, which also binds methylated CpG sites (51). The binding of MBDs to methylated CpG sites allows for the recruitment of repressive proteins, such as HDACs and Suv39h that facilitate the establishment of a heterochromatic state. MBDs are also thought to compete in binding with methylation-insensitive transcription factors such as Sp1 and CTCF (52).

Methylation of CpG sites within promoters may also affect nucleosome occupancy at the transcription start sites of genes, thereby altering transcriptional activation. The presence of nucleosomes within the transcription start region of genes can decrease the binding of transcription factors and RNA polymerase II (53), whereas, the absence of nucleosomes at these regions allows binding (54, 55). Davey *et al.*(56) used an *in vitro* study to show that methylation of CpG sites could affect nucleosome positioning at a particular sequence, although the exact mechanism behind this event is not fully understood.

Two associations have been observed *in vivo* that could potentially target nucleosomes to a promoter region containing methylated CpG sites. Chromatin remodeling complexes use ATP hydrolysis to alter the structure and position of nucleosomes (57). MeCP2 associates with Brahma, the catalytic subunit of the chromatin-remodeling complex SWI/SNF, in addition, DNMT3B interacts with another chromatin remodeler, hSNF2h (58, 59). These interactions suggest that chromatin remodelers may be directly targeted to these sites by MBDs as well as DNMTs.

Crosstalk Between DNA Methylation Histone Modifications

In mammalian cells, both DNA methylation and histone modifications are involved in the silencing of chromatin. These two processes are believed to be interdependent, thus one can target the function of the other to specific regions of the genome. Three models have been proposed for how DNA methylation and histone modification might influence each other.

DNA methylation patterns are established through *de novo* methylation by DNMT3A and 3B, and are maintained by DNMT1. A direct link between DNA methylation and chromatin structure has been suggested by the observation that MBDs interact with HDACs and function by recruiting HDACs to methylated promoters to promote gene silencing through the removal of acetyl groups from histones (58, 60, 61) (Figure 5). Through unknown mechanisms, histone methyltransferases (HMT) (e.g. Suv39h or G9a) are then attracted to the chromatin, which methylate H3K9 locking the chromatin in an inactive state.

Alternatively, H3K9 methylation acts as a signal for inactive chromatin by recruiting HP1 to methylated histones, which might in turn recruit DNMTs directly or indirectly to the silent chromatin to stabilize the inactive chromatin (62, 63) (Figure 5). HP1, a well-known silencing protein found at pericentromeres and telomeres, is involved in the establishment and maintenance of higher order chromatin structures. HP1

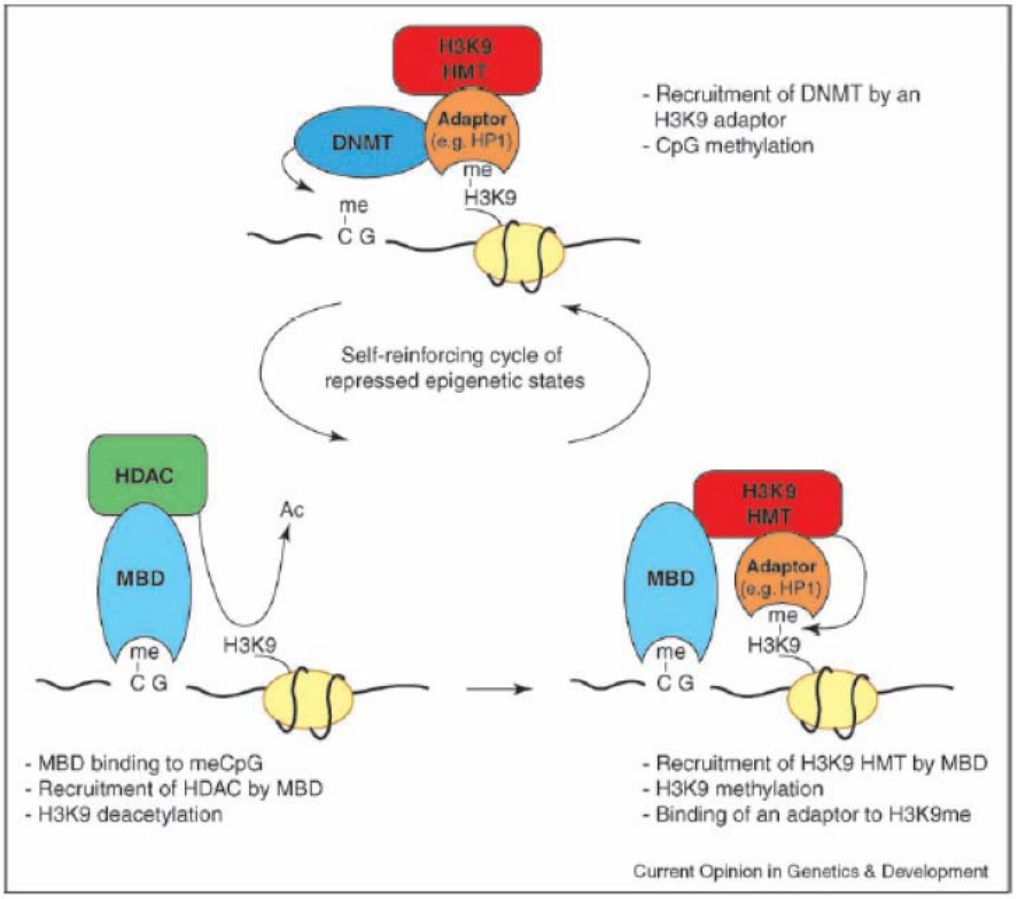

Figure 5: *Model for a self-reinforcing cycle that might maintain a repressed chromatin state.* H3K9me would allow for the binding of HP1 and subsequent recruitment of DNMT1 to deposit methyl groups on surrounding DNA sequences. The generation of methylated DNA would in turn allow the binding of MBD and recruitment of HDAC complexes. The presence of HDACs would cause the deactylation of H3K9, providing a substrate for H3K9 HMT. Consequently, H3K9me would allow the binding of HP1 thus continuing the cycle. This allows a consistent flow of information from the histone to the DNA and back. Adapted from Fuks *et. al.* 2005: DNA methylation and histone modification: teaming up to silence genes.

specifically recognizes and binds to methylated histone H3 at lysines 9 and 27 (64, 65), converting the nucleosomes to a compact configuration.

On the other hand, ATP-dependent chromatin-remodeling and DNA-helicase activities of proteins might facilitate DNA methylation and histone modification by unwinding nucleosomal DNA to increase accessibility to DNMT, HDACs, and HMTs. In plants, it has been shown that disruptions of these proteins impairs both DNA methylation and histone methylation (66, 67). In total, the observations from these experiments provide evidence that DNA methylation is linked to histone deacetylation in the repression of transcription, as well as, evidence that H3K9 methylation is required for DNA methylation.

Cancer Statistics

The American Cancer Society reported 7.6 million people died from cancer in the world during 2007 and 25% of all deaths in the U.S. were due to this major health problem. Seven percent of these cancer related deaths in the U.S. were due to breast cancer (68). Women in the Unites States have the highest incidence rates of breast cancer in the world. Among women in the U.S., breast cancer is the most common form of cancer and the second most common cause of cancer death after lung cancer. American women have a 1 in 8 (12.5%) lifetime chance of developing invasive breast cancer and a 1 in 35 (3%) chance of breast cancer causing their death (68). In recent years, studies have shown a racial disparity in the diagnosis and treatment of breast cancer. Several studies have demonstrated that African-American women in the U.S. are more likely to

die from breast cancer, even though Caucasian women are more likely to be diagnosed with the disease. Even after diagnosis, African-American women are less likely to get treatment compared to Caucasian women (69, 70).

There are several theories for the above mentioned disparities. The African-American women involved in these studies might have had inadequate access to screening thus the cancer was not detected at an early stage of development or may have had a reduced availability to the most advanced surgical and medical techniques due to socio-economic limitations. There is some biological characteristics of the disease yet to be identified in the African-American population. Triple negative breast cancer, a highly aggressive and difficult form of cancer to treat, has been demonstrated to be highly prevalent in African-American women in comparison to Caucasian women (71). However, the underlying cause for the susceptibility to a more aggressive form of the disease is unknown. Research is still ongoing to define the contribution of both biological and cultural factors in the racial disparity of breast cancer.

Altered Epigenetics in Cancer

It has become increasingly apparent that an understanding of epigenetic inheritance will be vital to our understanding of the differences between growing, senescent and immortal cells, tumor and normal cells and various differentiated cells. Compared to normal cells, genomic methylation patterns are drastically altered in tumorgenic cells with global hypomethylation and regional specific hypermethylation events occurring over time (72). More than a decade ago it was shown that DNA

methylation patterns are altered in human cancers (73, 74). The bulk of the genome becomes hypomethylated, specifically the normally hypermethylated and silent regions containing repetitive elements, such as centromeric satellite repeats are substantially demethylated. In experimental animal models of carcinogenesis, the decrease in the number of methyl groups appears to begin early in tumor progression and before tumor formation (75, 76). In 1983, Feinberg and Vogelstein reported a decrease of methylation in the promoter regions of c-Ha-ras and c-Ki-ras in lung and colon cancers (77). The inappropriate activation of these small GTPases could play a central role in malignant transformation. Due to this observation, activation of oncogenes was proposed as a possible result due to the decrease in DNA methylation. However, evidence for activation of oncogenes by specific gene demethylation is poor and has been reported for only a few oncogenes (78).

Two consequences of loss of methylation during tumorigenesis have been proposed. First, a weakening of transcriptional repression in normally silent regions of the genome could cause the potential harmful expression of inserted viral genes (e.g. intracisternal A particle (IAP) retrovirus present in the mouse genome), repeat elements and normally silenced genes (79, 80). Loss of promoter methylation and transcriptional activation of long interspersed nuclear elements (LINE), the most abundant retrotransposons in the human genome, has been reported in a variety of cancers (81, 82). The harmful effects of hypomethylation of LINE retrotransposons in cancer have been linked with both mutational disruption of genes as well as the potential disruption of nearby genes. Second, hypomethylation of DNA not associated with gene promoters could affect the functional stability of the chromosome in cancer. Studies performed in

mouse ES cells that contain a homozygous deletion of Dnmt1 have shown a direct link between chromosome instability and genome hypomethylation (83). The mutant ES cells have a significantly increased mutation rate predominantly involving genomic deletion. Furthermore, chromosome instability is a phenotype of ICF (immunodeficiency, centromeric instability, facial anomalies) patients, a genetic disorder in humans caused by mutations in the DNA methyltransferase DNMT3B (84).

At present, the mechanism responsible for genomic hypomethylation in cancer remains unclear. Several possibilities have been proposed, including insufficient dietary folate or a possible mutation in the folate metabolic process. A model was established in which the livers taken from rats fed folate-deficient diets exhibited genome hypomethylation and increased DNA strand breaks leading to the development of liver cancer (85, 86). This model of genomic hypomethylation is considered a "passive" model of demethylation in which the availability of the substrate (methyl groups) is rate limiting. It is also possible that hypomethylation is a result of deregulation of an unidentified DNA demethylase enzyme or conversely, a dysfunction of the DNMT enzymes. Although the pathways leading to hypomethylation are currently unknown, it is clear that hypomethylation is a hallmark of most cancer genomes.

.Hypermethylation/Inactivation of tumor suppressor genes

In conjunction with hypomethylation cancer cells also show a regional hypermethylation of CpG dinucleotides within CpG island regions. Often, the normally unmethylated expressing CpG island associated genes become hypermethylated and

inactivated. The mechanism responsible for genomic hypo- and hypermethylation in cancer is unclear. Heritable gene silencing by CpG hypermethylation at the promoters of tumor suppressor genes is one of the most fundamental epigenetic events in carcinogenesis and is probably the most common mechanism of tumor suppressor inactivation in cancer. Loss of tumor suppressor protein expression due to gene silencing has been shown to disrupt all of the cell's protective pathways to prevent cancer (87). Genes involved in cell cycle regulation, DNA repair, apoptosis, angiogenesis and metastasis have all been identified as being susceptible to hypermethylation in different cancers (30).

Over the past decade, a large body of knowledge has been obtained regarding how and where these methylation changes occur. Methylation of CpG-islands located near transcription start sites of tumor suppressor genes is associated with gene silencing, while methylation of the downstream gene sequence generally has no influence on gene expression (88, 89). As defined by Knudson *et al.*(90), disruption of the function of a tumor suppressor gene requires a complete loss of function of both copies of the involved gene. This loss of function can occur through hypermethylation of both alleles or a combination of methylation, mutation or chromosomal deletion (12) (Figure 6). Consequently, the loss of both alleles of a tumor suppressor gene sets the scene for malignant transformation of the cell. The number of cancer related genes affected by epigenetic inactivation equals or exceeds that inactivated by mutation (91-95).

Cancer-related genes silenced by aberrant methylation of CpG islands in various types of tumors indicate that epigenetic events play a key role in tumorigenesis. Although there are now a number of well-documented examples of epigenetic gene

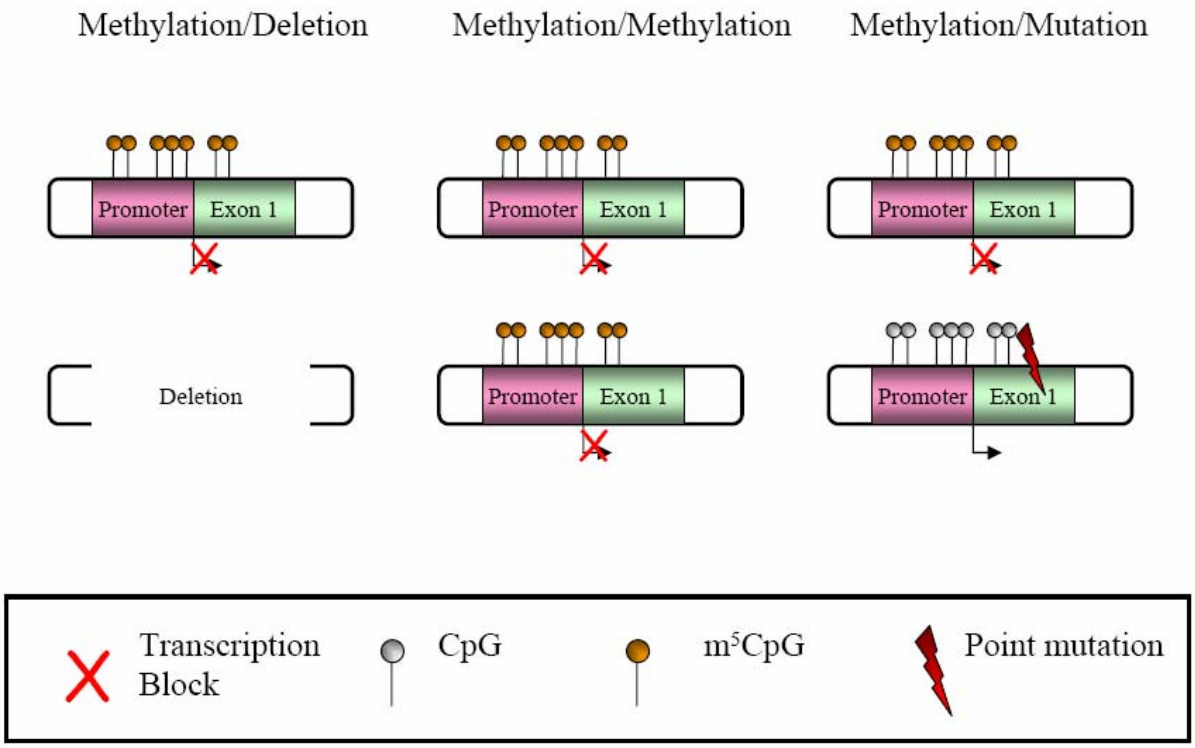

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Figure 6: *Knudson's two-hit hypothesis for the inactivation of tumor suppressor genes.* Disruption of the function of a tumor suppressor gene requires a complete loss of function of both copies of the involved gene. Depicted are the general mechanisms in which loss of function is gained. The sequence of inactivating events can occur in any order. Adapted from Herman *et al* 2003:Gene silencing in cancer in association with promoter hypermethylation.

silencing in human tumors, the mechanism in which such genes become aberrantly methylated in cancer remains unclear. A critical unanswered question is how are CpG islands protected from methylation in normal cells and how does this mechanism break down in cancer? It is thought that CpG islands spanning housekeeping genes are protected from methylation either through (1) the process of active transcription, (2) replication timing, or (3) local chromatin structure that may inhibit access to the DNA methyltransferase (96, 97). The importance of gene activity as the mode of protection is represented by the fact that the CpG islands on the inactive chromosome are silenced by methylation (98). Therefore CpG islands are not inherently unable to be methylated in the early stages of development.

As previously discussed, DNA methylation and chromatin modifications are epigenetics events that act in unison to silence the expression of genes. Research indicates that aberrant DNA methylation of promoters of tumor-suppressor genes in cancer is accompanied by a shift to a more compact chromatin structure characterized by hypoacetylated histones H3 and H4 and a shift in the methylation pattern from H3 methylated at lysines 4,36, and/or 79 to H3 methylated at lysines 9, 27 and/or 20 (99, 100) . Studies have reported that DNMT1 directly represses transcription in cooperation with HDACs (101). The association of HDACs with DNMT1 could allow for the maintenance of a transcriptionally silent state through the incorporation of hypoacetylated histones into newly formed nucleosomes. This would allow the propagation of a silent chromatin state following each round of DNA synthesis. A mechanism such as this would be beneficial for the maintenance of constitutively heterochromatic regions of the genome. A link between DNA methylation and histone

methylation has also been observed. Dmnt3a has been shown to localize with HP1, which binds the repressive mark of H3K9me (102). This localization could have important implications for the targeting of *de novo* DNA methyltransferase activity in order to establish new methylation patterns.

It is not easy to address the activation of hypermethylation in tumor tissue because DNA methylation is an early event in tumorgenesis. However, a model for hypermethylation has been put forth which involves "seeding and silencing". Clark *et al.* (103), proposed that in cancer, gene silencing comes first and triggers the hypermethylation of the CpG island associated gene. In a normal cell, CpG island-associated genes are actively transcribed and remain essentially unmethylated whereas in a cancer cell the genes are silent and associated with CpG island hypermethylation. Their model suggests that in a normal cell, CpG islands are subjected to a steady, but light "seeding" of *de novo* methylation, but remain methylation free due to demethylation promoted by active gene transcription. Supporting data includes bisulfite sequencing data that revealed not all CpG sites in a CpG island are unmethylated in normal cells (89, 104, 105). However, in a cancer cell, gene silencing is a prerequisite and that in combination with "seeding" allows methylation to shift in favor of *de novo* methylation of the island and silencing of the gene.

An alternative model has been introduced by Issa *et. al.* 2004 (106) for targeted hypermethylation and silencing of CpG island associated genes. In this model, unmethylated CpG island DNA is bound by proteins which inhibit the establishment of DNA methylation within the region as well as oppose the spreading of methylation in to the region. By an undetermined mechanism, DNMTs are recruited to the borders of the

CpG island DNA where methylation is able to occur creating methylation pressure at the 5' and 3' ends of these islands. Consequently, a balance is achieved at the CpG island promoter region between the protective proteins bound at the CpG island DNA and the surrounding hypermethylated DNA. A loss of balance would result in the spread of methylation into the transcription start area and trigger silencing (106). The disruption of this balance could occur in two ways. First, there could be a loss of protective proteins due to mutations or epigenetic silencing of said proteins. Second, the balance could be disrupted by overactive DNA methyltransferases (e.g. overexpression of DNMT1).

Work from our lab supports the model proposed by Issa et. al. 2004 (106) in that in cells that actively express TMS1, the associated CpG island is unmethylated and surrounded by heavily methylated DNA at the 5' and 3' borders. The chromatin structure of TMS1 is associated with three hypersensitive sites which form depending upon the methylation status of the DNA. When the CpG island is unmethylated and the locus is expressed, HS1 and HS3 form directly at the boundaries demarcating the unmethylated CpG island DNA from the heavily methylated surrounding DNA with HS2 forming in the center. Potentially, proteins are bound at HS1 and HS3 that provides a protective barrier from the surrounding heterochromatic marks that allows for active transcription of the locus. However, when DNMT1 is overexpressed, the TMS1 locus undergoes aberrant methylation, loss of CpG-island associated HS sites and transcriptional silencing. The overexpression of DNMT1 potentially disrupts the balance between protective proteins bound at the TMS1 CpG island and the surrounding hypermethylated DNA resulting in the spread of DNA methylation into the locus triggering silencing.

An additional model is that CpG island chromatin bears a particular 'histone code' that prevents DNA methylation. To this end, it is noteworthy that CpG island chromatin is marked by H3K4me2/3 and loss of this mark is universally associated with the absence of DNA methylation in genome-wide studies (107, 108). H3K4me blocks the interaction of DNMT3L with DNA and plays a role in preventing *de novo* methylation (109).

Aberrant methylation of cytosine residues and histone modifications in chromatin are epigenetic changes that act in unison to silence the expression of genes and occur frequently in tumor development (103). Even though these alterations have been well characterized, the mechanism responsible for eliciting these changes is still not well understood. Research is actively ongoing to decipher the mechanisms by which epigenetic modifications are regulated in normal cells, and disrupted in cancer cells. An understanding of this mechanism and other epigenetic events which leads to carcinogenesis is an important area of research in the fight against cancer.

Identification and Characterization of TMS1

Studies to determine the molecular mechanisms that underlie aberrant DNA methylation and epigenetic silencing in cancer identified a novel CpG-island associated gene called TMS1 (Target of Methylation-induced Silencing-1). TMS1 was initially identified in a PCR-based subtractive cDNA screen to isolate transcripts that were down-regulated in human fibroblast overexpressing DNMT1 (110). TMS1 is expressed normally in mammary epithelial cells and in immortalized, non-tumorgenic breast

epithelial cells in which its promoter-associated CpG island is unmethylated (111). Interestingly, 44% (5 of 11) of breast carcinoma cell lines fail to express TMS1 and silencing correlates with the methylation status of the TMS1 promoter (111). Subsequent studies have shown the methylation-associated silencing of TMS1 in 40% of breast carcinomas (111), 11% of gastric carcinomas (112), 40-41% of small cell and non small cell lung carcinomas (113), and 44% of primary glioblastomas (114). The loss of TMS1 expression also accompanies the transition from DCIS to invasive carcinomas in primary breast lesions (115).

TMS1 contains a caspase recruitment domain (CARD) at the C-terminus and a pyrin domain (PYD) at the N-terminus (111) (Figure 7). The protein structure of TMS1 is intriguing due to the fact that it contains both a CARD domain as well as a PYD domain. The death domain fold (DDF) is a unifying structural motif of a superfamily of protein domains comprising the pyrin domain (PYD), death domain (DD), and death effector domain (DED), all of which function primarily in regulation of apoptosis and inflammatory responses. DDF motifs function to maintain the balance between cell survival and death through the regulation of nuclear factor-κB (NF-κB) activities and caspase activation. Disruptions in these pathways ultimately lead to disease phenotypes. TMS1 is a bipartite protein that functions as an intracellular signaling adapter with roles in apoptosis and inflammation. TMS1 was initially characterized to participate in both the intrinsic and extrinsic apoptotic pathways. Inducible expression of TMS1 in HEK-293 cells induces characteristic apoptotic events which were inhibited by a dominant negative form of caspase-9. A dominant negative form of caspase-8 was unable to

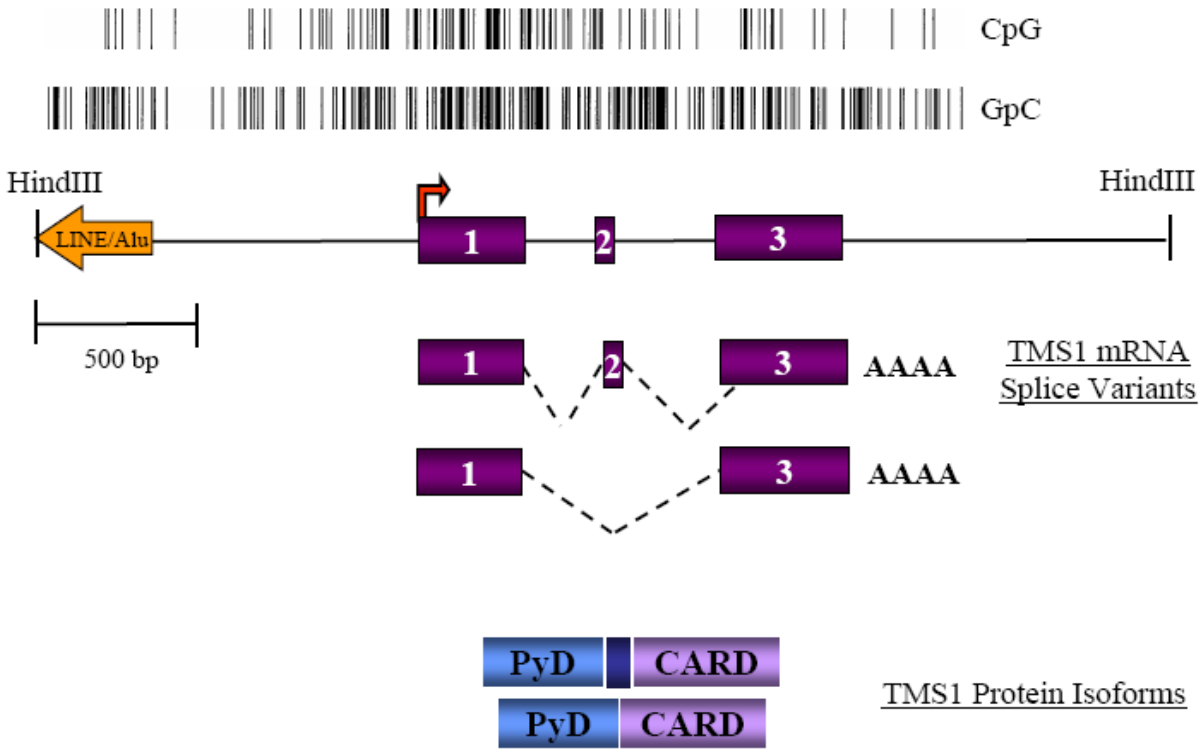

Figure 7: *TMS1 gene structure.* The TMS1 gene is located within a HindIII fragment on chromosome 16p11.2-12.1 and contains three exons with an associated CpG island surrounding exon 1. The mRNA is subject to alternative splicing to either include or exclude exon 2. Exon 1 codes for a pyrin domain (PyD) and exon 3 codes for a caspase recruitment domain (CARD). These protein-protein interaction domains are found in molecules involved in inflammation and apoptosis, respectively.

suppress the apoptotic events indicating that TMS1 functions through the activation of caspsase-9 (116).

TMS1 is highly expressed in monocytes and macrophages where it plays a critical role in the innate immune response (117, 118). Oligomerization of TMS1 induced by interactions with intracellular pathogen sensors such as the NALPs, or a decrease in potassium levels mediates the assembly of a macromolecular structure, termed the inflammasome. The formation of this structure ultimately leads to the activation of caspase-1, maturation and release of cytokines IL-1β and IL-18, and the induction of "pyrotopsis", a proinflammatory form of programmed cell death that occurs in response to invading pathogens (119, 120)

TMS1 is upregulated in epithelial cells in response to cell stress stimuli; such as death ligands TNFα and TRAIL in a JNK and NF-κB dependent manner (111, 121). Although not required for caspase-8 cleavage in response to death receptor stimulation, overexpression or forced oligomerization of TMS1 promotes caspase-8 cleavage and death receptor independent cell death (121). Recent work from our lab indicates that TMS1 plays a critical role in anoikis, or apoptosis induced in response to the loss of integrin-mediated contacts with the substratum, in breast epithelial cells. TMS1 is upregulated in response to detachment and is necessary for the subsequent upregulation of the BH3-only protein Bim, cleavage of procaspase-8 and detachment-induced cell death (115). These data suggest that the epigenetic silencing of TMS1 would confer a survival advantage to cancerous cells by allowing them to escape apoptosis activated through numerous pathways.

GA-Binding Protein

Ets factors are a family of related transcription factors involved in many cellular functions such as development, cellular differentiation, apoptosis and carcinogenesis (122). There are approximately 30 distinct mammalian ets factors found in species as diverse as *C.elegans* and humans. The v-ets oncogene, the first member of the ets gene family to be described, was discovered in the E26 acutely transforming retrovirus of chicken, from which it derives its name (123, 124). Members of this family were originally identified on the basis of a region of primary sequence homology with the protein product of the v-ets oncogene (125). Subsequent studies determine this region of conserved sequence homology also correlated with conservation at the structural level. It was determined that this region is a divergent member of the "winged-helix-turn-helix" superfamily of DNA binding proteins. This region, which covers approximately 85 amino acids, has no structural homology to other known DNA binding motifs such as the zinc finger, homeodomain, leucine-zipper or helix-turn-helix motifs (125). The conserved region was termed the ets-domain, and corresponds to the DNA-binding domain of these proteins. The target genes for ets transcription factors include oncogenes, tumor suppressor genes, apoptosis-related genes, differentiation-related genes, angiogenesis-related genes and invasion and metastasis-related genes (126-128). Consequently, aberrant expression of ets genes contribute to malignant transformation and tumor progression (129).

GABP is unique among the ets transcription factors in that its transcriptional activity requires complexation of two unrelated proteins, GABPα and GABPβ. GABPα

contains the evolutionary conserved ets DNA binding domain (DBD) while GABPβ contributes the transcriptional activation domain (TAD) as well as the nuclear localization signal that influences the efficiency of GABPα transport to the nucleus (130, 131) (Figure 8). GABPα is the only ets factor that can recruit GABPβ to the DNA (122). The GABPβ gene encodes four isoforms that arise from differential splicing. These isoforms differ in the presence or absence of a C-terminal TAD/leucine zipper domain as well as one internal domain (132). The TAD and leucine zipper domains are critical for transcription and formation of the GABP heterotetramer (Figure 8). These domains are present in two of the longer isoforms but are lacking in the shorter isoforms (131). The internal domain of GABPβ comprises 12 amino-acids which contain two pairs of adjacent serine residues (122). Even though this region has no known function, the presence of these serines raise the possibility that this site may serve as a site for phosphorylation. Transcription of GABP responsive genes is dependent upon which isoform of GABPβ is recruited to the DNA. The GABPβ1 isoform contains all domains necessary for heterotetramer formation, transcription activation and potential phosphorylation (131).

The GABPα/β1 complex exists in solution as a heterodimer but often binds tandem binding sites separated by 10-30bp. Homotypic interactions between the leucine zipper domains of GABPβ1 allow for the formation of a heterotetramer with 10-20 fold greater affinity for DNA (122, 131) (Figure 8) . Of note, the GABP heterotetramer is only able

to form in the presence of DNA.

GABPα was originally identified in studies of viral gene transcription (130, 133, 134). However, it has been discovered to play a critical role in the regulation of a wide

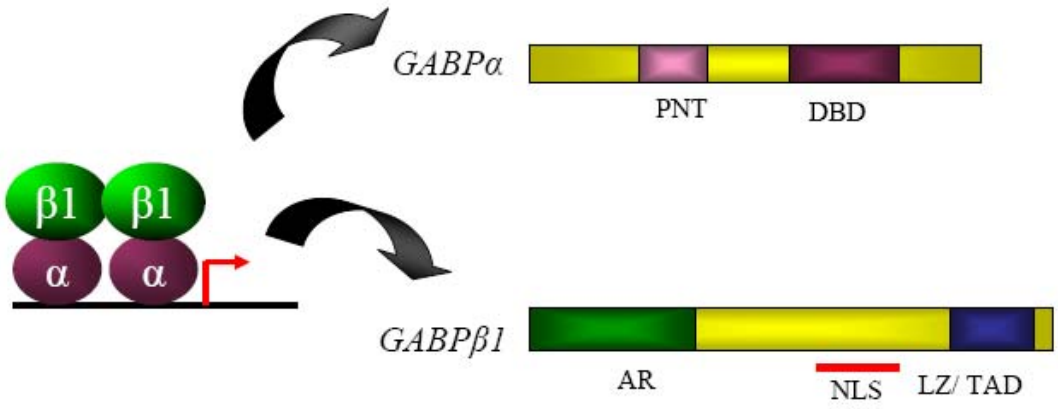

Figure 8: *Schematic of the GABP heterotetramer and component proteins.* GABPα constitutes the DNA binding component whereas GABPβ contains the nuclear localization signal (NLS) and the transcriptional activation domain (TAD). GABPα contains a pointed domain (PNT) at the N-terminus and an ets DNA binding domain (DBD) near the carboxy terminus. GABPβ contains an N-terminal ankryin repeat (AR) which mediates its interaction with GABPα, a transcriptional activation domain (TAD) and a C-terminus leucine zipper domain (LZ), which mediates homodimerization of GABPβ subunits.

array of genes involved in both core cellular processes (135), and more specialized functions, including several genes with known roles in breast cell biology and breast carcinogenesis, such as prolactin, oxytocin receptor, BRCA1, and Her2/neu (135-137). Recently, GABP has been shown to play a major role in regulating genes that control cell growth as well as being a necessary factor for cells transitioning from quiescence to cell-cycle re-entry in a pathway distinct from that of D-type cyclins and CDKs (138, 139). The importance of GABP is also underscored by the phenotype of Gabpα knock out mice which show an early preimplantation lethal phenotype (140).

Objectives

Aberrant methylation of CpG sites within CpG islands is associated with inappropriate gene silencing and has been implicated in the inactivation of tumor suppressor genes in human cancers. However, the mechanism responsible for eliciting these changes has yet to be elucidated. Methylation-mediated silencing of TMS1 may contribute to the pathogenesis of human cancers by conferring a survival advantage in the early stages of cancer progression. Previous work in our lab has shown the TMS1 gene covers ~1.5 kb and is localized to chromosome 16p11.2-12.1. The promoter region lacks a defined TATA box, but contains a 600bp CpG island that extends from ~200bp upstream to 400b downstream of a single transcription start site (16) (Figure 9). In normal cells and breast cells that retain TMS1 expression, the TMS1 CpG island is unmethylated, and exhibits an “active” chromatin signature characterized by hyperacetylated histones H3 and H4, enrichment of H3K4me2, and positioned

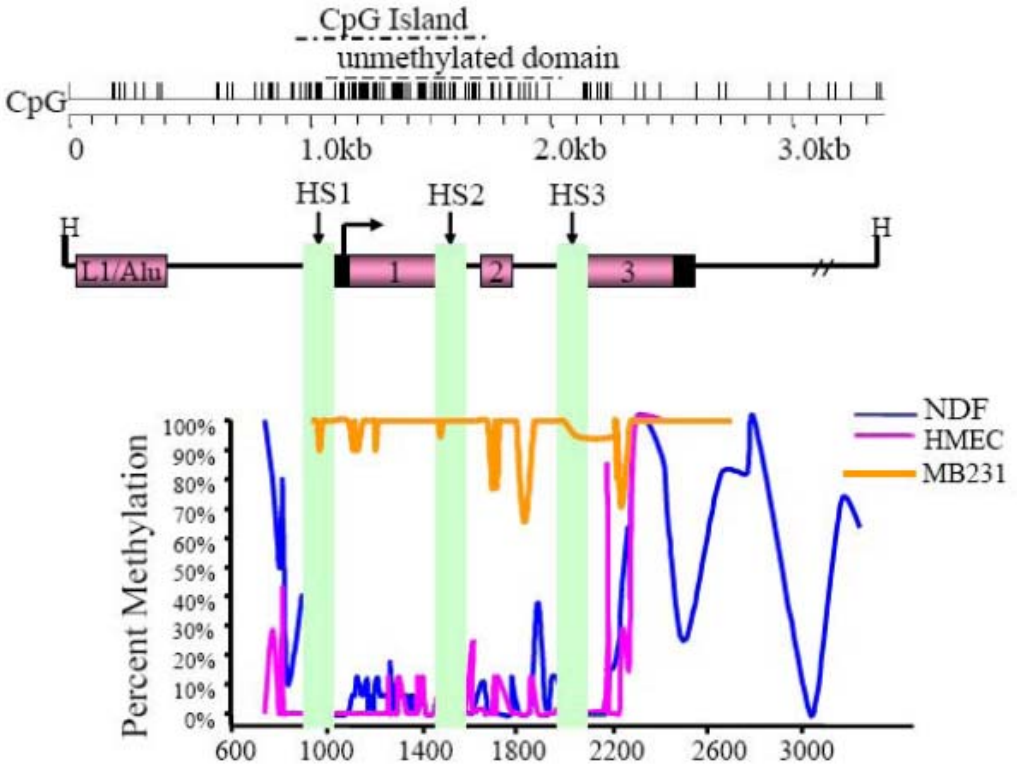

Figure 9: *Overview of the TMS1 locus.* Shown is the percent of methylated alleles at each CpG site as determined by bisulfite sequencing of DNA from normal human diploid fibroblast (NDF), human mammary epithelial cells (HMEC), or MDA-MB231 breast cancer cell line and the position of DNAseI hypersensitive sites (HS). Data represents the percent of methylated alleles based on the analysis of 8-10 individual alleles. Note the hypermethylation of the CpG island in MDA-MB231 cells in which TMS1 is methylated and silenced. Loss of CpG island associated HS is asociated with promoter hypermethylation.

nucleosomes (16, 111, 141). Three DNAseI hypersensitive sites (HS) span the CpG island in the active state; HS1 and HS3 mark the boundaries between unmethylated CpG island DNA and densely methylated flanking DNA while HS2 forms at the center of the CpG island (16) (Figure 9). Epigenetic silencing of TMS1 in breast cancer is accompanied by the remodeling of the CpG island associated HS, hypoacetylation of histones, a shift in histone methylation status, and hypermethylation of DNA (16). These changes are confined to the CpG island domain and do not affect DNA methylation or chromatin structure at a fourth HS located 1kb downstream of the CpG island. These studies have lead to the proposal that there maybe an activity and/or structural barrier occurring at the level of chromatin, marked by HS that protect the TMS1 CpG island from methylation in normal cells. Loss of function at these sites could lead to aberrant methylation in cancer.

At the time this project began preliminary studies identified GABPα/β1 as a heterotetrameric complex which bound a 55bp region of HS2 *in vitro*. However, beyond these initial studies, little was known about the factors that bind the TMS1 locus and regulate expression nor had relationship between GABP binding and methylation of TMS1 *in vivo* been established. The goal of this project was to characterize the role of GABP in the regulation of TMS1 expression and chromatin structure.

There are two mechanisms by which GABPα/β1 could regulate TMS1 expression and its epigenetic state (Figure 10). The binding of GABPα/β1 to HS2 might have a direct effect on TMS1 promoter activity. The first objective focused on determining whether GABP is recruited to the TMS1 locus *in vivo* and the relationship of this binding to methylation and expression at the locus (Chapter 2). Subsequently, the second

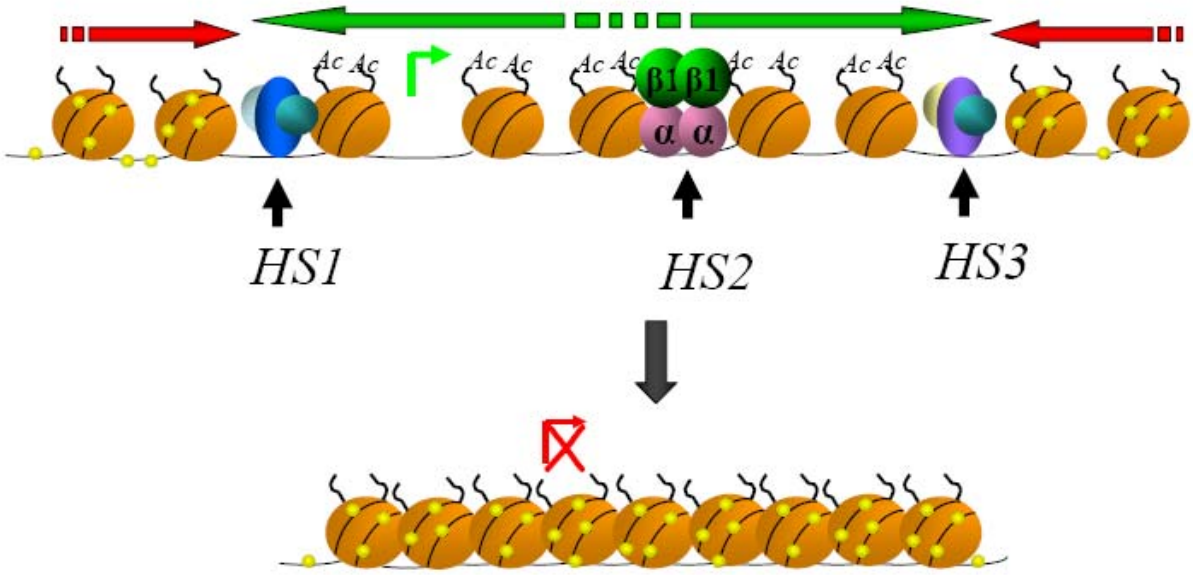

Figure 10: *Model for TMS1 silencing.* There are DNA binding protein defined by the hypersensitive sites which protect the CpG island from methylation in normal cells. Loss of function at these sites in cancer cells allows for aberrant DNA methylation (yellow circles), associated changes in chromatin structure (removal of acetyl groups (Ac) from histone tails) and gene silencing. GABP is a positive regulator of TMS1 expression by potentially two mechanisms. The binding of GABPα/β1 may have a direct effect on TMS1 promoter activity through interactions with basal transcription machinery or an indirect effect through the recruitment of chromatin modifying enzymes to the locus maintaining an open permissive state.

objective encompassed examining the impact of HS2 and GABP on TMS1 promoter activity (Chapter 2). Alternatively, GABPα/β1 may regulate the expression of TMS1 by maintaining local chromatin structure, thus protecting TMS1 from aberrant methylation and silencing. The third objective focused on determining the role of GABP in the maintenance of a methylation free, accessible chromatin domain (Chapter 3). These studies will characterized a potential regulator of the TMS1 locus and help decipher the elusive pathway of TMS1 activation.

Chapter 2

GABP α Binds the TMS1 Locus at HS2 in vivo in a Methylation Sensitive Manner and acts a Positive Regulator

I performed all major experiments in the chapter with the exception of IMR90 and HMT.1E1 chromatin immunoprecipitations (D.R. Powell). Figures 2-8 have been published in : M.L. Lucas et. al. (2009). Methylation-Sensitive Regulation of TMS1/ASC by the ets factors, GABPα (142).

Introduction

TMS1, also known as ASC/ PYCARD, is a proapoptotic protein identified in an effort to isolate transcripts down-regulated due to the overexpression of DNMT1 (110). The TMS1 promoter region lacks a defined TATA box, but contains a 600bp associated CpG island which surrounds a single transcription start site (16). In previous studies the transcriptional start site of TMS1 in breast cells was mapped to be 79bp upstream of the translation start site (143). Further investigation of the TMS1 promoter region showed that a 1254bp region directly upstream from translation start possessed the ability to drive expression of a luciferase reporter gene (89). Through deletion analysis it was determined that most promoter activity resided within a 263bp region directly upstream of transcription start (89).

The CpG island of TMS1 is unmethylated in normal somatic cells and is separated from densely methylated flanking DNA by distinct boundaries at the 5' and 3' ends (16). Aberrant methylation of the TMS1 CpG-island in cancer cells is accompanied by the loss of CpG island-specific DNAseI HS sites and gene silencing, indicating that local alteration in chromatin structure play an important role in the methylation-dependent silencing of TMS1 (16).

In the course of our studies on the function of the HS region, a complex was identified that bind to HS2, and localized to a 55bp fragment containing two CpG sites. Methylation of these CpG sites was inversely correlated with complex binding. The CpG sites were also located within a consensus sequence known to be bound by ets transcription factors. The complex was purified and MALDI tandem MS/MS analysis

determined that the methylation-sensitive complex contained GABPα. In *in vitro* binding assays, it was discovered that antibodies to the GABPβ1 isoform "supershifted" the methylation-sensitive binding activity, indicating that GABPβ1 is one other component of the complex. HS2 is located within the center of the TMS1 CpG island and forms only when the CpG island is unmethylated (16). These findings suggest that the GABP complex is potentially responsible for the formation of HS2, and may serve as a methylation-sensitive regulator of TMS1 expression.

The goal of this chapter is to determine the role of GABP in the regulation of TMS1. Little is known about the transcriptional regulation of TMS1, nor has a putative transcriptional regulator been identified. Initially, we examined if the *in vitro* methylation-sensitive binding activity of GABP at TMS1 is recapitulated *in vivo*. Subsequently, reporter assays were utilized to determine if the presence of GABP at HS2 has an effect on transcription from the minimal TMS1 promoter. Previous reports have shown GABPα/β1 to either activate or repress transcription in transient transfection assays (144, 145). Therefore, it is of interest to determine if the GABP complex is playing an active or repressive role in expression at the TMS1 locus.

Materials and Methods

Quantitative Reverse Transcriptase PCR: Total RNA was prepared using the RNeasy kit (Qiagen) and reverse transcribed using MMLV-RT (Invitrogen) as described (111). The cDNA was amplified using primers specific for GABPα, GABPβ1, TMS1, or 18S rRNA, as an internal control (Table 1). PCR reactions were monitored in real-time using SYBR Green dye detection and relative starting quantities calculated by comparison to a common standard curve generated with MCF7 cDNA.

Plasmid constructs and Luciferase Reporter Assays: A genomic *Sma*I-*Nco*I fragment containing 263 bp upstream of the TMS1 translation start codon was cloned in-frame into the pGL3 luciferase reporter plasmid (Promoega) to form the construct pTMS1min. A 236 bp fragment containing HS2 was amplified by PCR and cloned into the SmaI site of pTMS1min upstream of the TMS promoter to create pTMS1-HS2sense. Constructs in which one or the other GABP binding site (pTMS1-HS2-m1, pTMS1 HS2-m2), or both GABP binding sites (pTMS1-HS2-dm) were mutated were created by deleting the 4bp central core (GGAA/T) of each site using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). All clones were sequenced to verify the orientation and sequence of inserts. Primers used to generate the mutation are listed in Table I.

MCF7 and IMR90 cells ($2x10^5$) seeded in 6 well plates and transfected the next day with 1ug of the various reporter plasmids using the Lipofectamine (Invitrogen) reagent. A Renilla luciferase reporter (200ng, pRL-TK, Promega) was included as a control for transfection efficiency. After overnight incubation the transfection complex

was removed and replaced with fresh media. After an additional 24h, cells were lysed and firefly luciferase and renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega).

For GABPα/β siRNA co-transfection experiments, MCF7 cells ($5x10^4$)were seeded in 24 well plates and transfected the following day with 200ng of the indicated plasmids plus either 10pmol of siRNA targeting GABPα and GABPβ **(**Invitrogen Stealth™ Select 3 RNAi Set, see Table 1 for siRNA sequences) or 20pmol scrambled siRNA using 1.5ul of Lipofectamine 2000. Renilla luciferase plasmid (pRL-TK, 40ng) was included as a control. After 48h luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega).

siRNA Transfections: MCF7 and IMR90 cells ($2x10^5$) were seeded in six-well plates and transfected the next day 200nM of the indicated siRNA using Oligofectamine (Invitrogen) according to the manufacturer's instructions. For repeat transfection, cells were transfected with 200nm of the indicated siRNA 48h after the initial siRNA treatment. Protein lysates were collected 48h post-transfection for immunoblot analysis. Stealth Select Set 3 siRNA duplexes were purchased from Invitrogen. Invitrogen scrambled siRNA Control or BRSK2 (non-specific) siRNA were used as controls.

Lentiviral shRNA Production and Infection: 293T packaging cells were seeded at $1.5x10^5$ cells/ml (6ml per plate) in DMEM+10%FBS in 6cm tissue culture plates. After 24h, cells were transfected with a mixture of the 3 transfection plasmids: packaging plasmid, psPAX2 (900ng); envelope plasmid, VSV-G (100ng), and the empty pLKO.1 vector (1ug) or pLKO.1 containing a short hairpin sequence targeting GABPα (#18288-18292,

Open-Biosystems) using the FuGENE6 reagent. After 18h, the transfection medium was removed and replaced with 6ml high serum growth media (DMEM+ 30% FBS). Supernatant containing lentiviral particles was harvested 24hr later. A second 24 hr harvest was performed and the two supernatants combined and stored in 1ml aliquots at -80°C.

MCF7 and IMR90 cells ($2x10^5$) were seeded in 6 well plates in DMEM+10%FBS. The next day, the growth media was removed and replaced with fresh media containing 8ug/mL polybrene. Viral supernate (0.5mL) was added to the appropriate wells and allowed to incubate overnight. At 24h post-infection the medium was replaced with selection medium (DMEM+10%FBS) containing 0.5ug/mL puromycin. After 5 days of selection, cells were harvested for immunoblot analysis.

Immunoblotting and antibodies: Whole cell lysates were prepared in RIPA buffer supplemented with protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche, Indianapolis, IN, USA), 1mM sodium orthovanadate, and 10 mM NaF (Sigma, St. Louis, MO, USA). Total protein (25ug) was separated on a 12% SDS-PAGE gel, transferred to PVDF (BioRad, Hercules, CA, USA), and probed with the indicated primary antibody. Immunocomplexes were detected by incubation with HRP-conjugated secondary antibody and chemiluminescence detection (Pierce, Rockford, IL, USA). The antibodies used were: anti-ASC (ProteinTech), GABPα (Santa-Cruz), GABPβ1/2 (Santa-Cruz), and GAPDH (Abcam).

Construction of L1-TMS1-GFP-1L: PCR was utilized to amplify TMS1 from IMR90 gDNA. The primers used to amplify a 2027-2460 bp region created flanking EcoR1 and BamH1 sites. The 3' region of TMS1 was digested with EcoR1/BamH1 and ligated into the peGFP-N2 plasmid. A TMS1 DNA construct was digested with EcoR1 and the resulting excised band ligated into the peGFP-N2 plasmid containing the 3' portion of TMS1. The resulting TMS1-GFP sequence in the peGFP-N2 vector was digested with HindIII/NotI and blunted with Klenow. The recipient pL1-1L vector was digested with EcoRV. The TMS1-GFP sequence was ligated into the pL1-1L vector and screened for orientation. The resulting L1-TMS1-GFP-IL (WT) construct has the TMS1-GFP sequence in the anti-sense orientation. Mutant constructs in which one GABP binding site (αm1) or both GABP binding sites (αdm) were created by deleting the 4bp central core (GGAA/T) of each site using the Quick Change® Site-Directed Mutagenesis Kit (Stratagene). All clones were sequenced to verify the deletion of the 4bp central core. Primers used to generate the mutations are listed in Table 1.

Transient L1-TMS1-GFP-1L WT and αDM: MCF7 cells (2x105) were seeded in 6 well plates and transfected the next day with 2ug of the indicated plasmid using FuGENE (Roche). 48 hours post-transfection cells underwent confocal microscopy, Western blot and qRT-PCR analysis for the expression of TMS1-GFP.

Chromatin Immunoprecipitation: Cells were cross linked for 10 min in 16% formaldehyde at room temp. ChIP was carried out essentially as described in the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Millipore) except that a two step lysis

procedure was incorporated in which nuclei were first harvested in Cell Lysis Buffer (5mM PIPES, pH 8.0, 85mM KCl, ,5% NP-40, 1X Protease Inhibitors) followed by lysis of nuclei in Nuclei Lysis Buffer (50mM Tris-HCl, pH 8.1, 10mM EDTA, 1% SDS, 1X Protease Inhibitors). DNA fragments immunoprecipitated with GABPα were analyzed by Real-Time PCR. DNA recovered from ChIP was analyzed by real-time PCR and SYBR Green detection using primer sets spanning the TMS1 locus (Table I). Reactions (25 uL) contained 1 uL diluted DNA sample, 0.2 uM primers and 12.5 uL of IQ SYBR Green Supermix (BioRad). The reaction was subjected to a hot start for 3 minutes at 95^{o}C and 35 cycles of 95^{o}C,10s; (55 ^{o}C -1520bp, 60 ^{o}C 263bp, 55 ^{o}C 1823bp), 30s; 72^{o}C, 30s. Melt curve analysis was performed to verify a single product species. Starting quantities were determined relative to a common standard curve generated using MCF7 genomic DNA. Percent enrichment in each pulldown was calculated relative to input DNA. Antibodies used were GABPα (sc-22810, Santa Cruz) and rabbit IgG (sc-2027, Santa Cruz).

Table 1:

PCR Primers for Chromatin Immunoprecipitations

1:
5'-GATGGAGTTTCGCTCTTGTTG-3'
5'-GGTGGGTGATCAGGAGTTTG-3'

2:
5'-AATTCTGGCTCCCCTAGGAA-3'
5'-AAAAGGGCAGAGAGTGCAAA-3'

3:
5'-CCAGCTGGAGGGCGCGA-3'
5'-GCAAAAGGCGCTTCCTTACT-3'

4:
5’-CGCCTTGGACCTCACCGAC-3’
5’-ATGTCGCGCAGCACGTTAGC-3’

5:
5’-CCCATATCCTTCCAGGCTCTG-3’
5’-CAGGCTGTGCAGGAGTGCG-3’

6:
5’-AGAGCTCAGCTATGCTTCAG-3’
5’-CCTGGGTCACAGGCTGTTA-3’

7:
5’-GGTCATAGGTTGCACTTTGC-3’
5’-GTTTTAAATGTTCCTTTCAAATGA-3’

PCR Primers for Mutagenesis

Deletion of GABPα binding site 1 (418-426bp):
5’-CGAATGGGGCGCCCGAGGAAAGACAAGGAGG-3’
5’-CCTCCTTGTCTTTCCTCGGGCGCCCCATTCG-3’

Deletion of GABPα binding site 2 (458-466bp):
5’-GGATTTGTTTCGACGAGCTTTCCTCCGAGGG-3’
5’-CCCTCGGAGGAAAGCTCGTCGAAACAAATCC-3’

siRNA sequences

α-1:
5’-UUUGCAGAGAAGCAGUAGCCAGAGC-3’
5’-GCUCUGGCUACUGCUUCUCUGCAAA-3’

α-2
5’-AUCAAUCUCAAUUUCUAUCAGCUCC-3’
5’-GGAGCUGAUAGAAAUUGAGAUUGAU-3’

α-3
5’-CAAUGCUUUCUUCUGUGCACUCUGC-3’
5’-GCAGAGUGCACAGAAGAAAGCAUUG-3’

GABPβ1:
5’-AAAUACGAACUUCAUCAUCUUGACC -3’
5’-GGUCAAGAUGAUGAAGUUCGUAUUU -3’

PCR Primers for Quantitative Real-Time PCR

GABPα:
5'- CCTGAACTGGTTGCACAGAA -3'
5'- ACAAATCATGTCCCCATCGT -3'

GABPβ1:
5'- AGTGTTCTCGGAAGGAGCAG -3'
5'- CCATATGCATTGGGTTTTCC -3'

TMS1:
5'- TCCAGCAGCCACTCAACG -3'
5'- GCACTTTATAGACCAGCA -3'

Ribosomal 18S:
5'-GAGGGAGCCTGAGAAACGG-3'
5'-GTCGGGAGTGGGTAATTTGC-3'

Results

We first determined the relationship between GABPα/β1 expression and TMS1 expression in human cell lines. Using Western blot techniques, GABPα protein was detected in IMR90 (normal diploid fibroblast), 90SV (IMR90 SV40 immortalized derivative) numerous breast cancer cell lines (ZR75-1, HS578t, MDA-MB231, MDA-468, MCF7, T47D) and Hela cells (cervical cancer cell) that were initially used to isolate and purify the methylation-sensitive complex (146) (Figure 1). TMS1 protein was detected in the IMR90, 90SV, MDA-MB468, MCF7 and T47D cell lines but not in the HMT.1E1, ZR75-1, HS578t, Hela and MDA-MB231 cell lines where it is known to be methylated and not expressed (Figure 1). Of note, TMS1 levels in the T47D cell line is decreased in comparison to the MCF7 and MB468 cell lines. Comparison of the levels of GABPα and TMS1 indicated no correlation between GABPα and TMS1 protein levels. Similar Western blot analysis of GABPβ1 proved to be difficult due to the fact that most commercially available antibodies are generated against peptides in the N-terminal region which produced numerous bands during immunoblot analysis (Data not shown). Therefore, GABPβ1 levels were only investigated at the RNA level with primers targeting the unique C-terminal region. At the RNA level, both GABPα and GABPβ1 were ubiquitously expressed in all cell lines tested (Figure 1). As expected, TMS1 was absent from cells lines where the gene is known to be methylated, such as HMT.1E1, ZR75-1, HS578T, and MDA-MB231 (89). As seen at the protein level, there was no relationship between GABP and TMS1 expression at the RNA level.

HS2 is located within the center of the TMS1 CpG island and forms only when the CpG island is unmethylated (16). Previous studies indicate that GABP is able to bind

a 55bp fragment of HS2 when CpG sites within the sequence are unmethylated (142). These findings suggest that the GABP complex is responsible for the formation of HS2, and may serve as a regulator of TMS1 transcription. To test this hypothesis we performed transient transfection assays to determine the impact of HS2 DNA sequence on TMS1 promoter activity. Luciferase reporter constructs were developed that contained the minimal TMS1 promoter (min-TMS1) driving expression of luciferase in the presence and absence of a 236 bp fragment encompassing HS2 in the sense and anti-sense orientation with respect to the promoter (HS2-sense and HS2-anti) (Figure 2). The resulting constructs, as well as a control pGL3 construct, were transfected into the MCF7 breast cancer cell line and the IMR90 normal diploid fibroblast cell line. Luciferase activity was measured as an indication of TMS1 promoter activity. We found that the HS2 sequence conferred a 2-3 fold increase in TMS1 promoter activity, irrespective of orientation, in IMR90 cells. However, in MCF7 cells, HS2 induced a 2-3 fold increase in TMS1 promoter activity only in the sense orientation (Figure 2).

To determine whether this effect was mediated by GABP binding sites, constructs were created in which one (HS2-sense m1 and HS2-sense m2), or both (HS2-dm) of the tandem GABPα binding sites in HS2-sense were deleted. Disruption of either GABPα binding site blocked HS2-mediated enhancement of TMS1 promoter activity (Figure 3). Interestingly, whereas deletion of the 5' ets site (HS2-sense m1), completely blocked HS2 enhancer activity, reporter constructs in which the 3' site (HS2-sense m2) retain some enhancer activity. This latter site deviates from the ets consensus in a key core position (from 'GGAA' to 'GGTA') shown to be critical for optimal DNA binding (122).

These results indicate that the HS2 region acts in *cis* to positively regulate the TMS1 locus and that this activity is dependent on the GABP binding sites.

A similar effect was observed when HS2 was tested in its natural position relative to the promoter in a plasmid setting. Constructs containing the TMS1 genomic locus were developed in which GFP was fused into exon 3 at the end of the coding region. This alternative approach allowed us to examine the impact of GABP binding sites within HS2 on TMS1 expression in its native state. TMS1-GFP constructs either contained the wild-type TMS1 locus (WT), the TMS1 locus with the 5' GABPα binding site deleted (αm1), or the TMS1 locus with both of the GABPα binding sites deleted (αdm). The resulting constructs were transfected into MCF7 cells and TMS1-GFP expression was analyzed by confocal microscopy, Western blot, and qRT-PCR. Robust GFP expression could be detected by confocal microscopy in cells transiently transfected with the WT construct. Deletion of GABPα binding sites had a negative impact on TMS1-GFP expression. GFP detection through confocal microscopy became increasingly difficult in cells transfected with mutant constructs in comparison to those transfected with the WT construct (~80% decrease in expression) (Figure 4). The levels of TMS1-GFP protein expression correlated with intact GABP binding sites in that the WT construct, had the most expression while the αdm construct had the least (Figure 4). Accordingly, TMS1-GFP protein and RNA levels were decreased in response to deletion of GABPα binding sites (Figure 4). Strikingly, a decrease in endogenous TMS1 levels at the protein and transcript level was also observed in the cells transfected with the mutant construct in which both GABPα binding sites were deleted (αdm) (Figure 4). The data from these experiments indicate that the GABP binding sites are necessary to maintain TMS1 expression.

To further test whether the effect of HS2 on TMS1 promoter activity is dependent on GABP and not another ets transcription factor able to bind the same GGAA/T core sequence, luciferase assays were performed in cells treated with siRNA targeting GABPα and GABPβ1 followed by transient transfection of the HS2-TMS1 promoter constructs. Depletion of endogenous GABPα/β1 abrogated the stimulation of promoter activity conferred by HS2 (Figure 5). These results indicate that the HS2 region acts in *cis* to positively regulate the TMS1 promoter and this activity is dependent on both the GABP binding sites, and the activity of GABPα/β1 in *trans*.

We next sought to determine if GABP plays a functional role at the TMS1 locus *in vivo*. We examined the impact of GABPα on endogenous TMS1 expression in MCF7 and IMR90 cells using a siRNA approach. Transient transfection of MCF7 cells with siRNA targeting GABPα resulted in an ~75% reduction in GABPα proteins levels and a corresponding decrease in TMS1 expression levels (Figure 6). Knockdown of TMS1 was less efficient in IMR90 cells and only achieved a ~30% reduction in GABPα protein levels (Figure 6). Nevertheless, there was a corresponding decrease in TMS1 expression. Similar results were obtained using an infection approach in which MCF7 breast cancer cells and IMR90 normal diploid fibroblast cells were infected with lentiviral shRNA constructs targeting multiple independent regions of the GABPα mRNA. There was a direct correlation between the degree of GABPα knock down and levels of TMS1 expression (Figure 7). Taken together, these data indicate that the intronic HS2 element acts in *cis* to positively regulate the TMS1 promoter and that this is mediated by the ets transcription factor GABP.

Previous reports from our lab have shown that the two CpG dinucleotides within consensus GABPα binding sites (GCTCTNCCG) are unmethylated in the MCF7 breast cancer cells and IMR90 diploid fibroblasts in which TMS1 is expressed but are fully methylated in MDA-MB231 breast cancer cells and HMT.1E1 DNMT1 overexpressing cells in which TMS1 is silent (89). Chromatin immunoprecipitation was performed with chromatin from all four cell lines using an antibody specific to GABPα and analyzed by quantitative real-time PCR using primers spanning the TMS1 locus (Figure 8). We found that GABPα was selectively enriched at the HS2 region of the TMS1 locus in cells in which TMS1 is unmethylated and expressed (IMR90 and MCF7) but not in cell lines in which TMS1 is methylated and silent (MDA-MB231 and HMT.1E1) (Figure 8). These data indicate that, GABP associates with the TMS1 locus *in vivo*, and that this association is inversely correlated with the methylation state of the CpG island.

Summary

GABP is expressed in a wide variety of cell types such as hematopoietic cells, liver, and muscle where it is involved in the regulation of a broad number of genes (144). Through the use of Western blot analysis and quantitative real-time PCR, GABPα and GABPβ1 levels were analyzed in normal fibroblast and cancer cell lines in order to determine if there was a relationship between GABPα/β1 and TMS1 expression. This was the first in depth analysis of GABPα/β1 levels in these commonly used cell lines. All cell lines tested here ubiquitously expressed GABPα and GABPβ1. However, there was little correlation between GABPα/β1 and TMS1 levels. GABP is a methylation-sensitive transcription factor that is unable to activate transcription from a locus in the presence of DNA methylation. Therefore, even in the presence of ubiquitously expressed GABP, GABP mediated activation of TMS1 is unable to occur in the presence of methylation.

GABP binding at HS2 of the TMS1 locus was confirmed as an activator of expression through reporter assays and TMS1-GFP transgene studies. This activation was dependent on the presence of GABPα/β1 in trans, as well as GABPα ability to bind its recognition sequence. In IMR90 cells, HS2 enhanced transcription from the minimal TMS1 promoter irrespective of orientation relative to the promoter whereas only HS2 in the sense orientation was able to stimulate transcription in the MCF7 transformed breast cancer cell line. These results raise the possibility that HS2-mediated enhancement may be context dependent. Nonetheless, this enhancement is dependent on both the intact GABPα binding sites and the presence of the GABPα/β1 complex.

HS2 resides within the center of the TMS1 CpG island and only forms when the locus is unmethylated and expressed. GABP is potentially responsible for the formation of HS2, and could provide an opposing force to the spread of heterochromatic marks into the TMS1 locus through the activation of transcription. RNAi against endogenous GABPα caused a corresponding decrease in TMS1 expression in the IMR90 normal fibroblast and MCF7 transformed breast cancer cell line suggesting that GABP plays a direct role in methylation-sensitive transcriptional regulation of TMS1 *in vivo.*

The interaction of GABPα with its recognition sequence has been shown to be blocked by DNA methylation (137). The methylation-sensitivity of GABP is emphasized by the fact that even though GABP is ubiquitously expressed in all cell lines analyzed, it is unable to stimulate transcription from TMS1 in cell lines that contain a hypermethylated locus (e.g MB231 and HMT.1E1). Our chromatin IP studies show that cell lines in which the TMS1 CpG island and the GABP binding sites are hypermethylated lost the ability to recruit GABP, thus transcription is inhibited which could allow for the propagation of silent histone marks and subsequent gene silencing. Spurious methylation of GABP binding sites is a prospective initiating step in the silencing of TMS1.

We determined that GABPα was selectively enriched at the HS2 region of the TMS1 locus in unmethylated and expressing cell lines (IMR90 and MCF7) but not in cell lines in which TMS1 is methylated and silent (MDA-MB231, HMT.1E1) (Figure 8). These data indicate that, GABP associated with the TMS1 locus *in vivo*, and that this association is inversely correlated with the methylation state and expression of the TMS1 locus. In regards to the methylation status of the TMS1 locus, the T47D cell line contains

a mixed methylation pattern composed of alleles that are predominantly methylated and those that are predominantly unmethylated (89). TMS1 expression in the T47D cell line is half of that detected in the MCF7 cell line (Figure 1) in which all analyzed alleles contained an unmethylated TMS1 locus (89). It is possible that in T47D cells, GABP is activating transcription from clones containing an unmethylated TMS1 locus allowing for TMS1 expression to be half that of a fully unmethylated cell line. Therefore, if GABP binding sites are unmethylated, GABP is able to bind HS2 and initiate transcription and protect the locus from silencing. However, if the TMS1 CpG island is methylated, GABP is no longer able to bind its consensus sequences and transcription does not occur. It would be interesting to perform ChIP analysis utilizing the T47D cell line in order to determine if the aforementioned hypothesis holds true. Further studies are necessary to determine the exact mechanisms behind the methylation-sensitive transcriptional regulation of TMS1 by GABPα/β1.

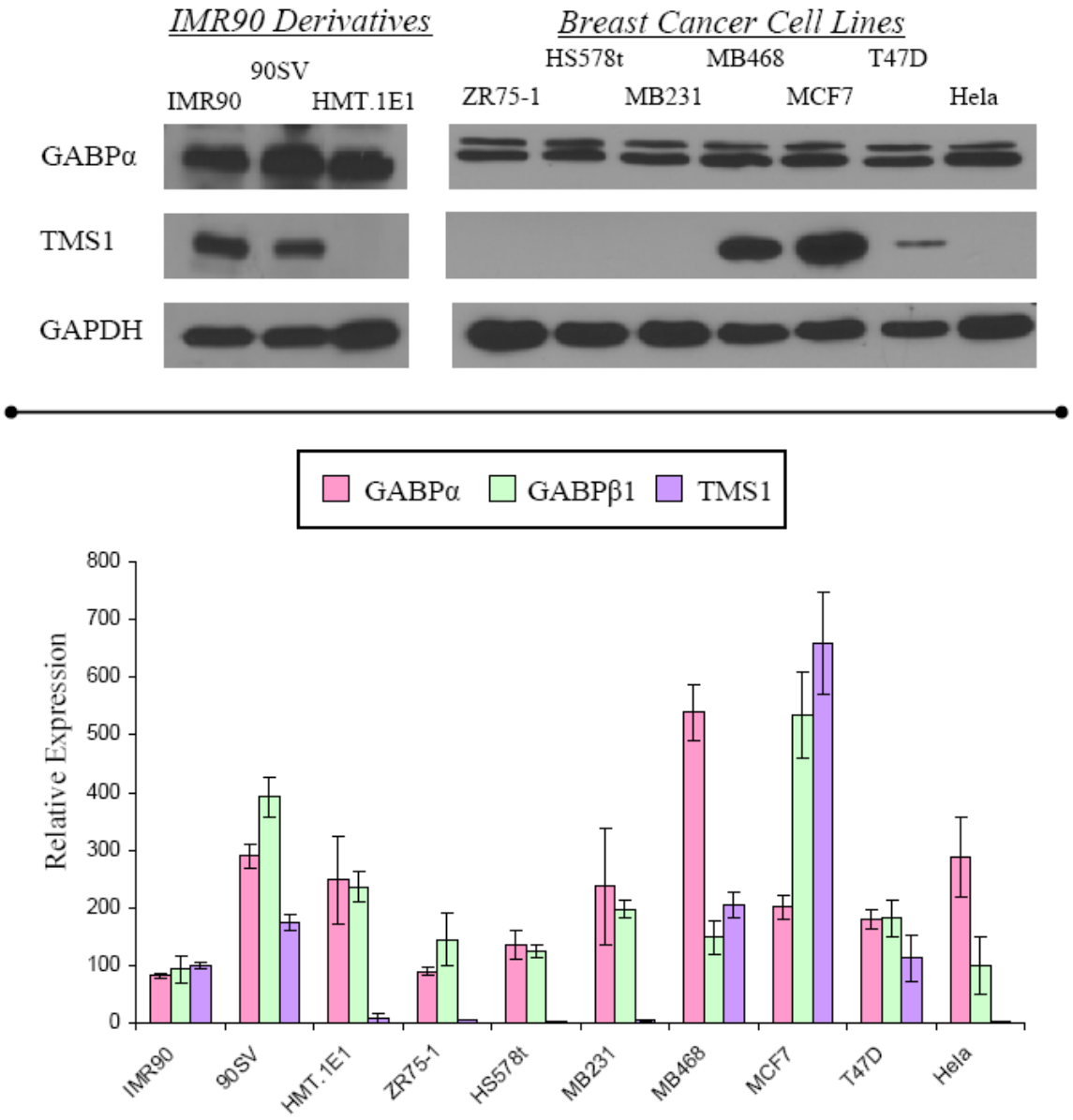

Figure 1: *Levels of GABPα, GABPβ1 and TMS1 human cell lines.* Protein lysates and cDNA from the indicated cell lines were subjected to Western blot analysis (top) and qRT-PCR (bottom). In qRT-PCR anaylsis, values were normalized to internal ribosomal 18s cDNA. There was no correlation between the expression of GABP proteins and TMS1. Data represents mean +/- SD of triplicate determinants.

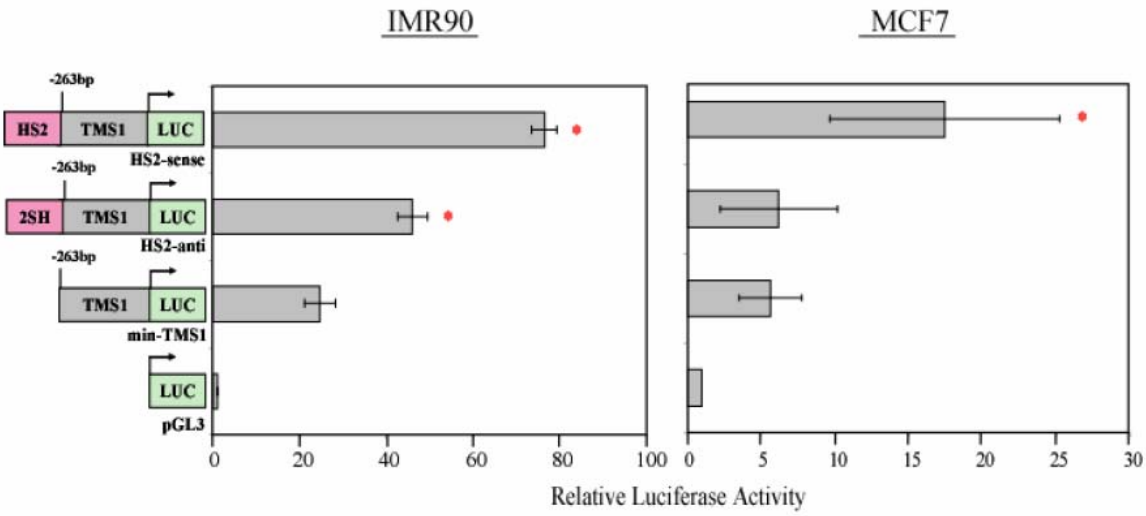

Figure 2: *HS2 enhances the minimal TMS1 promoter.* IMR90 and MCF7 cells were transfected with the indicated plasmids (5ug) and a Renilla luciferase reporter plasmid (pRL-TK) (50ng) as an internal transfection control. pGL3, control vector; min-TMS1, minimal TMS1 promoter (-263bp to +77 relative to transcription start); HS2-anti, HS2-236 bp fused to the minimal TMS1 promoter in the anti-sense orientation with respect to the promoter; HS2-sense, HS2-236bp fused to the minimal TMS1 promoter in the sense orientation with respect to the promoter. Luciferase activity was measured after 48h using the Dual Luciferase reporter assay. Data are represented as fold over pGL3 control after normalization to Renilla luciferase activity. Shown is the mean ± standard deviation of three (IMR90) and six (MCF7) independent experiments performed in triplicate. *, significant compared to min-TMS1 ($p < .05$, Student's *t* test).

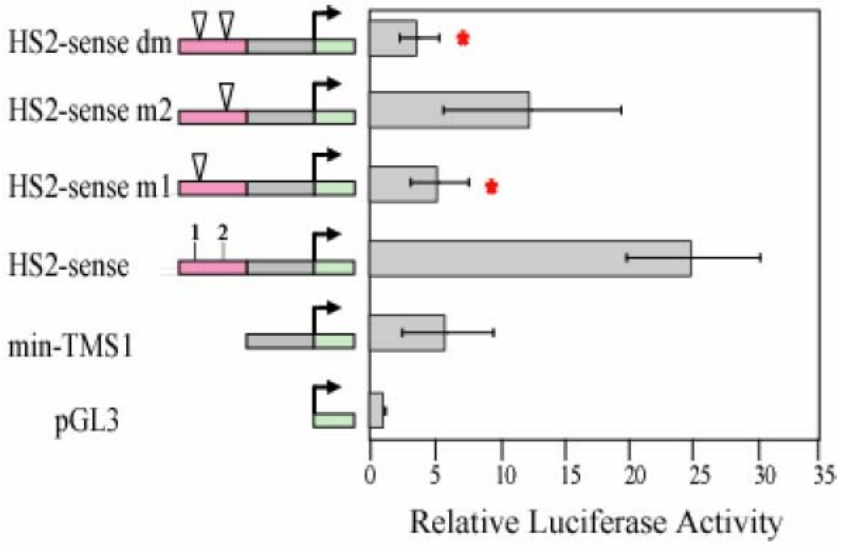

Figure 3: *GABPα binding sites are required for HS2 mediated activation of TMS1.* MCF7 cells were transfected with pGL3 or pGL3 constructs containing the TMS1 promoter alone (min-TMS1), in the presence of a 236bp HS2 fragment (HS2-sense) or HS2 fragment in which either the 5' (HS2-sense m1), 3' (HS2-sense m2) or both (HS2-sense dm) GABP binding sites have been deleted. A Renilla luciferase reporter plasmid (pRL-TK) was included as an internal transfection control. Luciferase activity was determined after 48 hr. Data are represented as fold over pGL3 control after normalization to Renilla luciferase activity. Shown are the means ± standard deviation of three independent experiments performed in triplicate. *, significant compared to HS2-sense ($p<0.05$, Student's *t* test)

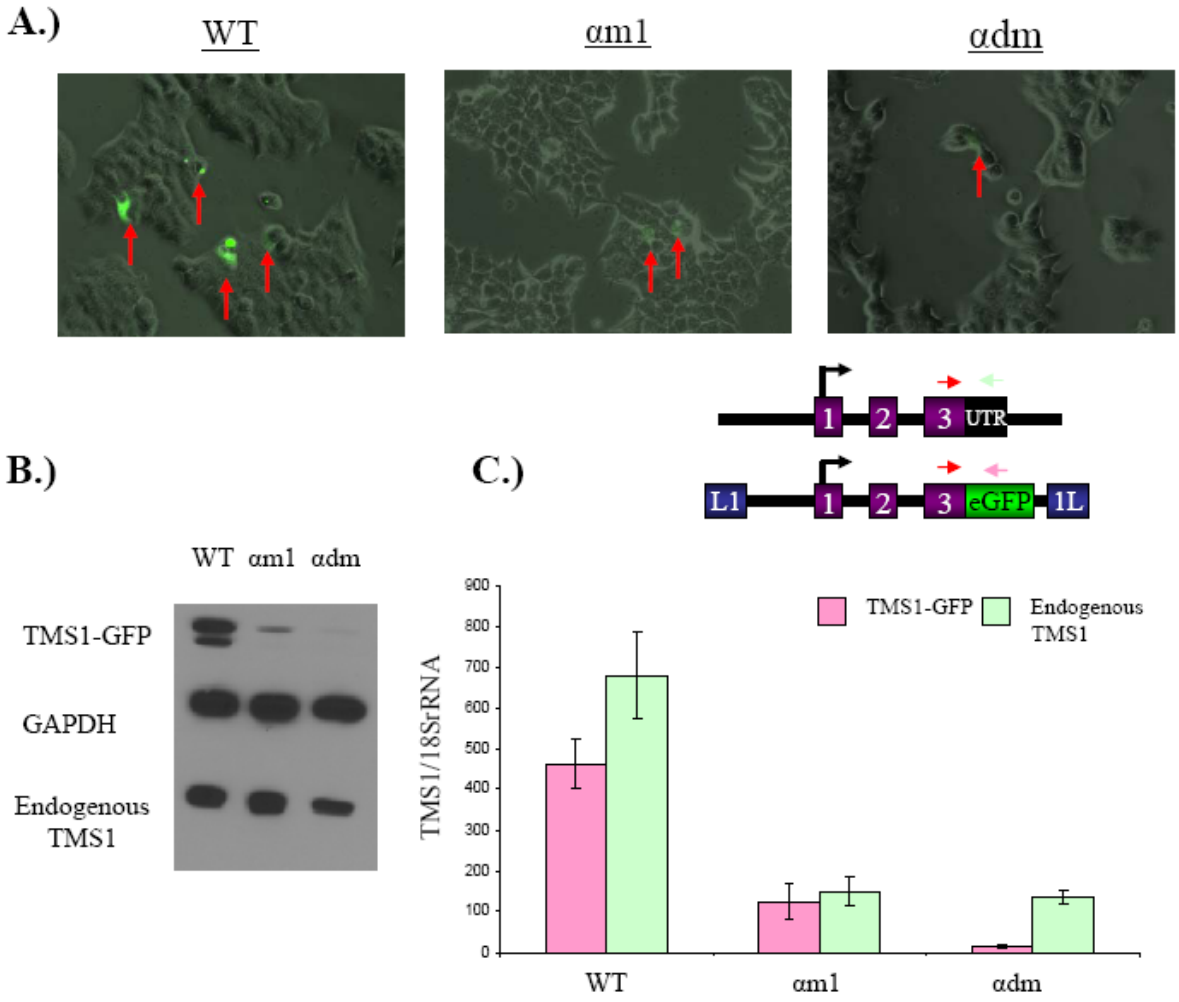

Figure 4: *Transient TMS1-GFP expression in MCF7 cells.* ***A.)*** Confocal images of MCF7 cells transfected with TMS1-GFP constructs. Images were taken 48h post-transfection. Note the decrease in GFP expression with deletion of GABPα binding sites (red arrows). **B., C.)** Immunoblot and qRT-PCR analysis of cells utilized in (A) for TMS1-GFP levels and endogenous TMS1 levels. **C.)** The TMS1 mRNA levels are normalized to 18s RNA. TMS1-GFP and endogenous TMS1 mRNA levels were able to be distinguished due to the antisense primer of each respective primer set annealing to either the untranslated region of exon 3 (pink primer) or GFP mRNA (green primer). Note the decrease in endogenous TMS1 levels detectable at the transcript levels in cells transfected with either mutant constructs. Data represents mean +/- SD of triplicate determinants.

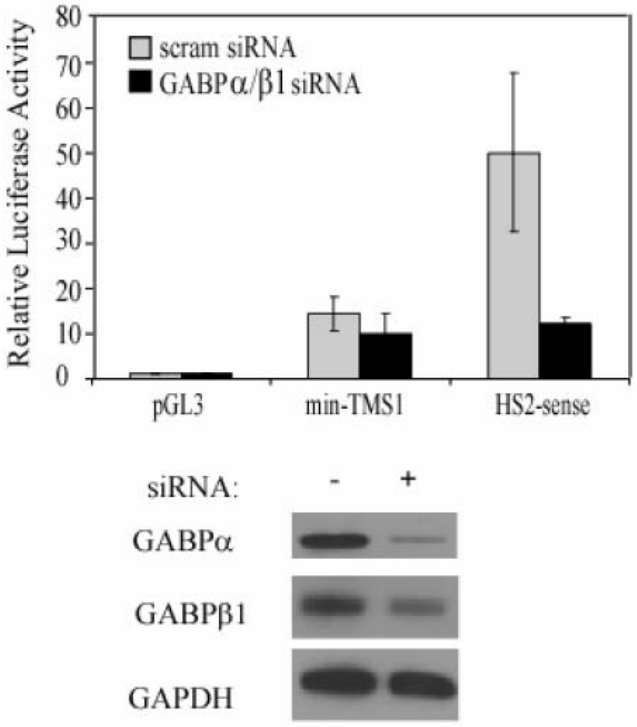

Figure 5 : *GABPα/β1 is responsible for HS2 mediated activation of TMS1.* MCF7 cells were transiently transfected with the indicated plasmid (5ug), and siRNAs targeting GABPα and GABPβ (100nmol/L each) or a scrambled siRNA control (200nmol/L) using the Lipofectamine 2000 reagent (Invitrogen). Renilla luciferase reporter plasmid (pRL-TK, 50ng) was included as an internal control. (*Left*) Luciferase activity was measured after 48h of culture. Data are represented as fold over pGL3 after normalization to the Renilla luciferase control. Shown is the mean ± standard deviation of three independent experiments performed in triplicate. (*Right*) Representative immunoblot showing GABPα, GABPβ1 and GAPDH levels in cells transfected with scrambled or both GABPα-1 and GABPβ siRNA in the same cell lysates used for luciferase assays.

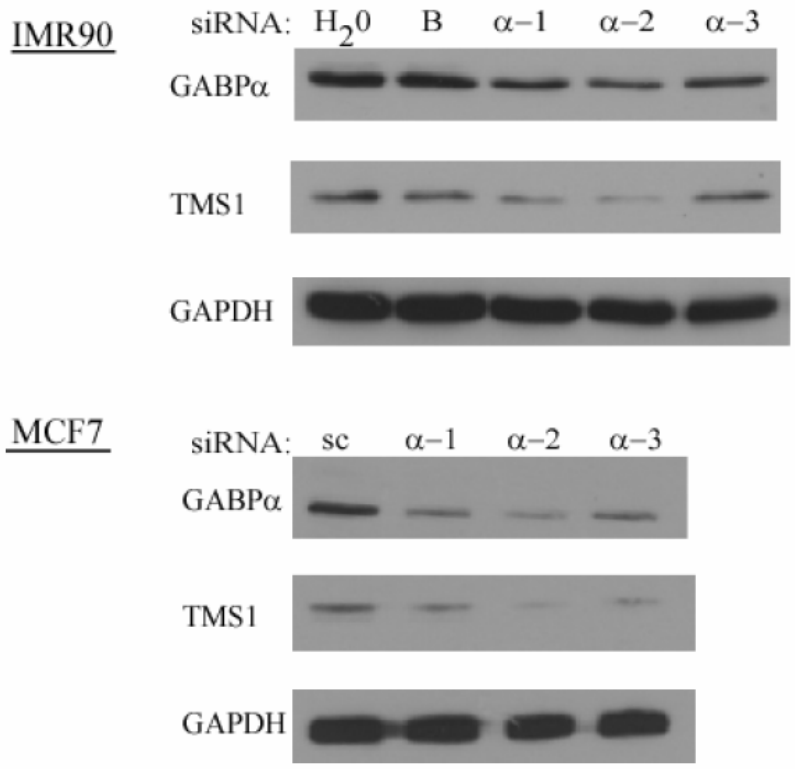

Figure 6 : *Effect of down-regulation of endogenous GABPα (siRNA) on TMS1 expression.* IMR90 and MCF7 cells were transfected with 200nmol/L of three independent siRNAs targeting GABPα, BRSK2 (B, non-specific control) or a scrambled siRNA control. MCF7 cells were harvested 48h later and analyzed for GABPα, TMS1, or GAPDH (loading control) protein expression by Western blot analysis. IMR90 cells underwent a repeat transfection (see materials and methods) before cells were harvested.

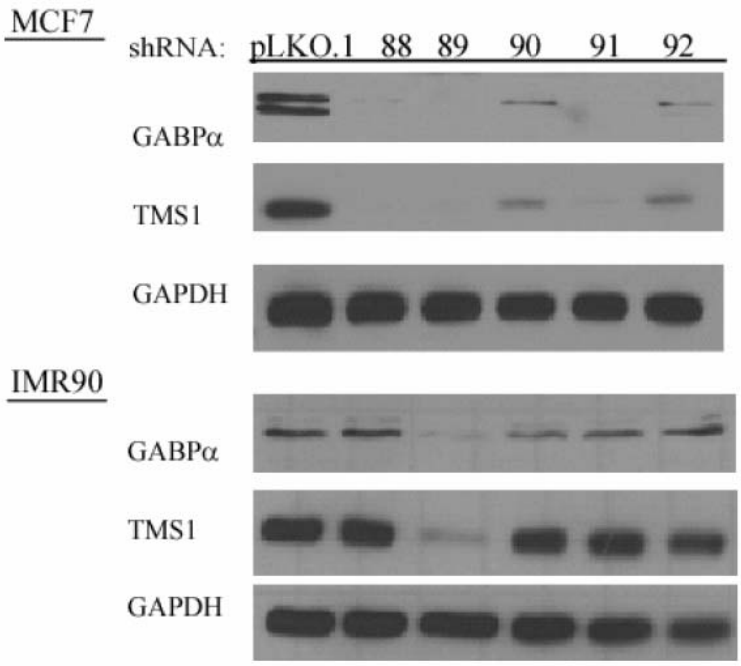

Figure 7 : *Effect of down-regulation of endogenous GABPα (lentiviral-shRNA) on TMS1 expression.* MCF7 and IMR90 cells were infected lentivirus carrying the empty pLKO.1 vector (pLKO.1) or pLKO.1 expressing GABPα shRNA (5 independent shRNA constructs, 18288-92) and selected with 0.5ug/ml puromycin for 5 days. Cells were harvested and analyzed for GABPα, TMS1, or GAPDH (loading control) protein expression by Western blot analysis. The doublet observed in the GABPα blot most likely arises from posttranslational modification as GABPα is known to be phosphorylated.

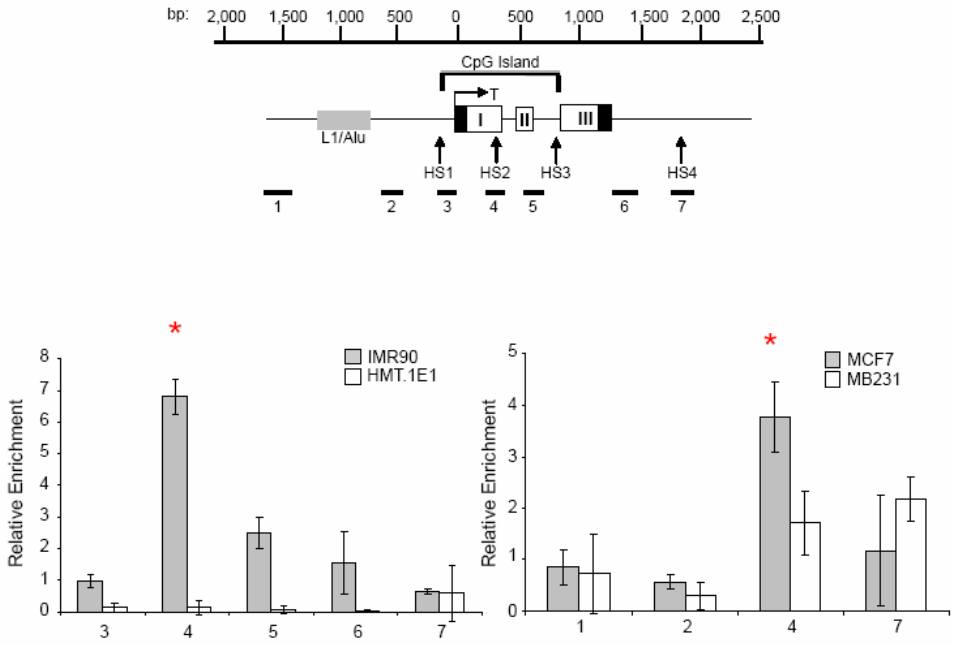

Figure 8: *In vivo occupancy of GABP at the endogenous TMS1 Locus is dependent on the methylation status of the DNA. Top*- Schematic of the TMS1 locus. *Black boxes,* noncoding regions. The nucleotide positions are numbered with respect to the transcription start site (T) and are shown above the gene. The location of the CpG island is denoted and spans from ~ -100 to + 900bp. Primer sets used (1-7) for real-time PCR in chromatin immunoprecipitation assays are shown. *Bottom* - Occupancy of GABP at the TMS1 locus was analyzed by chromatin immunoprecipitation in human fibroblasts (IMR90,HMT.1E1) and human breast cancer cell lines (MCF7, MDA-MB231) differing in their expression and methylation status at the TMS1 locus. Chromatin immunoprecipitation with the indicated cell lines was performed as described in "Experimental Procedures" using an antibody against GABPα or a non-specific antibody control. Immunoprecipated DNA was quantified by real-time PCR using the indicated primer sets. (Primer set 4 contains the HS2-55bp probe DNA). Relative enrichment was determined as the level of amplification precipitated by the GABPα antibody relative to that precipitated by the non-specific antibody at the same location. Shown are the mean ± standard deviation of data from three independent experiments. *, significant difference compared to all other points analyzed ($p<0.01$, Student's *t* test).

Chapter 3

Impact of GABP on Chromatin Structure at the TMS1 Locus.

I performed all experiments included in this chapter with the exception of chromatin immunoprecipitations (P. Kapoor-Vaziranti). In preparation.

Introduction

TMS1 (Target of Methylation-induced Silencing-1) is a novel CpG-island associated gene that is subject to aberrant methylation and epigenetic silencing in breast and others cancers (114, 147). Little is known about the transcriptional regulation of TMS1, and the mechanisms underlying its epigenetic silencing during tumorigenesis remain unclear. In normal cells and breast cancer cells that retain TMS1 expression, the TMS1 CpG island is unmethylated and exhibits an 'active' chromatin signature characterized by hyperacetylated histones H3 and H4, enrichment of H3K4me2, and positioned nucleosomes (141). Three DNAseI hypersensitive sites span the CpG island in the active state with HS1 and HS3 forming at the boundaries between unmethylated CpG island DNA and densely methylated flanking DNA, while HS2 forms at the center of the CpG island (16). Epigenetic silencing of TMS1 in breast cancer is accompanied by the loss of the CpG island-associated HS sites, hypoacetylation of histones, a shift in histone methylation status, and hypermethylation of DNA (16). These findings led us to propose that the HS sites might protect the TMS1 CpG island from methylation in normal cells by forming a structural barrier at the chromatin level. Loss of function at these sites might allow for aberrant methylation, changes in chromatin structure and gene silencing.

The GABP transcription factor complex was identified as a factor that binds to a 55bp region in HS2 in a methylation sensitive manner *in vitro* and *in vivo* and plays an activating role at the TMS1 locus through this interaction (Chapter 2). We hypothesize that the GABP complex plays a role in maintaining the unmethylated, open chromatin structure across the CpG island domain in normal cells, either through direct effects on TMS1 transcription or by recruiting other factors that mark the CpG island domain with a

permissive histone code. GABP has been shown to interact with the histone acetyltransferase, CBP/p300 (148). CBP/p300 acetylates multiple lysine residues on histones which establishes an open chromatin structure and permits active gene transcription. Studies have shown that actively transcribed genes are enriched with hyperacetylated histones and this acetylation is not present when the gene is silent (16, 17). We therefore sought to determine whether long-term inhibition of GABP binding at the TMS1 locus leads to alterations consistent with the epigenetic silencing of TMS1 using two independent approaches. Initially, we attempted to create a cell line depleted of GABPα through the use of lentiviral shRNA. Subsequently, a transgene approach was used wherein cell lines were created that contained either the wild-type TMS1 locus fused to GFP or a mutant form of the TMS1 locus containing GABPα binding site deletions stably integrated within the genome. These studies will help determine the impact of GABP binding on chromatin structure at the TMS1 locus.

Materials and Methods

Generation of MCF7 L1-TMS1-GFP-1L WT and αDM cell lines: MCF7 cells containing L1-HYTK-1L stably integrated into the genome were transfected with 15ug of L1-TMS1-GFP-1L WT and αDM plasmid DNA and 1.5ug Cre using the Cell Line Nucleofector™ Kit V. Cell media was changed after 48 hours followed by an additional 48 hr incubation. Cells were trypsinized and plated at a cell density of $1x10^5$ in 15cm dishes. Media containing 2uM ganciclovir was added 24 hours after plating. Cells underwent 2 weeks of selection with media refreshed every four days. Visible colonies were picked after selection and placed in individual wells of a 24-well plate. Cells were expanded over time until they reached confluency in a T75 flask (Passage 1). Genomic DNA was extracted from clones and screened for the presence of GFP and absence of the HYTK. Primer sequences are located in Table 1.

Chromatin Immunoprecipitation: Chromatin immunoprecipitation was carried out as described in the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Millipore). DNA recovered from chromatin immunoprecipitation was analyzed by real-time PCR. The reaction mixture (25uL) contained 1uL of the appropriately diluted DNA sample, 0.2 umol/L primers, and 12.5uL of IQ SYBR Green Supermix (Bio-Rad). The reaction was subjected to a hot start for 3 minutes at 95^{o}C and 35 cycles of 95^{o}C,10s; 60 ^{o}C, 30s; 72^{o}C, 30s. Melt curve analysis was performed to verify a single product species. Starting quantities were determined relative to a common standard curve generated using MCF7 genomic DNA. Percent enrichment in each pulldown was calculated relative to input

DNA. Primer pairs used for real-time analysis are listed in the Table 1. Antibodies used were rabbit IgG (Santa Cruz), histone H3 acetylated at lysine 9 and 14 (H3K9/14 Ac; Millipore) and demethylated histone at lysine 4 (H3K4me; Abcam).

Table 1:

PCR Primers for GFP/HYTK PCR

GFP:

5'- GTAAACGGCCACAAGTTCAG-3'
5'- GATGTTGCCGTCCTCCTTG -3'

HYTK:

5'- TTTCGGCTCCAACAATGTCC -3'
5'- ATGTTGGCGACCTCGTATTG-3'

PCR Primers for Chromatin Immunoprecipitations

TMS1-GFP:

5'- AGTCCCAGTCCTACCTGGTG -3'
5'- CGTGAACTTGTGGCCGTTTAC -3'

Endogenous TMS1:

5'- AGAGCTCAGCTATGCTTCAG -3'
5'- CTGGGTCACAGGCTGTTATC -3'

PCR Primers for quantitative Real-Time PCR

TMS1-GFP:

5'- AGTCCCAGTCCTACCTGGTG -3'
5'- CGTGAACTTGTGGCCGTTTAC -3

Endogenous TMS1:

5'- TCTCCAGGTAGAAGCTGACCAG -3'
5'- GCCGAGGAGCTCAAGAAGTTC -3'

Results

To determine the impact of GABP on DNA methylation and chromatin structure at the TMS1 locus, we generated MCF7 breast cancer cells stably knocked down for GABPα. MCF7 cells were infected with lentiviral shRNA directed against GABPα, as well as a pLKO.1 control. Stable pools of infected cells were created by selection for resistance to puromycin (0.5ug/ml) and designated MCF7-shGABP and MCF7-shPLKO.1. MCF7-shGABP and MCF7-shPLKO.1 cells were passaged under continuous selection, and GABPα and TMS1 protein and RNA levels analyzed at each passage. Initially, shRNA targeting of GABPα caused a knockdown of ~90% of GABPα protein with a correlated decrease in TMS1 protein levels (Figure 1). This correlation between GABP and TMS1 expression was also detected at the RNA level (Figure 1). Surprisingly, GABPα levels returned to baseline in MCF7-shGABP cells with continuous culture, and there was a concomitant increase in TMS1 levels even in the presence of puromycin (Figure 1). These data confirm previous findings (Chapter 2), and further support a direct role for GABP complex in the regulation of TMS1 expression in that knockdown of GABPα protein and RNA levels and the subsequent reappearance directly correlated with TMS1 expression levels.

The inability to create a stable cell line that maintained GABPα suppression for more than a few passages precluded the to evaluation of the impact of GABP on TMS1 chromatin structure. We therefore targeted the binding sites of GABPα using an integrated transgene approach. Recombinase Mediated Cassette Exchange was used to introduce TMS1-GFP transgenes into a tagged genomic site (Figure 2). The RMCE technique is beneficial because it allows for the efficient integration of different transgene

constructs into a common genomic site using Cre/LoxP recombination and a positive /negative selection scheme. The RMCE approach allows for direct comparison of different modified transgenes at a common genomic site, eliminating potential differences due to position effects. It also allows for the insertion of sequences without a selectable marker, which can potentially select against gene silencing events. Transgenes were constructed containing GFP fused to the TMS1 locus at the end of the coding region in exon 3 (Figure 2). A wild type locus (WT), containing intact GABP binding sites and a mutant locus (αDM), containing GABP binding deletions were compared for changes in TMS1-GFP expression and chromatin structure.

MCF7 cells containing a single copy of the HYTK cassette flanked by L1 sites was transfected with exchange constructs and a plasmid expressing Cre (Figure 2). Cells were subjected to selection with ganciclovir for two weeks to isolate clones in which the HYTK cassette has been lost. Isolated single cell clones were screened for cassette exchange as indicated by the absence of HYTK sequences and presence of GFP sequences in genomic DNA by PCR and Southern blot analysis (Figure 3A). Clones containing the wild type locus stably integrated within the genome (WT-5, WT-9, Hela-GFP) were generated previously and were used as positive controls during analysis of αDM clones (Figure 3B, lanes 1 and 2). Two rounds of RMCE were performed in which seven clones were isolated. Upon PCR examination, two of these clones (αDM-1, αDM-2) were determined to be positive for the presence of GFP and to lack HYTK confirming that the exchange had occurred (Figure 3B, lanes 5 and 9). Wild type (WT-5, -9) and mutant (αDM-1, -2) clones containing the exchange cassette were monitored over time for changes in GFP expression and chromatin modifications at the integrated transgene.

Upon stable integration, TMS1-GFP expression could not be detected in either WT nor αDM cells using confocal microscopy (data not shown). However, TMS1-GFP expression was detected by Western blot analysis using an antibody against TMS1 or by qRT-PCR using primers designed specifically against the TMS1-GFP fusion transcript (Figure 4). Western analysis as well as qRT-PCR demonstrated that deletion of GABPα binding sites correlated with a dramatic decrease in TMS-GFP expression (Figure 4). In fact, TMS1-GFP levels were undetectable at the transcript and protein levels in GABP deletion clones in comparison to wild type clones as early as passage 1(e.g. first expansion to ~ $5x10^5$ cells)(Figure 4). Interestingly, similar to what was observed in transient transfection studies (Chapter 2, Figure 4), there appeared to be an impact of the mutant construct on the expression of endogenous TMS1 (Figure 4).

Subsequent examination of clones over time yielded similar results as those detected at passage 1. Clones at passage 2 underwent Western blot and qRT-PCR analysis to determine TMS1-GFP expression levels. Deletion of the GABPα binding sites correlated with a decrease in TMS1-GFP expression at the protein and RNA levels (Figure 5). The impact of the mutant construct on expression of endogenous TMS1 that was detected in passage 1 was not observed in the later passage 2 (Figure 5). Of note, comparison of TMS1-GFP expression from the wild type locus appeared to decrease over time (compare relative levels of TMS1-GFP in WT clones to endogenous TMS1 levels, Figures 4 and 5). Consequently, TMS1-GFP expression was no longer detectable in the wild type clones by passage 4 (data not shown). Together these data suggest that GABP binding sites are necessary to maintain transcriptional integrity at the transgene locus.

However, over time, even the WT integrants lose TMS1-GFP expression through a mechanism independent of GABP.

We next determined the impact of GABP binding on chromatin structure at the transgene locus. Initially, we determined the status of histone modification at the integrated transgene locus and its relationship to the endogenous TMS1 locus. Chromatin immunoprecipitation was performed utilizing a PCR strategy devised to distinguish between the TMS1-GFP transgene and the endogenous TMS1 locus by using primers spanning the TMS1-GFP junction or the 3' untranslated region (see Figure 6). ChIP analysis indicated that the WT transgene locus is enriched in histone H3 acetylated at lysine 9 and 14 (H3Ac), as well as histone H3 dimethylated at lysine 4 (H3K4me2) at levels comparable to the endogenous TMS1 locus (Figure 6). Therefore, the TMS1-GFP transgene adopts a chromatin pattern consistent with the "active", unmethylated, endogenous TMS1 locus in MCF7 cells.

Subsequently, ChIPs were performed comparing H3Ac and H3K4me2 levels at the wild type or mutant TMS1-GFP transgenes. As described above, a decrease in TMS1-GFP expression was observed in αDM clones in comparison to wild type clones (Figure 5). In addition, the levels of H3Ac were decreased in αDM clones relative to the WT clones (Figure 7). However there was no difference in the levels of H3K4me2 (Figure 7). In contrast, there was no difference in H3Ac or H3K4me2 levels at the endogenous TMS1 locus between WT and αDM clones. These data support the hypothesis that the inhibition of GABPα binding leads to epigenetic changes, specifically a decrease in H3K9/14 acetylation.

As noted above, continuous passage of RMCE clones resulted in the loss of TMS1-GFP expression in wild type clones at later passages (>4). The mechanism that results in this silencing is unknown. To determine whether this silencing was due to changes in the chromatin structure, chromatin from wild type and mutant clones in which TMS1-GFP expression was no longer detectable (passage 6) was analyzed. These experiments showed again that deletion of GABP binding sites resulted in a decrease in H3Ac at lysine 9 and 14, but had no impact on the chromatin structure of the endogenous TMS1 locus or levels of H3K4 methylation at the TMS1-GFP transgene locus (Figure 8). Interestingly, despite the complete loss of detectable TMS1-GFP expression in these late passage clones, there was still considerable levels of H3Ac (Figure 8) at the wild type transgene locus. Therefore, we can conclude that GABP binding at HS2 is necessary to maintain the hyperacetylated state of the TMS1-GFP transgene. However, the mechanism for the silencing of the wild type transgene that occurs over time does not appear to be due to the loss of "active" histone marks (e.g. H3Ac, H3K4me2) and still needs to be further examined.

Summary

In order to determine the effect of GABP on chromatin structure at the TMS1 locus, Recombinase Mediate Cassette Exchange (RMCE) was employed to directly compare TMS1-GFP transgene loci which contained either intact GABP binding sites (WT) or GABP binding site deletions (αDM). The results from studies demonstrated the necessity of GABP binding at the TMS1 locus for transcriptional competency of the integrated transgene. The GABP mutant clones consistently showed a decrease in TMS1-GFP expression in comparison to wild type clones. Deletion of the GABP binding sites also had an affect on chromatin structure at the transgene locus. Levels of histone H3 acetylation were significantly decreased in the absence of GABP binding. Interestingly, TMS1-GFP expression from the wild type transgene also decreased over time via mechanisms that did not involve loss of histone H3acetylation.

Deacetylation of histone H3 along with the accumulation of H3K9 trimethylation has been shown to precede DNA methylation in the progressive inactivation of the RASSF1A gene during immortalization of human mammary epithelial cells (HMEC) (149). It has been proposed that during continuous culture a decrease in transcription is a requisite event in silencing followed by the accumulation of repressive histone marks and DNA methylation as the stabilizing event. One can propose a model in which the loss of GABP binding, due to spurious methylation and epigenetic changes at the TMS1 locus, may predispose the locus to silencing and ultimately hypermethylation of CpG island DNA.

GABP was demonstrated to have a direct effect on the levels of H3Ac at the TMS1-GFP transgene locus, but had no effect on levels of H3K4me2. Knockdown of another regulatory protein of TMS1, the histone acetyltransferase hMOF, led to a decrease in levels H4K16Ac with no effect detected on levels of H3Ac and H3K4me2 (141). Interestingly, knock down of hMOF caused a correlated decrease in TMS1 expression at levels comparable to knock down of GABPα. Potentially, hMOF, GABP, and a H3K4 methyltransferase work in concert to maintain the TMS1 locus in an open active state. Co-immunoprecipitation studies would be beneficial to determine if there is a physical interaction between GABP and hMOF as knockdown of GABP or hMOF has similar effects on TMS1 expression (141, 150). GABP potentially could recruit hMOF to the locus through its DNA binding capabilities. Subsequently, ChIP analysis would also be able to determine if knock down of GABP has an effect on acetylation of H4K16. These potential studies would better define the GABP complex role as a regulator of TMS1 expression by epigenetic mechanisms.

GABP has many co-activators that it interacts with and could potentially recruit to the TMS1 locus. Sp1, Elf and PU.1 are known transcription factors that work in concert with GABP in the regulation of target genes in numerous cell types (151, 152). A loss of GABP binding at the TMS1 locus could lead to the loss of recruitment of these or other co-activators which help the TMS1 locus maintain an open, expressing chromatin state. For example, CBP/p300 acetylates the lysine residues 9 and 14 at histone H3 (153). If GABP recruits CBP/p300, this may explain why the loss of GABP binding sites correlates with a decrease in acetylation at H3K9/14 detected at the mutant transgene

loci. A more defined examination of proteins with known roles in TMS1 expression will help in the identification factors with putative activating and repressive roles.

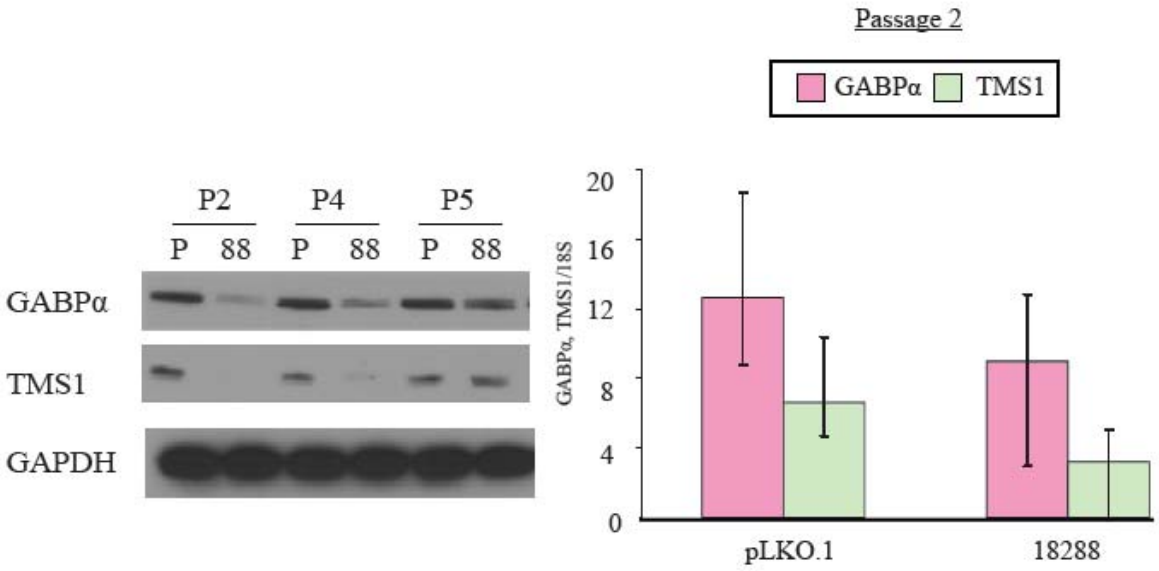

Figure 1: *Re-expression of GABPα in the presence of puromycin*. MCF7 cells were infected with lentivirus supernate containing shRNA targeting GABPα. Cells were harvested 5-days post infection and puromycin selection (0.5ug/ml). Cells were analyzed for GABPα, TMS1, or GAPDH (loading control) protein expression by Western blot analysis (left) or for GABPα, TMS1 or 18S (internal control) RNA expression by real time PCR (right). The levels of expression of GABPα and TMS1 mRNA are expressed relative to that obtained in cells treated with pLKO.1 control virus, after normalization to 18S. Data represents mean +/- SD of triplicate determinants from a single experiment.

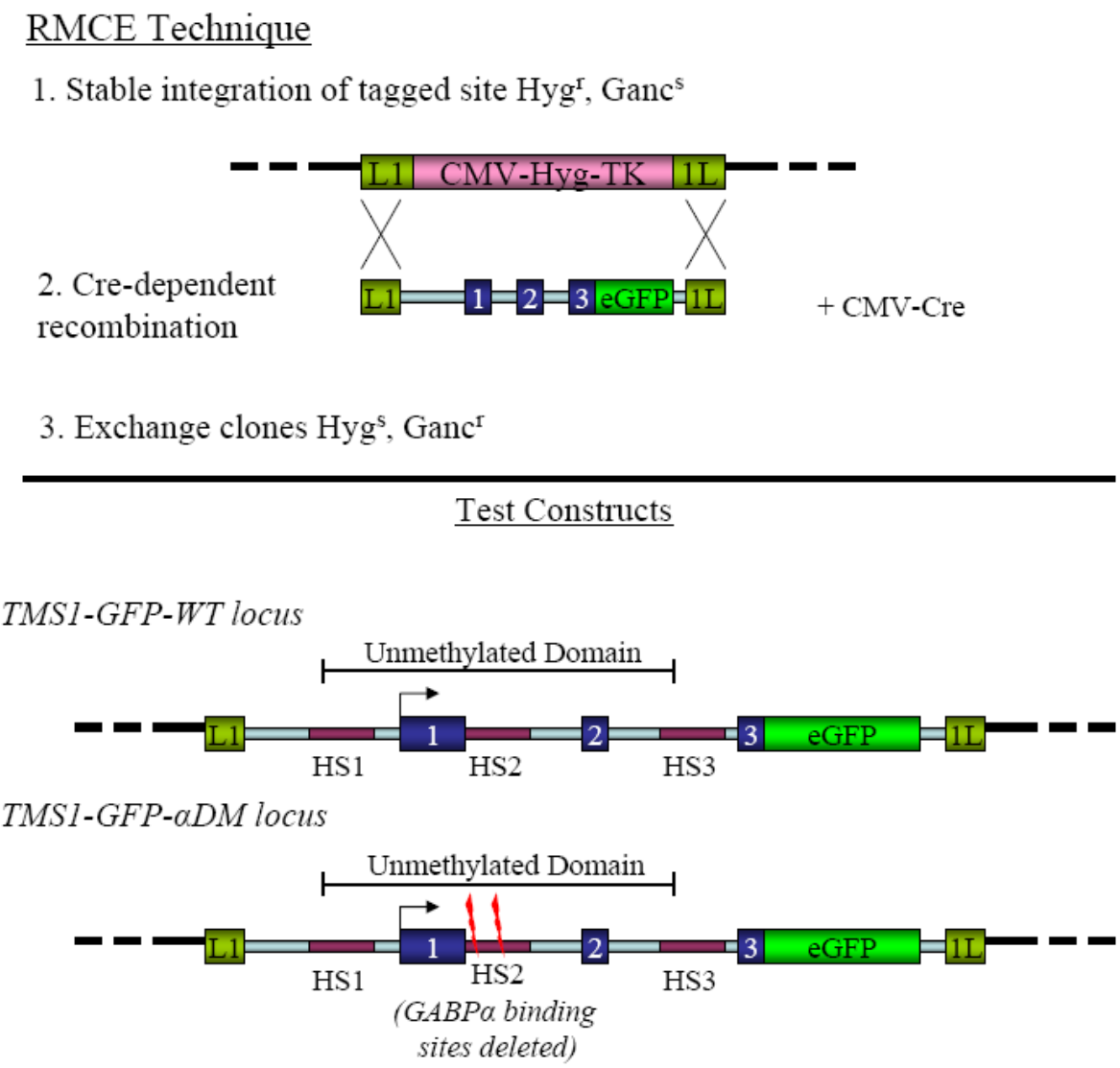

Figure 2: *Schematic of Recombinase Mediated Cassette Exchange (RMCE).* (*Top*) Cells containing a single integrated copy of the Hyg-TK cassette within the genome are transfected with TMS-GFP transgene constructs containing flanking L1-1L recombination sites along with CMV-Cre. Homologous recombination occurs at the L1-1L sites allowing the transgene construct to replace Hyg-TK. Cells are placed under resistance to ganciclovir to identify cells in which the exchange occurred. (*Bottom*) Schematic of TMS1-GFP transgene constructs WT (TMS1-GFP-WT) and mutant (TMS1-GFP-αDM). *(CMV-Hyg-TK: cassette encodes a fusion protein with hygromycin-resistance (HYG) and thymidine kinase (TK) activities under the control of the CMV promoter; red lines in TMS1-GFP-αDM schematic denote GABPα binding site deletions)*

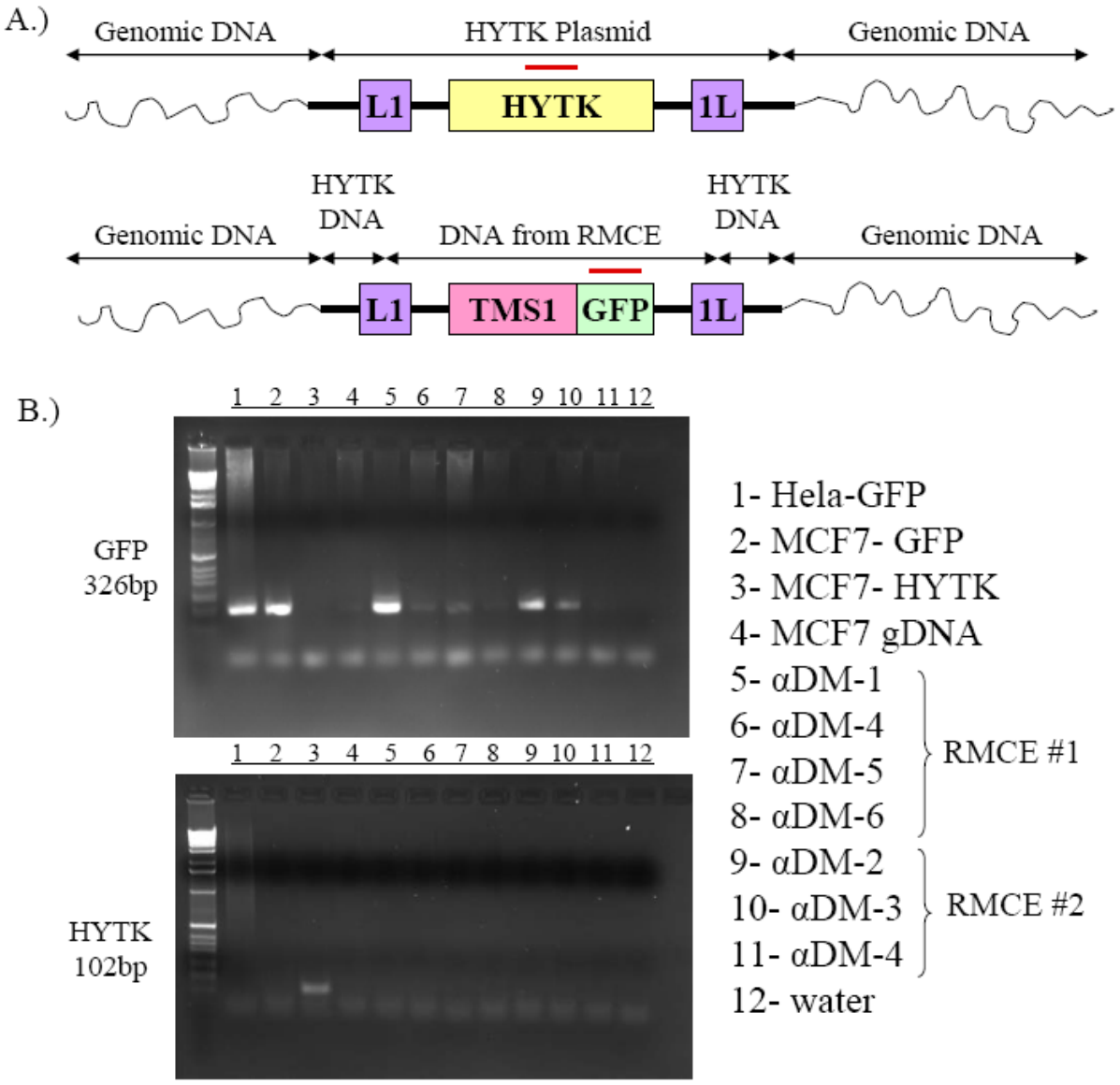

Figure 3: *Screening for positive clones: GFP/HYTK PCR.* Genomic DNA from two independent RMCE single cell colony clone isolations (see Materials and Methods) underwent PCR analysis to confirm the presence of GFP and absence of HYTK. A.) Schematic of integrated HYTK and TMS1-GFP loci. Position of PCR primers utilized to screen for the presence/absence of HYTK or GFP are indicated in red. B.) Genomic DNA from the indicated clone was screened by PCR for the presence of GFP (top) and HYTK (bottom). αDM-1 and αDM-2 were identified as mutant positive clones.

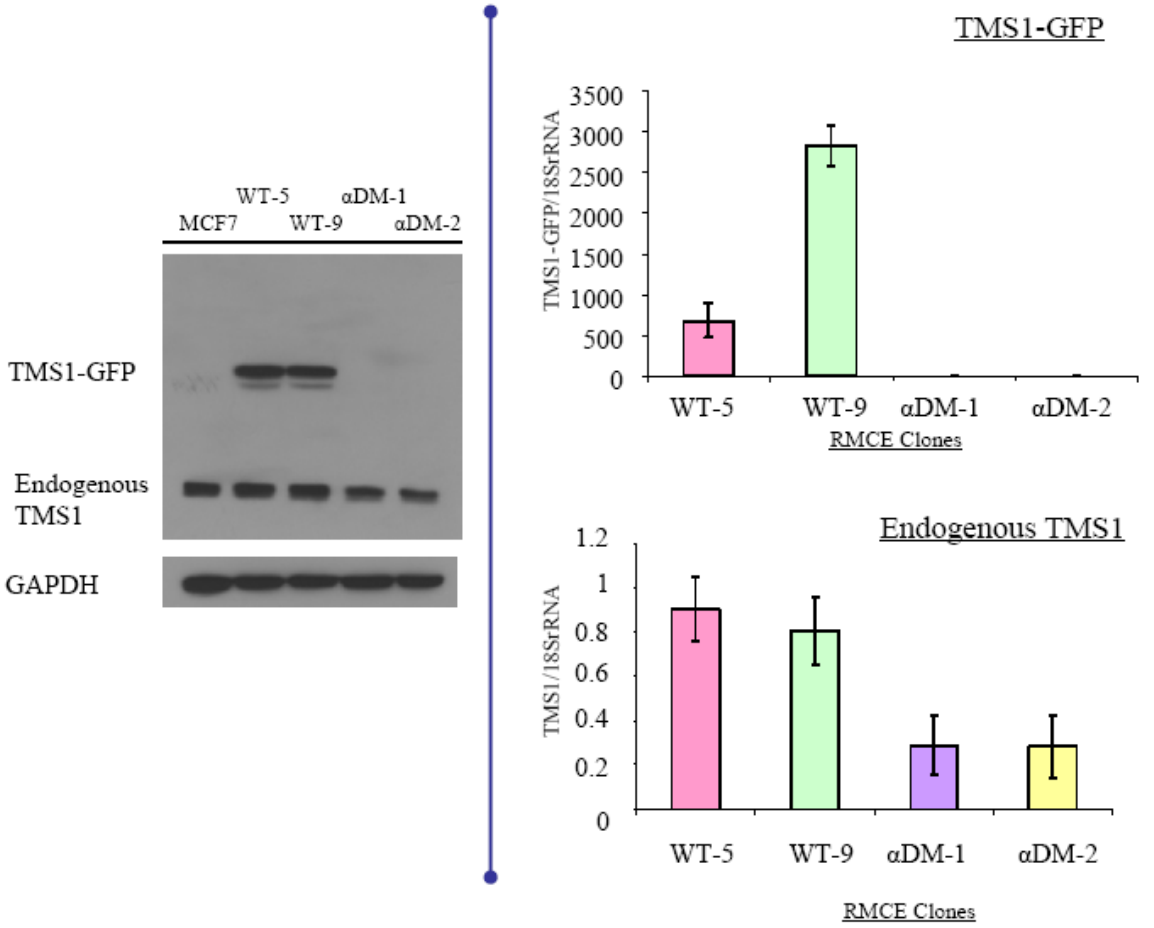

Figure 4: *Effect of GABP binding sites on TMS1-GFP expression at early passage (P1).* RMCE clones were analyzed for TMS1-GFP, endogenous TMS1, or GAPDH (loading control) protein expression by Western blot analysis (left), or for TMS1-GFP, endogenous TMS1, or 18S (internal control) RNA expression by real time PCR (right). The TMS1 mRNA levels are normalized to 18S rRNA. Data represents mean +/- SD of triplicate determinants from a single experiment.

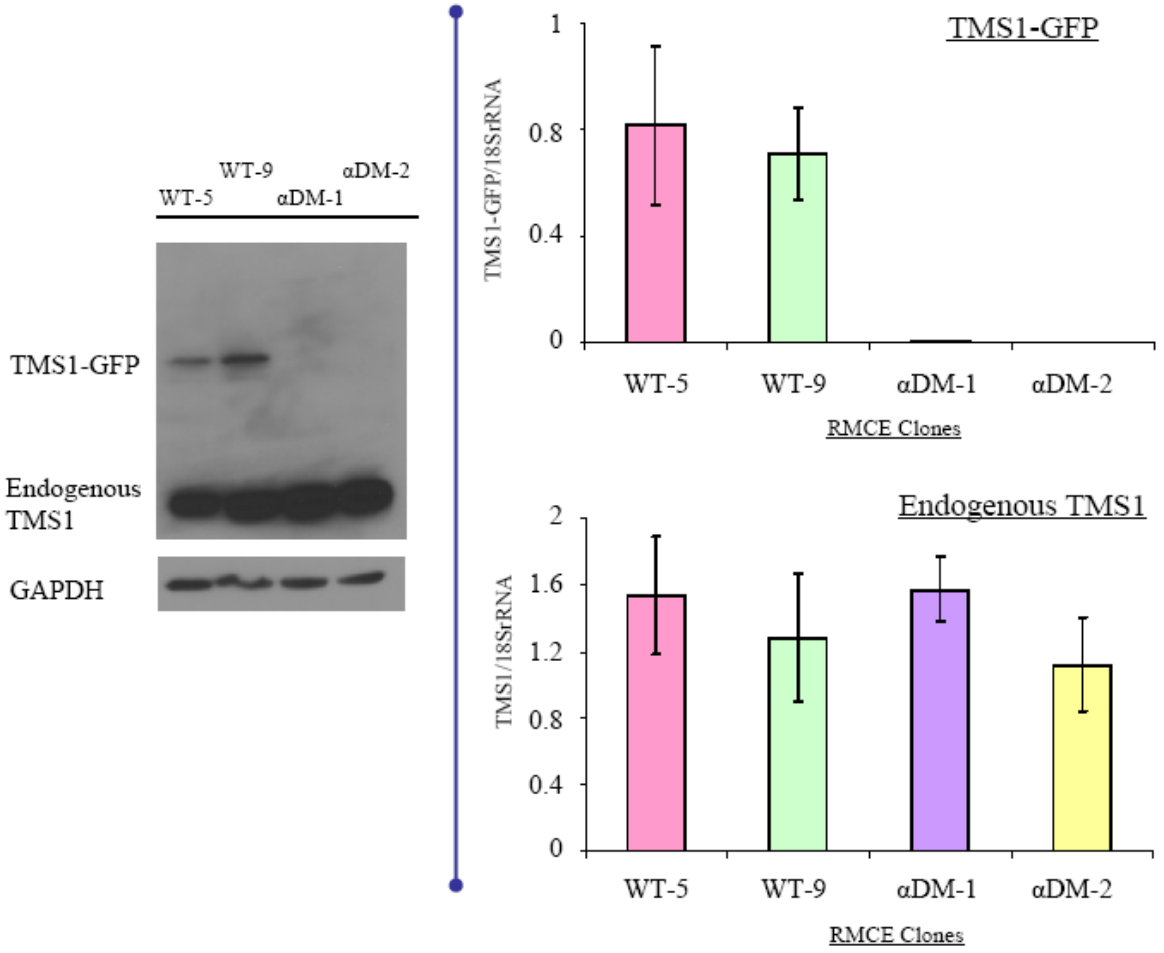

Figure 5: *TMS1-GFP expression at passage 2.* RMCE clones were analyzed for TMS1-GFP, endogenous TMS1, or GAPDH (loading control) protein expression by Western blot analysis (left), or for TMS1-GFP, endogenous TMS1, or 18S (internal control) RNA expression by real time PCR (right). The TMS1 mRNA levels are normalized to 18S rRNA. Data represents mean +/- SD of triplicate determinants from a single experiment. Levels of endogenous TMS1 are unaffected by the presence of the mutant TMS1-GFP locus. (Compare passage 1 (Figure 4) and passage 2 endogenous TMS1 levels in mutant clones).

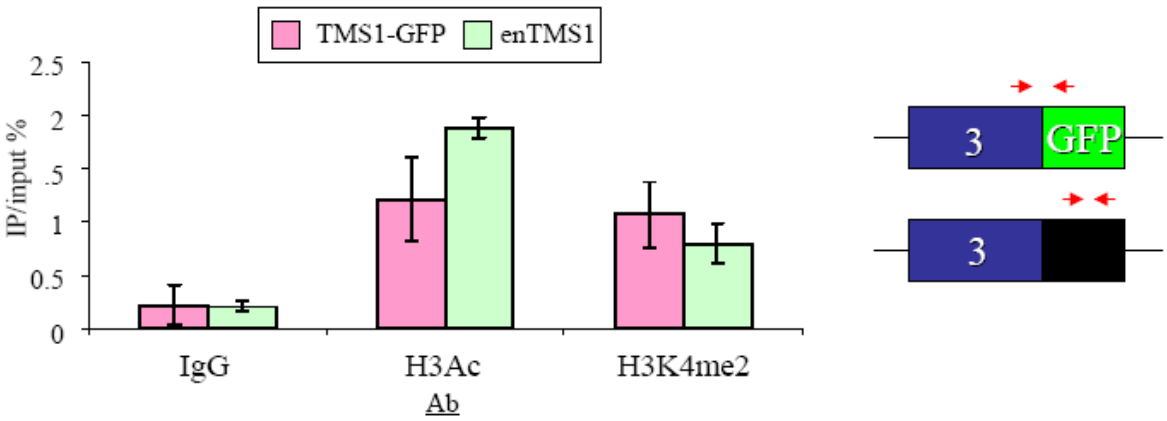

Figure 6: *Chromatin structure of the TMS1-GFP transgene locus.* Chromatin from WT-5 clones underwent chromatin immunoprecipitation with antibodies against rabbit IgG, H3Ac and H3K4me2. Immunoprecipitated DNA was amplified by real time PCR with primer sets (red arrows) indicated to the right. Data represents the mean percent input DNA recovered (*left*) +/- SD of triplicate determinants from a single experiment. Levels of H3Ac and H3K4me2 at the TMS1 transgene locus are comparable to the levels at the endogenous TMS1 locus.

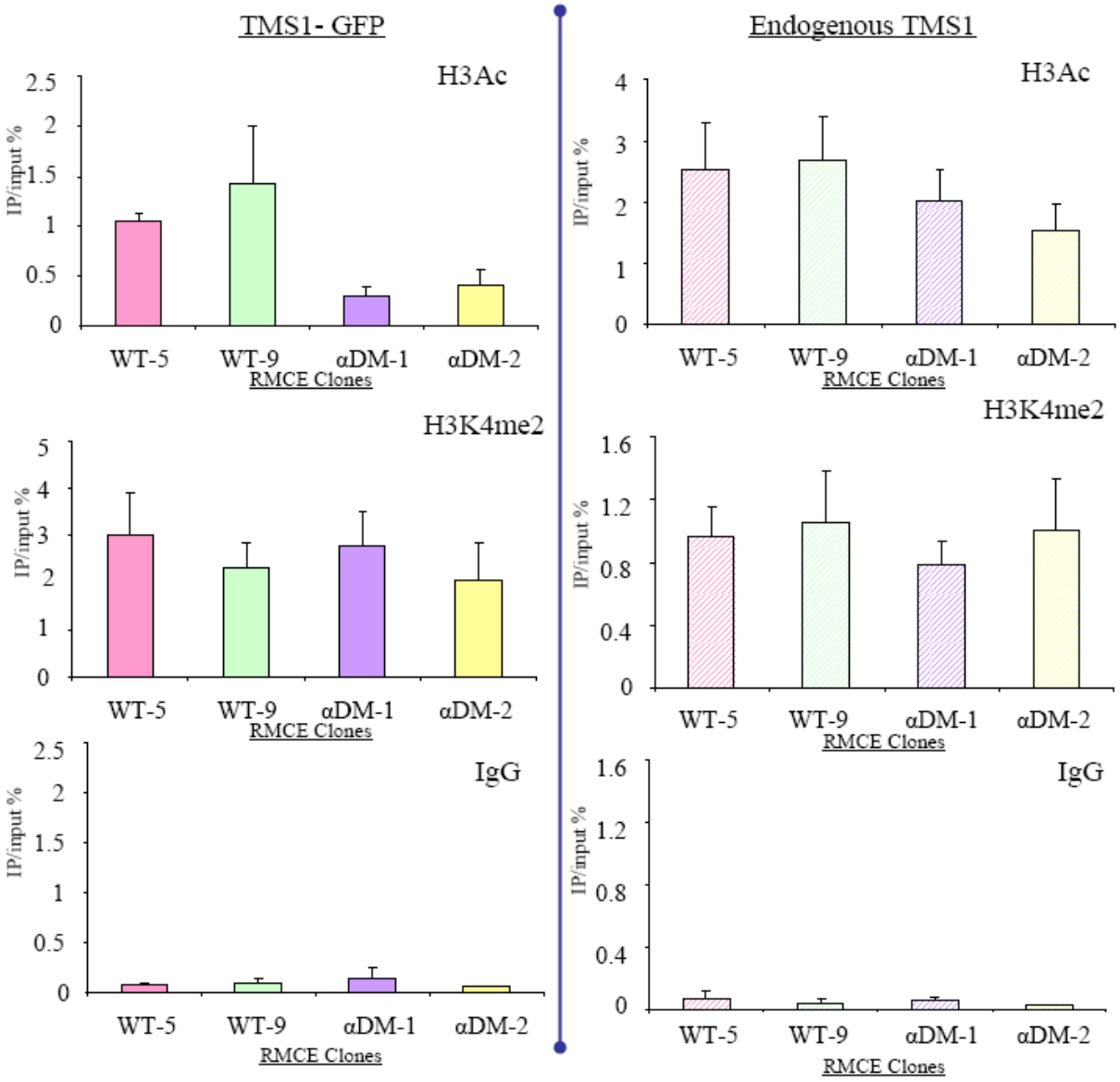

Figure 7: *Effect of GABP binding site deletions on chromatin structure at the mutant transgene locus.* Chromatin from RMCE clones (WT-5, WT-9, αDM-1 and αDM-2) underwent chromatin immunoprecipitation with antibodies against rabbit IgG, H3Ac and H3K4me2. Immunoprecipitated DNA was amplified by real time PCR with primer sets indicated in Figure 6. Data represents the mean percent input DNA recovered +/- SD of triplicate determinants from a representative experiment. Each chromatin immunoprecipitation experiment was repeated at least twice with reproducible results. Deletion of GABP binding sites has an effect on levels of acetylation at the transgene locus, however, no effect is seen at the endogenous locus.

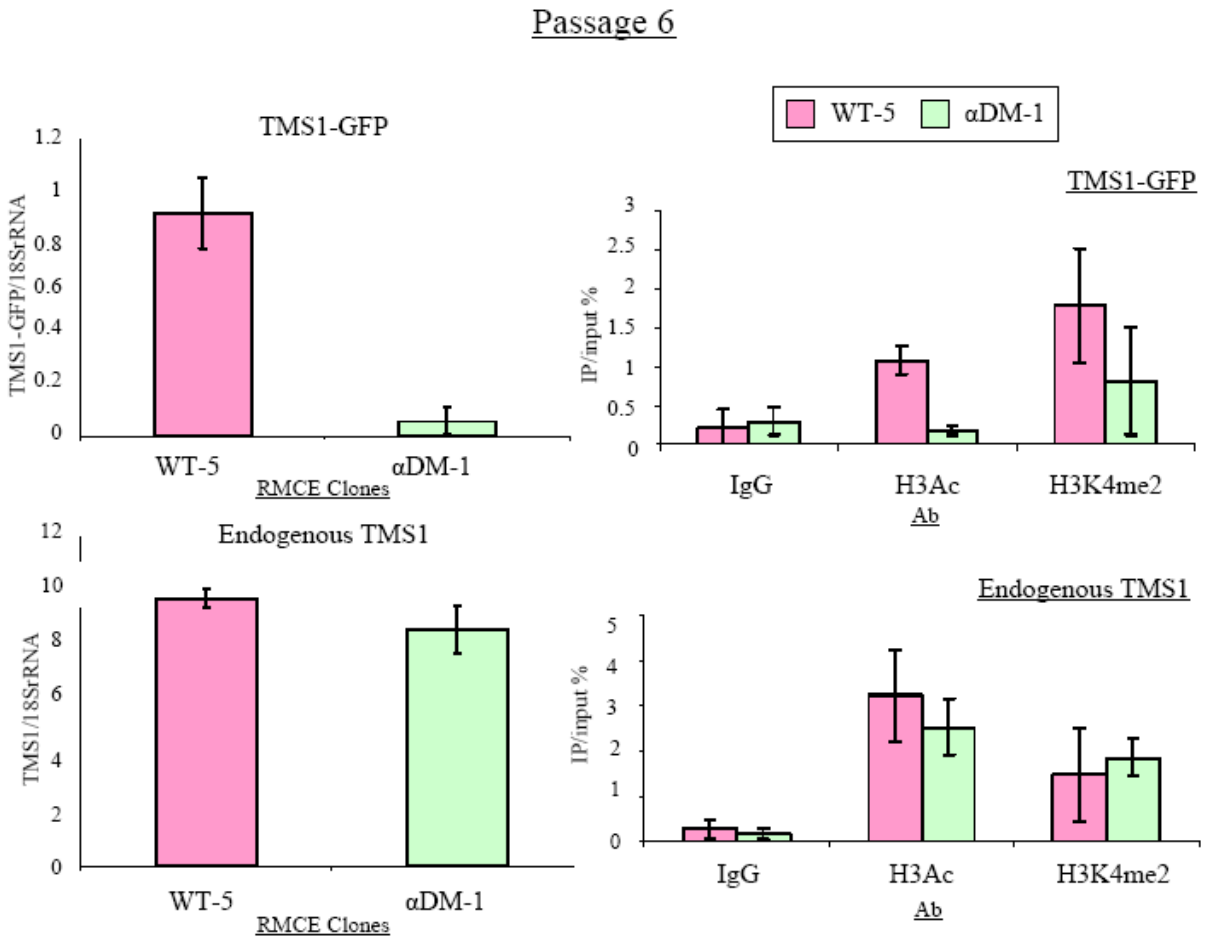

Figure 8: *Chromatin structure at the TMS1-GFP locus in late passage clones.* A.) WT-5 and αDM-1 RMCE clones were analyzed from TMS1-GFP, endogenous TMS1, or 18S (internal control) RNA expression by real time PCR (left). The levels of TMS1 mRNA expression are normalized to 18S rRNA. Data represents the mean +/- SD of triplicate determinants from a single experiment. Note the dramatic decrease in TMS1-GFP expression from WT-5 cells at passage 6 in comparison to passage 1 (Figure 4) or passage 2 (Figure 5). B.) Chromatin from WT-5 (pink) and αDM-1 (green) cells underwent chromatin immunoprecipitation with antibodies against rabbit IgG, H3Ac (K9/14), and H3K4me2. Immunoprecipitated DNA was amplified by real time PCR using primer sets specific to the integrated TMS1-GFP locus or endogenous TMS1 (see Figure 6). Data represents the mean percent input DNA recovered +/- SD of triplicate determinants from a single experiment.

Chapter 4

Discussion

Discussion

In this study, we demonstrate a role for GA-binding protein (GABP) in the regulation of TMS1 gene expression. In previous studies, a biochemical approach was utilized to purify and identify GABP as a factor that bound a 55bp region coincident with a DNAseI hypersensitive site (HS2) located in intron 1 of the TMS1 gene (150). ChIP experiments confirmed enrichment of GABP over this region *in vivo* in cells where TMS1 is expressed. Reporter assays utilizing the minimal TMS1 promoter fused to HS2 demonstrated that GABP is an activator of TMS1 expression in that deletion of GABP binding sites or depletion of GABP protein from the cell through RNAi abrogates transcriptional activation. Additionally, knockdown of endogenous GABPα correlates with a corresponding decrease in endogenous TMS1 levels. We also demonstrate a role for GABP in the maintenance of H3Ac at the TMS1 locus through the use of transgene TMS1-GFP constructs stably integrated into the MCF7 genome that are either wild type or mutant for GABP binding sites.

GABP is a member of the ets transcription factor family and is composed of two proteins that either contain a DBD (GABPα) or TAD (GABPβ1). The GABPα/β1 complex can exist as either a heterodimer (GABPα/β1) or heterotetramer ($GABP\alpha_2/\beta_2$). Both forms have the ability to either activate or repress transcription from GABP responsive genes, however, the heterotetramer is a stronger activator/repressor than the heterodimer (122). Our studies have determined that the intronic HS2 region containing GABP binding sites acts as an enhancer of TMS1 promoter activity. One of the two tandem ets consensus sites in HS2 deviates from the GABPα consensus “GGAA” in a

key core position (GGTA) shown to be critical for optimal DNA binding (122). Interestingly, our reporter assays showed that deletion of the 5' perfect ets consensus site (HS2-sense m1) completely abolished enhancement of TMS1 promoter activity, whereas deletion of this latter site (HS2-sense-m2) had less of an impact on HS2 enhancer activity. Therefore, the GABP heterotetramer, as well as the heterodimer, is able to activate transcription form the minimal TMS1 promoter in luciferase assays. However , the heterodimer is not able to activate transcription at levels comparable to the heterotetramer (compare activity from HS2-m2 and HS2-sense, Chapter 2). This data is evidence of GABP interaction with HS2 and its ability to regulate TMS1 expression in either the heterodimer or heterotetramer form.

At present the exact mechanism by which GABPα/β1 complex binding at HS2 promotes TMS1 expression is unknown. There are potentially two mechanisms by which GABPα/β1 could regulate TMS1 expression and its epigenetic state. The binding of GABPα/β1 to HS2 may have a direct effect on TMS1 promoter activity through interactions with the basal transcriptional machinery and/or other factors bound at the promoter to enhance transcription. The human TRAP/Mediator complex is an evolutionarily conserved multisubunit coactivator that plays a central role in regulating transcription from protein-encoding genes by serving as an adaptor between promoter-bound activators and the basal transcription machinery (154). Expression of the Aurora-A kinase gene requires a physical interaction between GABP and the targeting subunit of the TRAP/Mediator complex, TRAP220/MED1, which serves as an adapter between GABP and RNA polymerase (155). Consistent with these studies, the HS2 mediated activation of TMS1 promoter activity is dependent on the presence of both tandem ets

sites and GABP expression. Thus, GABP could be recruiting proteins to the TMS1 locus that serve as adapters to the basal transcription machinery.

Alternatively, GABP binding at HS2 could promote recruitment of chromatin modifying complexes to establish a permissive mark on the CpG island chromatin that either prevents the recruitment of repressive factors or provides a signal that opposes the spread of heterochromatin and DNA methylation. We have previously shown that the TMS1 CpG island is marked by hyperacetylated histones H3 and H4, and H3K4me2 in cells expressing TMS1, including the IMR90 fibroblast and MCF7 breast cancer cells (16, 141). Recently, our lab showed that the histone acetyltransferase hMOF and the MSL complex are recruited to and play a critical role in nucleosome positioning and maintenance of gene expression at the TMS1 locus (141). hMOF-mediated acetylation of H4K16 at two strongly positioned nucleosomes flanking the TMS1 CpG island and coincident with HS1 and HS3, is necessary to maintain nucleosome positioning and gene expression. Interestingly, knockdown of MSL1 of hMOF in MCF7 cells led to a loss of H4K16 acetylation and reversible silencing of TMS1 gene expression but had no impact on H3K9/14 acetylation or H3K4me2 at the CpG island, suggesting that other factors are involved in maintaining a permissive chromatin structure at the TMS1 CpG island (141).

The work described here identifies the GABP complex as one such potential factor necessary for the maintenance of a permissive chromatin structure at the TMS1 locus. GABP is enriched at HS2 in the center of the CpG island in cells where the gene is expressed, and downregulation of GABP leads to a concomitant loss in TMS1 expression. Furthermore, our preliminary studies on the TMS1-GFP transgenes suggest that GABP binding is necessary to maintain H3K9/14 acetylation at the TMS1 locus.

Levels of H3Ac were dramatically decreased at the mutant transgene locus in which GABPα binding sites are deleted relative to the wild type transgene. Loss of GABP binding sites had little effect on H3K4me2. This data suggest GABP may recruit HATs to the TMS1 locus that can acetylate histone H3 at lysine 9 and 14. GABP interacts with the histone acetyltransferase CBP/p300 (148, 153) and may serve to establish the zone of H3K9/14 acetylation detected across the TMS1 CpG island. The GABP complex interaction with CBP/p300 could have two interdependent effects on transcription from the TMS1 locus. GABP potentially recruits CBP/p300 to the TMS1 locus establishing a hyperacetylated chromatin structure which permits transcription. In addition, CBP/p300 could act as a scaffold between GABP and the basal transcription machinery located at the promoter. In this manner, GABP could be directly connected to the epigenetic changes at the locus and transcription.

A model where long-term absence of GABP binding leads to a subsequent shift in histone modification patterns and the acquisition of DNA methylation has proven difficult to test, as we have been unable to establish cell lines stably knocked down for GABPα. This difficulty is likely due to the central role of GABP in cell cycle progression and cell viability (138, 140, 156). The generation of RMCE cell lines (Chapter 3) with wild type and mutant TMS1-GFP loci stably integrated within the genome would be an alternate approach to answer this question, however, experiments thus far indicate that the transgenes are silenced within relatively few passages even in the presence of intact GABP sites. Therefore, the wild-type transgene locus can be silenced through a mechanism that is independent of GABP binding.

The interaction of GABPα with its recognition sequence is blocked by CpG methylation (45, 157-159). Yokomori *et. al.* (159) suggested that the sensitivity of GABPα to DNA methylation may be a mechanism for regulating sex-specific expression at the mouse Cyp 2d-9 locus, which is differentially methylated in males and females. The same group demonstrated an inverse relationship between DNA methylation, GABP binding and expression of the rat thyrotropin receptor gene (TSHR) in thyroid cells (45). Methylation of GABPα binding sites within a downstream enhancer has also been implicated in the regulation of the mouse M-lysozyme gene. During macrophage differentiation, the formation of a DNAse I hypersensitive site at a downstream enhancer correlates with DNA demethylation, binding of GABPα/β1 heterotetramer and transcriptional activation (158-160).

We similarly find that binding of GABP to HS2-55bp element is blocked by methylation. Methylation of CpG dinucleotides within either ets site blocked complex formation in DNA binding assays, and whereas GABP was enriched at HS2 in cells where the TMS1 CpG island is unmethylated (IMR90, MCF7), it was absent from cells in which the TMS1 CpG island is densely methylated (HMT.1E1, MDA-MB231). Interestingly, transgene experiments in mice have shown that the M-lysozyme downstream enhancer (MLDE) containing the GABP binding sites, which is genetically programmed to remain unmethylated during mouse development maintains a zone of open chromatin marked by hyperacetylated histones H3/H4 and H3K4me2 (161). Programmed *de novo* methylation of the element allows for the hypoacetylation of histones H3/H4, hypermethylation of H3K9 and silencing of a linked transgene.

There are some interesting parallels between the MLDE element and the chromatin structure of the TMS1 locus. In normal cells expressing TMS1, the CpG island bears a chromatin signature marked by hyperacetylated histones H3 and H4, H3K4me2 and lacking H3K9me2/3. The domain is bounded by distinct 5' and 3' boundaries that separate unmethylated CpG island DNA from densely methylated flanking DNA and manifest biochemically as DNAse I HS (HS1 and HS3). *De novo* methylation of the CpG island is accompanied by remodeling of the CpG-island specific hypersensitive sites, hypoacetylation of histones H3 and H4, hypomethylation of H3K4, hypermethylation of H3K9 and gene silencing (16). Of note, GABP binding at both the MLDE locus and the TMS1 locus occurs within a DNAse I hypersensitive site and correlates with gene activation.

Epigenetic silencing of TMS1 has been implicated in the pathogenesis of breast and a number of other tumor types, including glioblastomas, prostate carcinomas, non small cell lung cancers and melanomas (111, 114, 147, 162, 163). Loss of GABP binding due to spurious methylation of CpG dinucleotides within its recognition sequence may result in transcriptional downregulation and potentially a loss of histone acetylation, a prerequisite to the acquisition of other silencing marks (e.g. H3K9 methylation), putting the locus at risk of further methylation and stable silencing. Such 'seeding' of methylation and transcriptional downregulation promotes *de novo* methylation of the glutathione-S-transferase gene (GSTP1) CpG island in prostate cancer cells (164). In this regard, it is noteworthy that in comprehensive bisulfite sequencing analysis of normal human mammary epithelial cells, one of the CpGs in the GABP binding sites was methylated at twice the frequency of any other CpG site in the CpG island (in 25% of

alleles analyzed; no other CpG in the CpG island was methylated in more than 12%) and in MCF7 cells, it was one of only two CpG sites that showed any methylation (89). These data suggest that the CpG dinucleotides within GABPα binding sites might be preferentially targeted for methylation.

Loss of GABP binding also may be a consequence of aberrant methylation of the CpG island domain and serve to ensure the maintenance of a hypoacetylated state. Heavily methylated flanking DNA that surrounds the TMS1 CpG island never encroaches into the unmethylated CpG island DNA past a certain point within the 5' or 3' CpG island boundary in cell lines that express TMS1 (89). Once methylation crosses this threshold, aberrant methylation spreads throughout the entire CpG island leading to gene silencing due to the formation of a condensed chromatin structure. The loss of GABP binding would abolish active transcription from the TMS1 locus which is an opposing force against surrounding heterochromatic marks (e.g. DNA methylation).

TMS1 is normally highly expressed in cells of the monocyte and macrophage lineage and most epithelial cell types (117, 118, 165), however, little is known about the regulation of TMS1 transcription. Previous studies have shown that TMS1 is upregulated in response to inflammatory stimuli, such as IL-1β, IFN-γ, and LPS in macrophage (119, 120), although the mechanism of regulation has not been determined. In breast epithelial cells, TMS1 is upregulated in response to stress stimuli, including proinflammatory cytokines, such as TNFα and TRAIL (121), and in response to detachment from the substratum (166). Whereas the former is dependent on the NF-κB and JNK pathways; upregulation in response to detachment is independent of these pathways (166). One study indicated that TMS1 may be directly upregulated by p53, and identified a putative

p53 binding site in the TMS1 promoter region (167). However, subsequent studies suggest that the regulation of TMS1 by p53 may be more complex in that although treatment of breast epithelial cells or breast cancer cells wild-type for p53 with DNA damaging agents exhibit a modest upregulation in TMS1 promoter expression (121), this appeared to be independent of p53 in siRNA experiments and had little impact on activity of TMS1 promoter fragments containing the putative p53 element in reporter assays (121). Here we identify the GABPα/β1 complex as a positive regulator of the TMS1 expression through methylation sensitive binding at HS2.

Upon close examination of the two independent approaches used to identify TMS1/ASC, a correlation between GABP regulation and TMS1 expression can be observed. Masumoto et. al. identified ASC (Apoptosis-associated Speck-like protein containing a CARD) due to the observed co-localization of the protein to perinuclear aggregates or "specks" upon treatment with retinoic acid or etoposide. They also suggested that ASC/TMS1 played a role in triggering apoptosis in response to various chemotherapeutics and differentiation agents (168). Data has previously been shown for GABP dependent regulation of a distal enhancer of the CD18 promoter in myeloid cells in a retinoic acid dependent manner through the formation of an enhanceosome complex (148). Treatment of specific cell types with differentiation agents, such as retinoic acid, could stimulate the formation of an enhanceosome-like complex at the TMS1 locus through the looping of chromatin mediated by GABP and additional co-factors, causing a dose-dependent enhancement of expression.

In a completely independent approach, TMS1 was identified in a screen to isolate transcripts downregulated in cells overexpressing DNMT1 (110). Loss of expression

correlated with hypermethylation of the TMS1 CpG island. The absence of TMS1 expression in breast cancer cell lines correlated with dense methylation of the TMS1 CpG island (89). The methylation status of the TMS1 CpG island correlates with expression, as well as the ability of GABPα to binds its consensus sites (150). These data would lead one to propose the demethylation of CpG dinucleotides within the GABP binding sequence allow for the binding of GABP and active transcription.

The data presented in this study is important to the field of cancer genetics because it presents another regulatory component of TMS1 expression, the GABPα/β1 complex. The fact that TMS1 is silenced by aberrant methylation in a wide range of human cancers demonstrates the functional importance of silencing in order for carcinogenesis to occur. In normal and cancer cells, TMS1 has been shown to be involved in both the intrinsic and extrinsic apoptotic pathways (116, 121), pyroptosis (a proinflammatory form of programmed cell death) (119), and anoikis (cell death due to detachment from the ECM) (166). These data suggest that epigenetic silencing of TMS1 may contribute to the pathogenesis of human cancers by allowing cells to bypass normal cell death cues in the numerous stages of cancer progression. Subsequently, these cells are allotted time to acquire additional necessary mutations to become a malignant cell with the ability to metastasize throughout the body (87). Considering the widespread role of TMS1 in cell death it is understandable why this gene is targeted for silencing in cancer development.

TMS1 expression is restored in silent cell lines after treatment with the DNA methyltransferase inhibitor 5-aza-2'deoxycytidine (5-aza-CdR) and the histone deacetylases inhibitor Trichostatin A (TSA) (16). We have also observed the

reappearance of H3Ac and re-expression of TMS1 in MDA-MB231 cells in which the TMS1 locus is hypermethylated and silent upon treatment with 5-aza-CdR ((89) and unpublished data). Interestingly, GABP binding has been shown to have a role in the regulation of H3Ac levels at the TMS1-GFP locus in our RMCE clones (Chapter 3). These data lead one to propose that the demethylation of CpG dinucleotides within the GABP binding sequence might allow for the reestablishment of GABP binding and increase in H3Ac levels due to the recruitment of an unidentified HAT, possibly CBP/p300. 5-aza-CdR treatment of tumorgenic cells might restore GABP binding and activate expression from the TMS1 locus, which in turn would allow for the restoration of apoptotic pathways within the malignant cells. The identification of GABP as a positive regulator helps to shed light on the molecular mechanism by which TMS1 is expressed and provide insight for targeting TMS1 re-expression in tumorgenic cells.

Future Studies

Future studies involving the GABP complex role in activation of transcription from the TMS1 locus will involve the characterization of GABP interaction at the TMS1 locus following treatment with 5-aza-CdR. Treatment of the MDA-MB231 cell line with the demethylating agent 5-aza-CdR resulted in partial demethylation and expression of TMS1 (111). Through the use of bisulfite sequencing it was determined that there was a preference towards demethylation of the 5' end of the CpG island in the region surrounding and upstream of the transcription start site. (89). Therefore it was suggested that demethylation of the region surrounding the transcription start site is sufficient for

re-expression of TMS1, as transcription of TMS1 could occur in the presence of dense methylation of downstream sequences (89). Recently the reappearance of histone H3 acetylation has been shown at the TMS1 locus in MDA-MB231 cells after 5-aza-CdR treatment (unpublished data, Kagey, J. and Kappor-Vazirani, P.). It would therefore be of interest to determine if the CpG dinucleotides within GABP ets sequence sites are preferentially demethylated with 5-aza-CdR treatment and whether demethylation can restore GABP binding at the TMS1 locus and the impact on H3 acetylation.

We speculate that GABP is involved in the recruitment of CBP/p300 to the TMS1 locus because GABP-CBP is a published interaction (148, 153). ChIP analysis needs to be performed to determine if CBP/p300 is recruited to the TMS1 locus, and whether this recruitment is dependent on GABP. It is possible that the loss of GABP binding might facilitate transcriptional downregulation and potentially through the loss of histone acetylation.

Additional future studies will involve the identification of other proteins that interact with the TMS1 locus and could possibly play a role with GABP in TMS1 expression. GABP has been shown to work in concert with other transcription factors such as Sp1, Elf, PU.1, HCF-1, YY1 and C/EBP alpha to regulate target genes (151, 152, 169, 170). It is highly unlikely that GABP induced enhancement of TMS1 is purely due to GABP acting in an independent manner. More likely than not, GABP is activating expression from the TMS1 promoter through the recruitment of multiple transcription factors to the TMS1 locus. GABP binding at the TMS1 locus potentially helps to establish a chromatin environment that is permissive for transcription allowing the

recruitment of multiple transcription factors and chromatin modifying enzymes which help maintain a euchromatic state at the TMS1 locus.

It will also be of interest to explore TMS1 potential self-regulation. Transient transfection of MCF7 cells with a mutant TMS1-GFP expression construct consistently caused a decrease in endogenous TMS1 levels at the protein and RNA levels. The major question is how does exogenous expression of TMS1 affect expression of the endogenous TMS1 locus? It is known that TMS1 forms higher order protein structures.
Recent data supports a nuclear role for TMS1 where it localizes primarily in resting human monocytes and macrophages. Upon pathogen infection, TMS1 rapidly redistributes to the cytosol where it is a component in the assembly of macromolecular structures (e.g. pyroptosome, inflammasome) (171). ChIP analysis with an antibody to GFP would be able to determine if nuclear TMS1-GFP is recruited to the endogenous TMS1 locus. The mechanism behind this possible self-regulation has yet to be elucidated or even confirmed. However, the data consistently shows that the transient expression of a mutant copy of TMS1-GFP harboring GABPα binding site deletions has a repressive effect on expression from the endogenous TMS1 locus.

Conclusion

Epigenetic changes, such as aberrant methylation of cytosine residues, is one mechanism leading to the inappropriate silencing of a number of important genes in sporadic breast cancers as well as other cancers. To date the mechanism behind how aberrant DNA methylation occurs and ultimately leads to gene silencing has yet to be elucidated. DNA methylation can interfere with gene expression directly by inhibiting the binding of methylation-sensitive transcription factors, or by recruiting methyl-CpG-binding proteins that preferentially bind methyl CpG sites and compete in binding with methylation-insensitive transcription factors (52). One model for the silencing of genes by aberrant DNA methylation involves the light 'seeding' of methylation (103). Susan Clarkes' data showed that induced light seeding in the promoter/CpG island coupled with the inhibition of transcription promoted the spread of methylation within the CpG island of the glutathione S-transferase gene, GSTP1, in human prostatic carcinomas (172). Bisulfite sequencing analysis of CpG dinucleotides within actively transcribed genes demonstrate that gene expression is able to occur in the presence DNA methylation within the gene body (89). It is proposed that active transcription acts as a protective barrier from DNA methylation. Spurious methylation of CpG dinucleotides within transcription factor binding sites could reduce expression from the locus thus allowing the encroachment of DNA methylation from surrounding regions into the promoter region and the establishment of a permanently repressed and mitotically heritable chromatin state. In support of this model, spurious methylation of GABP binding sites would inhibit transcription from the locus, thus removing a protective barrier from the CpG island that would allow the propagation of heterochromatic marks into the locus and

gene silencing. CpG dinulceotides within GABP binding sites are methylated at a higher frequency than other CpG dinucleotides within the CpG island in breast cancer cells that retain TMS1 expression (150). In normal cells, these CpG sites are void of methylation. The methylation status of these CpG sites is critical to the binding of GABP at HS2 and subsequent gene expression.

Alternatively, proteins could be bound at the CpG island of actively transcribed genes that inhibit the establishment of DNA methylation within the region as well as oppose the spread of methylation into the region (106). In support of the second model, the chromatin structure of the TMS1 locus contains HS1 and HS3 forming directly at the boundaries between unmethylated CpG island DNA and the heavily methylated flanking DNA within the gene body. Gene expression occurs even in the presence of dense methylation surrounding the CpG island (16). The locus contains prominent peaks of H4K16Ac at HS1 and HS3, hyperacetylated histone H3K9/14 and H3K4me2 across the CpG island domain. GABP potentially recruits proteins to the locus to facilitate the establishment of an active open chromatin structure. The binding of these protein complexes would provide a euchromatic force within the CpG island DNA to oppose the heterochromatic forces at the CpG island boundaries.

Hypermethylation of tumor-suppressor genes is a common mechanism of inactivation which promotes carcinogenesis (12, 173). Genes subjected to epigenetic silencing, such as TMS1, remain structurally intact, thus the potential exists for re-expression. TMS1 was initially characterized as having a role in initiating apoptosis in response to various chemotherapeutics and differentiating agents (168). Subsequent studies have determined TMS1 has roles in both the intrinsic and extrinsic pathways of

apoptosis, anoikis (cell death due to detachment from the extracellular matrix), and pyroptosis (a proinflammatory form of cell death that occurs in response to invading pathogens) (116, 119-121, 166). The methylation-induced silencing of TMS1 in cancer cells would allow these cells to acquire drug resistance as well as confer a survival advantage by allowing them to escape from apoptosis triggered by various stimuli.

5-aza-2'-deoxycytidine has been shown to restore the expression of TMS1 in breast cancer cell lines, while 5-aza-2'-deoxycytidine and Zebularine in combination restores TMS1 expression in prostate cancer cells in which the TMS1 locus is methylated and silenced (16, 89, 162). Studies in which 5-aza-2'-deoxycytidine and Trichostatin A are used in combination determined that the addition of an HDAC inhibitor did not have a significant effect on gene re-expression in comparison to 5-aza-2'-deoxycytidine treatment alone (16). These data indicate that DNA methylation plays a primary role in the silencing of CpG-island associated genes. DNA-demethylating agents potentially can be used to achieve the re-expression of TMS1, which could re-sensitize tumors to anticancer agents and allow the activation of apoptosis.

In summary, I propose a model (Figure 1) in which in normal cells the TMS1 locus is unmethylated, hyperacetylated, and contains three CpG-island associated hypersensitive sites with two forming at the boundaries between heavily methylated flanking DNA and unmethylated CpG island DNA. The GABP complex binds HS2 in the unmethylated state and acts a positive regulator of transcription. GABP binding sites within HS2 and the binding of GABP may have a role in the maintenance of hyperacetylated histones at the TMS1 locus in that deletion of these sites correlates with a decrease in levels of acetylated histones detected at the locus. The binding of GABP at

HS2 potentially acts to protect the CpG island from aberrant methylation and maintain a hyperacetylated chromatin state that is permissive for transcription through the recruitment of HATs. It is possible that transcription from the locus provides an opposing force to the heterochromatic hypermethylated DNA surrounding the CpG island (Figure 1). Spurious methylation of GABP binding sites would inhibit complex binding at the locus, thus eradicating the opposing force against heterochromatin provided by gene expression, and allow the propagation of hypoacetylated histones and consequently gene silencing. The loss of H3Ac at K9 would provide a substrate for subsequent methylation by an H3K9 methyltransferase like G9a, bringing about the heterochromatic mark of H3K9me at the locus. Indeed, this mark is enriched at the TMS1 locus in cells in which the locus is hypermethylated, hypoacetylated and silenced (141). Our previous studies, the studies contained in this body of work, as well as future studies will help to elucidate the role cis-acting sequences and trans-acting factors in preventing genes from aberrant methylation and silencing, and may be representative of the events at other CpG-island associated genes silenced by methylation in cancer.

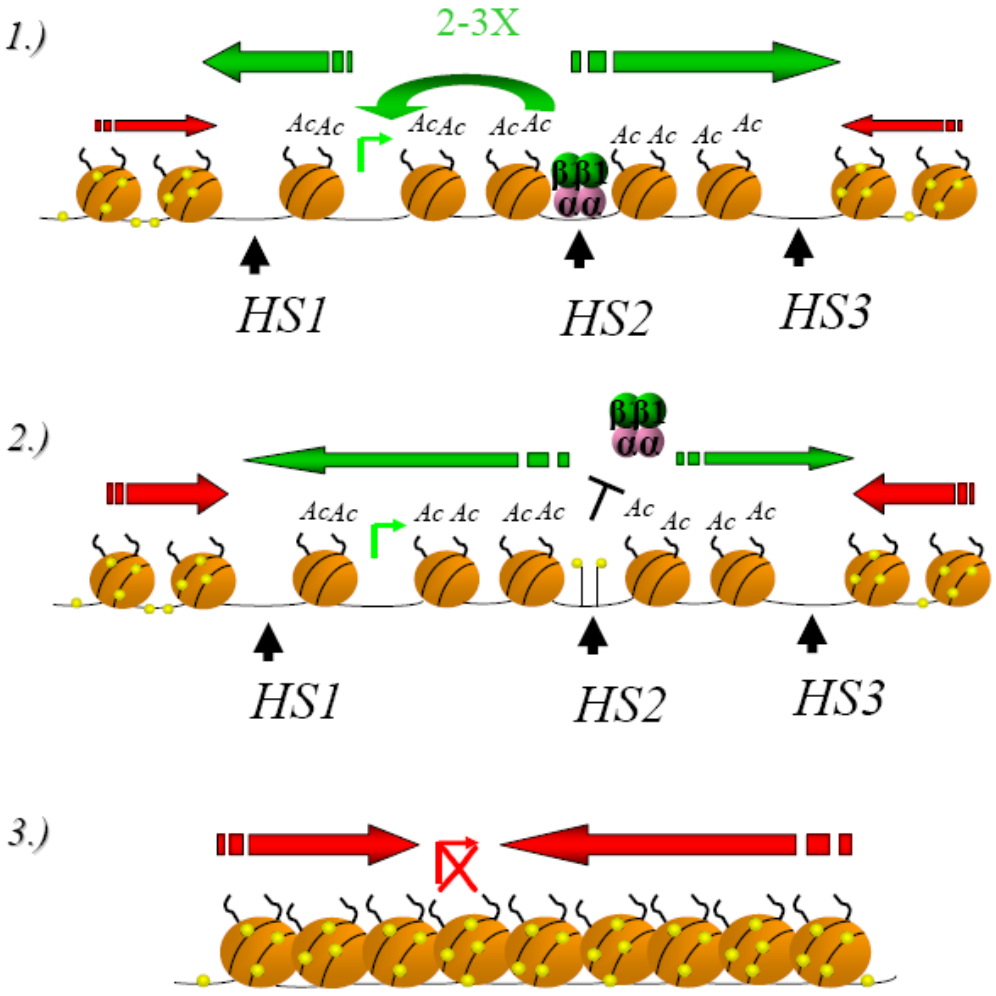

Figure 1: *Model for GABP functional role at the TMS1 locus.* 1.) GABP binds a 55bp region of HS2 at the TMS1 locus *in vitro* and *in vivo*. GABP activates transcription from the TMS1 promoter 2-3 fold in reporter assays. Transcription from the TMS1 locus is an opposing force (green arrows) to the heterochromatic hypermethylated DNA (red arrows) surrounding the CpG island DNA. 2.) Methylation of CpG dinucleotides within GABP binding sites inhibits GABP binding and causes a decrease in transcription. Consequently, the opposing force to heterochromatic DNA is diminished which shifts the balance in favor of repression. 3.) Due to the loss of GABP binding, transcription is decrease which allows the propagation of heterochromatic marks into the locus (e.g. hypoacetylated histones, DNA methylation, loss of HS) and subsequent gene silencing. Ac-acetylation, yellow circles-DNA methylation

Literature Cited

1. Plass C (2002) Cancer epigenomics *Hum Mol Genet* 11:2479-2488.
2. Bird A (2002) DNA methylation patterns and epigenetic memory *Genes Dev* 16:6-21.
3. Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development *Nat Rev Genet* 3:662-673.
4. Morgan HD, Santos F, Green K, Dean W,Reik W (2005) Epigenetic reprogramming in mammals *Hum Mol Genet* 14 Spec No 1:R47-58.
5. Turner BM (2007) Defining an epigenetic code *Nat Cell Biol* 9:2-6.
6. Penny GD, Kay GF, Sheardown SA, Rastan S,Brockdorff N (1996) Requirement for Xist in X chromosome inactivation *Nature* 379:131-137.
7. Cam HP, Chen ES,Grewal SI (2009) Transcriptional scaffolds for heterochromatin assembly *Cell* 136:610-614.
8. Boggs BA*, et al.* (2002) Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes *Nat Genet* 30:73-76.
9. Heard E*, et al.* (2001) Methylation of histone H3 at Lys-9 is an early mark on the X chromosome during X inactivation *Cell* 107:727-738.
10. Csankovszki G, Nagy A,Jaenisch R (2001) Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation *J Cell Biol* 153:773-784.
11. Richards EJ,Elgin SC (2002) Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects *Cell* 108:489-500.
12. Herman JG,Baylin SB (2003) Gene silencing in cancer in association with promoter hypermethylation *N Engl J Med* 349:2042-2054.
13. Isenberg I (1979) Histones *Annu Rev Biochem* 48:159-191.
14. Strahl BD,Allis CD (2000) The language of covalent histone modifications *Nature* 403:41-45.
15. Wolffe AP,Hayes JJ (1999) Chromatin disruption and modification *Nucleic Acids Res* 27:711-720.
16. Stimson KM,Vertino PM (2002) Methylation-mediated silencing of TMS1/ASC is accompanied by histone hypoacetylation and CpG island-localized changes in chromatin architecture *J Biol Chem* 277:4951-4958.
17. Bachman KE*, et al.* (2003) Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene *Cancer Cell* 3:89-95.
18. Berger SL (2007) The complex language of chromatin regulation during transcription *Nature* 447:407-412.
19. Mayer W, Niveleau A, Walter J, Fundele R,Haaf T (2000) Demethylation of the zygotic paternal genome *Nature* 403:501-502.
20. Oswald J*, et al.* (2000) Active demethylation of the paternal genome in the mouse zygote *Curr Biol* 10:475-478.
21. Howlett SK,Reik W (1991) Methylation levels of maternal and paternal genomes during preimplantation development *Development* 113:119-127.

22. Kafri T*, et al.* (1992) Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line *Genes Dev* 6:705-714.
23. Rougier N*, et al.* (1998) Chromosome methylation patterns during mammalian preimplantation development *Genes Dev* 12:2108-2113.
24. Li E, Bestor TH,Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality *Cell* 69:915-926.
25. Okano M, Bell DW, Haber DA,Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development *Cell* 99:247-257.
26. Larsen F, Gundersen G, Lopez R,Prydz H (1992) CpG islands as gene markers in the human genome *Genomics* 13:1095-1107.
27. Takai D,Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22 *Proc Natl Acad Sci U S A* 99:3740-3745.
28. Cross SH,Bird AP (1995) CpG islands and genes *Curr Opin Genet Dev* 5:309-314.
29. Coulondre C, Miller JH, Farabaugh PJ,Gilbert W (1978) Molecular basis of base substitution hotspots in Escherichia coli *Nature* 274:775-780.
30. Momparler RL (2003) Cancer epigenetics *Oncogene* 22:6479-6483.
31. Monk M (1990) Changes in DNA methylation during mouse embryonic development in relation to X-chromosome activity and imprinting *Philos Trans R Soc Lond B Biol Sci* 326:299-312.
32. Razin A,Cedar H (1993) DNA methylation and embryogenesis *EXS* 64:343-357.
33. Robertson KD (2001) DNA methylation, methyltransferases, and cancer *Oncogene* 20:3139-3155.
34. Goll MG*, et al.* (2006) Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2 *Science* 311:395-398.
35. Kareta MS, Botello ZM, Ennis JJ, Chou C,Chedin F (2006) Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L *J Biol Chem* 281:25893-25902.
36. Robertson KD*, et al.* (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors *Nucleic Acids Res* 27:2291-2298.
37. Chuang LS*, et al.* (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1 *Science* 277:1996-2000.
38. Pradhan S, Bacolla A, Wells RD,Roberts RJ (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation *J Biol Chem* 274:33002-33010.
39. Pradhan S*, et al.* (1997) Baculovirus-mediated expression and characterization of the full-length murine DNA methyltransferase *Nucleic Acids Res* 25:4666-4673.
40. Beard C, Li E,Jaenisch R (1995) Loss of methylation activates Xist in somatic but not in embryonic cells *Genes Dev* 9:2325-2334.
41. Li E, Beard C,Jaenisch R (1993) Role for DNA methylation in genomic imprinting *Nature* 366:362-365.
42. Bachman KE, Rountree MR,Baylin SB (2001) Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin *J Biol Chem* 276:32282-32287.

43. Kaneda M*, et al.* (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting *Nature* 429:900-903.
44. Hata K, Kusumi M, Yokomine T, Li E,Sasaki H (2006) Meiotic and epigenetic aberrations in Dnmt3L-deficient male germ cells *Mol Reprod Dev* 73:116-122.
45. Yokomori N, Tawata M, Saito T, Shimura H,Onaya T (1998) Regulation of the rat thyrotropin receptor gene by the methylation-sensitive transcription factor GA-binding protein *Mol Endocrinol* 12:1241-1249.
46. Bednarik DP*, et al.* (1991) DNA CpG methylation inhibits binding of NF-kappa B proteins to the HIV-1 long terminal repeat cognate DNA motifs *New Biol* 3:969-976.
47. Comb M,Goodman HM (1990) CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2 *Nucleic Acids Res* 18:3975-3982.
48. Iguchi-Ariga SM,Schaffner W (1989) CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation *Genes Dev* 3:612-619.
49. Kovesdi I, Reichel R,Nevins JR (1987) Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control *Proc Natl Acad Sci U S A* 84:2180-2184.
50. Prendergast GC, Lawe D,Ziff EB (1991) Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation *Cell* 65:395-407.
51. Sansom OJ, Maddison K,Clarke AR (2007) Mechanisms of disease: methyl-binding domain proteins as potential therapeutic targets in cancer *Nat Clin Pract Oncol* 4:305-315.
52. Ben-Hattar J, Beard P,Jiricny J (1989) Cytosine methylation in CTF and Sp1 recognition sites of an HSV tk promoter: effects on transcription in vivo and on factor binding in vitro *Nucleic Acids Res* 17:10179-10190.
53. Li B, Carey M,Workman JL (2007) The role of chromatin during transcription *Cell* 128:707-719.
54. Gal-Yam EN*, et al.* (2006) Constitutive nucleosome depletion and ordered factor assembly at the GRP78 promoter revealed by single molecule footprinting *PLoS Genet* 2:e160.
55. Ozsolak F, Song JS, Liu XS,Fisher DE (2007) High-throughput mapping of the chromatin structure of human promoters *Nat Biotechnol* 25:244-248.
56. Davey C, Pennings S,Allan J (1997) CpG methylation remodels chromatin structure in vitro *J Mol Biol* 267:276-288.
57. Reisman D, Glaros S,Thompson EA (2009) The SWI/SNF complex and cancer *Oncogene* 28:1653-1668.
58. Harikrishnan KN*, et al.* (2005) Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing *Nat Genet* 37:254-264.
59. Geiman TM, Sankpal UT, Robertson AK, Zhao Y,Robertson KD (2004) DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system *Biochem Biophys Res Commun* 318:544-555.

60. Jones PL*, et al.* (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription *Nat Genet* 19:187-191.
61. Nan X*, et al.* (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex *Nature* 393:386-389.
62. Jackson JP, Lindroth AM, Cao X,Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase *Nature* 416:556-560.
63. Tamaru H,Selker EU (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa *Nature* 414:277-283.
64. Bannister AJ*, et al.* (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain *Nature* 410:120-124.
65. Lachner M, O'Carroll D, Rea S, Mechtler K,Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins *Nature* 410:116-120.
66. Gendrel AV, Lippman Z, Yordan C, Colot V,Martienssen RA (2002) Dependence of heterochromatic histone H3 methylation patterns on the Arabidopsis gene DDM1 *Science* 297:1871-1873.
67. Jeddeloh JA, Stokes TL,Richards EJ (1999) Maintenance of genomic methylation requires a SWI2/SNF2-like protein *Nat Genet* 22:94-97.
68. Jemal A*, et al.* (2008) Cancer statistics, 2008 *CA Cancer J Clin* 58:71-96.
69. Hirschman J, Whitman S,Ansell D (2007) The black:white disparity in breast cancer mortality: the example of Chicago *Cancer Causes Control* 18:323-333.
70. Tammemagi CM (2007) Racial/ethnic disparities in breast and gynecologic cancer treatment and outcomes *Curr Opin Obstet Gynecol* 19:31-36.
71. Lund MJ*, et al.* (2009) Race and triple negative threats to breast cancer survival: a population-based study in Atlanta, GA *Breast Cancer Res Treat* 113:357-370.
72. Baylin SB (2005) DNA methylation and gene silencing in cancer *Nat Clin Pract Oncol* 2 Suppl 1:S4-11.
73. Gama-Sosa MA*, et al.* (1983) The 5-methylcytosine content of DNA from human tumors *Nucleic Acids Res* 11:6883-6894.
74. Feinberg AP, Gehrke CW, Kuo KC,Ehrlich M (1988) Reduced genomic 5-methylcytosine content in human colonic neoplasia *Cancer Res* 48:1159-1161.
75. Christman JK, Sheikhnejad G, Dizik M, Abileah S,Wainfan E (1993) Reversibility of changes in nucleic acid methylation and gene expression induced in rat liver by severe dietary methyl deficiency *Carcinogenesis* 14:551-557.
76. Pogribny IP*, et al.* (1995) Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl-deficient rats *Cancer Res* 55:1894-1901.
77. Feinberg AP,Vogelstein B (1983) Hypomethylation of ras oncogenes in primary human cancers *Biochem Biophys Res Commun* 111:47-54.
78. Fang JY*, et al.* (1996) Studies on the hypomethylation of c-myc, c-Ha-ras oncogenes and histopathological changes in human gastric carcinoma *J Gastroenterol Hepatol* 11:1079-1082.
79. Cui H*, et al.* (2003) Loss of IGF2 imprinting: a potential marker of colorectal cancer risk *Science* 299:1753-1755.
80. Walsh CP, Chaillet JR,Bestor TH (1998) Transcription of IAP endogenous retroviruses is constrained by cytosine methylation *Nat Genet* 20:116-117.

81. Alves G, Tatro A,Fanning T (1996) Differential methylation of human LINE-1 retrotransposons in malignant cells *Gene* 176:39-44.
82. Thayer RE, Singer MF,Fanning TG (1993) Undermethylation of specific LINE-1 sequences in human cells producing a LINE-1-encoded protein *Gene* 133:273-277.
83. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L,Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates *Nature* 395:89-93.
84. Xu GL*, et al.* (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene *Nature* 402:187-191.
85. Pogribny IP, Miller BJ,James SJ (1997) Alterations in hepatic p53 gene methylation patterns during tumor progression with folate/methyl deficiency in the rat *Cancer Lett* 115:31-38.
86. Maniatis T*, et al.* (1975) Recognition sequences of repressor and polymerase in the operators of bacteriophage lambda *Cell* 5:109-113.
87. Hanahan D,Weinberg RA (2000) The hallmarks of cancer *Cell* 100:57-70.
88. Jones PA (1999) The DNA methylation paradox *Trends Genet* 15:34-37.
89. Levine JJ, Stimson-Crider KM,Vertino PM (2003) Effects of methylation on expression of TMS1/ASC in human breast cancer cells *Oncogene* 22:3475-3488.
90. Knudson AG (2001) Two genetic hits (more or less) to cancer *Nat Rev Cancer* 1:157-162.
91. Jones PA,Laird PW (1999) Cancer epigenetics comes of age *Nat Genet* 21:163-167.
92. Jones PA,Baylin SB (2002) The fundamental role of epigenetic events in cancer *Nat Rev Genet* 3:415-428.
93. Herman JG (1999) Hypermethylation of tumor suppressor genes in cancer *Semin Cancer Biol* 9:359-367.
94. Merlo A*, et al.* (1995) 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers *Nat Med* 1:686-692.
95. Herman JG*, et al.* (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma *Proc Natl Acad Sci U S A* 91:9700-9704.
96. Jost JP,Bruhat A (1997) The formation of DNA methylation patterns and the silencing of genes *Prog Nucleic Acid Res Mol Biol* 57:217-248.
97. Kass SU, Pruss D,Wolffe AP (1997) How does DNA methylation repress transcription? *Trends Genet* 13:444-449.
98. Pfeifer GP, Tanguay RL, Steigerwald SD,Riggs AD (1990) In vivo footprint and methylation analysis by PCR-aided genomic sequencing: comparison of active and inactive X chromosomal DNA at the CpG island and promoter of human PGK-1 *Genes Dev* 4:1277-1287.
99. Kouzarides T (2007) Chromatin modifications and their function *Cell* 128:693-705.
100. Millar CB,Grunstein M (2006) Genome-wide patterns of histone modifications in yeast *Nat Rev Mol Cell Biol* 7:657-666.
101. Burgers WA, Fuks F,Kouzarides T (2002) DNA methyltransferases get connected to chromatin *Trends Genet* 18:275-277.

102. El Gazzar M*, et al.* (2008) G9a and HP1 couple histone and DNA methylation to TNFalpha transcription silencing during endotoxin tolerance *J Biol Chem* 283:32198-32208.
103. Clark SJ,Melki J (2002) DNA methylation and gene silencing in cancer: which is the guilty party? *Oncogene* 21:5380-5387.
104. Cameron EE, Baylin SB,Herman JG (1999) p15(INK4B) CpG island methylation in primary acute leukemia is heterogeneous and suggests density as a critical factor for transcriptional silencing *Blood* 94:2445-2451.
105. Melki JR, Vincent PC,Clark SJ (1999) Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia *Cancer Res* 59:3730-3740.
106. Issa JP (2004) CpG island methylator phenotype in cancer *Nat Rev Cancer* 4:988-993.
107. Fouse SD*, et al.* (2008) Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation *Cell Stem Cell* 2:160-169.
108. Weber M,Schubeler D (2007) Genomic patterns of DNA methylation: targets and function of an epigenetic mark *Curr Opin Cell Biol* 19:273-280.
109. Ooi SK*, et al.* (2007) DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA *Nature* 448:714-717.
110. Vertino PM, Yen RW, Gao J,Baylin SB (1996) De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase *Mol Cell Biol* 16:4555-4565.
111. Conway KE*, et al.* (2000) TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers *Cancer Res* 60:6236-6242.
112. Moriai R*, et al.* (2002) A proapoptotic caspase recruitment domain protein gene, TMS1, is hypermethylated in human breast and gastric cancers *Anticancer Res* 22:4163-4168.
113. Virmani A*, et al.* (2003) Aberrant methylation of the cyclin D2 promoter in primary small cell, nonsmall cell lung and breast cancers *Int J Cancer* 107:341-345.
114. Stone AR*, et al.* (2004) Aberrant methylation and down-regulation of TMS1/ASC in human glioblastoma *Am J Pathol* 165:1151-1161.
115. Parsons MJ, Patel P, Brat DJ, Colbert L,Vertino PM (2009) Silencing of TMS1/ASC promotes resistance to anoikis in breast epithelial cells *Cancer Res* 69:1706-1711.
116. McConnell BB,Vertino PM (2000) Activation of a caspase-9-mediated apoptotic pathway by subcellular redistribution of the novel caspase recruitment domain protein TMS1 *Cancer Res* 60:6243-6247.
117. Shiohara M*, et al.* (2002) ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils *Biochem Biophys Res Commun* 293:1314-1318.
118. Stehlik C*, et al.* (2003) Apoptosis-associated speck-like protein containing a caspase recruitment domain is a regulator of procaspase-1 activation *J Immunol* 171:6154-6163.

119. Fernandes-Alnemri T*, et al.* (2007) The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation *Cell Death Differ* 14:1590-1604.
120. Fernandes-Alnemri T, Yu JW, Datta P, Wu J,Alnemri ES (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA *Nature*
121. Parsons MJ,Vertino PM (2006) Dual role of TMS1/ASC in death receptor signaling *Oncogene* 25:6948-6958.
122. Rosmarin AG, Resendes KK, Yang Z, McMillan JN,Fleming SL (2004) GA-binding protein transcription factor: a review of GABP as an integrator of intracellular signaling and protein-protein interactions *Blood Cells Mol Dis* 32:143-154.
123. Leprince D*, et al.* (1983) A putative second cell-derived oncogene of the avian leukaemia retrovirus E26 *Nature* 306:395-397.
124. Watson DK, Ascione R,Papas TS (1990) Molecular analysis of the ets genes and their products *Crit Rev Oncog* 1:409-436.
125. Karim FD*, et al.* (1990) The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence *Genes Dev* 4:1451-1453.
126. Bassuk AG,Leiden JM (1997) The role of Ets transcription factors in the development and function of the mammalian immune system *Adv Immunol* 64:65-104.
127. Graves BJ,Petersen JM (1998) Specificity within the ets family of transcription factors *Adv Cancer Res* 75:1-55.
128. Oikawa T,Yamada T (2003) Molecular biology of the Ets family of transcription factors *Gene* 303:11-34.
129. Oikawa T (2004) ETS transcription factors: possible targets for cancer therapy *Cancer Sci* 95:626-633.
130. Brown TA,McKnight SL (1992) Specificities of protein-protein and protein-DNA interaction of GABP alpha and two newly defined ets-related proteins *Genes Dev* 6:2502-2512.
131. Sawa C*, et al.* (1996) Functional domains of transcription factor hGABP beta1/E4TF1-53 required for nuclear localization and transcription activation *Nucleic Acids Res* 24:4954-4961.
132. Gugneja S, Virbasius JV,Scarpulla RC (1995) Four structurally distinct, non-DNA-binding subunits of human nuclear respiratory factor 2 share a conserved transcriptional activation domain *Mol Cell Biol* 15:102-111.
133. Aurrekoetxea-Hernandez K,Buetti E (2000) Synergistic action of GA-binding protein and glucocorticoid receptor in transcription from the mouse mammary tumor virus promoter *J Virol* 74:4988-4998.
134. Flory E, Hoffmeyer A, Smola U, Rapp UR,Bruder JT (1996) Raf-1 kinase targets GA-binding protein in transcriptional regulation of the human immunodeficiency virus type 1 promoter *J Virol* 70:2260-2268.
135. Atlas E, Stramwasser M,Mueller CR (2001) A CREB site in the BRCA1 proximal promoter acts as a constitutive transcriptional element *Oncogene* 20:7110-7114.
136. Hoare S*, et al.* (1999) Identification of a GABP alpha/beta binding site involved in the induction of oxytocin receptor gene expression in human breast cells, potentiation by c-Fos/c-Jun *Endocrinology* 140:2268-2279.

137. Schweppe RE*, et al.* (2003) Purification and mass spectrometric identification of GA-binding protein (GABP) as the functional pituitary Ets factor binding to the basal transcription element of the prolactin promoter *J Biol Chem* 278:16863-16872.
138. Yang ZF, Mott S,Rosmarin AG (2007) The Ets transcription factor GABP is required for cell-cycle progression *Nat Cell Biol* 9:339-346.
139. Crook MF*, et al.* (2008) GA-binding protein regulates KIS gene expression, cell migration, and cell cycle progression *FASEB J* 22:225-235.
140. Ristevski S*, et al.* (2004) The ETS transcription factor GABPalpha is essential for early embryogenesis *Mol Cell Biol* 24:5844-5849.
141. Kapoor-Vazirani P, Kagey JD, Powell DR,Vertino PM (2008) Role of hMOF-dependent histone H4 lysine 16 acetylation in the maintenance of TMS1/ASC gene activity *Cancer Res* 68:6810-6821.
142. Lucas ME, Crider KS, Powell DR, Kapoor-Vazirani P,Vertino PM (2009) Methylation-sensitive regulation of TMS1/ASC by the ETS factor, GA-binding protein-alpha *J Biol Chem*
143. Levine J (2003) in *Genetics/ Molecular Biology* (Emory University, Atlanta), Vol. Ph.D., pp. 119.
144. Bannert N, Avots A, Baier M, Serfling E,Kurth R (1999) GA-binding protein factors, in concert with the coactivator CREB binding protein/p300, control the induction of the interleukin 16 promoter in T lymphocytes *Proc Natl Acad Sci U S A* 96:1541-1546.
145. Genuario RR,Perry RP (1996) The GA-binding protein can serve as both an activator and repressor of ribosomal protein gene transcription *J Biol Chem* 271:4388-4395.
146. Lucas ME, Stimson KM, Powell DR, Kapoor-Vazirani P,Vertino PM (2009) Regulation of TMS1/ASC by the ets factor, GABP *J Biol Chem*
147. Virmani A*, et al.* (2003) Aberrant methylation of TMS1 in small cell, non small cell lung cancer and breast cancer *Int J Cancer* 106:198-204.
148. Resendes KK,Rosmarin AG (2006) GA-binding protein and p300 are essential components of a retinoic acid-induced enhanceosome in myeloid cells *Mol Cell Biol* 26:3060-3070.
149. Strunnikova M*, et al.* (2005) Chromatin inactivation precedes de novo DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter *Mol Cell Biol* 25:3923-3933.
150. Lucas M.E., Crider K.S., Kapoor-Vizarani P., Powell D.R.,P.M. V (2009) Methylation-Sensitive Regulation of TMS1/ASC by the ets factor, GABP *Journal of Biological Chemistry*
151. DeKoter RP*, et al.* (2007) Regulation of the interleukin-7 receptor alpha promoter by the Ets transcription factors PU.1 and GA-binding protein in developing B cells *J Biol Chem* 282:14194-14204.
152. Takahashi K*, et al.* (2008) Cooperative regulation of Fc receptor gamma-chain gene expression by multiple transcription factors, including Sp1, GABP, and Elf-1 *J Biol Chem* 283:15134-15141.

153. Kang HS*, et al.* (2008) Identification and structural characterization of a CBP/p300-binding domain from the ETS family transcription factor GABP alpha *J Mol Biol* 377:636-646.
154. Malik S,Roeder RG (2000) Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells *Trends Biochem Sci* 25:277-283.
155. Udayakumar TS, Belakavadi M, Choi KH, Pandey PK,Fondell JD (2006) Regulation of Aurora-A kinase gene expression via GABP recruitment of TRAP220/MED1 *J Biol Chem* 281:14691-14699.
156. Imaki H*, et al.* (2003) Cell cycle-dependent regulation of the Skp2 promoter by GA-binding protein *Cancer Res* 63:4607-4613.
157. Cardot P*, et al.* (1994) Purification and characterization of nuclear factors binding to the negative regulatory element D of human apolipoprotein A-II promoter: a negative regulatory effect is reversed by GABP, an Ets-related protein *Biochemistry* 33:12139-12148.
158. Nickel J, Short ML, Schmitz A, Eggert M,Renkawitz R (1995) Methylation of the mouse M-lysozyme downstream enhancer inhibits heterotetrameric GABP binding *Nucleic Acids Res* 23:4785-4792.
159. Yokomori N, Kobayashi R, Moore R, Sueyoshi T,Negishi M (1995) A DNA methylation site in the male-specific P450 (Cyp 2d-9) promoter and binding of the heteromeric transcription factor GABP *Mol Cell Biol* 15:5355-5362.
160. Ammerpohl O, Short ML, Asbrand C, Schmitz A,Renkawitz R (1997) Complex protein binding to the mouse M-lysozyme gene downstream enhancer involves single-stranded DNA binding *Gene* 200:75-84.
161. Hashimshony T, Zhang J, Keshet I, Bustin M,Cedar H (2003) The role of DNA methylation in setting up chromatin structure during development *Nat Genet* 34:187-192.
162. Collard RL, Harya NS, Monzon FA, Maier CE,O'Keefe DS (2006) Methylation of the ASC gene promoter is associated with aggressive prostate cancer *Prostate* 66:687-695.
163. Guan X*, et al.* (2003) ASC/TMS1, a caspase-1 activating adaptor, is downregulated by aberrant methylation in human melanoma *Int J Cancer* 107:202-208.
164. Song JZ, Stirzaker C, Harrison J, Melki JR,Clark SJ (2002) Hypermethylation trigger of the glutathione-S-transferase gene (GSTP1) in prostate cancer cells *Oncogene* 21:1048-1061.
165. McConnell BB,Vertino PM (2004) TMS1/ASC: the cancer connection *Apoptosis* 9:5-18.
166. Parsons MJ, Patel, P., Brat, D. J., Colbert, L. and Vertino, P. M. (2009) Silencing of TMS1/ASC promotes resistance to anoikis in breast epithelial cells *Cancer Research* In Press:
167. Ohtsuka T*, et al.* (2004) ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway *Nat Cell Biol* 6:121-128.
168. Masumoto J*, et al.* (2003) ASC is an activating adaptor for NF-kappa B and caspase-8-dependent apoptosis *Biochem Biophys Res Commun* 303:69-73.
169. Delehouzee S*, et al.* (2005) GABP, HCF-1 and YY1 are involved in Rb gene expression during myogenesis *Genes Cells* 10:717-731.

170. Shimokawa T,Ra C (2005) C/EBPalpha functionally and physically interacts with GABP to activate the human myeloid IgA Fc receptor (Fc alphaR, CD89) gene promoter *Blood* 106:2534-2542.
171. Bryan NB, Dorfleutner A, Rojanasakul Y,Stehlik C (2009) Activation of inflammasomes requires intracellular redistribution of the apoptotic speck-like protein containing a caspase recruitment domain *J Immunol* 182:3173-3182.
172. Lin X*, et al.* (2001) GSTP1 CpG island hypermethylation is responsible for the absence of GSTP1 expression in human prostate cancer cells *Am J Pathol* 159:1815-1826.
173. Cortez CC,Jones PA (2008) Chromatin, cancer and drug therapies *Mutat Res* 647:44-51.