

*MBD2 and DNA Methylation-Associated GSTP1 Repression*

## **Methyl-CpG Binding Domain Protein 2 Represses Transcription from Hypermethylated $\pi$ -Class Glutathione S-Transferase Gene Promoters in Hepatocellular Carcinoma Cells\***

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## Summary

During the pathogenesis of human hepatocellular carcinoma (HCC), the CpG island encompassing the  $\pi$ -class glutathione *S*-transferase gene (*GSTP1*) becomes hypermethylated. Repression of transcription accompanying CpG island hypermethylation has been proposed to be mediated by methyl-CpG binding domain (MBD) proteins. We report here that inhibition of transcription from hypermethylated *GSTP1* promoters in Hep3B HCC cells, which fail to express *GSTP1* mRNA or GSTP1 polypeptides, appears to be mediated by MBD2. Treatment of Hep3B cells with 5-aza-deoxycytidine (5-aza-dC), a methyltransferase inhibitor, activated *GSTP1* expression, while treatment with trichostatin A (TSA), a histone deacetylase inhibitor, had little effect. To more precisely assess the contribution of the pattern of *GSTP1* CpG island methylation on *GSTP1* mRNA expression, Hep3B cells were treated for 72 hours with 5-aza-dC and then subjected to limiting dilution cloning. Bisulfite sequencing was used to map the methylation patterns of the *GSTP1* promoter region in *GSTP1* expressing and non-expressing clones. In the clones expressed *GSTP1* mRNA by Northern blot analysis and quantitative RT-PCR, widespread demethylation of at least one *GSTP1* allele was evident. Chromatin immunoprecipitation experiments revealed the presence of MBD2, but not Sp1, at the *GSTP1* promoter in Hep3B cells. In contrast, Sp1 was detected at the *GSTP1* promoter in a *GSTP1*-expressing Hep3B 5-aza-dC subclone. To test whether MBD2 might be responsible for the inhibition of *GSTP1* transcription from hypermethylated *GSTP1* promoters, siRNAs were used to reduce MBD2 polypeptide levels in Hep3B cells. *SssI*-catalyzed methylation of *GSTP1* promoter sequences resulted in diminished luciferase reporter activity after transfection into Hep3B

cells. However, when hypermethylated *GSTP1* promoter sequences were transfected into Hep3B cells that had been treated with siRNA targeting *MBD2* mRNA, no repression of luciferase reporter expression was evident. These findings implicate MBD2 in the repression of *GSTP1* expression associated with *GSTP1* CpG island hypermethylation in HCC cells.

DNA methylation changes stereotypically accompany carcinogenesis. While global DNA methylation levels decrease in cancer, CpG island sequences tend to be targets for hypermethylation (1,2). Hypermethylation of CpG islands appears responsible for the transcriptional silencing of critical genes, including caretaker genes and tumor suppressor genes, that may be selected during the development of cancer and during cancer progression in a variety of human cancers (3). Normal CpG dinucleotide methylation patterns are thought to be established during embryonic development, and maintained by DNMT1, a DNA methyltransferase targeted to DNA replication sites via interaction with PCNA<sup>1</sup> (4-6). In hepatocellular carcinoma (HCC), a number of genes are known to accumulate aberrant CpG island hypermethylation changes, including *GSTP1*, *p16*, and *E-Cadherin* (7-12). The mechanism by which CpG island hypermethylation, amidst global hypomethylation, appears in HCC, or in other human cancers, has not been established.

The mechanism by which hypermethylation at CpG islands acts to suppress the transcription of genes is an area of active research. Methyl-CpG-binding domain (MBD) family proteins have been identified as candidate mediators of this process. All MBD

proteins contain a conserved methyl-CpG-binding domain, first identified in MeCP2 (13-15). MeCP2 is capable of binding DNA containing a single 5-mCpG. MeCP2 also contains a transcriptional repression domain (TRD) that permits interaction with Sin3a and histone deacetylase (HDAC) to form one postulated 5-mCpG-dependent transcriptional repression complex (13,16,17). MBD2, which also binds DNA containing 5-mCpG, has been shown to be a part of another transcriptional repression complex, containing HDACs, MBD3, and Mi-2/NuRD proteins (18). The Mi-2/NuRD complex appears capable of disrupting histone-DNA interactions to promote chromatin remodeling (19). For cancer genes inactivated by somatic CpG island hypermethylation, the role of HDACs in transcriptional silencing is unclear. For some genes, treatment with trichostatin A, an HDAC inhibitor, is sufficient to reverse the repression associated with CpG island hypermethylation, while for other genes, TSA treatment alone is unable to restore gene expression (17,20,21). Treatment with a combination of TSA and a DNMT inhibitor has been reported to trigger the reactivation of some cancer genes carrying somatic CpG island hypermethylation (20).

*GSTP1*, encoding the human  $\pi$ -class glutathione *S*-transferase, has been reported to be targeted for somatic CpG island hypermethylation in 85% of HCCs, as well as in 30% of breast cancers and in >90% of prostate cancers (7,22-24). Hep3B cells, a human HCC line, have been shown to contain densely hypermethylated *GSTP1* CpG island sequences, and to be devoid of *GSTP1* mRNA (7). We report here that in Hep3B HCC cells, repression of *GSTP1* associated with CpG island hypermethylation was reversed by treatment with 5-aza-

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deoxycytidine (5-aza-dC), but was unaffected by treatment with TSA. Furthermore, when Hep3B cells were treated with 5-az-dC for 72 hours, subjected to limiting dilution cloning, and then assessed by quantitative RT-PCR for *GSTP1* mRNA and by bisulfite genomic sequencing for *GSTP1* CpG island methylation, Hep3B-5-aza-dC clones which express *GSTP1* mRNA all contained at least one unmethylated *GSTP1* CpG island allele. Hep3B-5-aza-dC clones that failed to reverse hypermethylation at the *GSTP1* CpG island failed to express *GSTP1* mRNA. Repression of transcription from hypermethylated *GSTP1* CpG island alleles in Hep3B cells appeared to be mediated by MBD2. Chromatin immunoprecipitation analysis of nucleoprotein complexes in Hep3B cells revealed a preferential association of MBD2, but not MeCP2, with hypermethylated *GSTP1* promoter sequences. Furthermore, when siRNAs targeting *MBD2* and *MeCP2* mRNAs were introduced by transfection into Hep3B cells, cells with reduced MBD2 levels, but not cells with reduced MeCP2 levels, were incapable of repressing transcription from *SssI*-methylated *GSTP1* promoters. All of the data collected suggest that MBD2, perhaps via an HDAC-independent pathway, acts to repress transcription from hypermethylated *GSTP1* promoters in HCC cells.

## EXPERIMENTAL PROCEDURES

*Culture of Hep3B cells and Treatment with 5-aza-dC and TSA*- Human Hep3B cells were

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propagated *in vitro* in MEM growth medium (Mediatech) supplemented with 1.0 mM sodium pyruvate and 10% fetal bovine serum (Invitrogen) (25). Treatment of Hep3B cells with 5-aza-dC (Sigma) and with TSA (Sigma) was accomplished by adding the drugs to complete growth medium at a concentration of 1 $\mu$ M for 5-aza-dC and 100ng/mL for TSA. Stock solutions of 5-aza-dC, 1 mM in DMSO, and TSA, 100 mg/mL in ethanol, were stored at –20°C. To isolate individual Hep3B clones with varying degrees of *GSTP1* CpG island methylation, Hep3B cells were treated with 5-aza-dC for 72 hours, and then maintained in complete growth medium without drug. The cells were then subjected to limiting dilution cloning in drug-free medium using 96-well culture plates. Eight Hep3B-5-aza-dC clones were isolated and maintained in complete growth medium without drug for at least three months before assessment for *GSTP1* expression and *GSTP1* CpG island hypermethylation.

#### *Detection of GSTP1 mRNA by Northern Blot Analysis and GSTP1 Polypeptides by*

*Immunoblot-* Total RNA was isolated from Hep3B cells and Hep3B-5-aza-dC clones using an RNeasy<sup>®</sup> RNA isolation kit (Qiagen), and quantified using an orcinol assay (26). Purified RNAs (20 $\mu$ g) were electrophoresed on 1.5% agarose gels in the presence of 2.2M formaldehyde, transferred to Zeta-Probe<sup>®</sup> GT (Bio-Rad) filters and then assessed for *GSTP1* mRNA levels by hybridization with specific <sup>32</sup>P-labeled *GSTP1* cDNA probes (ATCC), prepared using Rediprime II DNA labelling system (Amersham). Following hybridization at 50°C for 3 hours in Quick-Hybe<sup>®</sup> (Stratagene) hybridization solution containing heat-denatured salmon sperm

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DNA (Sigma) at 200 µg/mL, blots were washed twice with 2X SSC (1X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS at room temperature and once with 0.1X SSC and 0.1% SDS at 60EC. Blot were exposed to X-OMAT" film (Kodak) at 70EC. Immunoblot analyses for GSTP1 polypeptides in total protein extracts from cultured HCC cells were accomplished as described previously (7,27).

#### *Detection of GSTP1 mRNA using Quantitative Reverse Transcriptase-Polymerase Chain*

*Reaction (RT-PCR)*- Total RNA from each of the Hep3B-5-aza-dC was subjected to quantitative RT-PCR for *GSTP1* mRNA using an iCycler iQ" multi-color real time PCR system (Bio-Rad) (28). Before PCR, cDNA was synthesized from 1 µg of RNA using and Omniscript® RT kit (Qiagen). PCR reactions included cDNA from 125 ng RNA, sense (5'-GGGCAGTGCCTTCACATAGT-3') and antisense (5'-GGAGACCTCACCTGTACCA-3') primers, and the Master Mix from a QuantiTect" SYBR Green® PCR kit (Qiagen). PCR cycles were 94EC for 30 seconds, 60EC for 30 seconds, and 72EC for 30 seconds. Cloned *GSTP1* cDNA was used as a standard for quantification. As an internal control, *TBP* mRNA, encoding the TATA binding protein, was also detected by quantitative RT-PCR, using specific sense (5'-CACGAACCACGGCACTGATT-3') and antisense (5'-TTTTCTTGCTGCCAGTCTGGAC-3') primers. PCR cycles for *TBP* cDNA detection were 94EC for 30 seconds, 55EC for 30 seconds, and 72EC for 30 seconds. Each of the PCR assays was run in triplicate and the *GSTP1* and *TBP* copy numbers were estimated from the threshold amplification cycle numbers using software supplied with the iCycler IQ" Thermal Cycler.

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### *Bisulfite Genomic Sequencing for Mapping GSTP1 CpG island DNA Methylation Patterns in*

*Genomic DNA*- Genomic DNA was isolated from Hep3B cells using the DNeasy" kit (Qiagen)

To map <sup>5-m</sup>CpG dinucleotides in the *GSTP1* CpG island region, a bisulfite genomic sequencing approach was undertaken (24,29). Purified DNAs (500 µg) were treated with *EcoRI*, admixed with salmon sperm DNA (2.5 µg), and then treated with sodium bisulfite as previously described

(27). The bisulfite-treated DNA was then subjected to two rounds of PCR to amplify *GSTP1*

CPG island alleles, using primers that recognize the antisense strand of *GSTP1* after bisulfite

conversion. First round PCR primers were: 5'-AC<sup>A</sup>/<sub>G</sub>CAACCTATAATTCCACCTACTC-3' and 5'-

GT<sup>T</sup>/<sub>C</sub>GGGAGTTGGGGTTTGATGTTG-3'; second round PCR primers were 5'-AACCTAAACCACAAC<sup>A</sup>/<sub>G</sub>TAAACAT

TTGGTTTTATGTTGGGAGTTTTGA-3'. PCR reaction conditions have been described previously. To permit

DNA sequencing of individual *GSTP1* CpG island alleles, PCR products were purified by

electrophoresis on 1% agarose gels, using the Qiaquick" gel extraction kit (Qiagen), ligated into

pCR2.1pTOPO<sup>®</sup> cloning vectors (Invitrogen), and then introduced into TOP 10<sup>®</sup> One Shot

competent bacteria (Invitrogen). Plasmid DNA, isolated using Qiaprep<sup>®</sup> Spin Miniprep kit, was

subjected to DNA sequence analysis using M13 sequencing primers.

*Chromatin Immunoprecipitation*- 8-10 × 10<sup>6</sup> growing Hep3B cells or Hep3B-5-aza-dC clone

#5 cells were fixed with 1% formaldehyde for 10 minutes at 37EC, washed twice in ice-cold

PBS containing protease inhibitor cocktail III (Calbiochem), and then recovered by scraping and

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centrifugation at 325g for 5 minutes (30). Cell pellets were resuspended in 200  $\mu$ L chromatin lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1), incubated for 10 minutes 4EC, and then sonicated for 40 seconds, using a Versonic micropipette sonicator, to reduce DNA fragment size to 400-600 bp. The sonicated chromatin lysates were clarified by centrifugation at 14,000g for 10 minutes at 4EC, and the supernates added to 10 mL precipitation buffer (0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, and 1.2mM EDTA in 16.7mM Tris-HCl, pH 8.1). After preclearing with 400  $\mu$ L ssDNA/protein A agarose (Upstate Biotech) by incubation at 4EC for 30 minutes with gentle agitation and then centrifugation at 325g for 1 minute, nucleoprotein complexes were aliquotted into 1 mL fractions for immunoprecipitation using specific antibodies to MBD2, MeCP2, Sp1, acetylated histone H4 (all from Upstate Biotechnology). 5-10  $\mu$ g of antibody solution was added to 1 mL of nucleoprotein complexes. Antibody-nucleoprotein complex mixtures were incubated at 4EC overnight with gentle agitation. Immunocomplexes were collected by the addition of 60  $\mu$ L salmon sperm DNA/protein A agarose (Upstate Biotech), incubation for 1 hour at 4EC with rotation, then centrifugation at 325g for 1 minute. Pelleted immunocomplexes were washed with low salt wash buffer (0.1% SDS, 0.1% Triton X-100, 150 mM NaCl, and 2 mM EDTA in 20 mM Tris-HCl, pH 8.1), high salt wash buffer, (0.1% SDS, 0.1% Triton X-100, 500 mM NaCl, and 2 mM EDTA in 20 mM Tris-HCl, pH 8.1), LiCl/NP-40/deoxycholate buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, and 1mM EDTA in 10mM Tris-HCl, pH 8.1), and with TE buffer (1 mM EDTA in 10 mM tris-HCl, pH 8.0). Nucleoprotein complexes were eluted from the final washed immunoprecipitates in 250  $\mu$ L of 1% SDS and 0.1 M NaHCO<sub>3</sub> by incubation at room temperature for 15 minutes. To reverse

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the cross-linking of protein to DNA, 20 µL of 5 M NaCL was added to the eluted immunoprecipitates and incubated at 65EC overnight. Proteins were digested by adding 2 µL proteinase K (10 mg/mL), 10 µL 0.5 M EDTA, and 20 µL Tris-HCl, pH 6.5, and incubating the mixture for 1 hour at 45EC. DNA was recovered by phenol/chloroform extraction and EtOH precipitation. To detect *GSTP1* CpG island DNA, quantitative PCR was undertaken using the iCycler iQ" multi-color real time PCR system (BioRad) and a Quantitect" SYBR® Green Master Mix. The PCR cycles were 95EC for 15 minutes, then 40 cycles of 94EC for 30 seconds, 60EC for 30seconds, and 72EC for 45 seconds. The *GSTP1* primers were sense: 5-GACCTGGGAAAGAGAGGGAAAG-3 and antisense: 5-ACTCACTGGTGGCGAAGACT-3. PCR assays were run in trip and *GSTP1* copy numbers were estimated from the threshold amplification cycle numbers using software supplied with the iCycler IQ" Thermal Cycler. The amount of *GSTP1* DNA recovered by immunoprecipitation with specific antibodies was expressed as a percent of the total amount of *GSTP1* DNA in nucleoprotein complexes before immunoprecipitation.

*siRNA "Knock-Down" Experiments-* siRNA duplexes were designed targeting AA(N<sub>19</sub>)UU sequences in the open reading frames of mRNA encoding MBD2 and MeCP2; siRNA targeting mRNA encoding lamin A was already available (Dharmacon) (31). Selected siRNA target sequences were also submitted to BLAST searches against other human genome sequences to ensure target specificity. 21-nt RNAs were chemically synthesized by Dharmacon and obtained in annealed form. The following target sequences were used: *MBD2* mRNA (5-AAGAGGAUGGAUUGCCCGGCC-3), *MeCP2* mRNA (target 5-AAGCAUGAGCCCGUGCAGCCA-3), and *lamin A* mRNA

AAGGACCUGGAGGCUCUGCUG-3). siRNAs were transfected into Hep3B cells using Oligofectamine" (Invitrogen). An additional siRNA transfection was undertaken 48 hours later to increase the efficiency of target protein "knock-down." The effectiveness of the target protein reduction was monitored by immunoblot analysis. Total protein extracts, prepared by lysing cells in 2% SDS, were electrophoresed on 10% polyacrylamide gels (NOVEX) in MES running buffer (NOVEX), transferred to nitrocellulose membranes (Invitrogen), and then probed with antibodies to MBD, MeCP2, and acetylated histone H4, and lamin A/C (Upstate Biotech, Calbiochem), using horseradish peroxidase-conjugated anti-IgG (Amersham), as previously described.

*Transient Transfection Analysis of the Effects of CpG Island Hypermethylation on GSTP1*

*Promoter Activity-* *GSTP1* promoter-*luciferase* reporter constructs (pGL3 vector-Promega), containing sequences from -408 5' of the *GSTP1* transcription start site to +36 , were treated with *SssI*, a CpG methylase, or left untreated, and then transfected into Hep3B cells using LipofectAMINE" (Invitrogen) (24). After 48 hours, the transfected cells were lysed using Passive Lysis Buffer (Promega). Luciferase reporter activity was assayed using a Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega) and a 1450 MicroBeta<sup>®</sup> JET luminometer (Wallac). A *CMV* promoter- $\beta$ -*galactosidase* reporter construct was used to monitor transfection efficiency.

## RESULTS

*Reactivation GSTP1 Expression from Hep3B Cells Containing Hypermethylated GSTP1 CpG Islands using a DNA Methyltransferase Inhibitor-* Although  $\pi$ -class glutathione *S*-transferases appear to be upregulated in rat models of HCC, the human  $\pi$ -class glutathione *S*-transferase is not expressed in human HCCs, or by the human HCC cell line, Hep3B (5). In normal human liver tissue, the *GSTP1* CpG island is unmethylated, even though *GSTP1* is usually not expressed (7,32). However, in Hep3B HCC cells, the *GSTP1* promoter has been previously shown to be heavily methylated (7). When we subjected Hep3B cells to treatment with 5-aza-dC, a DNMT inhibitor, or with TSA, an HDAC inhibitor, *GSTP1* expression was evident only in cells treated with 5-aza-dC within 72 hours (Fig. 1). To ascertain whether combinations of 5-aza-dC and TSA might be more effective at restoring *GSTP1* expression than 5-aza-dC alone, Hep3B cells were treated sequentially for 48 hours with 5-aza-dC and/or TSA to a total 96 hours of drug treatment (Fig.2). Prior exposure of Hep3B cells to TSA did not potentiate the effect of 5-aza-dC on increasing *GSTP1* mRNA levels, nor did exposure to TSA after 5-aza-dC treatment have any synergistic effect on restoring *GSTP1* expression. These findings are consistent with a role for *GSTP1* CpG island hypermethylation in the silencing of *GSTP1* transcriptional in Hep3B cells, and further suggest that the mechanism of methylation-associated inhibition of *GSTP1* transcription may not require HDACs.

*Bisulfite Genomic Sequencing Analysis of Individual Hep3B clones Isolated after 5-aza-dC Treatment-* To better characterize the effect of CpG island hypermethylation on *GSTP1* expression in Hep3B cells, we treated the cells for 72 hours with 5-aza-dC, and then isolated individual Hep3B-5-aza-dC subclones by limiting dilution cloning. Eight Hep3B-5-aza-dC

clones were recovered, and three of the clones expressed significant levels of *GSTP1* mRNA by Northern blot (Fig. 3) and quantitative RT-PCR analyses (Fig. 4). DNMT inhibitors have been reported to restore the expression of many genes repressed by CpG island hypermethylation in cancer cells; however, a reduction in gene expression and a remethylation of CpG island sequences after prolonged passage in cell culture have been described for some such genes (33). In T24 bladder cancer cells, restoration of *p16* mRNA expression by treatment with 5-aza-dC was completely reversed after 21 population doublings in the absence of the inhibitor (33). Remarkably, in Hep3B-5-aza-dC clones #2, #5, and #7, *GSTP1* mRNA expression remained stable for at least 8 months during continuous cell culture in the absence of 5-aza-dC (not shown). Whether the apparent differences in propensity for CpG island remethylation between *p16* in T24 cells and *GSTP1* in Hep3B cells can be attributed to differences in selection for loss of *p16* versus *GSTP1* expression, or to some mechanism, has not been established (24). When genomic DNA from each of the clones was subjected to bisulfite genomic sequencing, capable of mapping 5-mCpG dinucleotides at the *GSTP1* transcriptional regulatory region, a reduction in *GSTP1* CpG island hypermethylation was evident only in Hep3B-5-aza-dC clones which expressed *GSTP1* mRNA (Fig. 5). Of interest, the PCR primers for bisulfite genomic sequence analysis flanked a polymorphic [ATAAA]<sub>n</sub> repeat located –506 bp 5' of the *GSTP1* transcription start site, permitting discrimination of CpG dinucleotide methylation patterns on individual *GSTP1* alleles. Hep3B cells, and four of the Hep3B-5-aza-dC clones, were found to contain three different [ATAAA]<sub>n</sub> repeat lengths, consistent with an instability of this polymorphic repeat at some point during the development, isolation, and propagation of Hep3B HCC cell line. Four of

the Hep3B-5-aza-dC clones appeared to have lost *GSTP1* allele 3 after 5-aza-dC treatment and limiting dilution cloning. For the three Hep3B-5-aza-dC clones with a reduction in *GSTP1* CpG island methylation, the reduction was restricted to one *GSTP1* allele. Furthermore, each of the three Hep3B-5-aza-dC subclones displayed reversal of *GSTP1* CpG island hypermethylation at different *GSTP1* alleles, suggesting no bias of 5-aza-dC action toward any specific *GSTP1* allele. Hep3B-5-aza-dC clones #2 and #5 appeared to have completely reversed *GSTP1* hypermethylation at a *GSTP1* allele; Hep3B-5-aza-dC clone #7 both only partially reversed the *GSTP1* CpG island hypermethylation. These experiments further support a direct correlation between *GSTP1* CpG island hypermethylation *GSTP1* repression.

*Chromatin Immunoprecipitation Analyses of Active and Inactive GSTP1 Promoters-* To ascertain whether MBD family proteins formed transcriptional repression complexes at hypermethylated *GSTP1* CpG islands, we performed chromatin immunoprecipitation analyses of Hep3B cells, which contain only hypermethylated *GSTP1* CpG islands and fail to express *GSTP1* mRNA, and of Hep3B-5-aza-dC clone #5 cells, which contain one unmethylated *GSTP1* CpG island allele and express high levels of *GSTP1* mRNA (Fig. 6). Antibodies to Sp1 and acetylated histone H4 were used to detect active transcription complexes, while antibodies to MBD2 and MeCP2 were used to detect repressive transcription complexes. For Hep3B cells, MBD2, and perhaps a small amount of MeCP2, but not Sp1 nor acetylated H4, were detected at the *GSTP1* promoter. In contrast, for Hep3B-5-aza-dC clone #5 cells, Sp1 and a small amount of acetylated histone H4 were detected at the *GSTP1* promoter on at least some *GSTP1* alleles, while reduced levels of MBD2 and MeCP2 were present. Differences in levels of *GSTP1*-

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MBD2 and *GSTP1*-MeCP2 nucleoprotein complexes in Hep3B cells versus Hep3B-5-aza-dC clone #5 cells were not attributable to differences in MBD2 or MeCP2 polypeptide levels, as both proteins were readily detected in protein extracts from both cell lines. Thus, the presence of at least one unmethylated *GSTP1* promoter allele permitted the assembly of a *GSTP1*-protein complexes containing the transcriptional *trans*-activator Sp1 and histone H4, while the exclusive presence of hypermethylated *GSTP1* promoter alleles only allowed the assembly of *GSTP1*-protein complexes containing MBD family proteins.

*SssI-Catalyzed CpG Methylation of GSTP1 Promoter Sequences Reduces GSTP1 Promoter Activity in Both Hep3B Cells and Hep3B-5-aza-dC Clone #5 Cells-* Although the stable high level *GSTP1* expression induced by brief treatment of Hep3B-5-aza-dC clone #5 cells with 5-aza-dC was correlated with reversal of *GSTP1* CpG island hypermethylation and with the assembly of an Sp1-containing complex at the *GSTP1* promoter, in principle, DNA methylation-independent increases in *trans*-activation activity might still contribute to the high level of *GSTP1* expression in the Hep3B-5-aza-dC clone #5 cells. Also, nucleoside DNMT inhibitors have been reported to increase the expression of some genes in the absence of alterations in DNA methylation (34-36). Nonetheless, when unmethylated *GSTP1* promoter sequences were transfected into Hep3B and Hep3B-5-aza-dC clone #5 cells, similar luciferase reporter expression levels, normalized to *CMV* promoter-driven  $\beta$ -galactosidase reporter expression levels, were seen (Fig. 4). Furthermore, 5-aza-dC treatment did not appear to increase the activity of unmethylated *GSTP1* promoters (Fig. 7). However, when *GSTP1* promoter sequences were treated with the CpG methylase *SssI* before transfection, a marked

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reduction in luciferase reporter expression in both Hep3B and Hep3B-5-aza-dC clone #5 cells was observed (Fig. 4).

#### *siRNA “Knock-down” of MBD2 and MeCP2 in Hep3B cells Implicates MBD2 in*

*Hypermethylation-Dependent GSTP1 Repression-* To test whether MBD2, MeCP2, or both MBD2 and MeCP2 were responsible for repression of transcription from hypermethylated *GSTP1* promoter alleles, the levels of the MBD family proteins were reduced in Hep3B cells by treatment with specific siRNAs, capable of degrading mRNA transcripts in a target specific manner (Fig. 8). The effectiveness of siRNA “knock-down” of MBD family proteins was monitored by immunoblot analysis. Remarkably, when an *SssI*-methylated *GSTP1* promoter was transfected into Hep3B cells treated with siRNA targeting *MBD2* mRNA, the reduction in MBD2 protein levels appeared to render the Hep3B cells incapable of repressing *GSTP1* transcription. Finally, a combined “knock-down” of MBD2 levels and MeCP2 levels in Hep3B cells was no better at reversing alleviating repression from hypermethylated *GSTP1* promoters as a “knock-down” of MBD2 alone. Considered along with the finding that MBD2 is located at hypermethylated *GSTP1* promoters in Hep3B cells, the lack of repression activity for hypermethylated *GSTP1* promoters in Hep3B cells with reduced MBD2 levels strongly suggest that MBD2 likely mediates CpG island hypermethylation-dependent repression of *GSTP1*.

### DISCUSSION

All of the data collected suggest that CpG island hypermethylation is responsible for

transcriptional silencing *GSTP1* in Hep3B cells. *GSTP1* repression was reversed by treatment with 5-aza-dC treatment, a DNMT inhibitor, but not with TSA, an HDAC inhibitor. For certain genes silenced by CpG island hypermethylation, treatment with TSA can activate gene expression, indicating the participation of HDACs in transcriptional repression, while for other genes, TSA alone is incapable of restoring gene function (17,20,21). In our chromatin immunoprecipitation experiments, we detected more acetylated histones in association with active *GSTP1* promoters, (in Hep3B-5-aza-dC clone #5 cells) than in association with inactive *GSTP1* promoters (in parent Hep3B cells), suggesting that histone acetylation likely accompanies *GSTP1* transcription. However, the absence of TSA stimulation of *GSTP1* expression from hypermethylated *GSTP1* promoters in Hep3B cells suggests that HDACs do not play a critical role in CpG island hypermethylation-associated *GSTP1* repression.

The mechanism by which aberrant methylation patterns develop in cancer cells has not been determined. Several cytosine methyltransferase genes have been identified and characterized. *Dnmt1*, *Dnmt3a*, and *Dnmt3b* are each essential for mouse development (37). DNMT1, thought to function as a maintenance methyltransferase in normal cells, is present at replication foci during the S-phase of the cell cycle (6). Under certain circumstances, DNMT1 may also promote *de novo* CpG dinucleotide methylation (38,39). In cancer cells, DNMT3a and DNMT3b may contribute to both *de novo* and to maintenance DNA methylation in some way. HCT116 colorectal carcinoma cells carrying disrupted *DNMT1* alleles display only a ~20% reduction in 5-mCpG (40). Furthermore, although DNMT3a and DNMT3b seem to be expressed at high levels during embryonic development and at low levels in normal adult tissues,

increased expression of *DNMT3a* and *DNMT3b* mRNA has been reported in human cancers (41-43). Nonetheless, DNMT1 has been more prominently implicated in the earliest stages of cancer development than other DNMTs. *Apc<sup>min/+</sup>* mice develop fewer intestinal polyps when crossed to a *Dnmt1<sup>+/-</sup>* background (44). Dnmt1 also appears essential for *fos* transformation of rat fibroblasts *in vitro*, as forced Dnmt1 over-expression recapitulates the *fos*-transformed phenotype and anti-sense *Dnmt1* cDNA inhibits transformation by *fos* (38). Despite these observations, whether DNMT1 acts to facilitate cancer development through catalyzing *de novo* CpG island methylation has not been irrefutably established. DNMT1 has been reported to act as a transcriptional repressor, independent of DNA methyltransferase activity, by forming complexes with HDAC2 and DMAP1 (45).

The methyl-CpG-binding domain proteins (MBDs) all contain sequences similar to a 60-80 amino acid motif shown in MeCP2 to be responsible for 5-mCpG binding . MeCP2, the first of these proteins to be identified, acts as a transcriptional repressor via interaction with Sin3A and HDACs. *MECP2* mutations are responsible for Rett syndrome, a neurodegenerative disorder in females, and for severe mental retardation and death in males. Targeted disruption of *Mecp2* leads to a similar phenotype in mice (46). In our studies, although we found a small amount of MeCP2 in association with hypermethylated *GSTP1* promoters in Hep3B cells by chromatin immunoprecipitation, we were unable to increase *GSTP1* promoter activity by treatment with siRNA targeting *MECP2* mRNA in the setting of *GSTP1* promoter hypermethylation. These data suggest that MeCP2 is not required for transcriptional repression

from hypermethylated *GSTP1* promoters in Hep3B cells. MeCP1, a multi-component transcriptional repression complex, contains MBD2, MBD3, and Mi-2/NuRD proteins (18). MBD2 most likely serves to recruit MeCP1 complex proteins to hypermethylated transcriptional promoters, because MBD3 does not bind 5-mCpG (15,47). Perhaps for this reason, cells from *Mbd2*<sup>-/-</sup> mice are unable to prevent transcription from exogenous hypermethylated SV40 promoters, while *Mbd3*<sup>-/-</sup> cells remain capable of promoter hypermethylation-associated repression (47). We detected MBD2 bound to hypermethylated *GSTP1* promoters in Hep3B cells by chromatin immunoprecipitation, and we showed that a reduction in MBD2 levels prevented repression of *GSTP1* associated with hypermethylation. Confirming these findings, preliminary data collected using MCF-7 breast cancer cells suggests that siRNA “knock-down” of MBD2 triggers induction of *GSTP1* mRNA expression despite the presence of hypermethylated *GSTP1* promoters.<sup>2</sup> The participation of MBD2 in the silencing of hypermethylated *GSTP1* promoters in Hep3B cells may provide a partial explanation for the failure of TSA to reactivate *GSTP1* expression. MeCP1 contains the SWI/SNF helicase Mi-2 as well as HDACs (18). Co-transfection of cDNA encoding a dominant-negative Mi-2 has been reported to alleviate repression from a model hypermethylated transcriptional promoter (18). For *GSTP1* in Hep3B cells, CpG island hypermethylation appears to cause transcriptional silencing by an MBD2-dependent, but HDAC-independent mechanism. Perhaps Mi-2, or some other MBD2-associated protein, may help MBD2 mediate repression from hypermethylated *GSTP1* promoters. In all, our findings support a critical role for MBD2 in the silencing of genes targeted

for somatic CpG island hypermethylation during cancer development.

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<sup>1</sup>The abbreviations used are: 5-aza-dC, 5-aza-deoxycytidine; TSA, trichostatin A; PCNA, proliferating cell nuclear antigen; TRD, transcriptional repression domain; HDAC, histone deacetylase.

<sup>2</sup>Lin, X. and Nelson, W.G., manuscript in preparation.

**Fig. 1. Activation of *GSTP1* expression in Hep3B cells by treatment with 5-aza-dC.** Hep3B cells were treated with 5-aza-dC (1 $\mu$ M) or TSA (100ng/mL ) for 24, 48, and 72 hours. Expression of *GSTP1* mRNA was monitored by Northern blot analysis using *GSTP1* cDNA as a probe (upper panel). Ethidium bromide staining of rRNA was used as a loading control (bottom panel).

**Fig. 2. Treatment with 5-aza-dC, but not with TSA, restores *GSTP1* expression in Hep3B cells.**

Hep3B cells were treated sequentially for two 48 hour periods with 5-aza-dC (1 $\mu$ M), with TSA (100ng/mL), or with neither drug. *GSTP1* expression was monitored by Northern blot analysis as described for Fig 1.

**Fig. 3. *GSTP1* expression by Hep3B clones isolated after 72 hours of 5-aza-dC exposure.**

Hep3B cells were treated with 5-aza-dC (1 $\mu$ M) for 72 hours, then maintained in complete growth medium without drug thereafter. The drug-treated cells were subjected to limiting dilution cloning and eight individual clones were isolated. *GSTP1* expression was monitored by Northern blot analysis as described for Fig. 1.

**Fig. 4. Quantitative RT-PCR for *GSTP1* mRNA in Hep3B-5-aza-dC clones.** Hep3B-5-aza-dC subclones were assessed for *GSTP1* mRNA levels using quantitative RT-PCR. Displayed in the ratio *GSTP1* mRNA/*TBP* mRNA for each Hep-3B-5-aza-dC subclone.

**Fig. 5. Bisulfite genomic sequencing for mapping of 5-mCpG in the *GSTP1* CpG island in Hep3B cells and in Hep3B-5-aza-dC clones.** Genomic DNA was isolated from Hep3B cells and Hep3B-5-aza-dC subclones, bisulfite treated, amplified by PCR targeting the *GSTP1* CpG island, and then subjected to DNA sequence analysis as described in the EXPERIMENTAL PROCEDURES. A minimum of 10 individual PCR product clones were sequenced for each cell type. PCR product clones with less than 85% conversion of non-CpG cytosines to thymines

were not considered. Sequences sharing [ATAAA]<sub>n</sub> repeat lengths were collected as individual *GSTP1* alleles; three alleles were present in Hep3B cells. For each *GSTP1* [ATAAA]<sub>n</sub> repeat allele in each cell type, a 5-mCpG map, with the percentage of clones containing 5-mCpG at specific sites, is displayed.

**Fig. 6. Chromatin immunoprecipitation targeting the *GSTP1* promoter in Hep3B and Hep3B-5-aza-dC clone #5 cells.** Nucleoprotein complexes recovered from Hep3B and Hep3B-5-aza-dC clone #5 cells were subjected to immunoprecipitation with antibodies against Sp1, acetylated H4, MBD2, and MeCP2. The immunoprecipitates were then analyzed by quantitative PCR targeting the *GSTP1* promoter; the amount of *GSTP1* promoter DNA in the immunoprecipitates is shown as a percentage of a total *GSTP1* promoter DNA. Levels of GSTP1, MeCP2, MBD2, and acetylated H4 in Hep3B and Hep3B-5-aza-dC clone #5 cells were monitored using immunoblot analysis.

**Fig. 7. Inhibition of *GSTP1* promoter activity in Hep3B cells by *SssI*-catalyzed CpG methylation.** Hep3B cells were transfected with unmethylated and methylated *GSTP1-P1* (a full-length *GSTP1* promoter-*luciferase* reporter construct), along with a *CMV* promoter- $\beta$ -*galactosidase* control. To determine whether 5-aza-dC triggered *trans*-activation of unmethylated *GSTP1* promoters, *GSTP-P1*-transfected Hep3B cells were treated with 5-aza-dC (1 $\mu$ M). Luciferase activity, normalized to  $\beta$ -galactosidase activity, assessed 48 hours after transfection, is shown.

**Fig. 8. Alleviation of repression from hypermethylated *GSTP1* promoters after targeted reduction of MBD2 using siRNA.** Hep3B cells were repeatedly transfected with siRNA targeting mRNA encoding lamin A, MBD2, and MeCP2. Reductions in the levels of targeted proteins were monitored by immunoblot analysis. After two siRNA treatments, *SssI*-methylated *GSTP-PI* promoter activity was assessed via transient transfection as described for Fig. 7.

**Figure 1**

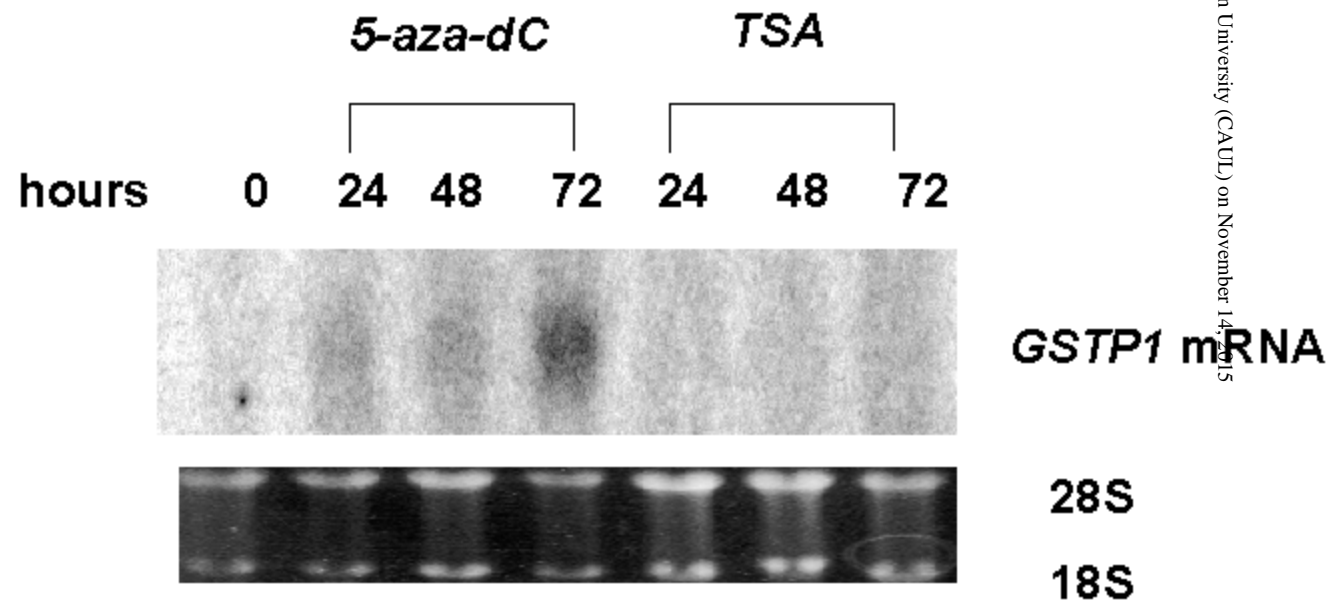


Figure 2

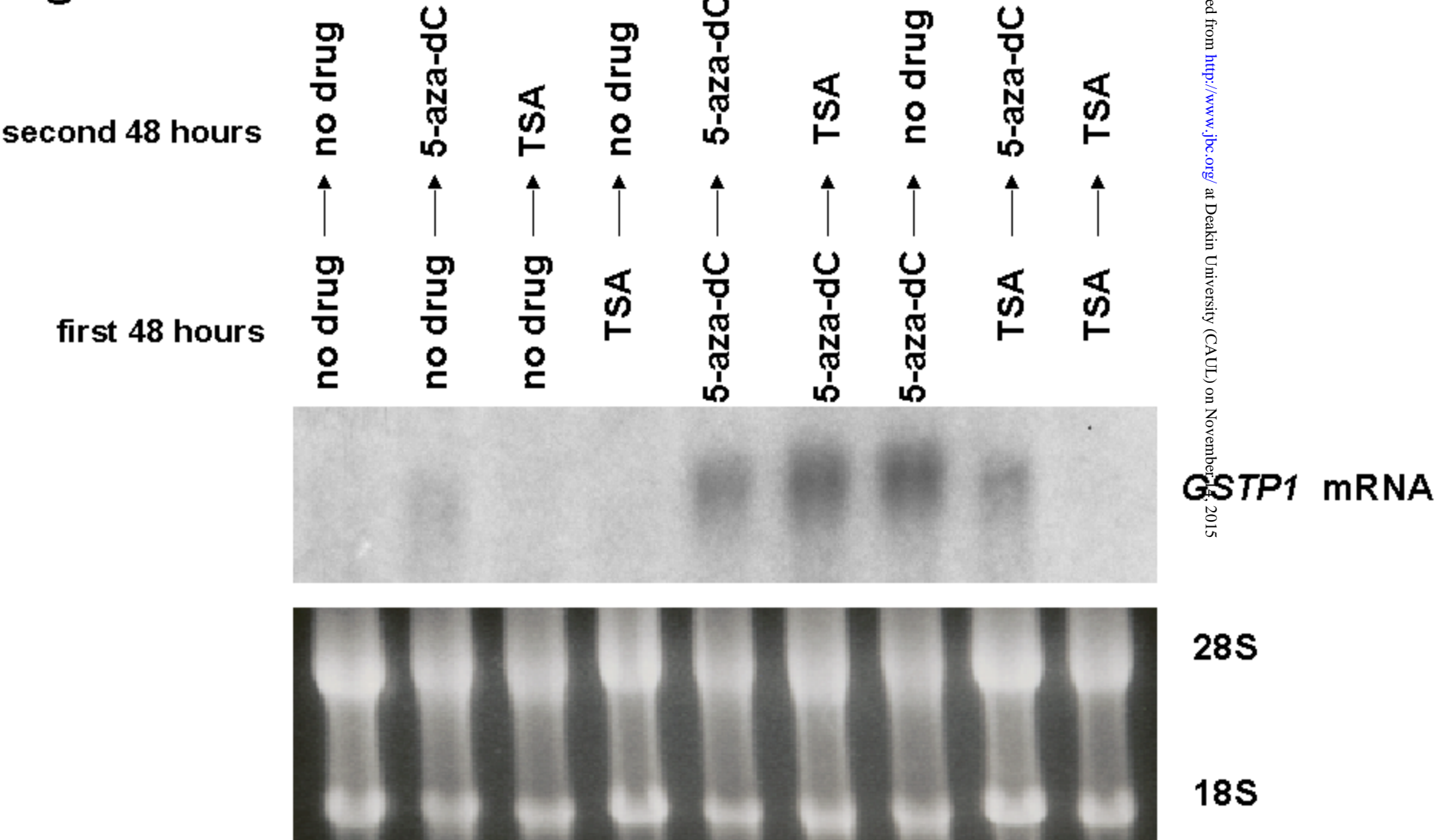


Figure 3

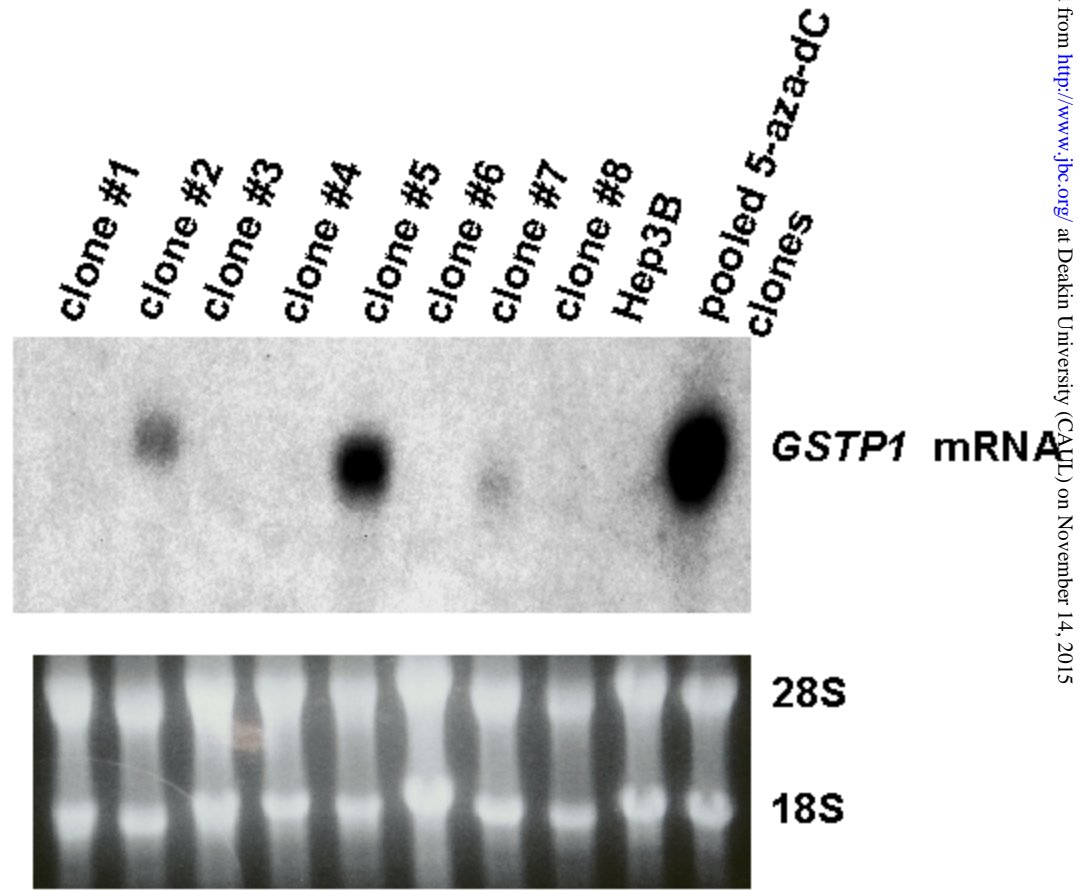
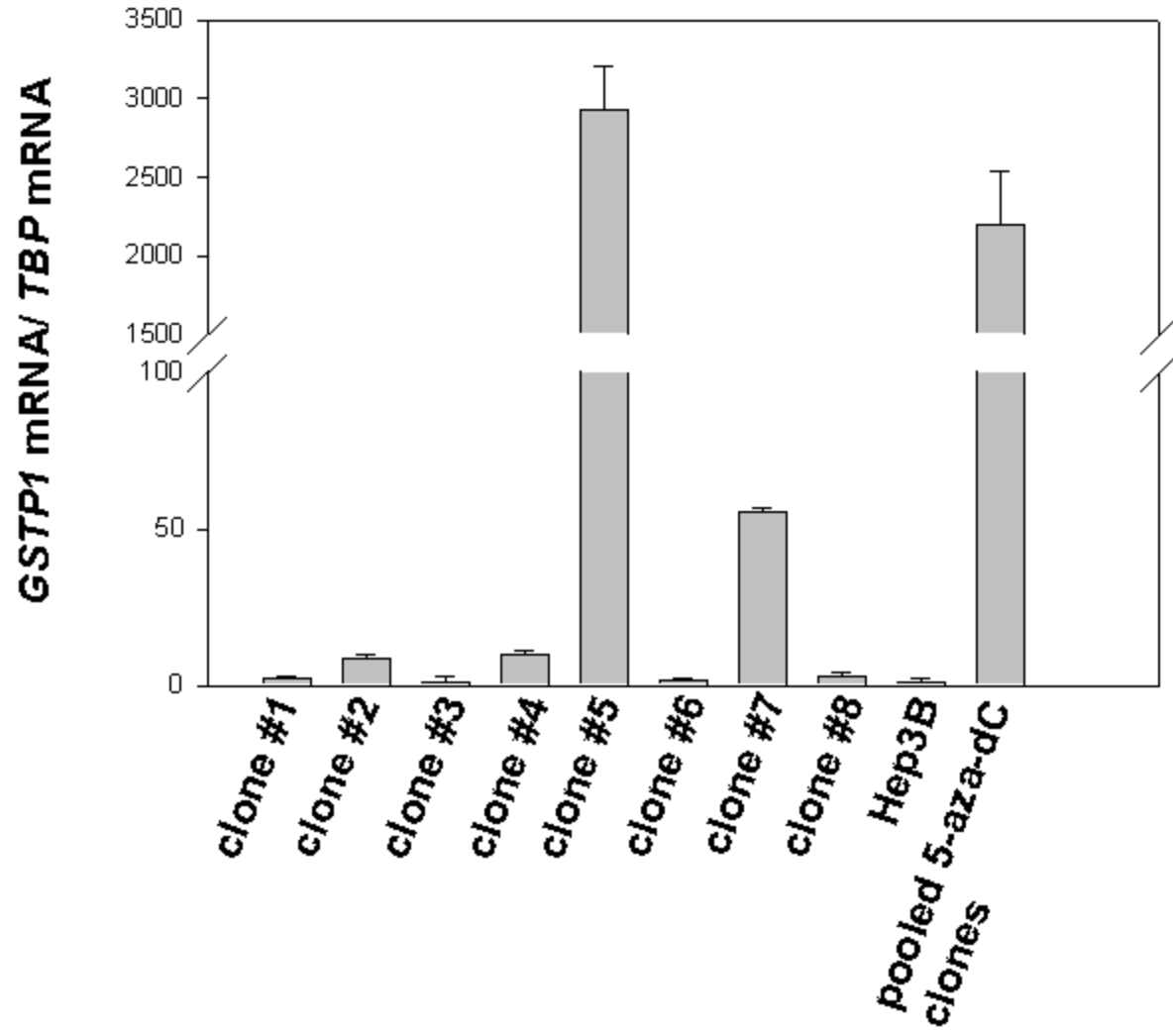
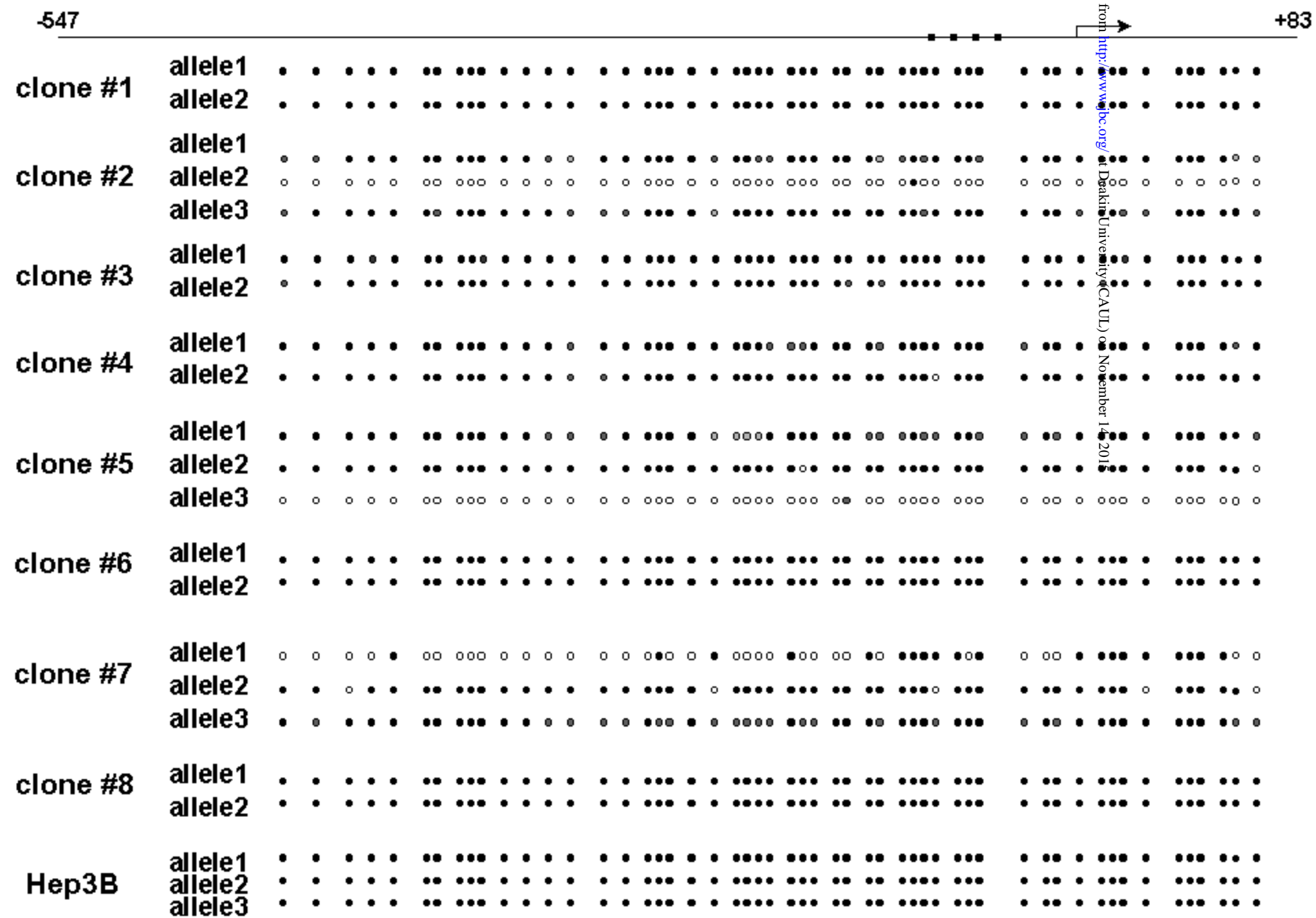


Figure 4



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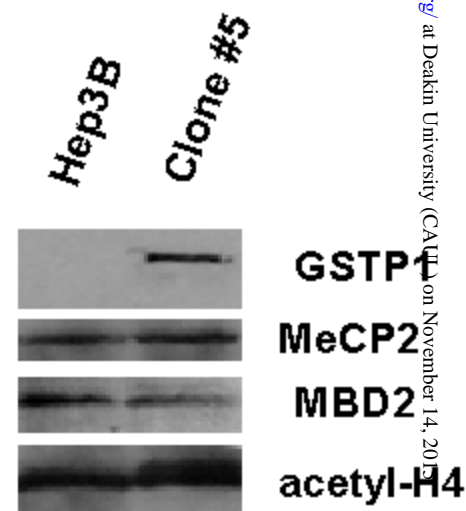
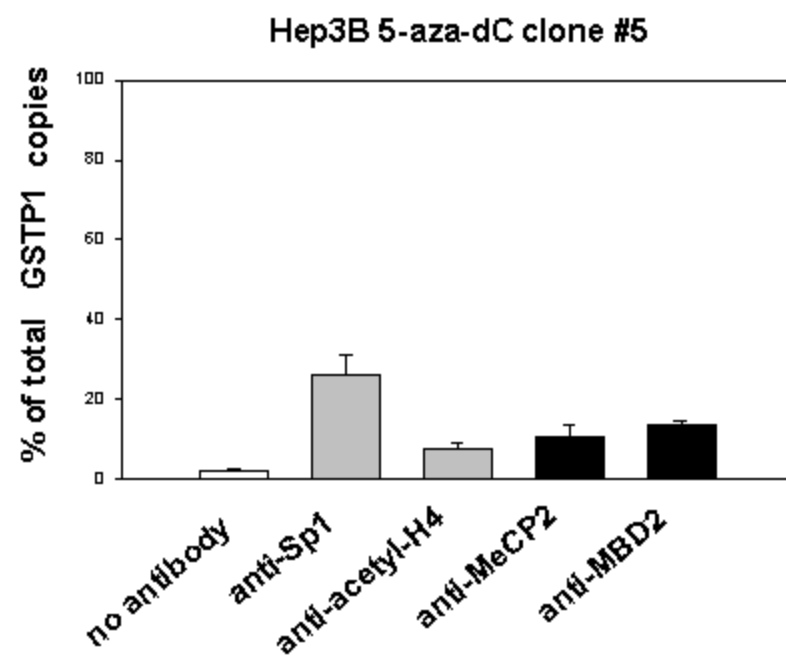
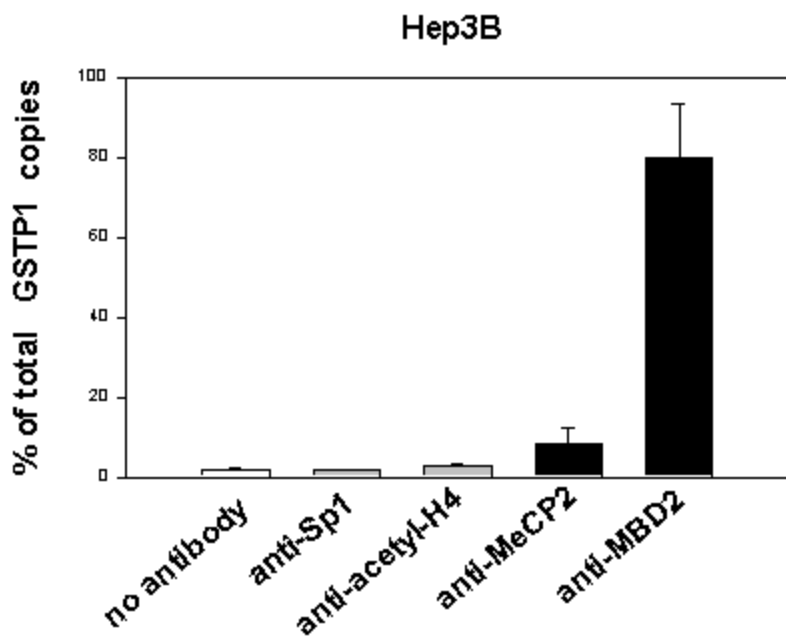


**Figure 5**

**Methylation percentage at each position**



**Figure 6**



**Figure 7**

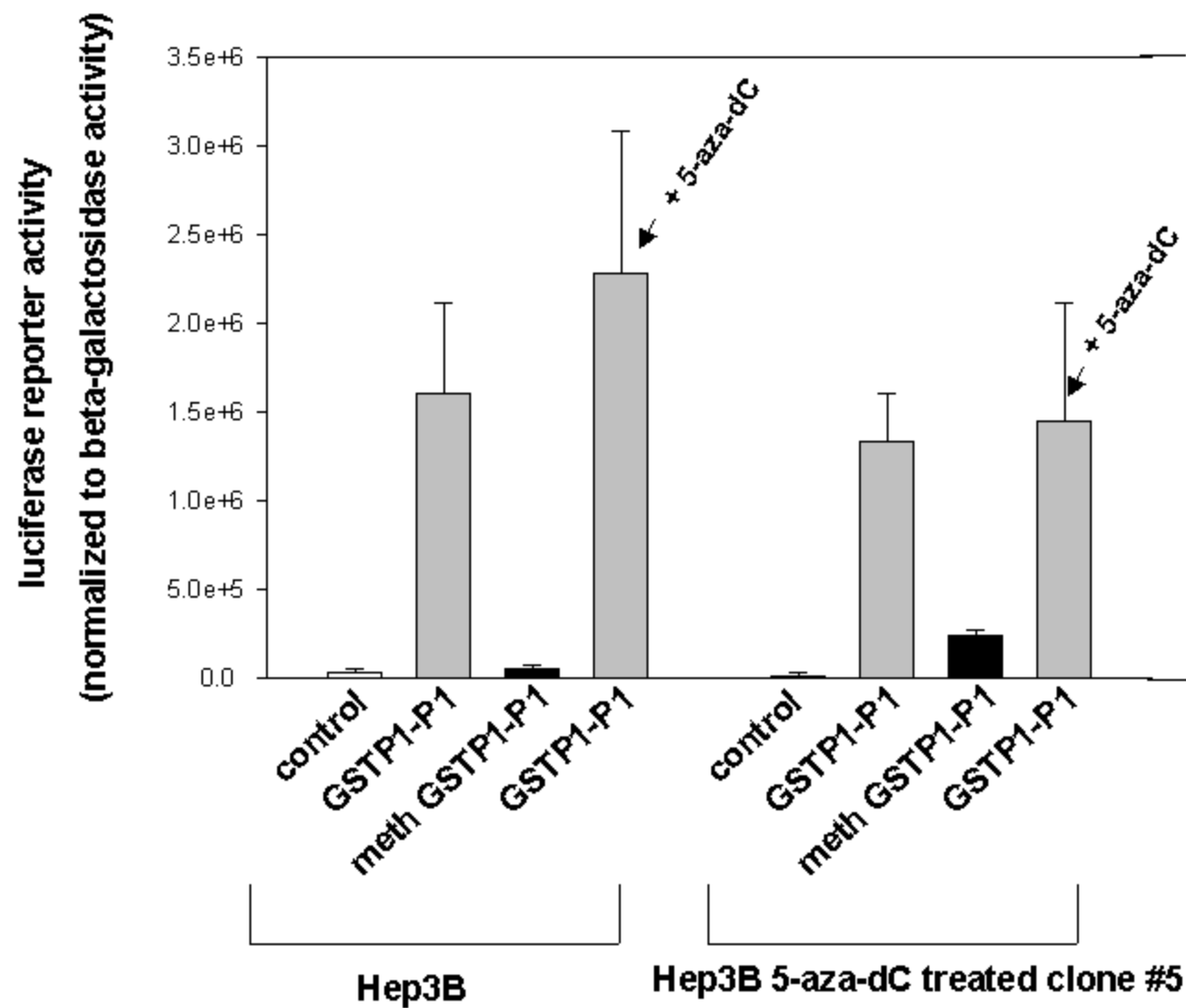


Figure 8

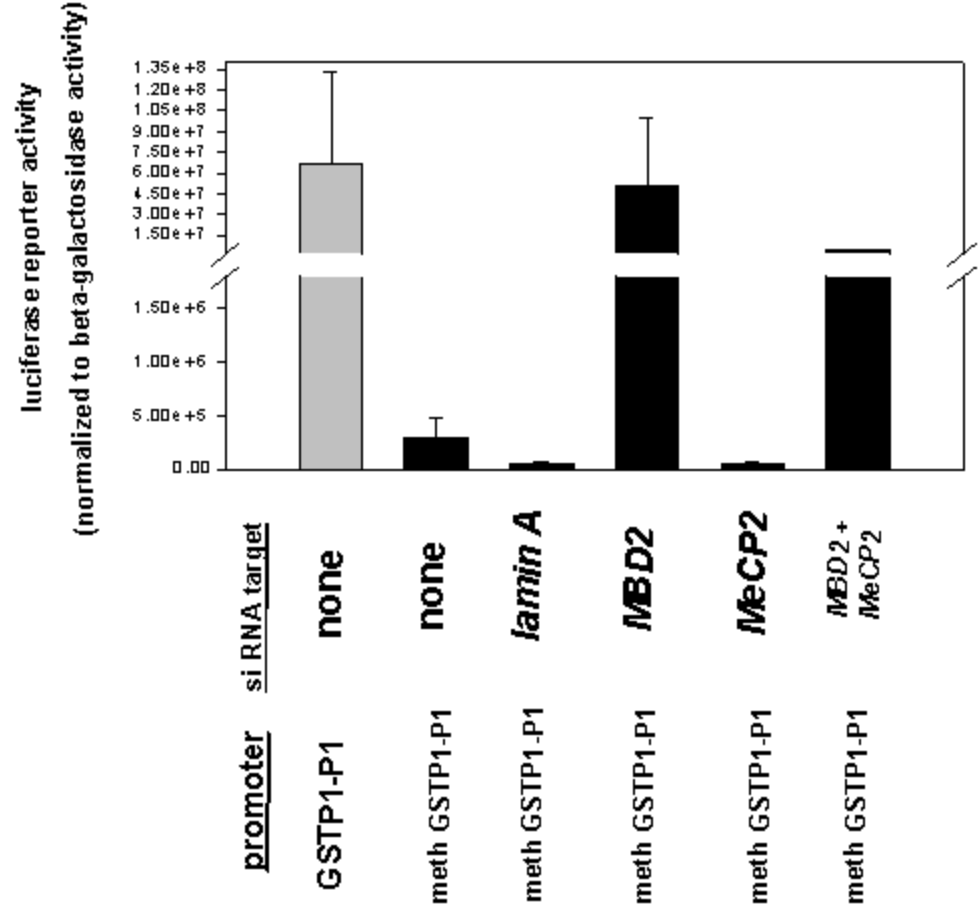
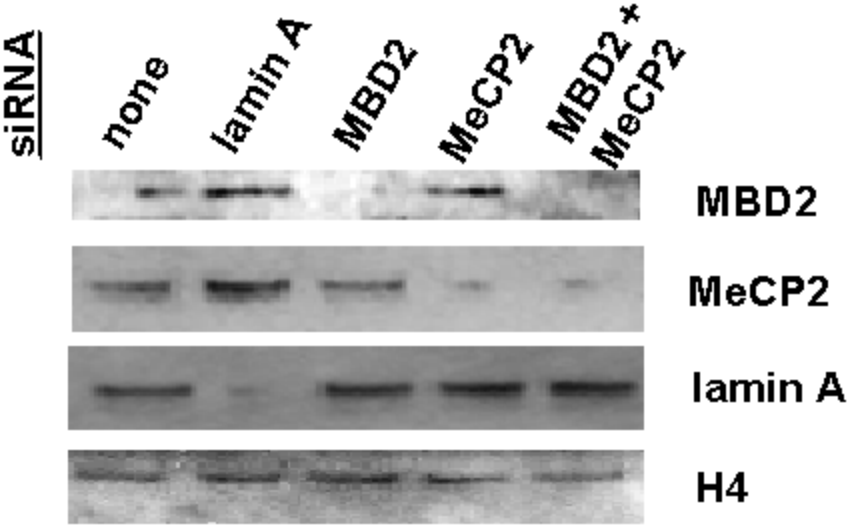


Figure 8



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**Genes Structure and Regulation:**  
**Methyl-CpG binding domain protein 2**  
**represses transcription from**  
**hypermethylated p-class glutathione**  
**S-transferase gene promoters in**  
**hepatocellular carcinoma cells**

Jila Bakker, Xiaohui Lin and William G.  
Nelson  
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