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The DNA methylation pattern is an important component of the epigenome that regulates and maintains gene expression programs. In this paper we test the hypothesis that vertebrate cells possess mechanisms protecting them from epigenomic stress similar to DNA damage checkpoints. We show that knock down of DNA methyltransferase1 (DNMT1) by an antisense oligonucleotide triggers an intra-S-phase arrest of DNA replication that is not observed with control oligonucleotide. The cells are arrested at different positions throughout the S-phase of the cell cycle suggesting that this response is not specific to distinct classes of origins of replication. The intra S phase arrest of DNA replication is proposed to protect the genome from extensive DNA demethylation that could come about by replication in the absence of DNMT1. This protective mechanism is not induced by 5-azadeoxycytidine (5-aza-CdR) a nucleoside analog that inhibits DNA methylation by trapping DNMT1 in the progressing replication fork, but does not reduce de novo synthesis of DNMT1. Our data therefore suggests that the intra-S-phase arrest is triggered by a reduction in DNMT1 and not by demethylation of DNA. DNMT1 knock down also leads to an induction of a set of genes that are implicated in genotoxic stress response such as NF-kB, JUN-B, ATF3 and GADD45. Based on this data we suggest that this stress response mechanism evolved to guard against build up of DNA methylation errors and to coordinate inheritance of genomic and epigenomic information.

1 Abbreviations: DNMT1-DNA methyltransferase 1; 5-aza-2’deoxycytidine-5-aza-CdR; GADD45-growth arrest DNA damage 45 gene
INTRODUCTION

Proper epigenomic regulation of gene expression is essential for the integrity of cell function. One critical component of the epigenome is the pattern of distribution of methylated cytosines in CG dinucleotide sequences in the genome (1). Methylation of CGs marks genes for inactivation by either interfering with the binding of methylated-DNA-sensitive transcription factors (2) or by recruiting methylated DNA binding proteins such as MeCP2, which in turn recruit co repressor complexes and histone deacetylases to the chromatin associated with the gene (3). The methylation pattern can thus determine the chromatin structure and state of activity of genes. Disruption in the proper maintenance of the DNA methylation pattern results in aberrant gene expression as is observed in tumor suppressor genes that are hypermethylated in cancer (4). Aberrant hypomethylation can also result in improper activation of genes (5).

The main enzyme responsible for replicating the DNA methylation pattern is DNMT1. This enzyme shows preference for hemimethylated DNA and is therefore believed to faithfully copy the DNA methylation pattern (6). Multiple mechanisms have been proposed to coordinate the inheritance of DNA methylation patterns with DNA replication. First, DNMT1 expression is regulated with the cell cycle (7, 8) and it is upregulated by protooncogenes Ras and Jun (9, 10) (11) Fos (12) and T antigen (13). Second, DNMT1 is localized to the replication fork (14) and is associated with the replication protein PCNA (15). Third, DNA methylation occurs concurrently with DNA replication (16). This temporal and physical association of DNMT1 with
DNA replication is believed to have evolved to guarantee concordant replication of DNA and its methylation pattern. Previous studies have shown that inhibition of DNMT1 can lead to inhibition of initiation of DNA replication (17) but it is not clear whether this response is a consequence of induction of tumor suppressor genes such as p21 (18) or p16 (19), leading to retreat from the cell cycle. A conditional knock out of murine *dnmt1* gene was also shown to reduce the rate of cell division (5), but it is still unclear whether inhibition of DNMT1 leads to a change in cell cycle kinetics similar to DNA damage response checkpoints.

Multiple mechanisms have been established to guard the integrity of the genome in response to DNA damage. For example, two parallel, cooperating mechanisms, both regulated by ATM, jointly contribute to the rapid and transient inhibition of firing of origins of DNA replication in response to ionizing radiation (20) (21) (22). This stalling of DNA synthesis is required to prevent genetic instability by coordinating replication and repair. We reasoned that similar mechanisms guard the integrity of epigenomic information in response to a disruption in the DNA methylation machinery.

In this paper we test this hypothesis by determining the response of human cell lines to a knockdown of *DNMT1* mRNA, encoding the enzyme responsible for the replication of the DNA methylation pattern. Our data suggests that cells respond to this epigenomic stress by an intra S phase arrest of DNA synthesis as well as by inducing a large number of stress response genes. The slow down in DNA synthesis
during S protects the DNA from a global loss of the methylation pattern. This mechanism is not triggered by 5-aza-CdR, which causes an extensive loss of DNA methylation.

MATERIALS AND METHODS

Cell Culture, antisense oligonucleotides, and 5-aza-CdR treatment- Both A549, a human non-small cell lung carcinoma cell line, and T24, a human bladder transitional carcinoma-derived cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). A549 cells were grown in Dulbecco's Modified Eagle's Medium (low glucose) supplemented with 10% fetal calf serum and 2 mM glutamine. T24 cells were maintained in McCoy’s medium supplemented with 10% FCS, 2 mM glutamine, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. 18-24 hours prior to treatment, cells were plated at a concentration of 3x10^5 cells/100-mm tissue culture dish or 5x10^4 cells/well in 6-well plate in the absence of antibiotics. The phosphorothioate oligodeoxynucleotides used in this study were MG88 (human DNMT1 antisense oligonucleotide) and its mismatch control MG208, which has a 6 base pair difference from MG88 (19). Oligonucleotides were transfected into cells with 6.25 µg/ml Lipofectin (Invitrogen) in serum free OptiMEM (Invitrogen). The oligonucleotide-containing OptiMEM medium was removed from the cells and replaced with regular growth medium after 4 hours. The treatment was repeated every 24 hours. The cells were harvested 24,48,72 and 96 hours following the first transfection. For 5-aza-CdR treatment, cells were grown in regular culture medium in
the presence of $10^{-6}$ M 5-aza-2’-deoxycytidine (5-aza-CdR) (Sigma) dissolved in DMSO. The 5-aza-CdR containing medium was freshly replaced every 24 hours.

**DNA Methyltransferase Activity Assay and Western blot analysis**- To determine the level of cellular DNA methyltransferase activity, nuclear extracts were prepared and DNA methyltransferase activity was assayed as described previously (8). For Western blot analysis of DNMT1, 50 µg nuclear protein was fractionated on a 5% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane and reacted with the polyclonal anti DNMT1 antibody (New England Biolabs) at a dilution of 1:2000 in the presence of 0.05% Tween and 5% milk, and was then reacted with anti rabbit IgG (Sigma) at a dilution of 1:5000. The amount of total protein per lane was determined by Amido black staining (23). The intensity of DNMT1 and total protein signal was measured by scanning densitometry and the ratio of DNMT1/total nuclear protein was calculated.

**RT-PCR**-Total RNA was extracted using the standard guanidium isothiocyanate method (24). cDNA was synthesized in a 20-µl reaction volume containing 2 µg of total RNA, 40 u MMuLV reverse transcriptase (MBI), 5µM random primer (Roche), 1 mM of each of the four deoxynucleotide triphosphates and 40 unit RNase inhibitor (Roche). mRNA was denatured for 5 min at 70°C, the random primers were annealed for 10 min at 25°C, and mRNA was reverse transcribed for 1 hr at 37°C. The reverse transcriptase was heat-inactivated for 10 min at 70°C and the products were stored at -20°C until use.
PCR was performed in a 50-µl reaction mixture containing 3 µl synthesized cDNA product, 5 µl 10x PCR buffer, 1.5-2.0 mM MgCl₂, 0.2 mM dNTP, 1 U Taq polymerase (all from MBI) and 0.5 µM of each primer. The primer sequences that were used for the different mRNAs were: GADD45β sense: 5’-GTGTACGAGTCGGCCAAGTT-3’, antisense: 5’-AGGAGACAATGCAGGTCTCG-3’; ATF-3 sense: 5’-AAGAGCTGAGGTTTGCACTC-3’, antisense: 5’-AGGTTTGGCCATC-3’; JunB sense: 5’-TGGAAACAGCCCTTCTACCAC-3’, antisense: 5’-GGAGTAGCTGCTGAGTGGGTGT-3’; ß-actin sense: 5’-GTTGCTAGCCAGGCTGTCTGGA-3’, antisense: 5’-GTGTACGAGTCGGCCAAGTT-3’, MAGEB2 sense: 5’-AGCGAGTGTAGGGGTGGGTGC-3’, antisense: 5’-GGAGTAGCTGCTGAGTGGGTGT-3’; BIK sense: 5’-GGCTGCTGTCTGTTATCTTT-3’, antisense: 5’-CCAGTAGATTCTTTGAG-3’; SSX2 sense: 5’-CAGAGTACGCACGGTCTG-3’, antisense: 5’-GGTCCACGGTTAGGGTCA-3’. Amplifications were performed in Biometra T3 Thermocycler (Biomedizinische Analytik GmbH) using the following programs: for GADD45β, first cycle 94°C for 3 min, 58°C for 1 min, and 72°C for 1 min, second cycle 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min followed by 37 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; for ATF-3 and JunB, an initial cycle of 94°C for 3 min 60°C for 1 min, and 72°C for 1 min, followed by 34 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; for ß-actin, first cycle 94°C for 3 min, 64°C for 1 min, and 72°C for 1 min, second cycle 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, and
72°C for 1 min; for MAGEB2, BIK and SSX2, first cycle 94°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec, second cycle 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and 30 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec. The numbers of cycles were selected and tested so that the PCR amplification remained in the linear phase. 10 µl of the PCR products were applied on a 1.2% agarose gel and visualized by ethidium bromide staining. Densitometric analysis was performed using Scion Imaging Software (Scion Inc., Frederick, MD).

**Competitive PCR for Quantification of DNMT1 mRNA Levels**- Total RNA (2 µg) was reverse transcribed as described above in the presence of 12.5 µCi of 35S-labeled dCTP (1250 Ci/mmol) (ICN) to quantify the efficiency of reverse transcription. Equal amounts of reverse transcribed cDNA (70 000 cpm as determined by the incorporation of 35S-labeled dCTP) were subjected to PCR amplification in the presence of increasing concentrations of a competitor DNA fragment that amplifies with the same set of primers but yields a product that is shorter by 48 base pairs. The following primers were used: 5’-ACCGCTTCTACTTCTCGAGGCCATA-3’ (*DNMT1* sense), 5’-GTTGCAGTCCTCTGTGAACACTGTGG-3’ (*DNMT1* anti-sense), and 5’-CGTCGAGG CCTAGAAACAAAGGGAAGGGCAAG (primer used to generate the competitor). PCR conditions were as follows: 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min. (33 cycles).

**Methylation-specific PCR (MSP)**- Genomic DNA was extracted with DNA extraction buffer (1% SDS, 5 mM EDTA, 150 mM NaCl) followed by proteinase K digestion, phenol/chloroform extractions and ethanol precipitations. Bisulfate treatment was
performed as described previously (25). The methylation status of the \textit{p16} gene was determined by methylation specific PCR (26) as modified by Palmisano et al., (27).

\textit{\textsuperscript{3}H} thymidine incorporation assay-Cells were plated in 6-well plate (5 x 10\textsuperscript{4}/well). For the final 4 hr of incubation, 1 µCi/ml [methyl-\textsuperscript{3}H]thymidine (NEN) was added to the medium. After washing twice with PBS, the cells were incubated in 10% trichloroacetic acid (TCA) for 30min at 4\degree C, washed twice with cold 10% TCA and then lysed with 1N NaOH and 1% SDS. \textsuperscript{\textsuperscript{3}}H-thymidine incorporation was measured using a liquid scintillation counter (LKB Wallac).

\textsuperscript{Flow cytometry analysis of 5-methyl-cytosine staining-} Global DNA methylation was evaluated by staining the cells with specific monoclonal antibody against 5-methyl-cytidine using the protocol described previously (28) with slight modifications. Briefly, cells were washed with phosphate buffered saline (PBS) supplemented with 0.1% Tween 20 and 1% bovine serum albumin (PBST-BSA), fixed with 0.25% paraformadehyde at 37\degree C for 10min and 88% methanol at -20\degree C for at least 30min. After 2 washes with PBST-BSA, the cells were treated with 2N HCl at 37\degree C for 30 min, and were then neutralized with 0.1M sodium borate (pH8.5). The cells were blocked with 10% donkey serum in PBST-BSA for 20min at 37\degree C, incubated with anti-5-methyl-cytidine antibody (1µg/ml) for 45 min at 37\degree C, followed by staining with donkey anti-mouse IgG conjugated with Rodamine Red-X (Jackson ImmunoResearch Laboratories). Finally, the cells were washed with PBS three times and were resuspended in PBS for flow cytometry analysis.
Microarray analysis - A549 cells were transfected with 200nM MG208 or MG88 or were treated with 1µM 5-aza-CdR or DMSO, for 48 hours. Total RNA was extracted with RNAeasy (Qiagen). Microarray analysis was performed as previously described (29). Briefly, 20 µg RNA was used for cDNA synthesis, followed by *in vitro* transcription with a T7 promoter primer having a poly-T tail. The resulting product was hybridized and processed with the GeneChip system (Affymetrix) to a HuGeneFL DNA microarray containing oligonucleotides specific for approximately 12,000 human transcripts. Data analysis, average difference and expression for each feature on the chip were computed using Affymetrix GeneChip Analysis Suite version 3.3 with default parameters. The gene expression analysis was performed by the Montreal Genome Center.

Double staining of BrdU and propidium iodide (PI) - Cells were incubated with 10 µM BrdU (Sigma) for the last 2 hr before harvesting. Incorporated BrdU was stained with anti-BrdU antibody conjugated with FITC (Roche) following the manufacturer’s protocol (30). After the last washing, the cells were resuspended in PBS containing 50 µg/ml propidium iodide and 10 µg/ml RNase A for 30 min at RT, and then analyzed with FACScan (BD Bioscience) for both FITC and PI fluorescence.

RESULTS

Knockdown of DNMT1 mRNA by the DNMT1 antisense oligonucleotide MG88 - DNMT1 activity is physically and temporally associated with the DNA replication machinery. The absence of DNMT1 from the replication fork could potentially lead to an epigenomic catastrophe. We have previously proposed that the coordination of
DNMT1 expression and DNA replication evolved as a mechanism to protect the coordinate inheritance of genetic and epigenetic information (31, 32). To test this hypothesis we determined the cellular response to a knockdown of DNMT1 protein. We took advantage of a previously described antisense oligonucleotide, which specifically knocks down $DNMT1$ mRNA (19) MG88, and its mismatch control MG208 (see Fig. 1A for sequence and alignment with human and mouse DNMT1 mRNA). We first optimized the time and concentration at which MG88 specifically knocks down $DNMT1$ activity in A549 cells in comparison to MG208. The results presented in Fig.1B show that MG88 reduces DNA methyltransferase activity in a dose and time-dependent manner relative to MG208. Inhibition of DNMT activity approximates 80% after 48 hours of MG88 treatment whereas no inhibition is observed following MG208 treatment. Therefore, for our further analysis we chose to treat the cells with 200nM of either MG88 or MG208 for 48 hours. We confirmed the antisense mechanism of action of MG88 by demonstrating that $DNMT1$ mRNA levels are knocked down following a 48 hours treatment with this oligonucleotide in comparison with MG208 treatment using a competitive RT-PCR assay for $DNMT1$ (Fig.1 C and D). To confirm that DNMT1 inhibition results in reduction of DNMT1 protein, nuclear extracts prepared from either MG208 or MG88 treated cells (200 nM for 48 hours) were subjected to a Western blot analysis and reacted with anti DNMT1 antibody (Fig. 1E). Quantification of the signal by densitometry reveals 85% reduction in protein levels. Inhibition of $DNMT1$ mRNA by MG88 was also confirmed in a gene array expression analysis presented in Table 2.
DNMT1 knock down reduces the fraction of cells that are in S. Several studies have previously demonstrated that inhibition of DNMT1 results in inhibition of cell growth (33). One possible explanation for the reduced cell growth is that knockdown of DNMT1 results in inhibition of firing of DNA replication origins (17). We therefore addressed the question of whether this inhibition of DNA replication reflects a distinct alteration in cell cycle kinetics, similar to the DNA damage checkpoints which trigger arrest at distinct phases of the cell cycle (34).

A549 cells were treated with either 200 nM of MG88 or MG208 for 24-96 hours as described in Materials and Methods. As observed in Fig. 2, DNMT1 knockdown results in a significant decrease in overall DNA synthetic capacity of A549 cells. This is illustrated by the reduced incorporation of [3H]-thymidine into DNA 24 hours after initiation of treatment, as has been previously reported (17). To exclude the possibility that inhibition of DNA synthesis by MG88 is independent of DNMT1 expression and is a toxic side effect of the sequence, we took advantage of the species specificity of MG88. As shown in Fig. 1A there is a 6 base pair mismatch between MG88 and the mouse dnmt1 mRNA. We therefore determined whether MG88 would inhibit DNA synthesis in a mouse adrenal carcinoma cell line Y1, which was previously shown by us to be responsive to a mouse dnmt1 antisense oligonucleotide (23). The results shown in Fig. 2B demonstrate that 48 hours treatment with 200 nM MG88 had no significant impact on the DNA synthetic capacity of Y1 cells in comparison with MG208, supporting the hypothesis that MG88 inhibition of DNA synthesis is DNMT1 dependent.
We then addressed the question of whether this inhibition in DNA synthesis represents a slow down in the rate of DNA synthesis or a reduction in the fraction of cells that are in the synthetic phase, which would indicate a change in cell cycle phase kinetics. We pulsed MG88 and MG208 treated cells with BrdU 48 hours after initiation of treatment and sorted the cells that incorporated BrdU using FACS as described in the Materials and Methods. As illustrated in Fig. 2C, DNMT1 knockdown reduces the fraction of cells that incorporate DNA (the M1 population). However, the reduction in the fraction of cells that synthesize DNA (up to 42%) does not account for the overall reduction in DNA replication shown by the $[^3H]$-thymidine incorporation assay which is >95%. Although there is no significant cell death that can account for this disparity, this difference might reflect the fact that cell number following MG88 treatment is reduced.

**DNMT1 knock down results in intra S arrest in DNA replication.** The reduction in the fraction of cells that incorporate DNA could be a consequence of a phase specific cell cycle arrest. However, preliminary results using flow cytometry sorting of propidium iodide stained cells failed to show either a significant G1 or G2 arrest that could explain the reduction in S phase cells. This raised the possibility that DNMT1 knockdown caused an intra S phase arrest similar to the previously described DNA damage checkpoint (35). To address this possibility, we treated A549 cells for 48 hours with either MG88 or MG208, and then pulsed with BrdU to mark cells that are actively replicating. We then stained the cells with propidium iodide to determine their total DNA content, which is indicative of their position in the cell cycle. The cells were analyzed simultaneously for both their BrdU incorporation and propidium
iodide staining by flow cytometry. The results of a representative analysis shown in Fig. 3A suggest a slight increase in subG1 population and a slight increase in cells found in the G2 phase of the cell cycle in response to DNMT1 knock down (9.76% in MG88 treated cells versus 7.71% in the MG208 treatment). However, an unexpected change in the cell cycle kinetics is a consequence of an intra-S phase DNA replication arrest. This arrest results in the partition of cells found in the S phase into two distinct populations, one that incorporates BrdU and another comprising 30% of the cells in the S that do not incorporate BrdU, in comparison with less than 7% of those in the MG 208 control. Similar observations were obtained in 5 independent experiments. The presence of a population of cells in S that does not incorporate BrdU in response to depletion of DNMT1 is consistent with a presence of an intra S checkpoint triggering cell cycle arrest. Cells that do not incorporate BrdU in S are distributed throughout the S phase of the cell cycle indicating that the arrest in DNA replication does not occur at a specific point in S phase. DNMT1 knockdown can lead to intra S arrest at any point in S. As has been previously observed for DNA damage checkpoints, the intra S phase arrest is transient since this partition of the S phase cells population disappears after longer treatment (10 days) (Fig. 3B).

The intra S phase arrest is not dependent on the extent of DNA demethylation. The intra S phase arrest in DNA replication might have been triggered by either the absence of DNMT1 from some replication forks or by demethylated DNA. To address this question we took advantage of a well-characterized inhibitor of DNA methylation 5-aza-deoxycytidine (5-aza-CdR) (36). 5-aza-CdR is a nucleoside analogue that is incorporated into DNA following its phosphorylation to the
trinucleotide form. It inhibits DNA methylation only once it is incorporated into DNA by trapping DNMT1 from the progressing replication fork. 5-aza-CdR does not inhibit either de novo synthesis of DNMT1 or its incorporation into the replication fork, but it inhibits DNA methylation during replication. It is dependent on DNA replication for its action in contrast to DNMT1 antisense, which reduces the availability of DNMT1 prior to the formation of the replication fork.

5-aza-CdR causes extensive DNA demethylation in A549 cells as well as many other cell lines and should cause a drastic change in cell cycle kinetics in S if the trigger for intra S-phase arrest in DNA replication is DNA demethylation. However, whereas 5-aza-CdR treatment, using a concentration that causes significant demethylation, results in limited decrease in the rate of DNA synthesis as indicated by thymidine incorporation analyses shown in Fig. 4A, this inhibition is considerably less than that observed with MG88 (Fig. 2A), and significant DNA synthesis occurs up to 96 hours after treatment with 5-aza-CdR. Similarly, the analysis of the distribution of BrdU incorporating cells in S (Fig. 4B) does not show a clear partition into two distinct populations as has been observed with MG88. 5-aza-CdR treated cells show a gradual limited decrease in DNA replication rate as indicated by the gradient of BrdU labeled cells in the S phase of the cell cycle. The continued synthesis of DNA in the presence of 5-aza-CdR can explain the ability of this agent to demethylate DNA. The main change in the cell cycle kinetics that is observed with 5-azaCdR is an increase in the G2 population (from 9.74% in the control to 28.99% in 5-aza-CdR treated cells). These results suggest that the intra S phase arrest is not correlated to the degree of inhibition of DNA methylation per se. Our data is consistent with the hypothesis that
the intra S phase arrest following DNMT1 knock down is a response to a reduction in the availability of DNMT1 in the replication fork rather than DNA demethylation.

Intra S phase arrest of DNA replication possibly protects the genome from global hypomethylation- One potential role of the intra S phase arrest triggered by reduction of DNMT1 is to protect the epigenome from global loss of the DNA methylation pattern. Using anti 5-methylcytosine antibodies that were previously described (37), we compared the state of methylation of A549 cells treated for 48 hours with either 200 nM MG88, which causes intra S phase arrest, or 5-azaCdR which does not trigger a distinct intra S-phase arrest. A549 cells treated with either the DNMT1 inhibitors (MG88 or 5-azaCdR) or their respective controls (MG208 or DMSO) were stained with either the 5-methylcytosine antibody, or the secondary antibody alone as a control, and were subjected to FACS analysis (Fig. 5A for MG88 versus MG208 and Fig. 5B for 5-aza-CdR versus DMSO control). MG88 treated cells are only slightly demethylated as indicated by the slight shift in the fluorescence intensity of the MG88 treated cells (Fig. 5A), whereas 5-azaCdR treatment results in extensive reduction in staining with the anti 5-methylcytosine antibody indicative of genome wide demethylation (Fig. 5B). The intra S-phase arrest of replication following MG88 treatment possibly protects A549 cells from genome wide loss of methylation. We have previously observed that hemimethylated inhibitors of DNMT1 that inhibit DNA replication also cause only limited demethylation of DNA (33).

Comparison of the kinetics of demethylation of the tumor suppressor p16 following DNMT1 knockdown by MG88 and 5-azaCdR trapping of DNMT1. We addressed the question of whether this difference in the kinetics of global DNA demethylation
between 5-azaCdR and DNMT1 antisense oligonucleotides is also observed when specific genes are examined. We focused on the methylated tumor suppressor gene p16 in the human bladder carcinoma cell line T24, since there is no well documented example of a methylated gene in A549 cells that is activated by pharmacological demethylation. The p16 gene is demethylated in response to both 5-azaCdR (38) and DNMT1 antisense (MG88) treatment (19).

We first verified that the DNMT1 antisense-triggered intra S phase arrest demonstrated above in A549 cells (Fig. 3) is also functional in T24 cells. A 48 hours treatment of T24 cells with MG88 results in an intra S phase arrest of DNA replication (Fig. 6A) similar to that observed in A549 cells (Fig. 3) as indicated by the partition of cells in S phase of the cell cycle to two distinct groups, those that incorporate BrdU (9.77% compared to 37.06% in the MG208 control group) and those that do not incorporate BrdU (12.4 % versus 5.5% in the control). Thus, 56% of the cells found in the S phase of the cell cycle do not synthesize DNA following MG88 treatment of T24 cells. On the other hand, 5-aza-CdR treatment results in an increase in the fraction of cells that are in G1 (72.06% in 5-aza-CdR treated versus 53.55% in the control) and a slow down of the rate of DNA synthesis in S phase cells as indicated by the gradient of the intensity of BrdU incorporation. However, there is no distinct partition of the population of S phase cells following 5-aza-CdR to two distinct groups as is observed following MG88 treatment.

We then assessed the global state of methylation of T24 cells following either MG88 or 5-aza-CdR treatment for 48h using a FACS analysis of 5-methylcytosine antibody stained cells. Fig. 6B demonstrates that similar to what is observed in A549 cells,
MG88 treatment results in very limited global hypomethylation in T24 cells, as indicated by the slight shift to the left in the intensity of staining with 5-methylcytosine antibodies. In contrast, 5-aza-CdR results in global hypomethylation as indicated by the considerable shift to the left of the population of 5-aza-CdR treated cells Fig. 6C.

We then determined the pattern of methylation of the p16 gene following either DNMT1 antisense or 5-aza-CdR treatments. The methylation pattern of the 5’ exon of p16 was studied by methylation specific-PCR that was previously described (26) (Fig. 6D and E). The results of this analysis show a dramatic difference in the kinetics of demethylation between MG88 (Fig. 6D) and 5-aza-CdR (Fig. 6E) treated cells. While p16 is significantly demethylated 24 hours after initiation of 5-aza-CdR treatment and is completely demethylated after 96 hours (Fig. 6E), p16 remains fully methylated 48 hours after MG88 treatment at the peak of the intra-S phase DNA replication arrest (Fig. 6D). Demethylation initiates only at 72 hours. The mechanism of this demethylation is unclear since passive demethylation requires DNA replication in the absence of DNA methyltransferase, while DNA replication is inhibited in T24 cells following MG88 treatment. It is possible that the demethylation of p16 is caused by an active demethylation mechanism or it might result from residual replication in the absence of DNMT1.

In summary, our data reveals that demethylation is delayed when DNA synthesis is arrested concomitantly with knock down of DNMT1. The signal for the intra S phase arrest following DNMT1 knockdown by MG88 is neither the extent of DNA demethylation nor the activation of p16, since DNA replication arrest precedes
demethylation. Furthermore, 5-aza-CdR, a potent inhibitor of DNA methylation that acts by a different mechanism than MG88, does not cause an intra-S phase arrest in DNA replication.

**Knockdown of DNMT1 by antisense, but not DNA methylation inhibition with 5-aza-CdR, induces expression of genotoxic stress-responsive genes** - Multiple genes have been shown in the past to be silenced by DNA methylation. A well accepted model is that global DNA demethylation results in misprogramming of gene expression by aberrant activation of genes that are normally silenced by methylation. A methodical analysis of genes that are induced following 5-aza-CdR treatment of a colorectal cancer cell line identified a group of genes that are silenced by DNA methylation and are demethylated by 5-aza-CdR. In addition, another group of genes that are not methylated and are activated by methylation independent mechanisms, were also shown to be induced by 5-aza-CdR (39). We used Affimexix 12K gene microarrays to repeat this analysis in our system. We compared the gene expression profile of A549 cells treated with 1µM 5-aza-CdR to the gene expression profile of A549 cells treated with DMSO for 48 hours. The list of genes induced more than 2.5 fold by 5-aza-CdR is shown in Table 1. Only genes that were induced in two separate experiments and did not show variation in expression within either the control or 5-aza-CdR replicates were included. The list of genes induced by 5-aza-CdR includes tissue specific genes such as smooth muscle ACTIN\(^\text{a2}\) and genes involved in interferon response such as INTERFERON\(^\text{b2}\), as well as the apoptosis promoter, the BCL2 interacting killer, BIK. BIK was previously shown to exhibit potent antitumor activity (40) and is induced by 5-aza-CdR and sodium butyrate in hepatic cancer cell
The induction of the IFN-response pathway was previously proposed to be a major cellular response to 5-Aza-CdR (42). However, in addition to upregulation of genes that are potentially antimitotic and proapoptotic, the most remarkable induction occurred in three groups of cancer/testis specific genes residing on the X chromosome, which are exclusively expressed in testis and a wide variety of tumors, but not in non tumor tissues. These are the GAGE (G antigen 7) family (43), the MAGE (44-48) family of melanoma antigens and the genes residing at the synovial sarcoma X breakpoint, SSX2-4 (49, 50). It is well documented that the MAGE (44-46) and GAGE family of genes as well as SSX2 are controlled by DNA methylation and are induced by DNA demethylating agents (51).

The upregulation of genes identified by gene array analysis was verified by semiquantitative RT-PCR shown in Fig. 7. The cancer/testis specific genes show a typical profile for methylated genes induced by demethylating agents. They are completely silenced in the control cells and are activated to clearly detectable levels following demethylation (Fig. 7). Methylation results in most cases in silencing of genes rather than a quantitative reduction in gene expression. In addition, expression levels are increased with time as expected from passive demethylation kinetics. Inhibition of DNA methyltransferase during new DNA synthesis results in a time dependent increase in the relative abundance of the population of newly replicated unmethylated DNA. On the other hand, genes such as BIK, which is expressed in control cells, are transiently induced by 5-aza-CdR, and their level of induction is reduced with time (Fig. 7). This profile of induction is consistent with a methylation-independent mechanism, which is also supported by the induction of BIK after
DNMT1 knockdown before any significant global demethylation is observed (two-fold induction 24 h after antisense treatment). BIK and other genes induced by a methylation independent mechanism were also induced by the deacetylase inhibitors Trichostatin A (39) and n-butyrate (41). In accordance with previous studies in colorectal cancer (39), our data shows both methylation dependent and independent induction by 5-aza-CdR in A549 cells (MAGEB2, SSX2, and BIK, respectively).

Based on the data presented above we predicted that in contrast to the response to 5-aza-CdR, knockdown of DNMT1 by antisense inhibition should not result in induction of methylation-silenced genes at the early time after treatment. However, since we have previously shown that inhibition of DNMT1 induces the expression of the p21 tumor suppressor gene by a mechanism that does not involve DNA demethylation (18), and since examples of such genes were identified in the recent analysis with 5-aza-CdR (39), we tested the possibility that the early response to DNMT1 knock down results in a programmed change in gene expression that precedes global hypomethylation and is possibly involved in the stress response. We therefore compared the gene expression profile of MG88 treated A549 cells to the gene expression profile of A549 cells treated with the control MG208 oligonucleotide for 48 hours using Affimetrix 12K genes microarrays.

We compared the normalized gene expression profile of the two treatment groups. 255 (2.1%) genes out of 12,000 genes were upregulated, while there were just 23 (0.19%) genes that were down-regulated. The experiment was repeated with similar results. *DNMT1* expression was 75% downregulated in two experiments, which is an internal validation of our gene expression analysis and antisense treatment. The
results are presented in Table 2. Only genes induced in both experiments are included. Amongst the genes that were induced, we did not identify genes that were previously characterized to be silenced by methylation such as SSX2 and MAGEB2. These two genes were shown to be induced with 5-aza-CdR, but were not induced following DNMT1 knockdown (Figure 7 and 8). However, a distinct group of genes that stood out was a set of previously characterized genotoxic-responsive genes such as \textit{ATF-3}, \textit{GADD45b} and \textit{JunB}. These three genes were not found to be induced by 5-aza-CdR treatment in the gene array analysis and this result was confirmed by RT-PCR (Figure 7). Their induction profiles following antisense treatment were verified using semiquantitative RT-PCR. The induction peaked at 48 h for \textit{GADD45b} and \textit{JUNB}, and at 72 hour for \textit{ATF-3}. This profile of induction is consistent with the hypothesis that the cell recognizes DNMT1 knockdown as a genotoxic challenge and reacts by inducing a stress response gene expression program. The kinetics of this response, early induction followed by mitigation of the response (Fig. 8), is inconsistent with the mechanism involving passive demethylation of DNA. Consistent with this hypothesis that MG88 action at 48 h is independent of DNA methylation is the fact that the genes induced by the DNA demethylating agent 5-aza-CdR (\textit{MAGEB2} and \textit{SSX2}) were not induced by MG88 treatment for 48h (Fig. 8).

\section*{DISCUSSION}

Multiple mechanisms regulate expression of DNMT1 within a cell (8) (7) (11) (32). In this paper we address the question of whether mammalian cells possess a
mechanism to respond to a sudden loss of DNMT1 and protect themselves from a
global loss of DNA methylation during replication in the absence of DNMT1. It is
well established that genotoxic challenges such as DNA damage evoke distinct
cellular responses resulting in a transient intra S phase arrest in DNA replication (20)
(21) (22). This intra S phase arrest guards against build-up of mutations during DNA
replication before the other checkpoints at G2/M and G1/S could take effect.
Similarly, relying on G2/M and G1/S checkpoints to respond to the absence of
DNMT1 in the fork during replication could result in a significant loss of DNA
methylation and a build-up of epigenomic errors.

We demonstrate here that following DNMT1 knock down, cells found in the S phase
of the cell cycle are partitioned into two groups, those that incorporate BrdU and
those that do not incorporate BrdU (Fig. 3). Our data is consistent with the presence
of an intra S phase checkpoint that arrests all the replication forks in a cell, as
illustrated by the appearance of a group of cells in S that do not incorporate any BrdU
as a response to a reduction in availability of DNMT1.

Different origins of replication replicate at discreet and well defined positions in the
cell cycle. Origins that replicate early in the cell cycle are associated with genomic
regions that are hypomethylated and are actively transcribed (52), while origins that
replicate late in S are associated with inactive genes, which are also known to be
hypermethylated (53). The results presented in Fig. 3 and Fig.6 show that DNA
replication is arrested at any point in the S phase of the cell cycle. This is inconsistent
with the hypothesis that DNMT1 knockdown affects only specific classes of origins.
What is the signal that triggers a stress response to antisense *DNMT1* knockdown? It is possible that the emergence of demethylated DNA caused by replication in the absence of DNMT1 triggers the intra S phase arrest. Alternatively, the signal is the inhibition of *de novo* synthesis of DNMT1 leading to its absence from DNA replication factories. The fact that 5-azaCdR, which causes a far more extensive demethylation than MG88, does not trigger the same magnitude of intra S phase arrest of replication (Fig. 3), suggests that it is not the demethylation that triggers the intra S phase arrest. Rather, our data is consistent with the hypothesis that it is the reduction in DNMT1 protein that triggers the intra S phase arrest observed after MG88 treatment.

We propose that the intra S phase arrest guarantees that no DNA is synthesized in the absence of DNMT1. However, this is a transient and incomplete protection and delayed demethylation is observed following extended MG88 treatment (Fig. 6). 5-aza-CdR bypasses this checkpoint to a large extent since it does not reduce DNMT1 synthesis but traps DNMT1 only once the replication fork has formed in the presence of DNMT1.

These differences in the mechanisms of action of these two inhibitors have important implications on the design and therapeutic utility of different DNA methylation inhibitors (31). Agents such as MG88, that reduce the availability of DNMT1 at the replication fork, are strong inhibitors of cell growth and should be effective in inhibiting tumor growth but will not cause extensive demethylation (33). There might be an advantage for therapeutic agents that do not cause extensive demethylation since extensive hypomethylation has been previously associated with
metastasis (54) and possibly induction of silenced repetitive elements (55, 56). The data presented in Table 1 illustrates the risks inherent in using DNA demethylating agents. In addition to induction of antimitotic and proapoptotic genes, 5-aza-CdR induces 3 families of testis/cancer specific antigens that were previously implicated in tumor progression and potentially tumor invasion and metastasis. It is interesting to note that expression of GAGE antigen family has been associated with poor prognosis in some cancers. G antigen 7 is expressed in prostate cancer (43) and G antigen 7c was proposed to be an antiapoptotic gene (57). Similarly, MAGE expression is associated with metastasis (53, 54). SSX2 was shown to be expressed in a wide variety of tumors (50) and was identified as one of 13 antigens that react exclusively with sera from colon cancer patients but not with sera from normal patients (58).

In addition to the change in cell kinetics, the cells respond to DNMT1 knock down by a change in the gene expression program. A significant fraction of the induced genes is known to be involved in genotoxic stress responses (Table 2). We have previously proposed that DNMT1 controls the expression of certain genes by a direct repression function that does not involve DNA methylation (18). DNMT1 was previously shown to interact with HDAC1 (59), HDAC2 (60) and Rb-E2F1 (61). We propose that some of the early genes induced by DNMT1 knock down are similarly controlled by the DNA methylation independent gene repression activities of DNMT1.
It remains to be seen whether the genes induced by DNMT1 knockdown are also involved in the intra S phase arrest or whether they are parallel responses that augment the protection against epigenomic loss. Recent studies have identified some of the players involved in the intra-S-phase checkpoint in response to ionizing DNA damage. ATM is activated by ionizing radiation, which in turn activates two signaling pathways, one leading to inactivation of cdk2 and intra S phase arrest, and the other leading to activation of p21 and G1 arrest (35). We have previously shown that DNMT1 inhibition can lead to transcriptional induction of p21, and here we show induction of GADD45β. The most established function of p21 is at the G1/S boundary (62) and GADD45β has been shown to play an important role in the G2/M check point in response to DNA damage (63), but their involvement in the intra S phase check point is unclear. However, it is possible that inhibition of Cdk2 by p21 can lead to S phase arrest. An additional possibility is that the assembly of the replication fork requires the presence of DNMT1 and that its absence from the fork is what signals arrest of DNA replication.

Although the precise mechanism by which reduction of DNMT1 causes intra S phase arrest is unknown, our data describes a new class of putative checkpoints that react to epigenomic stress caused by reduction of DNMT1 levels. We propose that this mechanism has evolved to protect the genome from unscheduled demethylation and to maintain the coordination of replication of the genome and the epigenome.

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Sadvakassova for technical assistance, and Nancy Detich for her critical review of the manuscript.

REFERENCES


Figure Legends

Fig. 1. Time and dose dependent inhibition of *DNMT1* mRNA and DNA methyltransferase activity by *DNMT1* antisense oligonucleotide MG88. A, The sequence of *DNMT1* antisense oligonucleotide MG88, and its mismatch control
oligonucleotide MG208 are aligned to the human and mouse *DNMT1* mRNA sequence. Mismatches with the human *DNMT1* mRNA are bolded and italicized. *B*, A549 cells were incubated with either 100 or 200nM of MG88 or MG208 for either 48 or 72 hours. DNA methyltransferase activity was determined for 3 µg of nuclear extracts using a hemimethylated substrate and [³H] S-adenosylmethionine as a methyl donor. The results presented are an average of 3 determinations ± SEM of triplicate determinations from three independent experiments. *C* and *D* are representative experiments from 5 similar experiments using competitive RT-PCR for quantification of *DNMT1* mRNA. 2 µg of RNA isolated from A549 cells treated with either MG88 or MG208 at 200nM for 48 hours was reverse transcribed into cDNA in the presence of ³⁵S-dCTP. Equal counts of labeled cDNA (target) and increasing amount of competitor molecules (from 10⁻¹⁶ to 10⁻¹² M) were used as templates for PCR reactions with primers targeted to *DNMT1* mRNA sequence. The PCR products were run on 1.2% agarose gel and quantified by densitometry, the logarithm of the ratio of target to competitor products were plotted against the –log of competitor concentration (*D*). *E*, Western blot analysis of DNMT1 expression in nuclear extracts prepared from either MG88 or MG208 treated A549 cells (200nM for 48 hours). In the right panel, the membrane was stained with Amido black to visualize the total protein transferred onto the membrane.

**Fig. 2.** MG88 knock down of DNMT1 inhibits DNA synthesis and reduces the fraction of cells that synthesize DNA in A549 cells. *A*, A549 cells were treated with MG208 and MG88 at 200nM for the indicated time intervals. [³H]-thymidine incorporation into DNA was quantified as described in Materials and Methods. The
results presented are Mean ± SD of triplicate determinations from one of three independent experiments that resulted in similar results. B, Y1 cells were treated with either MG208 or MG88 at 200nM for 48 hours. [3H] -thymidine incorporation into DNA was quantified as in A. C, A549 cells were incubated with either MG208 or MG88 at 200nM for 48 hours. In the last 2 hours of the experiment, the cells were pulsed with 10µM BrdU and were stained with anti-BrdU Ab and sorted by flow cytometry. The left peak represents cells that are negative for anti-BrdU staining, the right peak (M1) represents cells that are positive for anti-BrdU staining. Similar profiles were obtained for three other independent experiments.

Fig.3. **Knock down of DNM1 triggers intra-S-phase arrest of DNA replication.**

A, A549 cells were treated with 200 nM of either MG208 or MG88 for 48 hours. Cells pulsed with BrdU (10µM) for 2 hours were stained with anti-BrdU antibody and propidium iodide, and then analysed with flow cytometry. The profiles of BrdU staining fluorescence versus propidium iodide staining fluorescence are depicted. The dots representing population of cells in the different phases of the cell cycle are boxed and color coded (green: G0/G1; pink: S phase with BrdU incorporation; orange: S phase without BrdU incorporation; blue: G2/M). A representative experiment of 5 independent experiments is presented. B, A549 cells treated with either MG88 or MG208 for 7 days were pulsed for 2 hours with BrdU, stained with anti BrdU antibodies and propidium iodide and subjected to flow cytometry. The profiles of BrdU staining fluorescence versus propidium iodide staining fluorescence are depicted.
Fig. 4. The effects of 5-aza-CdR on DNA replication and cell cycle kinetics in A549 cells. A, A549 cells were treated with 1 μM 5-aza-CdR (solid bar) and the vehicle control DMSO (open bar) for 24 – 96 hr. [3H]-thymidine incorporation into DNA was quantified as described in Materials and Methods. The results presented are mean ± S.D. of triplicate determinations from one of three independent experiments with similar results. B, A549 cells treated with either 1 μM 5-aza-CdR or DMSO for 48 hours were pulsed with BrdU (10μM) for 2 hours and stained with both anti-BrdU Ab and propidium iodide, and then analysed with flow cytometry. The profiles of BrdU staining fluorescence versus propidium iodide staining fluorescence are depicted. The positions of G1 (1n DNA staining), S phase (1-2n) and G2 (2n staining) cells are indicated. This experiment is a representative of similar 3 experiments.

Fig. 5. The state of methylation of A549 cells following DNMT1 antisense or 5-azaCdR treatment. A, A549 cells were treated with 200nM of either MG208 (red) or MG88 (green) for 48 hours. Cells were harvested, stained with anti-5-mC antibody and anti-mouse IgG conjugated with Rodamine Red X, and subjected to flow cytometry. Cells stained with secondary Ab only, are used as a blank control (black line). B, A549 cells were treated with either DMSO (red) or 5-aza-CdR (green) for 48 hours and subjected to flow cytometry as in A.

Fig 6. Time course of global and p16 DNA demethylation in T24 cells treated with either DNMT1 antisense or 5-aza-CdR.
A, DNMT1 antisense treatment causes an intra S phase arrest in T24 cells. T24 cells were treated with 200nM of either MG208 or MG88, DMSO or 1 µM 5-aza-CdR for 48 hours and were then pulsed for 2 hours with BrdU. The cells were stained with anti-BrdU Ab and propidium iodide and subjected to flow cytometry. The profile of BrdU staining versus propidium iodide staining is shown for each of the treatment groups as described in the legend to Fig. 3.

B, T24 cells were treated with 200nM of either MG208 (red) or MG88 (green) for 48 hours. Cells were harvested, stained with anti-5-mC Ab and anti-mouse IgG conjugated with Rodamine X, and subjected to flow cytometry. Cells stained only with secondary antibody are used as a blank control (black line).

C, T24 cells were treated with either DMSO (red) or 5-aza-CdR (green) for 48 hours and subjected to flow cytometry as in B. T24 cells were treated for 48 hr with 200nM of either MG208 or MG88 (D), or 1µM 5-aza-C or DMSO (E). DNA was extracted and treated with sodium bisulfite. Two stage PCR was performed as described in Materials and Methods. The PCR products were separated on 2% agarose gel. One of two independent experiments with identical results is shown. L: ladder, 208: MG208 treated cells, 88: MG88 treated cells; Con: DMSO treated cells; Aza: 5-aza-CdR treated cells. U: unmethylated; M: methylated.

Fig. 7. 5-aza-CdR treatment induces expression of the proapoptotic gene BIK and cancer/testis specific genes MAGEB2 and SSX2. A, Total RNA was isolated from A549 cells treated with either 5-aza-CdR (1µM) or DMSO as a control for 24 to 96 hours. RT-PCR was performed with primers for the indicated genes as described
in Materials and Methods. 10 µl PCR products were run on 1.2% agarose gel. B, PCR products were quantified by densitometry, normalized to β-actin, and presented as arbitrary units for SSX2 and MAGEB2, or as a ratio of 5-aza-CdR (Aza) to DMSO for BIK, ATF-3, GADD45 and JUN-B. Representative data from two separate experiments are shown.

Fig. 8. *DNMT1* antisense knock down induces expression of the genotoxic stress response genes ATF-3, JunB and GADD45. A, Total RNA was isolated from A549 cells treated with 200nM of either MG208 or MG88 for 24 to 96 hrs. RT-PCR was performed with primers for the indicated genes as described in Materials and Methods. 10 µl PCR products were run on 1.2% agarose gel. In the case of MAGEB2, SSX2 and BIK, RT-PCR was also performed on A549 cells treated with 5-aza-CdR (1µM) for 72 hours, as an expression control. B, PCR products were quantified by densitometry and normalized to β-actin product. The relative ratio of MG88 to MG208 treated cells were then calculated and presented in panel B. Representative data from two to four separate experiments are shown.

Table. 1. Genes upregulated by 5-aza-CdR after 48 hours of treatment.

A549 cells were treated with 1 µM 5-aza-CdR or DMSO for 48 hours. Total RNA was subjected to a differential expression microarray analysis using HuGeneFL DNA microarrays containing oligonucleotides specific for approximately 12,000 human transcripts as described in Materials and Methods. The first column indicates the fold difference of the normalized expression of the indicated genes in 5-aza-CdR versus
DMSO treated A549 cells. The second column lists the accession numbers of the
genes. The third column lists the names of the genes and the last column provides
their abbreviated names.

Table. 2. **Stress responsive genes upregulated by MG88 after 48 hours of
treatment.**

A549 cells were transfected with 200nM of either MG208 or MG88 for 48 hours.
Total RNA was subjected to a differential expression microarray analysis using
HuGeneFL DNA microarrays containing oligonucleotides specific for approximately
12,000 human transcripts as described in Materials and Methods. The first column
indicates the fold difference of the normalized expression of the indicated genes in
MG88 versus MG208 treated A549 cells. The second column lists the accession
numbers of the genes. The third column lists the names of the genes and the last
column provides their abbreviated names.
Szyf_Fig. 1

A  

human mRNA 5' G G A G A A C G G T G C T C A T G C T - T 3'
MG88 3' C C T C T T G C C A C G A G T A C G A - A 5'
MG208 3' C C T G T T C C C A G G A C T A G C A - A 5'

mouse mRNA 5' G G A G A A C G G A A C A C A C A C T C T 3'

B  

Concentration and time of treatment

C  

D  

E
Szyf Fig. 4

A

**Graph A**

![Graph showing DNA content incorporation over time](image)

B

**Graph B**

![Graph showing BrdU incorporation over DNA content](image)
Fig. 5.  

5-mC staining
**Szyf_Fig. 6**

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**DNA content**

**BrdU incorporation**

**5- mC staining**

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Szyf_Fig. 7

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- SSX2
- MAGEB2
- BIK
- actin

B

- SSX2
- MAGEB2
- BIK
- actin

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- ATF-3
- JunB
- GADD45

ratio Aza/DMSO
Szyf_Fig. 8

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ATF-3
JunB
GADD45
actin

B

ATF-3
JunB
GADD45

MG88/MG208

24 48 72 96

MG88/MG208

24 48 72

MG88/MG208

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**Table 1**
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**Table 2**
Epigenomic stress response: Knock-down of DNA methyltransferase 1 triggers an intra S-phase arrest of DNA replication and induction of stress response genes
Snezana Milutinovic, Qianli Zhuang, Alain Niveleau and Moshe Szyf

J. Biol. Chem. published online February 7, 2003

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