DNA Methyltransferase 1 Knock Down Induces Gene Expression by a Mechanism Independent of DNA Methylation and Histone Deacetylation*

Snezana Milutinovic, Shelley E. Brown, Qianli Zhuang, and Moshe Szyf‡
From the Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec H3G 1Y6, Canada

DNA methyltransferase 1 (DNMT1) catalyzes the post-replication methylation of DNA and is responsible for maintaining the DNA methylation pattern during cell division. A long list of data supports a role for DNMT1 in cellular transformation and inhibitors of DNMT1 were shown to have antitumorigenic effects. It was long believed that DNMT1 promoted tumorigenesis by maintaining the hypermethylated and silenced state of tumor suppressor genes. We have previously shown that DNMT1 knock down by either antisense oligonucleotides directed at DNMT1 or expressed antisense activates a number of genes involved in stress response and cell cycle arrest by a DNA methylation-independent mechanism. In this report we demonstrate that antisense knock down of DNMT1 in human lung carcinoma A549 and embryonal kidney HEK293 cells induces gene expression by a mechanism that does not involve either of the known epigenetic mechanisms, DNA methylation, histone acetylation, or histone methylation. The mechanism of activation of the cell cycle inhibitor p21 and apoptosis inducer BIK by DNMT1 inhibition is independent of the mechanism of activation of the same genes by histone deacetylase inhibition. We determine whether DNMT1 knock down activates one of the nodal transcription activation pathways in the cell and demonstrate that DNMT1 activates Sp1 response elements. This activation of Sp1 response does not involve an increase in either Sp1 or Sp3 protein levels in the cell or the occupancy of the Sp1 elements with these proteins. The methylation-independent regulation of Sp1 elements by DNMT1 unravels a novel function for DNMT1 in gene regulation. DNA methylation was believed to be a mechanism for suppression of CG-rich Sp1-bearing promoters. Our data suggest a fundamentally different and surprising role for DNMT1 regulation of CG-rich genes by a mechanism independent of DNA methylation and histone acetylation. The implications of our data on the biological roles of DNMT1 and the therapeutic potential of DNMT1 inhibitors as anticancer agents are discussed.

DNA modification by methylation of cytosines residing at the dinucleotide sequence CG plays an important role in epigenomic programming of gene expression (1). Not all CGs are methylated, and the pattern of distribution of methylated and unmethylated CGs is cell type-specific (2). DNA methylation in regulatory regions of genes plays a role in silencing genes either by directly inhibiting the interaction of transcription factors with their regulatory sequences (3, 4) or by attracting methylated DNA-binding proteins, which in turn recruit histone deacetylases and histone methyltransferases, resulting in an inactive chromatin structure (5, 6). DNA methylation is catalyzed by DNA methyltransferases DNMTs,1 which transfer the methyl moiety from the methyl donor S-adenosylmethionine to 5th position on the cytosine ring (7). DNMT1 is responsible for maintaining the DNA methylation pattern during embryonal development and cell division (8, 9). DNMT1 deregulation was proposed to play a critical role in cellular transformation (10). Forced expression of DNMT1 was shown to transform NIH 3T3 cells (11), DNMT1−/−knockouts are resistant to colorectal tumorigenesis (12), and antisense knock down of DNMT1 reverses tumorigenesis in vitro (13, 14) and in vivo (15). The mechanisms through which DNMT1 causes cellular transformation and through which inhibition of DNMT1 reverses cellular transformation are unknown (16). The most obvious mechanism is that aberrant expression of DNMT1 causes methylation and silencing of tumor suppressor genes (17, 18). This hypothesis is supported by numerous documentations of methylated tumor suppressor genes in tumors (19). In accordance with this hypothesis, knock down of DNMT1 by either antisense or siRNA results in demethylation and activation of tumor suppressor genes such as p16 (14, 20).

However, it was surprisingly previously shown that knock down of DNMT1 results in induction of the unmethylated tumor suppressor gene p21 by a mechanism that does not involve DNA methylation (21). More recently it was shown that DNMT1 interacts with histone deacetylases (HDACs) 1 (22) and 2 (23) as well as histone methyltransferase Suv39H1 (24), suggesting that DNMT1 silences gene expression by recruiting chromatin-modifying enzymes. It was also shown that ectopic expression of DNMT1 could suppress exogenous genes bearing E2F1 sites by recruiting Rb/E2F1/HDAC1 complex (25). However, it is not clear whether DNMT1 regulates endogenous genes by these mechanisms.

We have previously shown that DNMT1 expression is regulated with the cell cycle (8, 26, 27) and that antisense knock down of DNMT1 results in an intra-S-phase arrest of DNA replication (28). This intra-S-phase arrest requires knock down of the DNMT1 protein rather than inhibition of DNA methyl-

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‡ To whom correspondence should be addressed: Dept. of Pharmacology and Therapeutics, McGill University, 3655 Sir William Osler Promenade, Montreal, Quebec H3G 1Y6, Canada. Tel.: 514-398-7107; Fax: 514-398-6690; E-mail: mszyf@pharma.mcgill.ca.

1 The abbreviations used are: DNMT, DNA methyltransferase; ODN, oligonucleotide; HSPA2, heat shock 70-kDa protein 2; BIK, Bcl-2-interacting killer; TSA, trichostatin A; HDAC, histone deacetylase; Suv39H1, suppression of variation 3–9; Drosophila, homolog of; RT, reverse transcriptase; MBI, Moloney murine leukemia virus reverse transcriptase; ChIP, chromatin immunoprecipitation; HSE, heat shock element; DAC, 5-aza-2′-deoxycytidine; HSP, heat shock protein.

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activation, suggesting that DNMT1 protein plays an important role in the regulatory circuitry independent of its methyltransferase activity (29). We and others have previously shown that antisense ODNs directed at DNMT1 exhibit sequence-specific down-regulation of DNMT1 and that they could be utilized to delineate the immediate consequences of knock down of the DNMT1 protein on gene expression and cellular regulation (14, 20, 28, 30, 31). In difference from agents such as 5-azacytidine that inhibit the catalytic activity of DNMT1 and, thus, mainly modify global DNA methylation-dependent roles of this protein (32), antisense knockout agents of DNMT1 causes an immediate arrest of DNA replication (28), resulting in limited passive demethylation. This allows us to study mainly DNA methyla-

Independent regulatory functions of DNMT1. We have previously shown that a number of stress response-related genes are activated by DNMT1 knock down (28). In this report we dissection the transcription factor pathway activated by DNMT1 knock down and show that surprisingly DNMT1 regu-

lates gene expression by a new mechanism that does not involve either DNA methylation or chromatin modification. We show that DNMT1 knock down and histone deacetylation inhi-

bates gene expression by independent mechanisms. These results have important implications on understanding the regulatory roles of DNMT1 in the cell cycle and cellular transformation, as well as on future approaches to targeting DNMT1 in anticancer therapy.

MATERIALS AND METHODS

Cell Culture, Antisense Oligonucleotides, and TSA Treatment—A549 cells, a human non-small cell lung carcinoma-derived cell line (33) (ATCC; CCL 185), were grown in Dulbecco’s modified Eagle’s medium (low glucose) supplemented with 10% fetal calf serum and 2 mM glutamine (low glucose) supplemented with 10% fetal calf serum and 2 mM glutamine.

DNA Methylation Analysis—50 µg of nuclear protein was fractionated on a 5% SDS-polycrylamide gel, transferred to polyvinylidene difluoride membrane, and reacted with the polyclonal anti-DNMT1 antibody (New England Biolabs) at a dilution of 1:2,000 in the presence of 0.05% Tween and 5% milk, and it was then reacted with anti-rabbit IgG (Sigma) at a dilution of 1:5,000 in the presence of 0.05% Tween and 5% milk. The protein loading was visualized in the same nuclear extracts. 50 µg of protein was fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and reacted with the monoclonal anti-β-actin antibody (Sigma, A5316) at a dilution of 1:50,000 in the presence of 0.05% Tween and 5% milk, and it was then reacted with anti-mouse IgG (Jackson Immuno-

Chemicals) at a dilution of 1:200,000 in the presence of 0.05% Tween.

RT-PCR—Total RNA was extracted using an RNaseasy kit (Qiagen). cDNA was synthesized in a 20-µl reaction volume containing 2 µg of total RNA, 40 units of Moloney murine leukemia virus reverse tran-

scriptase (MBI), 5 µM random primer (Roche Applied Science), 1 mM of each of the deoxyribonucleotide triphosphates, and 40 units of RNAse inhi-

bit (Promega) at 50 °C for 30 s. The cDNA synthesized was denatured at 95 °C for 5 s, the random primers were annealed for 10 min at 25 °C, and the mRNA was reverse-transcribed for 1 h at 37 °C. The reverse trans-

criptase was heat-inactivated for 10 min at 70 °C, and the products were stored at −20°C until use. PCRs were performed in a 40-µl reaction mixture containing 2 µl of synthesized cDNA product, 4 µl of 10× PCR buffer with (NH4)2SO4, 0.2 mM dNTPs, 1.5 unit of Taq polymerase (all from MBI), and 0.4 µl of each primer. Amplifications were performed in a Biometra T3 thermocycler (Bio-

medizine Analytik GmbH). The primer sequences and the amplifi-

cation protocols were as follows: p21 (cyclin-dependent kinase inhibitor 1) (sense, 5′-GGCCACTGGACGGCAGCAG-3′; antisense, 5′-GGCCG-

GGGTTGGAAGTGGTAGA-3′) 5′ 8°C 5 min, one cycle (95°C, 88°C, and 72°C for 2 s each), one cycle (95°C, 66°C, 72°C for 30 s each), 24 cycles (95°C, 65°C, and 72°C 30 s each), 72°C 5 min; BIK (BCL2-

interacting killer) (sense, 5′-GGCCGCTGCTGTTATCTTCT-3′; anti-

sense, 5′-CCAGTAGATTCTTTGCGAG-3′) 5′ 8°C 5 min, one cycle (95°C, 62°C, and 72°C for 30 s each), one cycle (95°C, 60°C, and 72°C for 30 s each), 29 cycles (95°C, 58°C, and 72°C 30 s each), 72°C 5 min; ODC (sense, 5′-CCGTTGGCAGAATTTCC-3′; antisense, 5′-ACTGGCAGCTGGGATCCTC-3′) 5′ 8°C 5 min, one cycle (95°C, 58°C, and 72°C for 30 s each), one cycle (95°C, 56°C, and 72°C for 30 s each), 26 cycles (95°C, 54°C, and 72°C for 30 s each), 72°C 5 min; β-actin (sense, 5′-GGTTCATGACGCTGTTGCT-3′; anti-

sense, 5′-CCGAGTGGGTCGTACACATC-3′) 5′ 8°C 5 min, one cycle (95°C, 66°C, and 72°C for 30 s each), one cycle (95°C, 64°C, and 72°C for 30 s each), one cycle (95°C, 60°C, and 72°C 30 s each), 72°C 5 min. The number of cycles was tested and selected so that the PCR amplification remained in the linear phase. 2 µl of the PCR products were run on a 1.2% gel and visualized by ethidium bromide staining. Denaturation and cooling were performed using MBI PEP-TAPEM software (Imaging Research Inc.).

Bisulphite Mapping of the BIK Promoter—A549 cells were treated with 10 nM MS88 or MG208 for 24 h. Bisulfite mapping was performed as described previously with minor modifications (34). 50 ng of sodium bisulfite-treated DNA samples were subjected to PCR amplification using the first set of primers (sense, 5′-GTAAAAAAGTTAGTTAGTT-GTG-3′; antisense, 5′-CTCACCTCTCTTTAAATCCT-3′). PCR products were used as templates for the nested PCR using the second set of primers (sense, 5′-AGGATTGGGGGAGGAGGAGG-3′; antisense, 5′-CAAC-

TACTCACACCACTAC-3′). The PCR products of the second reaction were subcloned into a TA cloning vector (Invitrogen), and the clones were sequenced using the T7 Sequencing kit (Amersham Biosciences).

Constructs analysis and reporter gene assay—The calcium phosphate precipitation method was used to transiently co-transfect HEK293 cells, plated in six-well dishes, with 2 µg of luciferase reporter con-

structs and 5 µg of either control pcDNA3.1 vector (Invitrogen) or a DNMT1 antisense (bp 396–5068) subcloned into pcDNA3.1 vector (as

DNMT1). The reporter constructs that were used were obtained either full p21 promoter (−2326 bp) or four different 5′ deletions (−1481, −883, −291, and −94) upstream from the luciferase gene (35). In addition, Sp1-responsive reporters were constructed using basic luciferase re-

porter containing only TATA box and initiator sequence (pGL2 T+1). An oligonucleotide containing consensus Sp1 binding site corresponding to 71–86 of the p21 promoter was synthesized and inserted in the reporter either once (1 × Sp1) or four times (4 × Sp1) into pGL2 T+1 vector (36). 48 h after transfection, the cells were lysed and luciferase activity was assayed using the Luciferase Assay System (Promega) according to the manufacturer’s instructions.

A549 cells plated in six-well dishes were transfected using 3.75 µg/ml Lipofectin (Invitrogen) and 0.5 µg of the reporter vectors containing different cis-acting enhancer elements upstream from the firefly lucif-

erase gene. All the reporters, except for the 4 × Sp1, belong to the Mercury Pathway Profiling System (Clontech). 24 h after transfection, the cells were either treated with 120 nM MS88 and MG208 for 24 h or with 1 µM TSA for 6 h. The luciferase activity was assayed using the Luciferase Assay System (Promega).

The full p21 promoter-luciferase reporter construct (−2326 p21) was used to create a construct with 3-bp mutations (CC→GA) within the Sp1 site corresponding to bases between −71 and −86 of the p21 promoter (−2326 p21 CC→GA). A QuickChange XLII site-directed mutagenesis Kit (Stratagene) was used with the following mutagenic primers: 5′-CCACTGGGCTCCGGG3′ and 5′-GGCCGGCTTACTAGGG-G3′. The amplification was performed as follows: 98°C 3 min, 18 cycles (98°C 50 s, 68°C 17 min), 68°C 7 min. A549 cells plated in six-well dishes were transfected using 3.75 µg/ml Lipofectin (Invitrogen) and 0.5 µg of either wild type or Sp1-mutated full p21 promoter construct. 24 h after transfection, the cells were treated with 120 nM MS88 and MG208 for 24 h, and the luciferase activity was assayed using the Luciferase Assay System (Promega).
harvested in cold phosphate-buffered saline containing protease inhibitors (Complete mini, Roche Applied Science), were pelleted, resuspended in 300 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), and incubated on ice for 10 min. Lysates were sonicated with six 10-s bursts. Debris was removed by centrifugation for 10 min at 13,000 rpm at 4°C. Supernatants were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 20 mM Tris-HCl, pH 8.1, 167 mM NaCl) and incubated with 80 μl of agarose G mix (50% agarose G, 0.2 mg/ml sonicated herring sperm, 0.5 mg/ml bovine serum albumin, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 30 min at 4°C. Beads were pelleted for 5 min at 3,000 rpm at 4°C. 100 μl of the supernatant was saved as input, and the rest was divided into equal aliquots and incubated by rocking with either no antibody (control) or specific antibody (95°C for 5 min, and 72°C for 30 s each) to the exogenous wild type and type p21 promoter. The FailSafe PCR system (Epicenter) was used with the pre-mix D. 0.5 μl of each primer, 0.5 μl of FailSafe polymerase, and 2 μl of ChIP product. The primer sequences and the amplification programs were as follows: p21 (324-bp region) (sense, 5′-ACCAAGCGAGGCGGCAGT-3′; antisense, 5′-CCGGCTCCATTTGAGA-3′), 95°C 5 min, one cycle (95°C, 64°C, and 72°C for 30 s each), one cycle (95°C, 62°C, and 72°C for 30 s each), 32 cycles (95°C, 60°C, and 72°C for 30 s each), 72°C 5 min; p21 (255-bp region) (sense, 5′-CTCGAGTTGTCGCAAGT-3′; antisense, 5′-GGAGGCTCCAGGTACCTTCGTA-3′), 95°C 5 min, one cycle (95°C, 64°C, 72°C for 30 s each), one cycle (95°C, 62°C, and 72°C for 30 s each), 32 cycles (95°C, 60°C, and 72°C for 30 s each), 72°C 5 min; ssDNA (sense, 5′-AGTTGACACCTTCCTACCGTG-3′; antisense, 5′-GGCCGCGGCTTCCTTGGTATC-3′), 95°C 5 min, one cycle (95°C, 60°C, and 72°C for 30 s each), one cycle (95°C, 58°C, and 72°C for 30 s each), 24 cycles (95°C, 56°C, and 72°C for 30 s each), 72°C 5 min; BIK (sense, 5′-GCACATTCCTTTGAACGGCTG-3′; antisense, 5′-GGTTCCCGCCTCCTTGGT-3′), 95°C 5 min, one cycle (95°C, 62°C, and 72°C for 30 s each), one cycle (95°C, 60°C, and 72°C for 30 s each), 32 cycles (95°C, 58°C, and 72°C for 30 s each), 72°C 5 min; protector of the bands was quantified by densitometric analysis using MCID software (Image Research Inc.). The intensities of the p21-amplified bands immunoprecipitated with the anti-Sp1 antibody were normalized to the intensities of the bands amplified from the input material.

Electrophoretic Mobility Shift Assays—Complementary oligonucleotides carrying either the Sp1 binding site corresponding to bases −71 and −80 of the wild type p21 promoter (5′-GGTCCCCGGCTCT-TGA-3′ and 5′-TCAAGGAGGCGGGAC-3′) or to a mutated Sp1 site containing 2-bp mismatch (CC-GA—5′-GGTCCCGGATCTTCCGA-3′ and 5′-TCAAGGATCCGGGAC-3′) were synthesized and annealed. 20 ng of the annealed oligonucleotides was end-labeled with [γ-32P]ATP and T4 polynucleotide kinase and gel-purified from 5% non-denaturing polyacrylamide gel. To measure the intensity of either the Sp1 site or its mutated version to Sp1 protein 1 × 10⁵ counts of the labeled oligonucleotides were used in a gel shift reaction containing 400 ng of the purified human recombinant Sp1 protein (Promega, catalog number E6391), 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)/(dI-dC), and the reaction was incubated for 20 min at 23°C. To demonstrate the presence of Sp1 in the DNA-protein complex, the Sp1-DNA reaction mixtures were preincubated with anti-Sp1 antibody (Santa Cruz Biotechnology, #sc-59) for 10 min before the addition of the labeled oligonucleotides. The complexes were resolved on a 5% non-denaturing polyacrylamide gel, and the gel was dried and analyzed by autoradiography.

RESULTS

DNMT1 Antisense and HDAC Inhibitor TSA Induce p21 and BIK Gene Expression; p21 and BIK Are Regulated by HDAC Activity—DNMT1 knock down was previously shown using a microarray gene expression analysis to induce expression of a cluster of genes involved in stress response (28). The kinetics of induction indicated that some of these genes were induced by a methylation-independent mechanism. All of the methylation-independent pathways of gene repression by DNMT1 that have been proposed to date are based on the ability of DNMT1 to interact with and possibly recruit histone deacetylases HDAC1 and HDAC2 (22, 25) (23) and histone methyltransferase SUV39H1 to promoters (24). However, the data supporting these interactions are based on ectopic expression experiments, and there is no evidence as of yet that an endogenous unmethylated gene is regulated by DNMT1-HDAC1 interaction through a histone acetylation-dependent mechanism. We therefore tested the hypothesis that DNMT1 knock down induces gene expression by a histone acetylation-dependent mechanism.

We focused on two genes that were demonstrated to be induced by DNMT1 knock down, the cell cycle inhibitor p21 and apoptosis inducer, Bcl-2 interacting killer, BIK. We have previously shown that the induction of p21 is methylation-independent (21), and the kinetics of BIK induction suggested that it is also induced by a methylation-independent mechanism (28).

We first tested the hypothesis that p21 and BIK genes are regulated by the state of histone acetylation by taking advantage of the general HDAC inhibitor trichostatin A (TSA). A549 cells were treated with 120 nM of either antisense to DNMT1 GM88, or its mismatch control MG208, for 12–42 h as well as with 1 μM TSA for 2–12 h. We show that both DNMT1 knock

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down and HDAC inhibition by TSA induce these genes to a comparable degree (4- to 5-fold). MG88 induces almost complete knock down of DNMT1 mRNA and protein 24 h after treatment (Fig. 1, A and B) at which point we observe peak induction of p21 and BIK mRNA (Fig. 1, B and C). TSA causes peak induction somewhat earlier, between 6 and 12 h (Fig. 2) as previously demonstrated (37). The slight delay in induction of BIK and p21 by DNMT1 knock down reflects the different mechanisms of action of these two inhibitors. TSA inhibits the catalytic activity of HDACs, whereas MG88 inhibits de novo synthesis of DNMT1 mRNA and protein. The delay in induction probably reflects the turnover rate of DNMT1 protein. The induction of p21 and BIK with TSA illustrates that these genes are down-regulated by histone deacetylation in their basal state, and it is therefore possible that knock down of DNMT1 affects the state of histone acetylation of these genes. We now address the question of whether induction of these genes by DNMT1 knock down and histone deacetylation inhibition act through identical or independent pathways.

**Knock Down of DNMT1 Does Not Cause Demethylation of BIK Promoter and Induces Its Expression by a Methylation-independent Pathway**—CG methylation is believed to be an on-off switch in gene expression. Hypermethylation of promoter regions is associated with compact chromatin inaccessi-
ble to the transcriptional machinery, which results in a stable suppression of gene expression (1). Hence, DNA methylation is unlikely to play a role in the regulation of \textit{p21} and \textit{BIK} genes, because they are expressed even in the untreated cells (Figs. 1 and 2). In accordance with this hypothesis, we have previously shown that the regulatory region of the \textit{p21} promoter upstream of transcription start site is unmethylated in A549 cells (21).

To rule out the possibility that induction of the \textit{BIK} expression by DNMT1 knock down involves demethylation of its promoter, we examined the state of methylation of the \textit{BIK} promoter after treatment with either MG88 or MG208. \textit{BIK} gene bears a CG island around the transcriptional start site, which renders it susceptible to control by methylation (Fig. 3A). We performed bisulfite mapping of the proximal part of this CG island and found that it is predominantly unmethylated, except for the sporadically methylated CG sites in both MG88- and MG208-treated A549 cells (Fig. 3B). These results suggest that DNMT1 knock down does not result in demethylation of the \textit{BIK} promoter. Thus, similar to \textit{p21} (16, 21), \textit{BIK} seems to be induced by a methylation-independent mechanism.

\textbf{Histone Acetylation of Promoters of \textit{p21} and \textit{BIK} Is Induced by TSA Treatment but Remains Unchanged after DNMT1 Antisense Knock down; DNMT1 and HDAC Control \textit{p21} and \textit{BIK} Expression by Independent Pathways—If DNMT1 knock down induces \textit{p21} and \textit{BIK} through either a histone deacetylase-dependent pathway (22) or a histone methyltransferase-depend-
Treatment for 6 h causes a 5–7 fold increase in the state of acetylation of both histones H3 and H4 associated with promoter regions of p21 and BIK. We conclude that the state of acetylation of histones associated with these regions is regulated by HDACs, but DNMT1 knock down does not affect either HDAC or histone methyltransferase activity on these histones.

HDAC inhibitors and DNMT1 knock down induce p21 and BIK by independent pathways.

The Sp1 Site Corresponding to Bases between −71 and −86 in the p21 Promoter Is Sufficient to Mediate the Induction by DNMT1 Knock down—Since we showed that DNMT1 knock down does not induce p21 and BIK by modifying their chroma-
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Inhibition of DNMT1 induces the activity of p21 promoter through Sp1 sites. HEK 293 cells were co-transfected with either a control vector (pcDNA3.1), or the same vector expressing DNMT1 mRNA in the antisense orientation (as DNMT1), as well as the luciferase reporter constructs depicted. A, serial deletions of the p21 promoter inserted upstream from the firefly luciferase gene and their activities are shown. The presence of the Sp1 and E2F sites in the first 94 bp of p21 promoter are shown (gray and black circles). The sequence of the Sp1 site crucial for the p21 activation is shown. B, basic luciferase expression vector containing only TATA box and the initiator sequence (pGL2 T+1) was used to create 1 × Sp1 and 4 × Sp1 constructs by inserting either one or four copies of the Sp1 response element (shown in A) upstream from TATA box. The luciferase activity from the whole cell lysate was assayed 48 h after the transfection. The induction of the luciferase reporters by antisense to DNMT1 is expressed as a percentage of the empty vector controls. The error bars are S.E. values of triplicate experiments.

Fig. 5. Inhibition of DNMT1 induces the activity of p21 promoter through Sp1 sites. HEK 293 cells were co-transfected with either a control vector (pcDNA3.1), or the same vector expressing DNMT1 mRNA in the antisense orientation (as DNMT1), as well as the luciferase reporter constructs depicted. A, serial deletions of the p21 promoter inserted upstream from the firefly luciferase gene and their activities are shown. The presence of the Sp1 and E2F sites in the first 94 bp of p21 promoter are shown (gray and black circles). The sequence of the Sp1 site crucial for the p21 activation is shown. B, basic luciferase expression vector containing only TATA box and the initiator sequence (pGL2 T+1) was used to create 1 × Sp1 and 4 × Sp1 constructs by inserting either one or four copies of the Sp1 response element (shown in A) upstream from TATA box. The luciferase activity from the whole cell lysate was assayed 48 h after the transfection. The induction of the luciferase reporters by antisense to DNMT1 is expressed as a percentage of the empty vector controls. The error bars are S.E. values of triplicate experiments.
Fig. 6. Mutation of the Sp1 site corresponding to bases between −71 and −86 of the p21 promoter precludes Sp1 binding and abolishes the activation produced by DNMT1 knock down. A, the physical maps of wild type (−2326 p21) and Sp1 mutated (−2326 p21 CC→GA) full p21 promoter luciferase reporter construct are shown. The sequence of the p21 promoter Sp1 site used for the gel shifts (C) is indicated, with the boldface letters representing the Sp1 recognition element and the underlined letters indicating the mutated bases. The arrows indicate the primers used for ChIP (D). B, A549 cells were transfected with 0.5 μg of either wild type (−2326 p21) or Sp1-mutated (−2326 p21 CC→GA) full p21 promoter luciferase reporter construct. 24 h post-transfection, the cells were treated with 120 nM MG88 and MG208 for 24 h, and the luciferase activity was measured and normalized as described under “Materials and Methods.” C, 400 ng of recombinant human Sp1 protein (hrSp1) was incubated with end-labeled oligonucleotides corresponding to bases between −71 and −86 of either a wild type or CC→GA mutated p21 promoter. A supershift was generated by incubating the reaction with 1 μg of anti-Sp1 antibody. The positions of the Sp1-DNA complex and the anti-Sp1-Sp1-DNA supershift are indicated with arrows. D, A549 cells were transfected with 1.5 μg of either −2326 p21 or −2326 p21 CC→GA construct. Chromatin was isolated and immunoprecipitated with anti-Sp1 antibody, followed by PCR amplification of the 256 bp of the proximal p21 promoter of the transfected luciferase reporters. The relative occupancy of the promoters with Sp1 is determined by densitometric quantification of PCR products in the anti-Sp1 immunoprecipitate and normalization to the input DNA. The normalized Sp1 occupancy of the wild type construct (−2326 p21) and the mutated construct (−2326 p21 CC→GA) is represented in arbitrary units. The error bars are S.E. values of the triplicates. There was no PCR amplification in no antibody control immunoprecipitations (data not shown).
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sively with the wild type Sp1 oligonucleotide but not with the CC→GA mutated Sp1 oligonucleotide (Fig. 6C). To determine that Sp1 is present in the complex, we demonstrated that an anti-Sp1 antibody supershifted the complex (Fig. 6C). To test whether this mutation precludes the binding of Sp1 to the p21 promoter in living cells, we transfected A549 cells with either wild type (−2326 p21) or Sp1-mutated (−2326 CC→GA p21) full p21 promoter reporter construct and performed a ChIP assay using anti-Sp1 antibody 48 h after transfection. The binding of the endogenous Sp1 protein to the Sp1-mutated construct was markedly reduced compared with the wild type p21 promoter construct (Fig. 6D). Some minute Sp1 binding to the −2326 CC→GA construct was detected suggesting that other putative Sp1 elements found in the p21 promoter might also mediate Sp1 protein binding in A549 cells. However, our results suggest that the occupancy of the p21 promoter with Sp1 is mostly determined by the Sp1 site at −71 to −86.

**DNMT1 Antisense and TSA Affect Different Transcription Activator Pathways**—Our previously published differential microarray gene expression analysis revealed that DNMT1 knock down results in induction of multiple genes, including many stress response genes (28). The downstream response of a cell to different physiological and toxic signals involves activation of distinct transcription factors, which in turn activate genes bearing their cognate response elements. To test which nodal transcription factor pathway in the cell is responsive to DNMT1 knock down, we utilized the Mercury Pathway Profiling System (Clontech). This system consists of a set of reporter constructs containing distinct enhancer elements upstream from the firefly luciferase gene. The description of the enhancer elements used in our study is shown in Fig. 7A. To further determine whether DNMT1 knock down acts through an HDAC-independent pathway, we compared the pathway profile induced by DNMT1 knock down to the profile elicited by HDAC inhibition. 24 h after transient transfection of A549 cells with each of these reporter vectors, we treated cells with 120 nM MG88 or MG208 for 24 h, or with 1 μM TSA for 6 h. We then determined the luciferase activity in these cells and found that the profiles of signal transduction pathway activation by MG88 and TSA were different. HDAC inhibition resulted in a general induction of 9 out of the 11 pathways tested, which is consistent with its general effects on histone acetylation. On the other hand out of 11 pathways tested, 4 were induced by MG88 treatment by at least 2-fold (Fig. 7B). These different profiles of responses to the two treatments are consistent with our results showing that MG88 induces unmethylated promoters independent of histone acetylation (Fig. 3). Two of the response elements activated by DNMT1 knock down, NF-κB and AP-1, were expected, because our gene array analysis revealed the induction of transcription factors binding to these elements, NF-κB and c-Jun, respectively (28). The induction of the heat shock elements (HSEs) was strong, and this is also in accordance with our gene array data showing that DNMT1 knock down induces stress response genes (28). However, one transcription factor that consistently stood out as the highest responder to DNMT1 knock down was Sp1. Based on our analysis of p21 and BIK promoters, which revealed the presence of multiple Sp1 elements (Fig. 4A), and based on our deletion analysis of the p21 promoter, we conclude that these elements are primarily responsible for mediating the effects of DNMT1 knock down. Interestingly, the Sp1 family of proteins contains zinc finger motifs that primarily bind to the CG-rich cis-elements, and it is well established that CG-rich promoters are silenced by DNA methylation. One excellent example of this silencing is the tumor suppressor p16. It has also been shown that binding of the methylated DNA-binding protein MeCP2 to methylated CG-rich promoters inhibits transactivation by Sp1 (38). Also, methyl-binding domain protein 1 can repress transactivation of a methylated promoter by Sp1 through its interactions with methyl-binding domain protein 1-containing chromatin-associated factor (39). Methylation of CGs adjacent to Sp1/Sp3 binding elements inhibits Sp1 binding and the activity of the p21 promoter (40). It is therefore important to note that all the reporters used in this profiling experiment were unmethylated, because they were all amplified in Escherichia coli, which does not express a CG DNA methyltransferase. We have also previously demonstrated that plasmids do not undergo de novo methylation in the cell (21). Thus, our assay detects exclusively the methylation-independent effects of DNMT1 knock down. The fact that DNMT1 regulates Sp1 responsiveness in the absence of DNA methylation might explain how DNMT1 regulates the expression of multiple housekeeping genes rich in unmethylated CG sites.

**MG88 Activates Sp1 Firing by a Mechanism, Which Is Independent of Histone Acetylation**—It has been previously demonstrated that induction of p21 by TSA requires the presence of
Sp1 response elements (37). We examined whether the induction of 4 × Sp1 reporter vector by DNMT1 knock down is acetylation-dependent or -independent, like the induction of endogenous p21 and BIK genes (Fig. 4). A549 cells were transiently transfected with 4 × Sp1 construct, and the cells were treated with either MG88 or MG208 for 24 h. The cells were then subjected to a ChIP assay using antibodies against acetylated H3 or acetylated H4 histones, followed by PCR amplification of the region containing four tandem Sp1 copies of this luciferase reporter. The results indicate that MG88 treatment does not cause a change in acetylation of either H3 or H4 histones associated with the transiently transfected 4 × Sp1 reporter construct, similar to the endogenous p21 and BIK promoters (Figs. 4 and 8). On the other hand, HDAC inhibitor TSA, which induces 4 × Sp1 reporter construct to the similar extent as the MG88 treatment (6- and 8-fold, respectively, Fig. 7), produces a marked induction of acetylation of both histones H3 and H4 associated with this reporter (Fig. 8). Thus, Sp1 element firing could be induced by either inhibition of histone deacetylation or by knock down of DNMT1 through independent mechanisms.

Knock down of DNMT1 Does Not Increase the Occupancy of Either p21 or BIK or the 4 × Sp1 Reporter Gene Promoter with Sp1/Sp3—One possible explanation for the increase in Sp1 promoter firing is that DNMT1 knock down results in an increase in either Sp1 mRNA transcription or Sp1 binding activity. The microarray gene expression analysis did not reveal an induction of any of the Sp1-binding proteins upon MG88 treatment (28). It was previously reported that Sp1 binding activity is increased after treating cells with a DNA methylation inhibitor 5-aza-2′-deoxycytidine (DAC) (41). We performed gel shift assays but did not detect any increase in either Sp1 or Sp3 binding to Sp1 recognition elements upon MG88 treatment (data not shown).

We then tested whether Sp1 or Sp3 bind to p21, BIK, and the 4 × Sp1 promoter, which is expected if DNMT1 knock down activates these promoters through Sp1 elements. Because it was previously shown that Sp3 binding might negatively affect promoters regulated by Sp1 (42, 43), we determined the occupancy of the p21 promoter with both transcription factors. ChIP assays using either anti-Sp1 or anti-Sp3 antibodies was performed on either MG88- or MG208-treated A549 cells, followed by PCR amplification of p21, BIK, and 4 × Sp1 promoters. The results in Fig. 7B show that both Sp1 and Sp3 interact with these promoters in A549 cells, which is consistent with either Sp1 or Sp3 mediating the effects of DNMT1 knock down. However, DNMT1 knock down does not change the occupancy of these promoters by either Sp1 or Sp3. In summary, DNMT1 knock down induces the promoters containing Sp1 recognition elements by a mechanism that does not involve increasing the occupancy of these promoters by Sp1 or Sp3. DNMT1 regulates CG-rich Sp1-containing promoters by a mechanism that does not involve DNA methylation.

DNMT1 Knock Down Induces Heat Shock 70-kDa Protein 2 by a Histone Acetylation-independent Mechanism—Our pathway profiling revealed that MG88 and TSA induce the firing of a promoter bearing the heat shock element (HSE) (Fig. 7). HSEs are found within the promoters of heat shock proteins (HSPs), and they bind heat shock factors resulting in gene activation (44). HSPs are a family of molecular chaperons that are involved in response to heat shock and a range of other cellular insults and might be involved in the epigenomic stress response, which is launched by DNMT1 knock down (28). To validate that the induction of firing of HSEs by DNMT1 knock down applies also to an endogenous HSP gene, we determined whether the gene encoding the nodal heat shock 70-kDa protein 2 (HSPA2) is induced by this treatment. The results presented in Fig. 9A illustrate that DNMT1 knock down induces HSPA2 3-fold. Induction of HSPA2 by DNMT1 knock down was also observed in the microarray gene expression analysis (data not shown).

We then addressed the question whether the acetylation-independent effect that DNMT1 knock down has on Sp1 elements is specific to these elements, or whether it applies to other nodal transcriptional regulatory pathways such as the heat shock response. We first demonstrated that the HSPA2 gene is partially suppressed by histone deacetylation in our system, as indicated by its induction with the HDAC inhibitor TSA (Fig. 9B). It has been previously shown that TSA induces Hsp70 gene expression in Drosophila (45). As expected, ChIP analysis of the HSPA2 promoter on A549 cells treated with 1 μM TSA for 6 h resulted in an increase in the state of acetylation of both H3 and H4 histones associated with this promoter. However, as is the case with Sp1 elements, DNMT1 knock down did not change the state of acetylation of HSPA2 pro-
moter (Fig. 10B). To test whether these effects on histone acetylation are carried through the gene downstream of the enhancer elements, we analyzed a region of the second exon of HSPA2 gene. We found that, unlike TSA treatment, which produces an increase in acetylation of both histones H3 and H4, DNMT1 knock down does not cause any change in histone acetylation associated with exon 2 (Fig. 10C).

In summary, DNMT1 knock down results in induction of two nodal transcriptional regulatory pathways in the cell by a mechanism that is surprisingly independent of both DNA methylation and histone acetylation. We propose a new function of DNMT1 that is independent of both its methyltransferase activity and its histone deacetylase-recruiting activity.

DISCUSSION

This report addresses the question of whether DNMT1 regulates gene expression by a DNA methylation independent pathway and whether this regulation is mediated by the state of chromatin modification through histone acetylation and methylation. We took advantage of well characterized second generation DNMT1 antisense ODNs (MG88) and their mismatch controls (MG208). We first show that our ODNs achieve a complete knock down of DNMT1 protein 24 h after initiation of treatment. In the same time frame, DNMT1 knock down induces the expression of critical cell-cycle regulatory genes such as p21 and BIK. The regulation of expression of either p21 or BIK does not involve a change in their DNA methylation state (Fig. 3) and previous data (21). Moreover, DNMT1 knock down results in activation of transiently transfected unmethylated reporter genes directed by tandem copies of distinct transcription factor recognition elements. Because it was previously shown that DNMT1 interacts with histone-modifying enzymes (22, 24), we tested the hypothesis that DNMT1 knock down effects are mediated by a change in histone acetylation. Both BIK and p21 are partially suppressed by HDACs in our system, because the inhibition of HDACs by TSA increases the state of acetylation of their promoters and induces their expression. Nevertheless, we demonstrate that DNMT1 regulates the expression of these genes without changing their state of acetylation (Fig. 4). Our data therefore unravel a third novel mechanism of regulation of gene expression by DNMT1 that does not involve either DNA methylation or histone modification, the two most established fundamental principles of epigenomic regulation.

Knock down of DNMT1 results in induction of many genes, which includes a class of genes encoding stress response proteins (28) suggesting that DNMT1 acts on a common transcriptional regulatory pathway. Our analysis of the p21 promoter revealed that the Sp1 element corresponding to the bases between −71 and −86 mediates the effects of DNMT1 knock down, because four copies of this site (4 × Sp1) were sufficient to mediate the induction of the luciferase reporter produced by DNMT1 knock down (Fig. 5B). In addition, 2-bp mutation (CC→GA) of this Sp1 site in the full-length p21 promoter abolished Sp1 binding and the induction by DNMT1 knock down. p21 promoter responds to both antisense ODN as well as a plasmid expressed DNMT1 antisense mRNA, demonstrating that this induction is not a consequence of nonspecific effects of the modified ODNs (Figs. 5 and 6). We further tested a number
of basic cis-acting elements that are known to respond to major regulatory pathways in the cell. Among these, E2F elements were previously shown to play an important role in regulating p21 gene expression (40–43). Our data exclude the possibility that DNMT1 acts on a pathway triggering E2Fs, because the E2F recognition element was unresponsive. This is surprising, because DNMT1 was shown to suppress exogenous E2F-responsive elements by recruiting HDACs into E2F1/Rb/DNMT1 complex (25). Our data, showing that histone deacetylation is not changed by DNMT1 knock down, further support our conclusion that E2F elements are not involved. Sp1 therefore appears to be the main transcription regulatory element triggered by DNMT1 knock down.

Induction of Sp1 response by DNMT1 knock down is not mediated by increased Sp1 expression or increased occupancy of the p21 and BIK promoters with either Sp1 or Sp3 (Fig. 8). DNMT1 must therefore be acting either directly or indirectly on proteins interacting with Sp1 or Sp3 and modulating their trans-activation activity. Identifying these proteins will require extensive future experiments. Sp1 protein contains zinc finger motifs that primarily bind to the GC-rich cis-elements that are widely distributed in the promoters, enhancers, and locus-control regions of housekeeping genes and some cell-specific genes (46). It was generally accepted that these genes escape regulation by DNMT1, because their promoters are especially enriched in unmethylated CG sites and are usually not methylated. The fact that DNMT1 regulates Sp1 responsiveness in the absence of DNA methylation might explain how DNMT1 regulates the expression of multiple housekeeping cell cycle regulatory genes (28). Whereas ectopic DNA methylation is known to suppress Sp1 responsive genes by attracting methylated DNA-binding proteins that suppress Sp1 activation (38–40), our data suggest an entirely distinctive mechanism through which DNMT1 suppresses Sp1-responsive genes.

The observation that DNMT1 regulates Sp1-responsive genes suggests that it may play a role in the regulation of the cell cycle. Sp1 elements are found in genes that perform contradictory cellular roles such as, thymidine kinase, which
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