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*Advances in Brief*

# p53-Mediated Repression of DNA Methyltransferase 1 Expression by Specific DNA Binding[1]

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## Abstract

**Cytosine methylation patterns in genomic DNA are significantly altered in cancer, and *de novo* CpG island methylation has been implicated in tumor suppressor gene silencing. Here we demonstrate a mechanistic link between the *p53* tumor suppressor gene and control of epigenetic regulation by genomic methylation. Deletion of *p53* in HCT116 human colon carcinoma cells and primary mouse astrocytes resulted in a 6-fold increase of DNA cytosine methyltransferase 1 (Dnmt1) mRNA and protein, suggesting relief of p53-mediated *Dnmt1* repression. A p53 consensus binding site in exon 1 of the human *Dnmt1* gene bound recombinant p53 *in vitro* and endogenous p53 *in vivo* in the absence of stimuli that activate p53, implying that p53 controls *Dnmt1* transcription through direct DNA binding. Interestingly, ionizing radiation or etoposide, both of which stabilize and activate p53, diminished p53 binding in chromatin immunoprecipitation assays, concomitant with a 5-fold increase in Dnmt1 levels. Our findings suggest that activation of p53 reduces binding and relieves transcriptional repression of the *Dnmt1* gene, whereas loss of p53, a frequent, early event in tumorigenesis, may significantly contribute to aberrant genomic methylation.**

## Introduction

Dnmt1[3] methylates cytosine residues at selected and specific CpG dinucleotides, a process associated with transcriptional silencing during mammalian development and differentiation (1, 2). In malignant cells, methylation patterns are significantly altered; the emerging generality appears to be global hypomethylation coupled with hypermethylation of CpG islands that are normally refractive to cytosine methylation (2). These changes in methylation patterns critically alter gene expression in tumors in ways that appear to drive tumor progression. Thus, increased methylation in promoter regions contributes to or causes the silencing of one allele of several tumor suppressor genes (2, 3). It has been shown recently that selective depletion of Dnmt1 results in lower maintenance methyltransferase activity, global and gene-specific demethylation, and reexpression of silenced tumor suppressor genes in human cancer cells (4). Reducing Dnmt1 expression using antisense technology appears to reverse the transformed phenotype of cultured cells (2). Conversely, forced overexpression of Dnmt1 induces transformation of NIH 3T3 cells and *de novo* methylation of CpG island sequences in human fibroblasts (2). Together, these observations suggest that *Dnmt1* expression levels are under strict regulatory control during development and that the balance of factors involved in this regulation appears to be disrupted during tumorigenesis.

p53 is a central tumor suppressor in humans and is one of the most frequently mutated genes in inherited and sporadic cancers (5). As a DNA sequence-specific transcription factor, p53 plays a critical role in both activation and repression of target genes (6). After genotoxic stress, p53 is stabilized by posttranslational modifications and accumulates in the nucleus, where it interacts with target loci (6). The consensus DNA sequence specific for p53 recognition consists of two copies of the inverted repeat sequence [RRRC(A/T)(A/T)GYYY] separated by 0–13 nucleotides (7), which binds a tetrameric p53 complex (8). Examination of the human *Dnmt1* genomic locus revealed candidate p53 binding sites in the 5′-flanking region and in exon 1. Several candidate p53 binding sites were also noted in the 5′ region of the mouse *Dnmt1* locus. We therefore asked whether p53 was involved in the regulation of *Dnmt1* expression and whether loss of p53 function might be a contributing factor in the reassignment of methylation patterns during oncogenesis.

## Materials and Methods

**Cell Culture.** HCT116 *p53*+/+ and *p53*−/− cells, obtained from Dr. Bert Vogelstein (Johns Hopkins University), were grown in RPMI 1640 supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere. Primary astrocytes were obtained from the brains of neonatal mice (9). Dissociated cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 100 μg/ml L-glutamine.

**EMSAs.** Oligonucleotide duplexes representing the following p53 response elements were end labeled using T4 polynucleotide kinase (New England Biolabs, Beverley, MA): $p21^{Waf1}$, 5′-AGCTTGAACATGTCCCAACATGGTGA-3′; mutant $p21^{Waf1}$, 5′-AGCTTGAAACTCTCCCAAACTCGTGA-3′; pDnmt1-1, 5′-TCCCAGACTCGTGCCAACATGGTGAAACCCTGTC-3′; pDnmt1-2, 5′-CTCTGGAGTAGTTTGGACTATGGGCACATGCCACCACGACTA GCTAATTT-3′; pDnmt1-3, 5′-CCTTGCGCATGCGTGTTCCCTGGGGCATGGCCGGCT-3′; and mutant pDnmt1-3, 5′-CCT TGCGACTCCGTGTTCCCTGGGGACTCGCCGGCT-3. EMSAs were performed in 20 μl containing 10 mM HEPES (pH 7.9), 75 mM KCl, 2.5 mM $MgCl_2$, 100 mM EDTA, 100 μM DTT, 3% Ficoll, 15 ng of poly(deoxyinosinic-deoxycytidylic acid), 1 ng of $^{32}$P-labeled oligonucleotide probe, and 50 ng of recombinant p53 protein (purified from bacculoviral-infected Sf9 cells; a gift of Dr. Sumitra Deb, Virginia Commonwealth University). Complexes were resolved on a 4% polyacrylamide gel (acrylamide: bisacrylamide, 35:1) containing 0.5x Tris-borate-EDTA at 150 V for 1 h at room temperature. Gels were dried and exposed for autoradiography. Competition assays incorporated up to a 200-fold molar excess of unlabeled oligonucleotide per reaction.

**Northern Blot Analysis.** Total cellular RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA), and mRNA was isolated using Oligotex (Qiagen, Valencia, CA). Five μg of polyadenylated RNA were denatured with glyoxal-DMSO, fractionated on a 1% agarose gel, and transferred to a nylon membrane. The membrane was prehybridized with 0.5 M sodium phosphate (pH 7.0), 1 mM EDTA, 1% BSA, and 7% SDS for 1 h and

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[3] The abbreviations used are: Dnmt1, DNA methyltransferase 1; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; IR, ionizing irradiation.

hybridized with $^{32}$P-labeled cDNA probe at 65°C for 18–20 h. The membranes were washed to a stringency of 0.2× SSC, 0.1% SDS solution at 50°C and exposed to X-ray film. The probes used included human Dnmt1 (1.2-kb *Eco*RI-*Bam*HI fragment from pKR11D-6; obtained from Dr. Peter Jones, University of Southern California), p21 (850-bp *Eco*RI fragment from p21-9C; obtained from Dr. K. Huppi, National Cancer Institute), GAPDH (750-bp *Pst*I-*Xba*I fragment from pHcGAP; obtained from American Type Culture Collection), and mouse Dnmt1 (600-bp *Eco*RI-*Bam*HI fragment from pMG; obtained from Dr. Timothy Bestor, Columbia University).

**Western Blot Analysis.** Cell pellets were lysed in 60 mM Tris-HCl (pH 7.4), 5% glycerol, 5% β-mercaptoethanol, and 2% SDS containing a mixture of protease inhibitors (Roche Applied Science, Indianapolis, IN). For Dnmt1 analysis, 200 μg of total protein were separated in a 5.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. Western blotting using a 1:1000 dilution of Dnmt1 $NH_2$-terminal antibody (New England Biolabs) was performed and analyzed with an enhanced chemiluminescence horseradish peroxidase-Western blotting analysis system (Supersignal; Pierce, Rockford, IL) according to the manufacturer's instructions.

**ChIP Assay.** ChIP experiments were performed essentially as described previously (10), with minor modifications. HCT116 cells ($4 \times 10^6$) were cross-linked with 1% formaldehyde for 10 min at room temperature. Immunoprecipitations were carried out in 150 mM NaCl, 25 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors. Equal aliquots of the precleared extract were used for a control lacking antibody and for immunoprecipitation with 2 μg of anti-p53 antibody (p53 Ab-6; Oncogene, Boston, MA). After incubation with protein A beads, the supernatants of control samples were used as the total chromatin "input" samples. After immunoprecipitation, beads were washed in radioimmunoprecipitation assay buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8), 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP40], High Salt Wash [500 mM NaCl, 50 mM Tris-HCl (pH 8), 0.1% SDS, and 1.0% NP40], and LiCl Wash [250 mM LiCl, 50 mM Tris-HCl (pH 8), 0.5% sodium deoxycholate, and 1.0% NP40]. After reversing the cross-links, proteins were removed by phenol chloroform extraction, and DNA was precipitated with ethanol and resuspended in 20 μl of $H_2O$. Subsequent PCR was carried out using 1 μl of immunoprecipitated target DNA. Primers used were as follows: *p21* promoter, 5′-GTGGCTCTGATTGGCTTTCTG-3′ (sense) and 3′-GAACCCGACGGACAAAAGTC-5′ (antisense); and *Dnmt1* promoter, 5′-ATCCCCATCACACCTGAAAG-3′ (sense) and 3′-CCTGTCCAGAAGGATGGAAC-5′ (antisense). Amplification was carried out for 30 cycles, which was determined to be within the linear range.

**Quantitative PCR.** Quantitative PCR was performed on the iCycler (Bio-Rad) using 10× Quantitect Sybr Green Master Mix (Qiagen), 3 μm each primer, and 1 μl of a 1:5 sample dilution of ChIP DNA template. Amplification was carried out in a total volume of 25 μl. The PCR cycles were 95°C for 15 min to activate the hot start Taq polymerase, followed by 94°C for 30 s, 57°C for 30 s (p21 and Dnmt1 primers), and 72°C for 30 s repeated 36 times. Error bars show the SD of triplicate measurements. At the end of the PCR, the temperature was increased from 55°C to 95°C at a rate of 0.5°C/10 s to construct the melting curve. Both primer sets generated only one amplification peak, demonstrating specificity. Primer sets used were as follows: *p21*, 5′-CTTTCCACCTTTCACCATTCC-3′ (sense) and 5′-AAGGACAAAATAGCCACCAGC-3′ (antisense); and *Dnmt1*, 5′-ATCCCCATCACACCGAAAG-3′ (sense) and 5′-CCTGTGCAGAAGGATGGAAC-3′ (antisense). Results are expressed as percentage input [Ab+/input ± SD (δX/X)]. $\delta X/X = \sqrt{(\delta A/A)^2 + (\delta B/B)^2}$.

## Results and Discussion

Examination of the human *Dnmt1* genomic locus revealed three candidate p53 binding sites in the 5′-flanking region and in exon 1 at positions –1196 to –1174 (pDnmt1-1), −555 to –533 (pDnmt1-2), and +30 to +56 (pDnmt1-3) relative to the transcriptional start site (Fig. 1*A*). The ability of recombinant p53 protein to bind these sites was determined by EMSA. An electrophoretically retarded complex indicative of p53 binding was observed in the presence of the consensus binding site located in exon 1 (pDnmt1-3), which was equivalent to that seen with the well-studied p53 binding site from $p21^{Waf\text{-}1}$ (Fig. 1*B*). p53 failed to show significant binding to the two consensus sites located upstream of the proximal promoter (pDnmt1-1 and pDnmt1-2; data not shown) or to mutant forms of either $p21^{Waf\text{-}1}$ or pDnmt1-3 oligonucleotides (Fig. 1*B*, *Lanes 5* and *7*). Oligonucleotide pDnmt1-3 can compete with the $p21^{Waf\text{-}1}$ oligonucleotide at 20-fold molar excess (Fig. 1*D*), whereas $p21^{Waf\text{-}1}$ requires 100-fold molar excess to efficiently compete with the pDnmt1-3 oligonucleotide (Fig. 1*E*). Thus, the affinity of p53 for the Dnmt1-3 element was higher than that of the p53 binding sequence from $p21^{Waf\text{-}1}$.

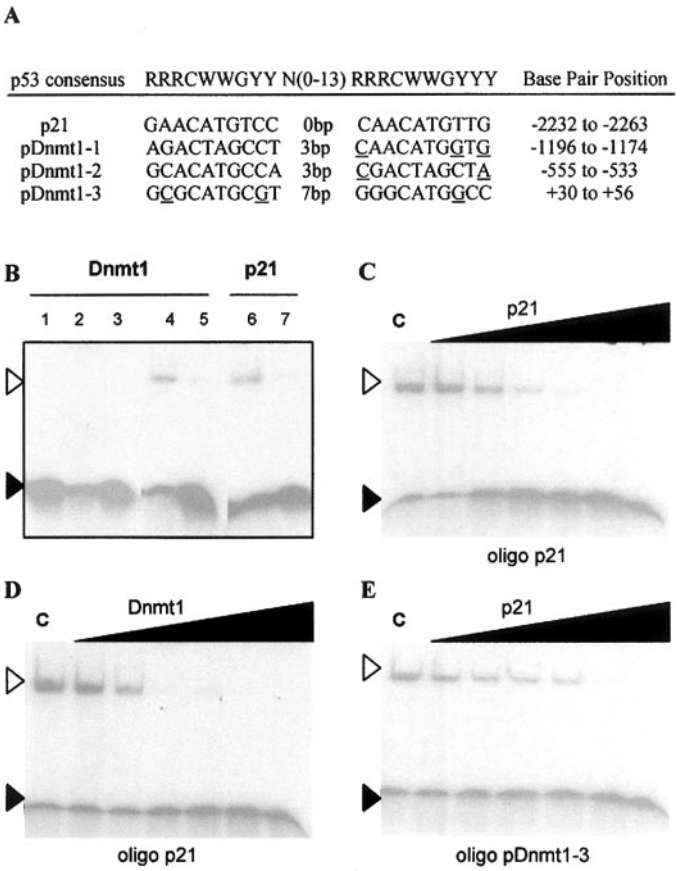


Fig. 1. p53 binds *in vitro* to a consensus sequence located in exon 1 of the Dnmt1 gene. *A*, candidate p53 binding sites within the human Dnmt1 locus. *R*, purine; *Y*, pyrimidine; *W*, A or T. Location of each candidate binding site is given relative to the transcription start site. *B*, EMSA of double-stranded oligonucleotides containing potential p53 binding sites within the Dnmt1 locus. A p53 binding site from $p21^{waf1}$ was used as a positive control, and mutant oligonucleotides for both p21 and pDnmt1–3 were used as negative binding controls. *Lane 1*, no p53 control; *Lane 2*, pDnmt1-1; *Lane 3*, pDnmt1-2; *Lane 4*, pDnmt1-3; *Lane 5*, mutant pDnmt1-3; *Lane 6*, p21 oligonucleotide; *Lane 7*, mutant p21 oligonucleotide. *C*–*E*, competition gel shift assays analyzing the relative binding affinity of p53 protein to p21 in comparison with Dnmt1. Equal amounts (5-, 20-, 50-, 100-, or 200-fold molar excess) of the indicated unlabeled oligonucleotide were added to the reaction. In each panel, *c* represents the control reaction in the absence of competitor DNA. The *open arrow* indicates retarded p53-DNA complexes. The *closed arrow* denotes unbound probe.

We used the isogenic human colon carcinoma cell lines HCT116 *p53*+/+ and HCT116 *p53*−/− to investigate the effect of p53 on endogenous *Dnmt1* expression. Northern analysis of Dnmt1 mRNA levels in these cell lines revealed a 6-fold elevated level of Dnmt1 in cells lacking p53 (Fig. 2*A*). Western analysis of whole cell lysates showed that Dnmt1 protein levels were similarly elevated (Fig. 2*B*). We confirmed the generality of these findings using astrocytes from *p53*+/+ and *p53*−/− knockout mice. Loss of *p53* is an initiating event in astrocyte transformation and an early step in the formation of astrocytic gliomas (11), which show a very high level of abnormal methylation patterns (12). A similar pattern of elevated Dnmt1 mRNA expression was seen in primary cultures of *p53*−/− astrocytes when

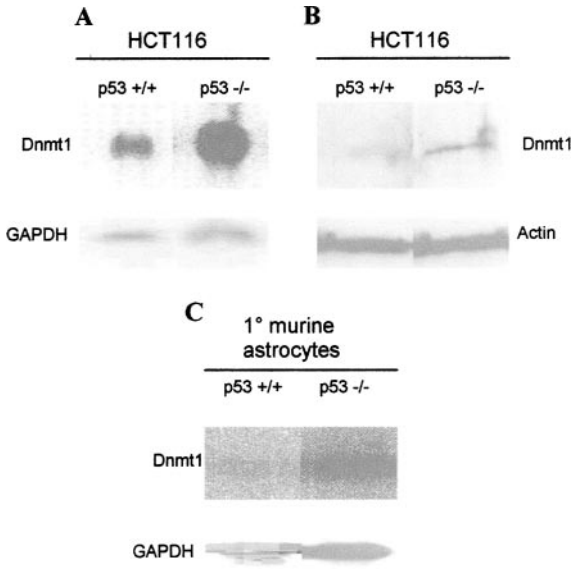


Fig. 2. Increased expression of Dnmt1 in cells lacking p53. Northern analysis of Dnmt1 mRNA levels is shown in (*A*) HCT116 human colon carcinoma cells compared with *p53*-null HCT116 cells (*p53*−/−) and (*C*) primary murine astrocyte cells and astrocytes from *p53*−/− mice. GAPDH was used as a loading control. *B*, Western analysis of Dnmt1 levels in HCT116 *p53*+/+ cells compared with *p53*−/− cells. Actin antibody was used to monitor for equal protein loading.

compared with their wild-type controls (Fig. 2*C*). Thus, endogenous p53 appears to repress transcription from the *Dnmt1* promoter by direct, sequence-specific binding to a p53 consensus site in exon 1. This occurs in the absence of any genotoxic stress that would activate p53.

DNA-damaging agents and cytotoxic drugs result in p53 modification and stabilization, leading to the induction of downstream target genes, such as $p21^{Waf\text{-}1}$. To determine whether Dnmt1 expression in *p53*+/+ cells changes when p53 is modified in response to DNA-damaging signals, HCT116 cells were treated with either IR or etoposide (VP16). Both IR and VP16 treatment induced Dnmt1 expression in *p53*+/+ cells (Fig. 3, *A* and *B*). It is well documented that $p21^{Waf\text{-}1}$ can be activated by DNA damage in both a p53-dependent and p53-independent manner (13). As shown in Fig. 3*A*, the levels of $p21^{Waf\text{-}1}$ increased in both HCT116 *p53*+/+ and *p53*−/− cells in response to VP16. In contrast, the level of Dnmt1 did not change after treatment of *p53*−/− cells with VP16, suggesting that the induction of Dnmt1 observed in *p53*+/+ cells was strictly a p53-dependent response. Changes in protein levels of Dnmt1 paralleled Dnmt1 mRNA expression (Fig. 3*C*). In HCT116 *p53*−/− cells, Dnmt1 levels were elevated 6-fold relative to *p53*+/+ cells but unchanged in response to IR, whereas Dnmt1 expression was low in HCT116 *p53*+/+ cells but increased dramatically in response to p53-dependent DNA damage signaling.

ChIP assays (10) allowed an assessment of p53 binding to the *Dnmt1* genomic locus *in vivo*. In untreated HCT116 *p53*+/+ cells, p53 bound efficiently to the *Dnmt1* proximal promoter *in vivo* (Fig. 4*A*). The amount of complex between p53 and the *Dnmt1* promoter *in vivo* decreased dramatically over the 24-h period after activation of p53 with 10 Gy of IR to a value that is 20% of that in untreated cultures (Fig. 4*B*). A similar decrease in p53 binding was observed in response to treatment with VP16. Importantly, these effects are in direct contrast to the increase in p53 binding to the *p21* promoter seen in the same cultures (Fig. 4*B*). Taken together, the data suggest that p53 has a repressive effect on *Dnmt1* gene expression when bound to the *Dnmt1* promoter. It is also evident that p53 is released from the *Dnmt1* promoter after DNA damage, allowing an increase in Dnmt1 levels.

Genes repressed by p53 fall into two general categories. In the first class of promoters, repression by p53 is mediated through interference with the action of upstream activators. In almost all of these cases, repression occurs through interaction of p53 with a promoter-bound transcriptional activator in the absence of direct DNA binding (14–16). In a variation of this mechanism, a few promoters appear to be repressed by direct p53-DNA interactions, with p53 binding to a consensus site that overlaps the binding site of a primary activator (17, 18). Although promoter-bound p53 still activates transcription, it also displaces the more potent activator, resulting in a net decrease in transcription, *i.e.*, apparent "repression." In the second class of promoters, p53 interacts with the TATA-binding protein and TATA-binding protein-associated factors, leading to the proposal that p53 inhibits transcription of this class of genes by interfering with assembly of the general transcriptional machinery (19). Whereas it is likely that repression of the *Dnmt1* promoter by p53 operates by a variation of one of these models, it is important to note that studies regarding activation or repression of target genes by p53 generally describe the properties of p53 that have been modified after DNA damage signaling. Stabilization of p53 by posttranslational modification after genotoxic stress has long been considered a prerequisite for binding to target gene promoters. However, recent studies have suggested that unmodified (latent) p53 is capable of binding to reassembled chromatin (20) or to chromatin in the nuclear environment (21). Our

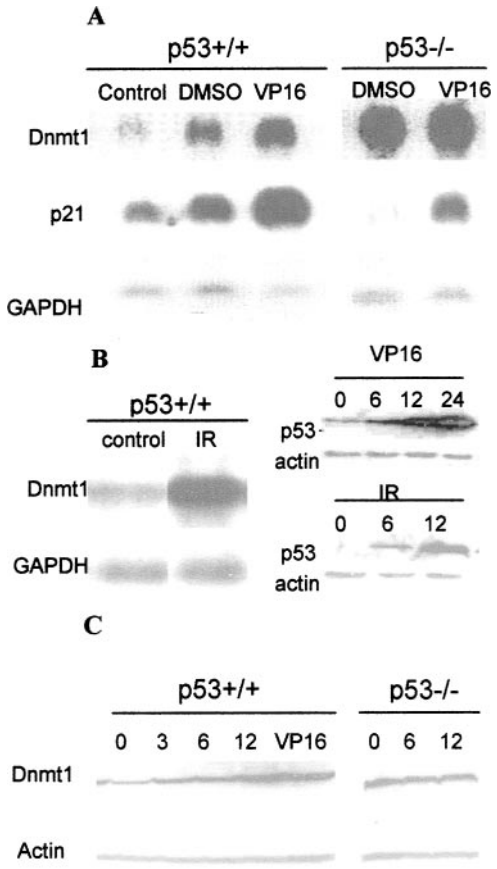


Fig. 3. Induction of Dnmt1 expression by etoposide (*VP16*) or IR. Northern blot of Dnmt1 and p21 mRNA levels is shown in HCT116 *p53*+/+ and *p53*−/− cells analyzed after treatment with (*A*) 5 μg/ml VP16 in DMSO and (*B*) 10 Gy of IR after 24-h incubation. Western blot figures (*right panels*) show p53 induction as a result of the above treatments. GAPDH and actin were used as loading controls. *C*, Dnmt1 protein levels in HCT116 *p53*+/+ and *p53*−/− cells treated with 10 Gy of IR and harvested at various times after treatment or treated with VP16 for 24 h. Actin was used as a loading control.

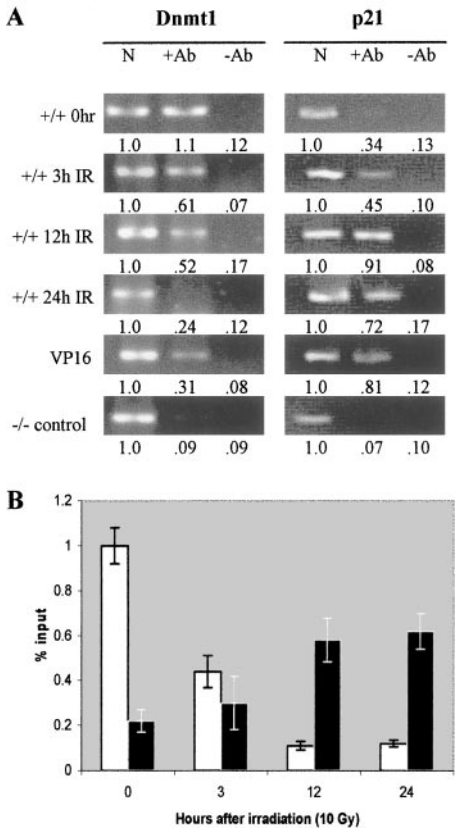


Fig. 4. *A*, p53 protein binds *in vivo* to its consensus site in exon 1 of the *Dnmt1* gene. HCT116 *p53*+/+ cells were treated with either 10 Gy of IR or 5 μg/ml etoposide (*VP16*), and *in vivo* binding of p53 protein to exon 1 of *Dnmt1* was analyzed by ChIP. *N*, input, or total chromatin sample; *Ab*+, samples immunoprecipitated with p53 antibody; *Ab*−, mock immunoprecipitation control without p53 antibody. HCT116 *p53*−/− cells were used as a negative p53 binding control. *p21* primers flanking a well-documented p53 consensus site were used as a positive binding control. The input PCR signal was set to 1.0, and the amount of ChIP Ab+ signal is expressed relative to the input signal. *B*, quantitation of irradiated ChIP samples using real-time PCR. The percentage of p53 bound to the promoter of control *p21* (■), or *Dnmt1* (□) is expressed as the ratio of the relative Ab+/input ± SD.

demonstration that the *Dnmt1* promoter is significantly occupied by p53 in the absence of genotoxic stress supports these studies, and demonstrates that unmodified p53 is capable of entering the nucleus and binding to the consensus binding site in the *Dnmt1* promoter.

We report a novel mechanism of repression by p53, mediated through specific DNA binding that appears to be conditional. When p53 is activated by DNA damage signaling, its repressive effects on *Dnmt1* expression are reduced, perhaps through modulation of binding affinities via posttranslational modifications, or through interaction with coactivators or corepressors. Release of repression by activated p53 is likely necessary to fulfill the requirement for elevated Dnmt1 during DNA repair. Furthermore, elevated levels of Dnmt1 resulting from the permanent loss of p53 function may be sufficient to drive the altered patterns of methylation seen in human cancer.

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