DNA Damage-induced Down-regulation of Human Cdc25C and Cdc2 Is Mediated by Cooperation between p53 and Maintenance DNA (Cytosine-5) Methyltransferase 1*§

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The Cdc25C phosphatase mediates cellular entry into mitosis in mammalian cells. Cdc25C activates Cdc2 for entry into mitosis by dephosphorylating Thr and Tyr at the site of inhibitory phosphorylation. The Cdc25C gene contains tumor suppressor p53 binding sites and is demonstrated to contribute to the p53-dependent cell cycle arrest upon DNA damage. Here we show that both Cdc25C and Cdc2 were down-regulated in wild-type HCT116 cells but not in p53-null, DNMT1-null or DNMT1- and DNMT3b-null cells, upon p53 stabilization following doxorubicin-mediated DNA damage. Furthermore, zebularine, a drug that selectively traps and depletes nuclear DNMT1 and DNMT3b, relieved p53-mediated repression of endogenous Cdc25C and Cdc2. Methylation analysis of the Cdc25C and Cdc2 promoters displayed internal CG methylation proximal to the p53 binding site upon DNA damage in a p53-dependent manner. Chromatin immunoprecipitation of doxorubicin treated wild-type HCT116 cells showed the presence of DNMT1, p53, H3K9me2, and the transcriptional repressor HDAC1 on the Cdc25C and Cdc2 promoters, suggesting their involvement as repressive complexes in Cdc25C and Cdc2 gene silencing. Thus, the general mechanism of p53-mediated gene repression may involve recruitment of other repressive factors.

Because the majority of CpG dinucleotides in the mammalian genome are methylated and methylation often dictates the transcriptional status of a gene, it has been demonstrated that DNA methylation is correlated with transcriptional inactivation of a gene and the reverse is true for gene activation, although recently it was reported that histone modification greatly influences the transcriptional status of a gene (3). Additionally, there are two more DNA (cytosine-5) methyltransferases in the mammalian genome known as de novo methyltransferases, DNMT3a and DNMT3b (4). These two enzymes participate in early embryonic development (4) and perhaps to a lesser extent in the genome wide maintenance of CpG methylation either alone or in conjunction with DNMT1 (5, 6).

Although DNMTs enforce gene silencing directly by DNA methylation, they also act as a platform for recruitment of transcriptional repressor complexes for gene silencing through their N-terminal regions (7). Transcriptional repressors such as MeCP2, methyl-binding proteins (MBDs) and histone deacetylases (HDACs) participate in gene silencing by DNMT1 recruitment (8, 9, 10). Recently another class of enzymes was shown to be involved in repressive chromatin modeling via specific lysine residues methylation on histones. These histone methyltransferases such as G9a, SUV39H1, SetDB1, and their homologues can also act as a recruitment center for several transcriptional repressors (11, 12, 13). The importance of DNA and histone methyltransferase gene products in cell survival was shown in genetic knock-out studies. Knock-out mice for the genes encoding enzymes for DNA methylation or histone methylation displayed growth retardation or embryonic lethality (14, 4, 15), demonstrating their importance in animal growth and development. Thus, specific gene expression patterns in mammalian cells are coordinated and conducted by a host of genetic and epigenetic factors. Interaction of these factors on a promoter or in its vicinity often leads to gene silencing or regulation. For example, Myc binds the corepressor DNMT3a and can target DNMT3a selectively to the promoter of p21Cip1 (16). Similarly, the leukemia-promoting PML-RAR fusion protein induces gene hypermethylation and silencing by recruiting DNA methyltransferases to target promoters and contributes to its leukemogenic potential (17).

A key protein in gene regulation during cellular stress is tumor suppressor p53. Intracellular stabilization and accumulation of p53 takes place when cells are stressed or their DNA is damaged (18). Tumor suppressor p53 protects mammals from neoplasia by selectively eliminating stressed cells or cells with

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3 The abbreviations used are: DNMT1, DNA methyltransferase 1; HDAC, histone deacetylase; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation assay; G6PDH, glucose-6-phosphate dehydrogenase; WT, wild type; CDK, cyclin-dependent kinase.
damaged DNA via programmed cell death. The p53-dependent growth arrest in response to damaged DNA occurs during both the G1, and G2 phases of the cell cycle (19, 20). Furthermore p53 can also arrest cell division if the nucleotide pool in the cell becomes a limiting factor. The p53 protein has a central DNA binding domain flanked by transactivation and negative regulation domains. Binding of p53 to the promoter results in transcriptional activation of several genes that control cellular responses to stress (21). For example, p53 causes G1 arrest in cells by stimulating cyclin-dependent kinase (CDK) inhibitor p21/Waf1, thus reducing the activity of CDK2, -4, and -6 that are required for progression from G1 to S phase (22, 23). Furthermore, p53 was shown to bind to the promoter of cell survival proteins such as survivin and down-regulate its expression by recruitment of epigenetic machinery such as DNMT1 and HDAC1 (24). These events link both histone and DNA methylation to gene silencing.

Mammalian cell division is controlled by a number of regulatory factors, which include, among others, the cyclins, the CDKs, and the tumor suppressor protein p53 (25). The interaction of p53 with the CDK inhibitor p21/Waf1 is essential for the cell cycle arrest that follows DNA damage (26). Binding of p53 to the promoter results in transcriptional activation of several genes that control cellular responses to stress (21). For example, p53 causes G1 arrest in cells by stimulating cyclin-dependent kinase (CDK) inhibitor p21/Waf1, thus reducing the activity of CDK2, -4, and -6 that are required for progression from G1 to S phase (22, 23). Furthermore, p53 was shown to bind to the promoter of cell survival proteins such as survivin and down-regulate its expression by recruitment of epigenetic machinery such as DNMT1 and HDAC1 (24). These events link both histone and DNA methylation to gene silencing.

EXPERIMENTAL PROCEDURES

Cell Culture—Parental HCT116 (colorectal carcinoma) cells were purchased from the American Type Culture Collection. DNMT1+/−/ and both DNMT1−/−/DNMT3b−/−/ HCT116 cells were kindly provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD). All cells were incubated at 37 °C in a 5% CO2 humidified atmosphere and propagated in Mac Coy’s 5A modified medium (ATCC) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin. 100 μg/ml hygromycin were added to the DNMT1−/−/ cells.

Drug Treatments—For zebularine treatment, cells were plated (5 × 105 cells/100-mm diameter dish) and treated with 1.5 10−4 M of zebularine (Calbiochem) 24 h post-plating. The medium was changed every 3 days, along with fresh zebularine treatment, for up to 8 days. For doxorubicin treatment, subconfluent cells were treated with 1 μM doxorubicin (Sigma) for 0, 2, 4, 24, and 48 h.

For mitotic index determination, HCT116 and DNMT1−/−/ cells were grown on coverslips. The cells were treated with doxorubicin and were fixed with paraformaldehyde 24- and 48-h post-treatment. The cells were permeabilized with 1× PBS supplemented with 0.2% Triton X-100 and incubated with Hoechst 33342. Mitotic cells were scored under UV using a Zeiss inverted microscope.

Western Blot Analysis—Subconfluent cultures of HCT116 cells were washed with 1× PBS, and lysed at 4 °C for 20 min with wash buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) supplemented with a protease inhibitor mixture (Sigma). Protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent based on the Bradford colorimetric method. 25–75 μg of proteins were electrophoresed on 4–20% SDS-polyacrylamide gels (Daichi) after boiling for 5 min in a SDS sample buffer supplemented with 100 mM dithiothreitol (New England Biolabs (NEB)). The proteins were transferred onto Protran® pure nitrocellulose membranes (0.2 μm) (Schleicher and Schuell). After overnight electroblotting at 4 °C, the membranes were incubated with Tris-buffered saline with 0.1% Tween-20, 5% nonfat dry milk (Bio-Rad) and rabbit polyclonal antibody against DNMT1 (NEB), DNMT3a, DNMT3b (NEB), p53, Cdc2, Cdc25C, Chk1, Chk2, Chk1 phos (Ser296), Chk2 phos (Thr68), rabbit monoclonal for Cdc25C phos (Ser116), mouse monoclonal for survivin (Cell Signaling Technology (CST)), and rabbit polyclonal antibody against actin (Sigma). Goat anti-mouse or anti-rabbit secondary antibodies, conjugated to horseradish peroxidase, were used in a chemiluminescence detection system as directed by the manufacturer (CST).

For zebularine experiments, cytoplasmic and nuclear extracts were separated and prepared using the NE-PER® Nuclear and Cytoplasmic Extraction reagents according to the manufacturer’s instructions (Pierce).

Total RNA Extraction and Quantification—Total RNA was isolated from subconfluent cells (cultured in 6-well plates) using RNAqueous® columns (Ambion) according to the manufacturer’s recommendations for mammalian cultured cells. Briefly, the RNA of lysed cells was absorbed to a silica matrix, washed, and eluted with 50 μl (40 + 10) of 70 °C preheated Rnase-free elution solution. Residual DNA contamination was eliminated using the DNA-freeTM kit (Ambion). RNA concentrations and purities were determined by diluting 20 μl of each RNA preparation in 480 μl of Tris-EDTA, pH 8.0 (1:25 dilution) and measuring the absorbance at 260 and 280 nm.

cDNA Synthesis—One microgram of purified total RNA was reverse-transcribed using the ProtoscriptTM First Strand cDNA synthesis kit (NEB) and oligo(dT)23 primer. cDNAs were diluted to 50 μl (1–2.5) prior use in quantitative PCR experiments.

Quantitative PCR Experiments—The cDNAs of interest and reference cDNA (G6PDH) were amplified from a separate mix. All reactions were prepared in a 25-μl final volume with indicated end-concentrations: 0.8 μM of forward and reverse primer, 1× iQ™ SYBR® Green Supermix (Bio-Rad) and 4% cDNAs (2 of 50 μl). For each mix a No Template Control (NTC) was performed with 1 μg of total RNA in place of cDNAs.

G6PDH primers were previously published (28). All the other primer sets (Table 1) were designed using the Primer3
program. These primers were selected to produce PCR fragments between 100 and 200 base pairs in length and in a narrow range of various primer annealing temperatures. All targets were thus amplified using a common PCR program as follows: a denaturation step at 95 °C for 2 min and 40 cycles with a 59 °C annealing temperature (95 °C for 20 s, 59 °C for 20 s, 72 °C for 20 s). All reactions were carried out using the i-Cycler instrument from Bio-Rad and double-strand DNA SYBR green incorporation that was measured during each elongation step.

Quantitative RT-PCR Analysis—Relative expression ratios were calculated using the mathematical model described by M. W. Paffl (29). Real-time PCR efficiency (E) of each of the target and reference gene (G6PDH) transcript was investigated from 40-ng to 64-pg cDNA dilutions (Table 1). Each cDNA sample was examined in triplicate.

**TABLE 1**

| GenBankTM accession no. | Primer sequences (5'-3') | Real-time PCR efficiency (E) |
|-------------------------|--------------------------|-----------------------------|
| G6PDH                   | X03674                    | attgcactacagttcgccga       | E = 1.98, R² = 0.997 |
| Survivin                | NM_001168                 | ttgctgctcaactgtccgga       | E = 2.01, R² = 0.999 |
| Cdc2                    | BT007004                  | gagacatctggttgctttgcttc    | E = 2.16, R² = 0.984 |
| Cdc25C                  | NM_001790                 | caccctgcggttaactctccta     | E = 1.97, R² = 0.998 |
| DNMT1                   | X63692                    | gtcctgcggttaactctccta     | E = 2.00, R² = 0.996 |
| DNMT3α                  | AF067972                  | caccctgcggttaactctccta     | E = 2.04, R² = 0.998 |
| DNMT3β                  | AF156487                  | caccctgcggttaactctccta     | E = 2.32, R² = 0.993 |

Luciferase Activity Assays—The pGL3 reporter plasmids were transiently transfected into wild-type and mutant HCT116 cells (cultured in 6-well plates) using the FuGENE method according to the manufacturer’s recommendations (Roche Applied Science) and using a 4:5:1:2 transfection reagent (µl)/DNA ratio (µg). Transfected DNA included 1.0 µg of the pcd2-luc or pcd25C-luc plasmid construction and 200 ng of a pSV-β-galactosidase vector (Promega). This second reporter vector was used to check the transfection efficiencies and normalize luciferase activity values. Twelve hours after transfection, culture medium was changed. Then, cells were washed three times in 1× PBS and lysed in 200 µl of the Promega Passive Lysis Buffer® with gentle shaking at room temperature for 20 min. The cell lysate was centrifuged at 13,800 × g for 2 min to pellet the cell debris. Luciferase and β-galactosidase activities were determined, from 100 and 25 µl of the supernatants respectively, according to the manufacturer’s protocols (Promega). For p53 expression pcDNA353 construct was used. This construct contains the wild-type p53 sequence under the CMV promoter.

Promoters Methylation Analysis—Genomic DNA from doxorubicin-treated or untreated cells was extracted using the Easy-DNA™ kit (Invitrogen), diluted in Tris-EDTA and exactly quantified using the PicoGreen® dsDNA Quantification kit (Molecular Probes) in a 200-µl volume assay. Then, 1 µg of each DNA sample was subjected to the restriction enzyme HpaII or BstUI digestion (NEB). Gene-specific primers flanking p53-binding site of Cdc25C and Cdc2 promoter amplified the digested DNAs.

Chromatin Immunoprecipitation Assay—HCT116 cells were grown on 150-mm dishes and treated with 1 µm of doxorubicin. After 24 h, proteins were cross-linked with DNA using 1% formaldehyde for 10 min at 37 °C. Cells were washed two times with ice-cold 1× PBS, harvested, and lysed with an SDS lysis buffer (UB) in the presence of a mixture of protease inhibitors (Sigma). The lysates were sonicated to shear DNA to lengths between 200 and 1000 bp. After 10-fold dilution of the sonicated cell supernatants in ChIP dilution buffer (Upstate Biotechnology, UB) containing protease inhibitors. Immunoprecipitations were carried out overnight at 4 °C with mixing by using 2 µg of DNMT1 (NEB), p53 (CST), HDAC1 (CST), H3K9me2 polyonc (UB), G9a (UB), Sp1 (UB), and control purified rabbit IgG antibodies (Calbiochem) to the extract. 40 µl of protein G-agarose beads pre-adsorbed with salmon sperm DNA were added and incubated for an hour at 4 °C with rotation. The beads were isolated and washed according to UB’s manual. DNA-protein complexes were eluted from the beads with a buffer containing 1% SDS and 0.1 M NaHCO3. The cross-links were reversed by incubating the eluates with NaCl (5 M) for 6 h at 65 °C. Proteinase K (NEB) was added for 1 h at 45 °C, and the DNA was recovered by phenol/chloroform extraction and ethanol precipitation. ChIP DNA was analyzed for the presence of Cdc2 gene promoter sequence by PCR with proximal (forward: 5′-AACCTGTCACAGGTGGAGA-3′ and reverse: 5′-AGCCAGCTTTTGAAGCCAAGT-3′) and distal (forward: 5′-TCCCGTCTGACATCGTGGG-3′) and reverse: 5′-CTTTTCTGACATCGTGGG-3′) primer sets using PCR of 30 cycles at 94 °C 30 s, 60 °C 30 s, and 72 °C 30 s. Similarly for Cdc25C promoter ChIP analysis PCR was performed with proximal (forward: 5′-GGCTGTGGGTTTGCGG-3′) and reverse: 5′-TTTCTGACATCGTGGG-3′) primer sets using PCR of 30 cycles at 94 °C 30 s, 60 °C 30 s, and 72 °C 30 s.

Cdc25C and Cdc2 Down-regulation by p53 and DNMT1
**RESULTS**

Repression of Cdc2 and Cdc25C Promoters Is Mediated by p53 and DNMT1—Endogenous Cdc2 and Cdc25C promoters contain DNA sequences for p53-binding. In the Cdc2 promoter, CCAAT boxes are responsible for p53-mediated repression of the promoter (30). Whereas in the human Cdc25C promoter a p53-binding site that physically recruits p53 has been identified (31). Our previous studies demonstrated p53 stabilization after treatment of HCT116 cells with DNA-damaging agent, doxorubicin, resulting in down-regulation of endogenous Cdc25C expression (24). A similar down-regulation of endogenous Cdc2 was reported elsewhere (30). To reconcile these findings and understand the mechanism of gene repression, reporters were constructed with the promoter of either Cdc2 and Cdc25C (pcdc2-luc or pcdc25C-luc) preceding the luciferase gene. These reporter constructs were used in transfection assays. These constructs contain essentially all of the features of the respective endogenous promoter including Sp1, NF-Y, CDE, and CHR binding sites (Fig. 1A). To investigate whether p53 can repress these promoters, the constructs were cotransfected with an increasing amount of p53 expression construct pcDNAP53 into HCT116 p53-null cells. In the control experiment, backbone pcDNA vector was used instead of pcDNAP53. Both Cdc2 and Cdc25C promoter-mediated luciferase expression were down-regulated in the transfected cells in a dose-dependent manner by pcDNAP53 (Fig. 1, B and C). To determine if DNMTs participate in this down-regulation event, either pcdc2-luc or pcdc25C-luc were transfected into HCT116 cells WT or into DNMT1-null or DNMT1 and DNMT3b-null background. After the transfection, endogenous p53 was induced via doxorubicin treatment. In the WT cells, luciferase expression was down-regulated. Both Cdc2 and Cdc25C promoters were repressed (∼75%) in the presence of p53. However, in DNMT1-null cells or DNMT1 and DNMT3b-null cells luciferase expression was not down-regulated (Fig. 1, D and E), suggesting a direct involvement of both DNMT1 and p53 in Cdc2 and Cdc25C promoter regulation as observed before for the survivin promoter (24). A similar derepression of luciferase reporter was observed in p53-null cells (Fig. 1, D and E). This suggests a plausible general mechanism of gene regulation of promoters containing a p53-binding site that are repressed in the presence of p53.

**DNA Damage-induced Down-regulation of Endogenous Cdc25C and Cdc2 Requires Both Tumor Suppressor p53 and DNA (Cytosine-5) Methyltransferase 1—**To evaluate the impact of p53-mediated repression of the endogenous Cdc2 and Cdc25C promoter, wild-type HCT116 cells were treated with doxorubicin and the level of tumor suppressor p53, DNMT1, Cdc2, and Cdc25C were measured at different time intervals (0-, 24-, and 48-h post-treatment) via Western blot analysis. A time-dependent accumulation of the tumor suppressor protein p53 was observed in 24- and 48-h post-treatment (Fig. 2A). Correlated with the accumulation of p53 the level of Cdc2 and Cdc25C decreased in the wild-type HCT116 extracts (Fig. 2A). At 48-h post-treatment there was a ∼90% reduction in Cdc2 and Cdc25C protein levels (Fig. 2A, right panels). As a positive control, survivin, another p53-repressed gene, was monitored and was down-regulated under identical conditions. The loading control, actin or PCNA (data not shown) level remained the same in
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FIGURE 2. Endogenous Cdc2 and Cdc25C genes require both p53 and DNMT1 for transcriptional repression. Western blot analysis of endogenous p53, DNMT1, survivin, Cdc2, Cdc25C, and actin expression levels in HCT116 (WT, A), HCT116 p53-null (p53−/−, B), HCT116 DNMT1-null (DNMT1−/−, C), and HCT116 DNMT1 plus DNMT3b-null (DNMT1−/−, DNMT3b−/−, D) cells in the presence of 1 μM doxorubicin. Time points post-treatment (in hours) are shown on the top, and the antibody used for detection is indicated below each blot. Densitometric scans of endogenous levels of Cdc2 and Cdc25C are shown at the right top and bottom panel, respectively, with protein expression at the left of each graph. Times of post-treatment in hours are indicated at the bottom of the graph.

Zebularine-mediated DNMT1 Entrapment Can Disrupt p53-mediated Repression of Cdc2 and Cdc25C—To determine if the derepression of endogenous Cdc2 and Cdc25C promoters is mediated by DNA damage in p53-null cells, DNMT1-null cells or in DNMT1 and DNMT3b-null cells are direct consequence of DNMT1 involvement, we treated the HCT116 cells with zebularine, a mechanistic inhibitor of DNMTs. Zebularine is a nucleotide analogue that can get incorporated to DNA during DNA synthesis and give rise to high affinity complexes with DNA (cytosine-5) methyltransferase (33). The incorporation of zebularine into DNA is believed to facilitate DNMT1 entrapment leading to depletion of the enzyme level in the cell, resulting in the demethylation of the genome (34).

Initially, to validate the specificity of zebularine, wild-type HCT116 cells were treated with a fixed amount of zebularine and the nuclear and cytoplasmic fractions of the cells were separated, Western-blotted and probed with antibodies specific for human DNMT1, DNMT3a, and DNMT3b (Fig. 3A). In the cytoplasmic fraction either the presence or absence of zebularine did not impact on the quantity of DNMT3a and DNMT3b, because the density of the signal remained consistent between the samples. A small decrease was observed for DNMT3a and DNMT3b in nuclear fraction with zebularine. Thus, in HCT116 cells zebularine treatment displayed only a small percentage of DNMT3a and DNMT3b, because the density of the signal remained consistent between the samples. A small decrease was observed for DNMT3a and DNMT3b in nuclear fraction with zebularine. Thus, in HCT116 cells zebularine treatment displayed only a small percentage of DNMT3a and DNMT3b entrapment. However, in the nuclear fraction of the untreated cells the DNMT1 level was intense as compared with the cytoplasmic fraction confirming the majority of DNMT1 is present in the nuclear compartment of the cell. No detectable amount of DNMT1 was observed in the nuclear fraction of the sample treated with zebularine suggesting DNMT1 was effectively trapped onto the DNA (Fig. 3A, top panel, lanes 3 and 4). Furthermore, real time PCR analysis of DNMT1-, DNMT3a- and DNMT3b-specific RNA levels in

all the different time points indicating consistent loading of extract in each lane. An identical experiment performed side by side with the HCT116 p53-null cells, HCT116 DNMT1-null cells or HCT116 DNMT1 and DNMT3b-null cells resulted in either no changes or a gradual increase with the level of Cdc2 or Cdc25C after doxorubicin treatment (Fig. 2, B–D). The positive control survivin level increased in all the null cell lines validating previously reported up-regulation of survivin (24). The actin level remained the same throughout. A lower time point, between 0 and 24 h, also demonstrated the same effect for survivin, Cdc2, Cdc25C in wild-type HCT116, HCT116 p53-null, and HCT116 DNMT1-null cell lines (data not shown). This argues in favor of the notion that down-regulation of endoge-
Depletion of DNMT1 via zebularine entrapment leads to repression of survivin, Cdc2, and Cdc25C genes despite the presence of significant quantity of p53 (Fig. 3C, lane 4). Real time PCR analysis confirmed that the down-regulation of survivin, Cdc2 and Cdc25C occurred at the RNA level after doxorubicin treatment (Fig. 3D), and a combination of doxorubicin and zebularine relieved this repression partially or fully (Fig. 3D, lane 4), thus complementing the Western blot observation in Fig. 3C. However, Cdc2 and survivin mRNA expression levels in doxorubicin plus zebularine-treated cells was 25–30% lesser than zebularine alone treated cells (Fig. 3D, lane 3 versus 4), although both Cdc2 and survivin protein levels revealed by Western blot (Fig. 3C, lane 2 versus 4) were comparable, suggesting turnover for both proteins are not correlated to mRNA expression.

We observed a similar derepression mechanism of luciferase gene expression in either pcdc2-luc or pcdc25C-luc in the presence of both zebularine and doxorubicin in transfected HCT116 cells. These observations confirm that both DNMT1 and p53-mediated repression on the isolated promoter fragments can be disrupted by DNMT1 inhibitor (data not shown), and the synergistic effect of gene repression is mediated by p53 and DNMT1.

Cdc2 and Cdc25C Promoter Silencing Requires p53-mediated Methylation—DNMT1 can down-regulate gene expression directly by methylating the promoter and/or recruiting transcriptional repressors to a particular promoter. The Cdc25C promoter contains several CpGs; DNMT1 target sites for methylation. These sequences also contain overlapping restriction enzyme BstUI and HhaI target sites (Fig. 4A). To validate the mechanisms, we induced p53 by doxorubicin in HCT116 wild-type or HCT116 p53-null cells. The
genomic DNA was isolated, cut with methyl-sensitive restriction enzymes HhaI and BstUI followed by PCR amplification of the promoters using two flanking primers. If the promoters were methylated at CpG sites embedded in the restriction enzyme recognition sequence CGCG (BstUI) or GCGC (HhaI), then a PCR product of 290 base pairs, corresponding to the Cdc25C promoter is expected. Indeed, doxorubicin-mediated methylation of the Cdc25C promoter was observed in HCT116 cells but not in HCT116 p53-null cells (Fig. 4B). A similar experiment was performed for the detection of endogenous DNA methylation of the Cdc2 promoter and was also methylated in the presence of doxorubicin in wild-type HCT116 cells but not in p53-null background (data not shown). This implicates p53-mediated methylation of the promoter in the presence of DNA damage.

FIGURE 4. Methylation analysis of the Cdc25C promoter upon doxorubicin treatment. A, schematic diagram of the endogenous Cdc25C promoter with a detailed sequence of the p53 binding site along with methyl sensitive restriction enzymes as shown. The recognition sequences are lined both above and below. The PCR primers are indicated by two arrows. B, PCR analysis of the methylation status of CpGs in the Cdc25C promoter. Doxorubicin treatment is indicated above each panel. Methyl-sensitive restriction enzyme digestion of genomic DNA by HhaI or BstUI is indicated on the top of each panel. PCR product analysis of genomic DNA from parental HCT116 and HCT116 p53-null cells are shown on four panels with the genome indicated at the right side.

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Repression Complex Analysis of the Cdc2 and Cdc25C Promoters—A detailed analysis of the physical association of p53, DNMT1 and other transacting repressor complexes on the Cdc2 and Cdc25C promoters was undertaken to elucidate the promoter silencing mechanism. Both promoters can be silenced in a p53-dependent and independent manner. For example, the Cdc25C promoter is regulated by direct binding of p53 to a site on it or by a direct interaction of CDE/CHR regulatory elements (30). Therefore, HCT116 wild-type cells were treated with doxorubicin for DNA damage, and the endogenous Cdc25C and Cdc2 promoter occupancy was examined on formaldehyde cross-linked chromatin by ChIP using specific antibodies for p53, DNMT1, HDAC1, dimethyl lysine 9 histone H3 (H3K9me2), G9a, and Sp1. In parallel, a nonspecific IgG was included to monitor the specificity of the reaction. Two different regions of the Cdc25C promoter were investigated by PCR, a proximal region without a p53 binding site, and a proximal region with p53 and Sp1 binding sites. Furthermore, parallel PCR was conducted with different amounts of input DNA to ensure the linearity of the PCR reaction. Indeed, the proximal part of the Cdc25C promoter was occupied by p53, DNMT1, HDAC1, and H3K9me2 as a part of the repressor complex only in the presence of DNA damage and p53 stabilization (Fig. 5), because the antibodies against them were able to pull-down repressor complexes along with the promoter. The distal part of the promoter did not contain any of these elements. Surprisingly, Sp1 a transcriptional activator of Cdc25C was also found to be associated with the promoter. Because Sp1 is a transcriptional activator of the Cdc25C promoter, its presence in the repressed promoter may have functions other than gene activation.

In the case of the Cdc2 promoter, HDAC1 levels did increased after doxorubicin treatment. However, DNA damage resulted in accumulation of G9a and its product H3K9me2, suggesting H3K9me2 may play a larger role in gene repression along with DNMT1 and p53 despite the presence of a robust amount of Sp1 (Fig. 6). This suggests that Sp1 may act as a platform for repressive complex recruitment or Sp1 may stabilize the repressive complex in the presence of doxorubicin as observed in the Cdc25C pro-
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However, doxorubicin-mediated DNA damage also elicits a variety of cellular response including arresting the cells at G2/M phase. Indeed the percentage of mitotic cells in HCT116 wild-type, DNMT1-null, p53-null, DNMT1- and DNMT3b-null cells remained between 8 and 10%. And following 24 or 48 h of doxorubicin treatment, all cells were arrested at G2/M (data not shown). This suggested that HCT116 wild-type cells and the null mutants behaved similarly after doxorubicin treatment. This brings up the question if the checkpoint response elements were affected in response to DNA damage between HCT116 wild-type and DNMT1-null cells. To examine this we Western-blotted and probed HCT116 and DNMT1-null cell extracts at 0, 2-, and 4-h post-doxorubicin treatment with anti-Chk1, anti-Chk2, anti-Cdc25C, anti-Chk1 phosphos (Ser209), anti-Chk2 phosphos (Thr68) and anti-Cdc25C phosphos (Ser216). The protein level between the wild-type and DNMT1-null cells did not change although the phosphorylation increased for Chk1, Chk2, and Cdc25C as expected (supplemental Fig. S1). Accumulation of phosphorylated Cdc25C and Chk2 demonstrate the cells are in interphase, but not in mitosis (41, 42). This suggests that both wild-type and null cells do respond to DNA damage in a similar manner implying that our proposed mechanism involving DNMT1 does not contribute to the cell cycle checkpoint function. The repression of Cdc25C or Cdc2 in wild-type HCT116 cells may be caused by epigenetic changes that may lead to long term growth suppression. Arresting the cells at G2/M may allow the p53-DNMT1-HDAC1 complex to establish on the transcriptionally active genes and thus triggering various epigenetic modification events and gene repression. However, a detailed study is required for validation.

In our current study, we have demonstrated that exogenous p53 can repress human Cdc25C and Cdc2 promoters in HCT116 p53-null cells, suggesting a strong link between the presence of p53 and gene repression. However, a minimal promoter of Cdc25C containing only the p53 binding site confers repression to key cellular promoters. p53 protein-mediated DNMT1 recruits DNMT1 indirectly or downstream of other factors that bind to the additional sequence. A closer examination of these sequences suggested that GC-rich motifs that flank and overlap the p53 binding site are indeed putative Sp1 transcription factor binding sites that can recruit DNMT1 to the survivin promoter (24).

In our current study, we have demonstrated that exogenous p53 can repress human Cdc25C and Cdc2 promoters in HCT116 p53-null cells, suggesting a strong link between the presence of p53 and gene repression. However, a minimal promoter of Cdc25C containing only the p53 binding site confers p53-mediated activation. Thus, an additional 8 GC-rich bases upstream of this minimal promoter can convert this p53-activating promoter to a repressor (31).

One of the possible explanations for this observation is the involvement of other factors that bind to the additional sequence. A closer examination of these sequences suggested that GC-rich motifs that flank and overlap the p53 binding site are indeed putative Sp1 transcription factor binding sites that can recruit DNMT1 indirectly or directly. The CpG richness of these sites make it an attractive target site for DNMT1 binding and methylation. There are several reports of protein-mediated DNMT1 recruitment to key cellular promoters. p53 can recruit DNMT1 to the survivin promoter following DNA damage to methylate the promoter (24). Thus, in general the p53-mediated
repression mechanism may involve DNMT1 recruitment onto promoters such as Cdc25C and Cdc2 because we observe some amount of DNA methylation upon doxorubicin treatment. Damaged DNA also facilitates DNMT1-mediated methylation of the promoter since it serves as a good substrate and often has a high rate of the reaction (43). Furthermore, recruitment of DNMT1 to the damaged sites (44) may also stall DNMT1, which in turn can recruit a transcriptional repressor such as HDAC1 (10) as seen in the chromatin immunoprecipitation experiment. Similar methylation-dependent and independent mechanisms have been observed for other target genes. For example, the gene promoters containing E2F transcription factor binding sites may be prone to retinoblastoma (Rb) tumor suppressor gene product mediated repression via E2F1, and HDAC1 and DNMT1 repressor complex (45). Similarly, the leukemia-promoting PML-RAR fusion protein induces gene hypermethylation and silencing by recruiting DNA methyltransferases, DNMT1 and DNMT3b, to target promoters and the resulting hypermethylation likely contributes to its leukemogenic potential (17).

We also found that removal of DNMT1 from the cellular pool via zebularine treatment relieved the p53-mediated repression of both the Cdc2 and Cdc25C genes in transfected plasmids as well as endogenous genes even in the presence of significant levels of p53 without significant loss of the respective transcript. This supports a crucial role of DNMT1 in gene repression in association with p53 in HCT116 cells. There are several reports of p53 recruitment of other transcriptional repressors either directly or indirectly such as HDAC1, Sp1, and MeCP2. There could be several mechanisms that can explain how DNMT1 and p53 may be mediating the repression of Cdc2 and Cdc25C. If Sp1 transcription factor is required for these gene expressions, p53 stabilization in response to DNA damage may aid a p53-Sp1 complex formation, as reported earlier (46). It is unlikely that p53 binding to the Cdc25C or Cdc2 promoter excludes Sp1 from the promoter. This is evident in the Cdc25C chromatin immunoprecipitation assay where in the presence of DNA damage; Sp1 was increased on the promoter, concurrent with p53 and DNMT1 accumulation. In the same sets of experiments, we observed an increase of H3K9me2 on the Cdc25C promoter although the enzyme that catalyzes this methylation, G9a, did not increase. This suggests that other H3K9 methyltransferase enzymes such as SetDB1 or EuHMT may participate in the accumulation of H3K9me2. This hypothesis needs further investigation. In a similar Cdc2 chromatin immunoprecipitation assay, Sp1 levels did not change, suggesting Sp1 may direct the repressive complexes or act as a recruitment platform. Thus, elements present in Cdc2, Cdc25C, and survivin promoters can mediate p53-dependent transcriptional down-regulation that is dependent on sequence-specific DNA binding by p53, Sp1, DNMT1, and HDAC1.

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