2688–2698 *Nucleic Acids Research, 2009, Vol. 37, No. 8* *Published online 9 March 2009*
doi:10.1093/nar/gkp110

DNA damage induced p53 downregulates *Cdc20* by direct binding to its promoter causing chromatin remodeling

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Received July 31, 2008; Accepted February 9, 2009

ABSTRACT

CDC20 is a critical molecule in the Spindle Assembly Checkpoint (SAC). It activates the Anaphase promoting complex and helps a dividing cell to proceed towards Anaphase. CDC20 is overexpressed in many tumor cells which cause chromosomal instability. There have been limited reports on the mechanism of SAC's response to genotoxic stress. We show that ectopically expressed p53 or DNA damage induced endogenous p53 can downregulate *Cdc20* transcriptionally. We have identified a consensus p53-binding site on the *Cdc20* promoter and have shown that it is being used by p53 to bind the promoter and bring about chromatin remodeling thereby repressing *Cdc20*. Additionally, p53 also downregulates *Cdc20* promoter through CDE/CHR element, but in a p21 independent manner. This CDE/CHR element-mediated downregulation occurs only under p53 overexpressed condition but not in the context of DNA damage. The present results suggest that the two CCAAT elements in the *Cdc20* promoter are not used by p53 to downregulate its activity, as reported earlier.

INTRODUCTION

The spindle assembly checkpoint (SAC) is the surveillance system that maintains genomic stability by ensuring the proper segregation of chromosomes during mitosis. SAC remains activated and cells are arrested at metaphase until all sister chromatids bind to the bipolar spindle. Mad2 and BubR1, key components of the Mitotic Checkpoint Complex (MCC), bind and inhibit CDC20 activity, which is necessary for the activation of anaphase promoting complex (APC) (1,2). Thus, the cell cycle is arrested at metaphase when the checkpoint detects any defects in microtubule–kinetochore attachment or in the tension of the spindles (3–5). Only after ensuring proper attachment of the sister chromatids, the MCC complex detach from CDC20 and free CDC20 can activate APC. Active APC^{CDC20} is the ubiquitin ligase that degrades Securin thereby releasing the nuclear protease Separase (2). Free and active Separase cleaves Cohesin, which till now held the two sister chromatids together (6). The chromatids then migrate to the two poles of the dividing cell thereby ensuing Anaphase.

The tumor suppressor gene *p53* is activated at the nucleus by a variety of genotoxic stresses such as DNA damage, hypoxia, oxidative stress and heat shock (7–9). Activated p53 protein directly and indirectly regulates transcription of many genes that are involved in cell cycle, apoptosis and cellular senescence, and subsequently inhibits malignant transformation and tumor progression. p53 has been implicated in various cell cycle checkpoints like the G1/S and G2/M (10,11). Few studies in fibroblasts and yeast have implicated p53 to function as a checkpoint at mitosis. When the mitotic spindle was disrupted in wild-type fibroblasts by drug treatment, it was observed that the cells arrested division, whereas in p53-deficient cells there was failure to arrest, instead a new round of cell division occurred resulting in polyploidy (12). This phenotype was similar to that observed in yeast strains that have inactivated SAC (13,14). Recently, p53 has also been reported to have role in the transcription of the SAC gene *Mad1L1* (6). It has been established that p53 directly binds to the *Mad1L1* promoter and represses its transcription, but no p53 consensus site was found. There have been recent reports of several genes which are being targeted for repression by p53 like DNA *topoisomerase II, cyclin B, Cdc2, Mmp-1 and -13, presenilin-1, myc, Map-4* etc, although, the actual mechanism of this repression remains largely unexplained (15–17). Transcriptional activation requires p53 to bind a consensus sequence consisting of two copies of the canonical site 5′RRRC (A/T) (T/A) GYYY 3′ separated by 0–13 bp (6,18,19). But, the repression mechanism by

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p53 is much obscure. In some cases DNA binding is required like *α-fetoprotein* and *Cdc2* (17,20). On the other hand, p53 can also repress indirectly by interfering with transcriptional factors which usually transactivate the genes (21).

There have been reports of CDC20 overexpression leading to aneuploidy and other chromosomal abnormalities (22,23). Also, CDC20 has been shown to be overexpressed in several cancer tissues (22,23). It has also been suggested that apart from MCC-mediated sequestration, spindle checkpoint also reduces CDC20 level below a certain threshold to ensure complete inhibition of APC before anaphase (22). In the present study we observed that the level of CDC20 decreased upon DNA damage with the subsequent increase of p53 within the cell. We show that wild-type p53 transcriptionally represses *Cdc20* in several human cancer cell lines. Moreover, our studies reveal that this repression is brought about by direct binding of p53 to a *bona fide* p53 consensus binding site in the promoter of *Cdc20* resulting in chromatin remodeling.

MATERIALS AND METHODS

Plasmids

Cdc20 promoter containing 999-bp upstream of the transcription start site was amplified from human genomic DNA with the primers 5′TCTGAGCACATTCATACA ATTCACTC3′ (forward) and 5′AACACGCCTGGCTT ACGCCTCT3′ (reverse). The amplified fragment was cloned into the linearized pTZ57R/T (Fermentas, Lithuania) utilizing T/A cloning method. The fragment was then subcloned into the mammalian expression vector pGL3 basic from Promega, (Madison, WI, USA) using the restriction enzymes KpnI and XhoI (New England Biolabs, Beverly, MA). The site directed mutations of the NFY, CDE and p53-binding sites on the 1kb *Cdc20* promoter were created using the Site Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA). The primer sets used for the purpose is listed in Supplementary Table 1. The wild-type p53 expression plasmid pCMVp53 was a kind gift of Prof Bert Vogelstein (Johns Hopkins University, Baltimore, USA). The hTERT.Luc expression plasmid was a kind gift of Dr Riccardo Dalla-Favera (Columbia University, NY, USA). All the clones were checked by sequencing using the ABI Big-Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) and the 3130xl Genetic Analyser (Applied Biosystems Foster City, CA, USA).

Cell lines, transfection and drug treatment

Human cell line HCT116 and MCF7 were purchased from American Type Culture Collection (Manassas, VA, USA). HCT116 ($p53^{-/-}$ and $p21^{-/-}$) cells were kind gifts of Dr S. Das (Massachusetts General Hospital and Harvard Medical School, Charlestown, MA). HepG2 cells were gifted by Dr S. Adhya (Indian Institute of Chemical Biology, India). All the cells were grown in DMEM (Dulbecco's Modified Eagle's Medium; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal calf serum. Transient transfections were done with various expression plasmids in different cell lines using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol and cells were harvested after 48 h. For induction of endogenous p53 protein, cells were treated with 1 μg/ml and 10 μg/ml of 5-fluoro uracil (5FU) and 10 μM, 25 μM and 50 μM of Etoposide (Sigma, St Louis, USA). For release of Histone Deacetylase-mediated gene repression the cells were treated with 100 ng/ml and 250 ng/ml doses of Trichostatin A (Calbiochem, San Diego, CA, USA) respectively. The proteasomal inhibitor MG115 (Sigma, St Louis, USA) was administered at a final concentration of 25 μM and cells were incubated for 5 h.

RT–PCR

Total RNA from cell lines was isolated using TRIZOL (Invitrogen, Carlsbad, USA) following manufacturer's protocol. Five micrograms of total RNA was treated with DNAse in a total of 10 μl reaction volume. Two microliters of this mixture was used for cDNA preparation using random hexamer and MMLV-RT from Promega (Madison, WI, USA). The cDNA was then PCR amplified using specific primers indicated in the Supplementary Table 1.

Western blot

Western blots were done as described in (22). The primary antibodies used were anti-p53 antibody in a dilution of 1:1000 from US Biologicals (Swampscott, MA, USA), anti-CDC20 antibody in a dilution of 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 antibody in a dilution of 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β actin antibody at a dilution of 1:2000 (Sigma, St Louis, USA).

Chromatin immunoprecipitation (ChIP)

ChIP assay was done using the Quick ChIP kit from Imgenex Corporation (San Diego, CA, USA) according to manufacturer's protocol. PCR amplification of precipitated chromatin was done using primers indicated in the Supplementary Table 1. Ten micrograms each of α-p53 antibody, α-HDAC1 antibody, α-mSin3A antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-$H3K9(Me)_3$ antibody (Abcam, Cambridge, MA, USA) were used for the immunoprecipitation

Luciferase assay

After transfection and/or treatment, the cells were washed with phosphate-buffered saline (PBS). The cells were then lysed in the luciferase cell culture lysis buffer provided with the Luciferase Assay Kit (Promega, Madison, USA). After a brief vortex, whole cell lysates were centrifuged in the cold at 12 000 rpm for 2 min. Supernatant was collected in a fresh tube and 20–30 μl of that was added to luciferase assay substrate (60–80 μl). Luminescence was measured as relative light units (RLU), twice for each lysate, taking the reading of luciferase assay substrate alone and then with lysate in, GLOMAX

(Promega, Madison, WI, USA). The value obtained for luciferase assay substrate without lysate was subtracted from respective RLU value for each lysate with luciferase assay reagent. The total protein concentration in each lysate was determined with a protein assay kit (Sigma, St Louis, USA) and subsequently used to normalize the luciferase activity. Each assay was done in duplicate and repeated for three times. Fold repression values were represented as mean of the three experiments.

Electrophoretic mobility shift assay

The double-stranded oligonucleotides were prepared by annealing sense and anti-sense oligonucleotides (Supplementary Table 1) and labeled with [γ^{32}P] ATP and T4 polynucleotide kinase (Promega, Madison, WI, USA). Nuclear extracts from HepG2 cells treated with 5FU were used in the assay. For competition assay a 100-fold molar excess of unlabeled DNA oligonucleotides were added before incubation. After incubation, each sample was electrophoresed in a native 7% polyacrylamide gel. The gels were dried and exposed for autoradiography at −20°C. For supershift assay 5 μg of anti-p53 antibody was used (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RESULT

Downregulation of CDC20 by wild-type p53

We examined the effect of p53 on *Cdc20* mRNA and protein expression. Negative regulation of *Cdc20* by ectopic expression of p53 was noticed both at the mRNA and protein levels in HCT116 $p53^{-/-}$ cells (Figure 1A and B). Next, we investigated whether endogenous p53 could suppress *Cdc20* expression. HepG2 cells were treated with two different DNA damaging drugs, 5-FU and etoposide. As shown in Figure 1C and D, accumulation of p53 and subsequent suppression of CDC20 was observed with 1 μg/ml concentration of 5FU and 10 μM of etoposide. To eliminate the possibility of the decrease in CDC20 level due to protein degradation, we treated HepG2 cells with the proteasomal inhibitor MG115 along with 5FU. As seen in Figure 1E, treatment with MG115 failed to restore CDC20 protein level, whereas degradation of endogenous p53 in the absence of 5FU was stopped by MG115 (Figure 1F).

p53 downregulates CDC20 at the transcription level

After eliminating the possibility of p53-mediated proteasomal degradation of CDC20, we asked whether the repression occurred at the level of transcription. Towards that end, we amplified ~1 kb [nt # 43254437 to nt # 43255456, Accession Number 113772 TRED (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home)] region of human *Cdc20* including the transcription start site by PCR and cloned it into a luciferase assay vector pGL3 basic. Because the pGL3 basic vector did not contain any promoter or enhancer sequences, detectable luciferase activity indicated that 1-kb sequence possessed the *Cdc20* promoter activity (Figure 2A and B). To confirm the effect of p53 on *Cdc20* promoter we cotransfected the p53 null cell line, HCT116 $p53^{-/-}$ with the reporter construct and increasing amounts of wild-type p53 expression vector pCMVp53. As observed in Figure 2B, with 250 ng concentration of pCMVp53 vector there is almost 2-fold decrease in promoter activity, indicating that the p53 induced suppressive effect on *Cdc20* is a transcriptional phenomenon. The downregulation of *Cdc20* promoter driven luciferase activity was also examined by inducing endogenous p53 in HepG2 cells treated with 5FU and Etoposide (Figure 2C and D). In both the cases a dose dependent decrease (2–3.5-fold) in luciferase activity was observed. These results indicated that p53 transcriptionally repressed the expression of CDC20 in a dose-dependent manner, and this effect was physiological and not cell type specific.

CDC20 is downregulated by direct binding of wild-type p53 to its promoter

Having observed that the downregulation of *Cdc20* by wild-type p53 is at the transcription level, we examined whether p53 is physically present in the transcription complex on the *Cdc20* promoter. We first scanned the upstream 1 kb region of the *Cdc20* promoter which we had cloned in the luciferase reporter assay system for the presence of a consensus p53-binding region. We could identify the presence of a putative consensus p53-binding site 689 bases upstream of the transcriptional start site comprising of two copies of the sequence, 5′-RRRC (A/T) (T/A) GYYY-3′ separated by 17 bp (Figure 3A). Next, we used synthetically prepared oligos containing the sequence corresponding to the putative p53-binding site and performed an EMSA using nuclear extract from HepG2 cells treated with 5FU. We found strong binding of the radioisotope-labeled oligo with the nuclear extract and this band disappeared when the nuclear extract was previously incubated with excess of unlabeled oligo thus proving the specificity of the binding (Figure 3B). Moreover, when a supershift assay was done using antibody against p53 protein there was a prominent shift in the band, thus indicating the presence of p53 in the complex, binding the sequence (Figure 3B). Furthermore, we wanted to check for the specificity and strength of binding of p53 with the sequence. Towards that, we incubated the nuclear extract from 5FU-treated HepG2 cells with the well-characterized *p21* promoter sequence that binds p53 (24). The complex thus formed was chased by excess of non-radiolabeled probes of both *p21* and *Cdc20* promoter sequences. In parallel, the complex formed between 5FU treated HepG2 nuclear extract and the Cdc20 promoter sequence could also be chased by excess of cold *p21* promoter oligo. In both the cases the chase were of comparable efficiency indicating that the *Cdc20* promoter sequence binds p53 with appreciable efficiency and the strength of binding is comparable to that of p21 promoter (Figure 3B). This finding was further validated by ChIP. In MCF7 cells, treated with 5FU, chromatin precipitation was done using anti-p53 antibody and the precipitated DNA was PCR amplified using primers specific for a region of the *Cdc20* promoter (Figure 3C). Amplification was observed in MCF7 cells

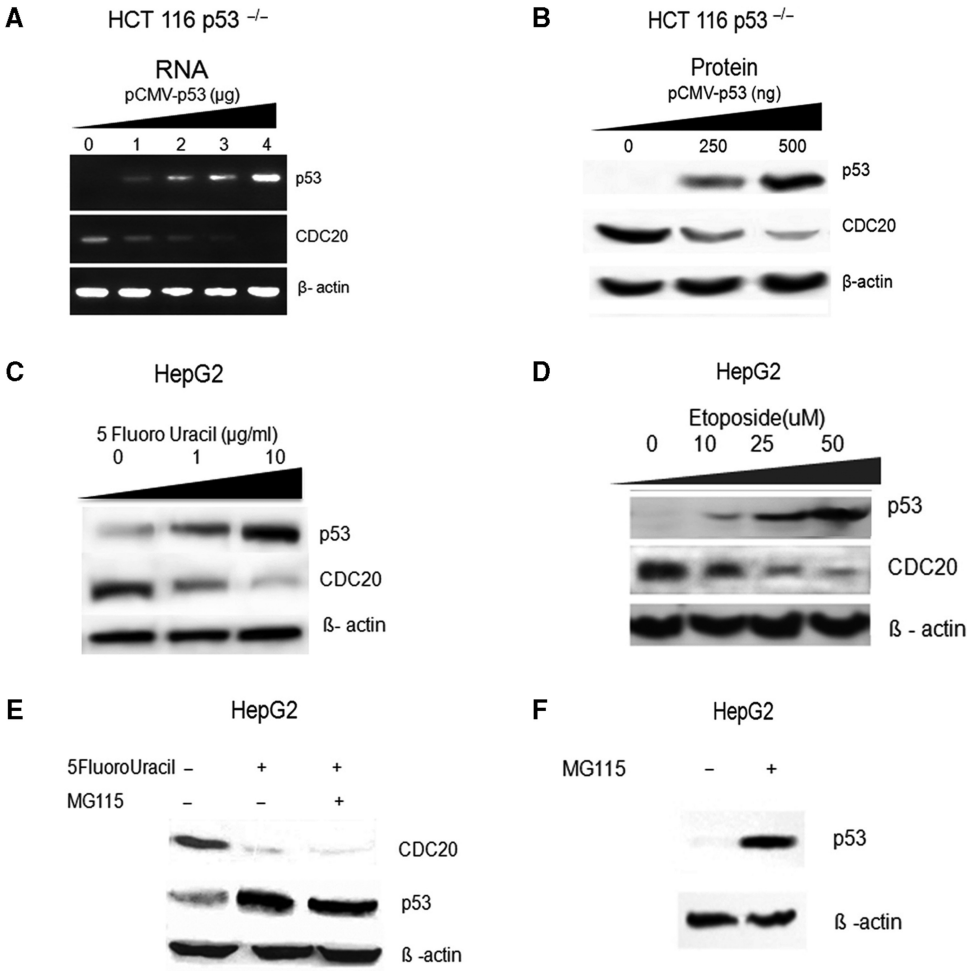

Figure 1. (**A**) HCT116 $p53^{-/-}$ cells were transiently transfected with 0, 1, 2, 3 and 4 μg of pCMVp53 expression plasmids. RT–PCR was done for *p53* and *Cdc20* and *β-actin* taken as internal control. It was seen that with increase in the level of *p53* mRNA, the *Cdc20* mRNA decreases. (**B**) HCT116 $p53^{-/-}$ cells transiently transfected with 0, 250 and 500 ng of pCMVp53 vector and the total protein isolated followed by western blot analysis with anti-p53, anti-CDC20 and anti-β-actin antibodies. It was observed that, with increase in p53 protein, the level of CDC20 protein decreased. (**C**) HepG2 cells were treated with 0, 1 and 10 μg/ml 5-FU for 48 h followed by western blot analysis. With increasing doses of the DNA damaging drug within the cell, endogenous p53 protein level increased with subsequent decrease in CDC20 protein level. (**D**) HepG2 cells were treated with 0, 10, 25 and 50 μM concentrations of Etoposide for 24 h followed by western blot analysis. It was observed that, with increasing drug, the endogenous p53 protein level increased with subsequent decrease in CDC20 protein level. (**E**) HepG2 cells expressing higher levels of p53 due to 5-FU treatment showed no increase in CDC20 protein level when treated with the proteasomal inhibitor MG115 indicating that the decrease in CDC20 protein with increasing p53 protein level was not due to proteasomal-mediated protein degradation. (**F**) Western blot analysis of HepG2 cells treated with MG115 in the absence of 5-FU showed inhibition of degradation of endogenous p53.

treated with 5FU indicating that p53 was present physically in the transcription complex on the *Cdc20* promoter. Also, HCT116 $p53^{-/-}$ cells were transfected with pCMVp53 construct followed by ChIP, and a positive amplification was observed when anti-p53 antibody was used for immunoprecipitation (Figure 3D).

There have been several reports of p53 repressing genes via the p21, NFY and E2F pathways (7,25). One such report also implicates the role of NFY and E2F in the repression of *Cdc20* by p53 (7). Therefore, we wanted to study whether p53 independently could use this newly identified binding site on *Cdc20* promoter to bring about the repression. We constructed several *Cdc20* promoter mutants where NFY, E2F (CDE/CHR) and p53-binding sites were mutated singly or in combinations (Figure 4A). These constructs were transfected in HCT116$p53^{-/-}$ cells in presence or absence of p53 expression vector pCMVp53. It was seen that even with both the NFY and CDE sites mutated (pTB3), ectopic expression of p53 could bring about the repression of CDC20, albeit to a lesser extent (Figure 4B). Notably, when the p53-binding site was mutated in conjunction with the NFY and CDE sites (pTB7) no effect of p53 overexpression was detected on the *Cdc20* promoter. These results indicated quite conclusively that p53 was using the direct binding site, independent of NFY and CDE. It was also seen that when HCT116 $p53^{-/-}$ cells were cotransfected with reporter constructs having mutations for both the NFY and p53 sites (pTB4) or only the p53 site (pTB5) along with pCMVp53, there was appreciable downregulation in promoter activity. These results suggest that p53 can also inhibit *Cdc20* promoter through an indirect

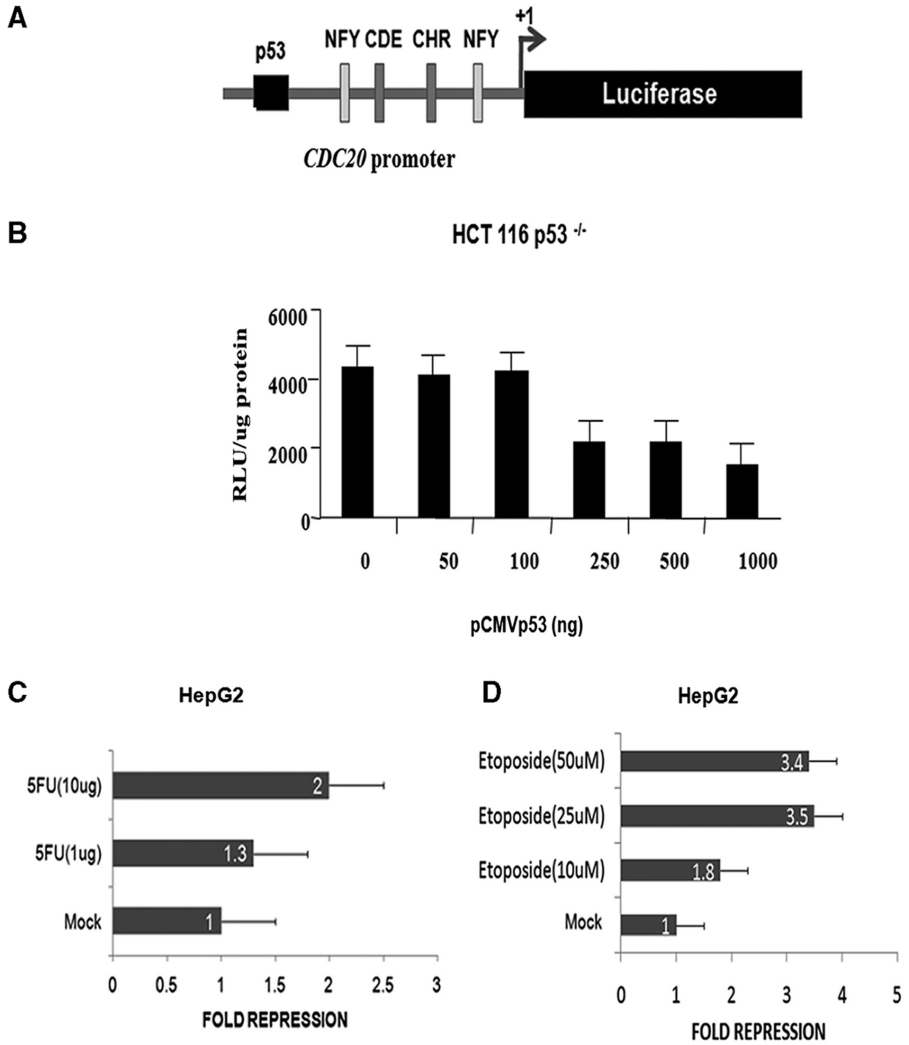

Figure 2. (**A**) 1 kb DNA sequence upstream of the start site of *Cdc20* transcription was cloned into the pGL3 basic vector such that it transcribes the luciferase gene. (**B**) HCT116 $p53^{-/-}$ cells were transiently co-transfected with pTB1 vector along with 0, 50,100, 250, 500 and 1000 ng of pCMVp53 vector followed by luciferase assay after 48 h. With increasing p53 expression the luciferase expression driven by the *Cdc20* promoter reduces. (**C**) HepG2 cells transfected with pTB1 were treated with 0, 1 and 10 μg/ml of 5FU followed by luciferase assay after 48 h. The luciferase activity decreased subsequently with increasing drug dose. (**D**) HepG2 cells transfected with pTB1 were treated with 0, 10, 25 and 50 μM of Etoposide followed by luciferase assay after 48 h. The *Cdc20* promoter driven luciferase activity decreased with increasing drug dose.

pathway, as suggested by others (Figure 4A). Another notable observation here is that the fold repression of pTB2 (with both NFY RE sites mutated) is comparable to that of the wild-type promoter construct pTB1 while in pTB6 having the p53 site and CDE mutated and only NFY site functional do not get repressed by p53. In all the cases NFY RE mutation does not have any additional effect on the p53-mediated repression, indicating that NFY does not have a role in p53-mediated CDC20 repression, contrary to what have been reported before (25).

These results were further validated by studying the effect of p53 on *Cdc20* promoter in a p21 null cell line HCT116 $p21^{-/-}$. Here also, we found that p53 could considerably repress the luciferase expression driven by wild-type (pTB1) or CDE and NFY mutated (pTB3) *Cdc20* promoter (Figure 4C), thus reinforcing the fact that there is a mechanism of p53-mediated repression of CDC20 independent of p21. Interestingly here, the NFY and p53 double mutant construct (pTB4) showed downregulation in promoter activity when p53 was over-expressed (Figure 4C). These results suggest that p53 can inhibit *Cdc20* promoter activity through CDE-binding site independent of p21 pathway.

We finally measured the mutant promoter activities by inducing endogenous p53 in the HCT116 $p21^{-/-}$ cells to mimic a more physiological condition (Figure 4D). As expected, wt *Cdc20* promoter luciferase construct (pTB1) exhibited considerable downregulation upon activation of endogenous p53 both by 5FU and etoposide. However, drug induced p53 failed to downregulate the *Cdc20* promoter which was mutated at the p53-binding site (pTB5) or both the NFY and p53-binding sites (pTB4) whereas, constructs with NFY (pTB2) and NFY-CDE mutations (pTB3) showed significant repression. Here what is

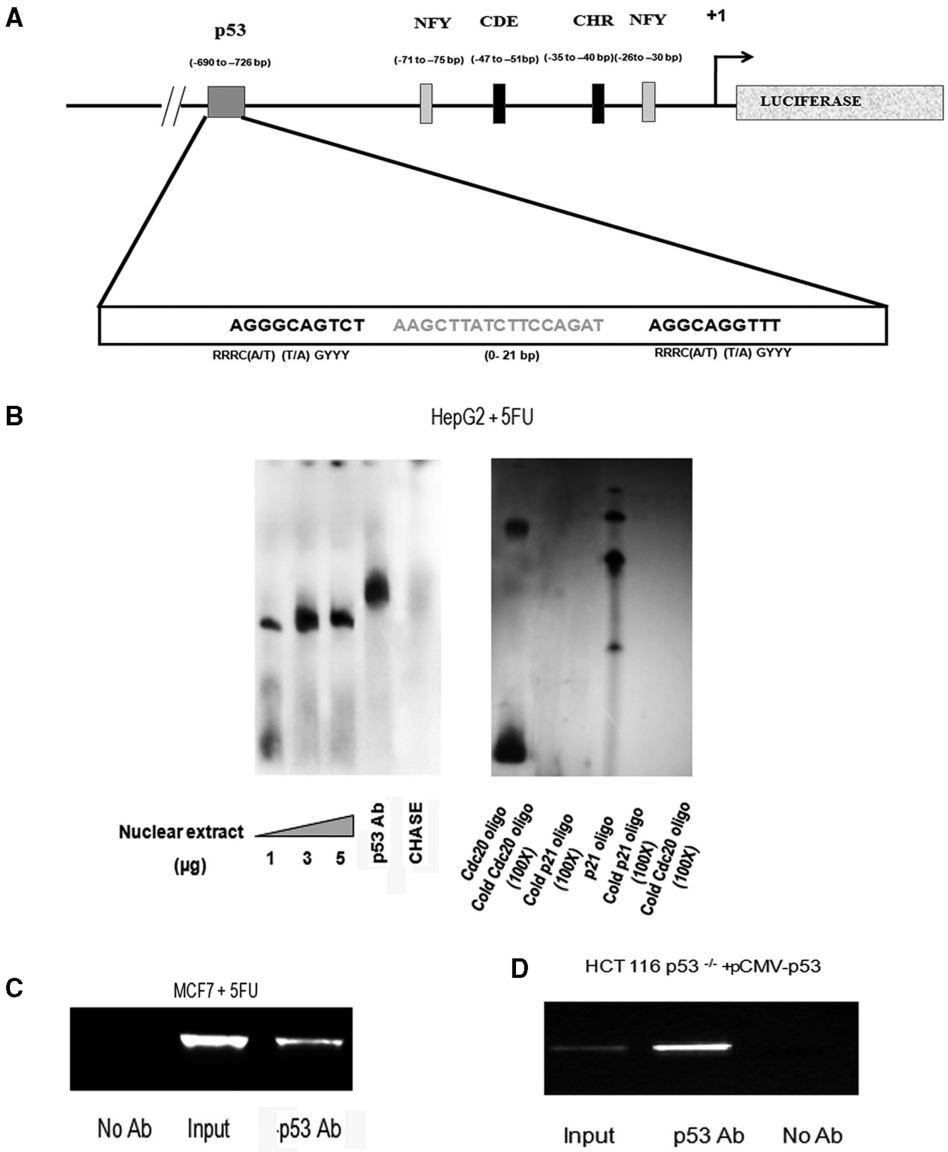

Figure 3. (**A**) Cis elements on the *Cdc20* promoter and the nucleotide sequence of the identified p53 direct binding site. (**B**) Electrophoretic mobility shift assay done with the oligonucleotide sequence corresponding to the p53-binding site and nuclear extract from HepG2 cells treated with 5FU. The first three lanes of the left panel show binding of the oligonucleotide and 1, 3 and 5 µg of the nuclear extract, the fourth lane shows the supershift with anti-p53 antibody and the last lane is the chase with unlabeled probe (100× molar excess). In the right panel, the first three lanes correspond to the binding of p53 from 5FU treated HepG2 nuclear extracts with Cdc20 promoter oligo and chase with 100× molar concentration of the non-radiolabeled Cdc20 promoter oligo and p21 promoter oligo respectively. The next three lanes correspond to the binding of p53 from 5FU-treated HepG2 nuclear extract with the well characterized p21 promoter oligo and chase with 100× molar concentration of non-radiolabeled p21 promoter oligo and Cdc20 promoter oligo respectively. (**C**) ChIP in MCF7 cells treated with 5FU. Anti-p53 antibody was used to precipitate the chromatin–protein complex and *Cdc20* promoter-specific primers were used to amplify the precipitated DNA. (**D**) ChIP in HCT116 $p53^{-/-}$ cells transfected with 1 µg of pCMVp53 vector. Anti-p53 antibody was used for immunoprecipitation and *Cdc20* promoter-specific primers were used for amplification of the precipitated DNA. A fraction of the input was loaded.

notable is that all the constructs having the wild-type p53-binding site (pTB1, pTB2 and pTB3), responded to the drug-mediated p53 induction whereas in those clones where the p53 site is mutated irrespective of the CDE site being intact (pTB4, pTB5) there is no repression. Taken together, these results suggest that physiological levels of p53 inhibits *Cdc20* promoter via the putative p53-binding site whereas inhibition of *Cdc20* promoter through indirect pathway using the CDE site requires much higher levels of p53 expression.

p53 downregulates CDC20 expression through chromatin remodeling

After establishing that p53 binds directly to the *Cdc20* promoter and represses its activity, we wanted to investigate whether this binding induces chromatin remodeling thereby causing the downregulation. We first, ectopically expressed p53 in HCT116 $p53^{-/-}$ cells and treated them with increasing doses of the HDAC inhibitor Trichostatin A (TSA). The luciferase activity driven by the *Cdc20*

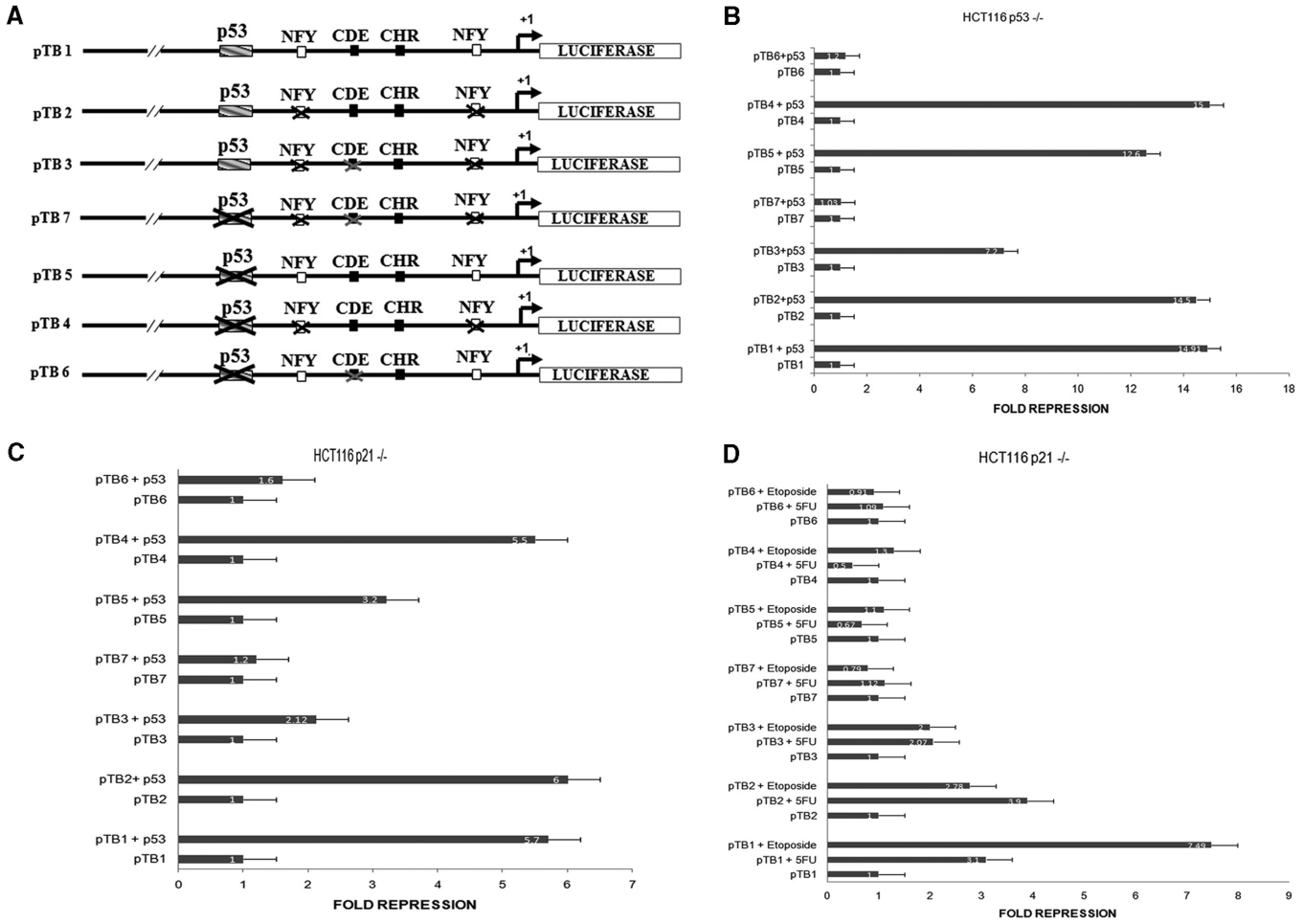

Figure 4. (**A**) The NFY and E2F response elements and putative p53-binding site on the *Cdc20* promoter have been mutated by site directed mutagenesis. The crossed boxes refer to the mutated responsive elements. (**B**) Luciferase assays done in HCT116 $p53^{-/-}$ cell with different mutants (as indicated) in presence or absence of wild-type p53 overexpression. (**C**) Luciferase assay performed in HCT116 $p21^{-/-}$ cells with the different mutants along with ectopic expression of wild-type p53. (**D**) Luciferase assay performed in HCT116 $p21^{-/-}$ cells with the different mutants in presence of the DNA damaging drugs 5FU and Etoposide.

promoter recovered as higher doses of TSA were administered, indicating a role of HDACs in the p53-mediated repression (Figure 5A). To eliminate the possibility of arbitrary release in repression by Trichostatin A we performed an identical experiment using the human telomerase reverse-transcriptase (hTERT) promoter, which is transcriptionally downregulated by p53 in a HDAC independent fashion (26,27). TSA could not release the repression on *hTERT* promoter brought about by p53 (Figure 5B), establishing the specificity of the drug action. We next performed ChIP in HepG2 cells treated with 5FU where immunoprecipitation was done using anti-p53 antibody, anti-HDAC1 antibody and the antibody against co-repressor mSin3A (Figure 5C). PCR amplification was observed in all the three cases indicating the chromatin remodelers HDAC1 and mSin3A to be present in the transcription complex of *Cdc20*. To establish that the recruitment of HDAC1 and mSin3A to the *Cdc20* promoter occurs only in the presence of p53, we treated HCT116 $p53^{-/-}$ cells with 5FU and then performed ChIP using p53, HDAC1 and mSin3A antibodies, but no amplification was observed (Figure 5D) indicating that the chromatin remodelers did not bind to the *Cdc20* promoter in the absence of p53. Furthermore, to verify whether this event was specific to the DNA damage context we performed ChIP with HCT116 cells, which were treated with or without 5FU. Immunoprecipitation was done using antibodies specific for p53 and HDAC1. Interestingly, we found that only in those cells which were treated with the DNA damaging drug and having higher levels of p53 protein and corresponding lower levels of CDC20 (Figure 5G), gave a positive PCR amplification for the *Cdc20* promoter when immunoprecipitated with the two antibodies (Figure 5E). Also Histone3 lysine 9 tri-methyl-specific antibody could precipitate the *Cdc20* promoter in 5FU treated HCT116 cells compared to the untreated one (Figure 5F). All these results taken together indicate that DNA damage within a cell induces p53 which is recruited to the *Cdc20* promoter and which in turn brings about transcriptional downregulation by histone deacetylation and histone methylation.

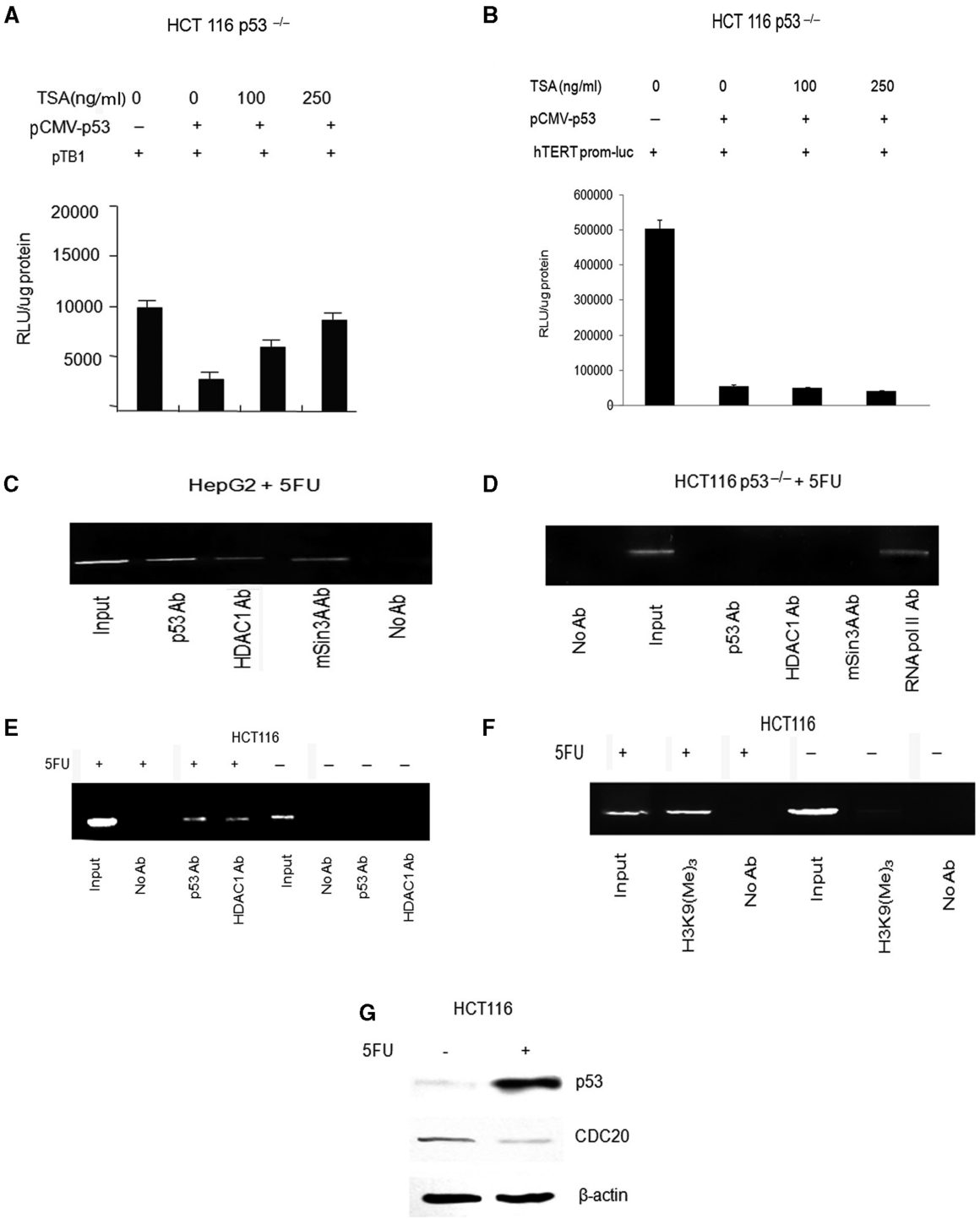

Figure 5. (**A**) HCT116 $p53^{-/-}$ cells cotransfected with pTB1 and pCMVp53 expression vectors were treated with increasing doses of the HDAC inhibitor Trichostatin A (TSA) and luciferase assay was performed after 48 h. Luciferase acivity is expressed as Relative Light Unit (RLU) per microgram of total protein. (**B**) HCT116 $p53^{-/-}$ cells cotransfected with hTERT prom-luc and pCMVp53 expression vectors were treated with increasing doses of TSA and luciferase assay was performed after 48 h. Luciferase activity is expressed as RLU per microgram of total protein. (**C**) ChIP assay was done in 5-FU treated HepG2 cells with anti-p53, anti-HDAC1 and anti-mSin3A antibodies and the precipitated chromatin amplified using primers specific for the *Cdc20* promoter. (D) ChIP assay was done in 5FU treated HCT116 $p53^{-/-}$with anti-p53, anti-HDAC1, anti-mSin3A and anti-RNA pol II antibodies and the precipitated DNA was amplified using primers specific for the *Cdc20* promoter. (**E**) ChIP done in HCT116 cells treated or untreated with 5-FU. Amplification with the *Cdc20* promoter specific primer was observed in chromatin precipitated with anti-p53 and anti-HDAC1 antibodies only in the treated cells but not in the untreated cells. (**F**) ChIP done in HCT116 cells treated with or without 5-FU. Immunoprecipitation was done using antibody against Histone3 lysine 9 tri-methylation and the precipitated DNA amplified using CDC20 promoter-specific primers. (**G**) Western blot analysis of HCT 116 cells treated with or without 5FU using anti-p53, anti-CDC20 and anti- β-actin antibodies showed an increase in endogenous p53 level with consequent decrease in CDC20 level upon drug treatment.

DISCUSSION

Extensive studies have established p53 to be a key gene, which is activated upon cellular stress or DNA damage (8,21,25,28). Activated p53 has been found to transactivate a number of genes (10) but there has been increasing evidence of several genes which are also repressed by p53 (6,8,9,29). There is report of 129 genes from both human and viral genomes which contain the p53 consensus response element (RE) (30). About 160 REs have been reported of the 129 identified p53 responsive genes (several genes have multiple REs). A number of genes like *stathmin, AbcB1, Birc5 (survivin), Hsp90β*, etc. are repressed by p53 via their responsive elements, upon several stress signals (28,29). The current consensus is that there are four criteria that are to be fulfilled for a particular sequence to be designated as a *bona fide* p53 direct binding responsive element and the gene to be a p53 responsive target (30). First, is the presence of a p53 RE in the DNA close to the gene or within the gene. Second, is to demonstrate that the particular gene is upregulated or downregulated at the RNA and protein level by wild-type p53 protein. The third line of evidence is to clone the RE upstream of any reporter gene like luciferase and show that p53 induction can regulate this test gene in the same fashion as the gene of interest. Finally, it has to be established by ChIP using specific p53 antibody, that p53 physically interacts with the RE and binds to the chromatin. In some cases a gel-shift assay is also done additionally to demonstrate the binding *in vitro*. Here, in this study we have fulfilled all the above criteria and have established the SAC gene *Cdc20* to be downregulated by p53 upon DNA damage where activated p53 directly binds to the RE located 689-bp upstream of the transcription start site (TSS).

The putative p53-binding site on the *Cdc20* promoter has two half sites conforming to the 5′-RRRCWWGYY-3′ (where R is a purine, Y is a pyramidine, W is either A or T, G is guanine and C cytosine) rule. These half sites are separated by an 17-bp spacer. Each quadrate of the half sites is arranged in the head to head orientation. All these are in conformation with the requirements of a sequence to be a p53 RE (30). However, careful analysis of the p53 RE of *Cdc20* promoter revealed few deviations from the consensus motif. It has been noted that the central CWWG sequence of the half site is highly conserved in experimentally verified p53 REs (18,19,30). The first C is replaced by G in the left half site of the *Cdc20* p53 RE (Figure 3A). Similarly, instead of WW this p53 RE contains CW and WG in the left and right half-site respectively. It is known that p53 RE is highly degenerate and deviations from the consensus sequence have been noted in many p53 responsive genes, including both which are activated or repressed by p53. Another important component of the p53 RE is the length of the spacer between the two half-sites. Recent analysis suggests that on an average, p53-repressor biding sites have significantly longer spacer length than that of p53 activator sites (30). The observed 17-bp spacer length between the two half-site of the *Cdc20* p53 RE is in conformity with these findings.

p53 downregulates genes both by direct and indirect mechanisms (21). At present, three generally accepted methods of direct p53-mediated repression are known: first, binding site overlap (steric interference) (20); second, p53 squelching of transcriptional activators (17); and third, p53-mediated recruitment of histone deacetylases (HDACs) (30,31). There are two generally accepted modes of indirect p53-mediated repression. The first is brought about by activation of CDKN1A ($p21^{WAF}$), which in turn inhibits cyclinD–CDK4 complex through direct binding (25,30). The consequence of this inhibition is the absence of hyperphosphorylation of the Rb protein. Unphosphorylated Rb represses the function of the E2F family of transcription factors. Also, on the other hand inhibited cyclinD–CDK4 complex cannot phosphorylate transcription factor NFY to perform its necessary functions (25). In the second mode of indirect repression, p53 binds to another transcription factor and represses the target gene without any p53-specific RE (6). There have been reports of the *Cdc20* gene being repressed by the indirect mechanism through CDKN1A activation (7,25). The reports have identified NFY and E2F responsive elements on the promoter region of *Cdc20*, which transactivate the gene under normal condition. Upon p53 induction, p21 level is increased within the cell, hence E2F and NFY can no longer activate *Cdc20* resulting in a decrease in CDC20 level. But, in our study we have given evidence of p53 direct binding to the *Cdc20* promoter in the context of DNA damage. We have performed ChIP assay to show that after DNA damage p53 binds to the *Cdc20* promoter, which in turn recruits the co-repressor mSin3A and the histone deacetylase HDAC1. Also, ChIP studies have shown histone methylation of the *Cdc20* promoter chromatin to occur in presence of p53. These are in agreement with the already established mechanism of p53-mediated direct repression of genes (30). We have also generated site-directed mutations of the NFY and E2F-binding sites of the *Cdc20* promoter and have done reporter assays with these mutants to show that even in absence of the indirect repression mechanism, p53 can bring about further repression of the *Cdc20* gene by direct binding. This has been further proved by performing the studies in a p21 null cell line. Thus, the existence of both the direct and the indirect pathway for p53-mediated downregulation of *Cdc20* gene is operating in DNA damaged cells. A few additional informations on the indirect mechanism of repression have emerged from this study. First, p53 represses *Cdc20* promoter via CDE/CHR element in p21 independent manner. The exact mechanism of CDE/CHR-mediated *Cdc20* promoter repression remains unclear. p21 independent CDE/CHR element-mediated gene repression by p53 has also been reported for another cell cycle checkpoint protein *Cdc25C* (21). Second, while p53-mediated direct repression of *Cdc20* promoter works at more physiological condition, the CDE/CHR-mediated indirect pathway operates at higher p53 levels (Figure 6). Finally, we see that contrary to what has been reported till now (25), the two CCAAT/NFY elements present on the *Cdc20* promoter do not contribute to the p53-mediated transcriptional repression of the gene.

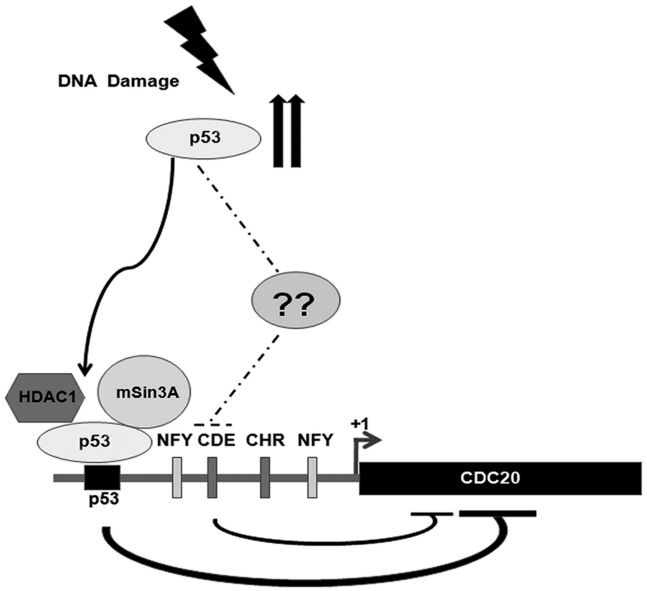

Figure 6. Schematic representation of the proposed model. DNA damage induces p53 within cells which directly bind to the *Cdc20* promoter and represses its transcription. HDAC1 and mSin3A are recruited causing heterochromatinization of the *Cdc20* promoter. The CDE element on the promoter seems to participate in p53 dependent repression only when there is a very high concentration of p53 present within the cell. This phenomenon is p21 independent.

Under cellular stress or DNA damage cell division is halted to allow time to correct the damage or overcome the stress by activating several cell-cycle checkpoints (2). Indeed major cell cycle checkpoints such as G1/S, G2/M and SAC are known to be regulated by activated p53 in response to DNA damage or other cellular stress (8,10,25). Although, the regulation of G1/S and G2/M by activated p53 is known in great detail, the regulation of SAC by p53 in response to DNA damage is poorly understood. As a dividing cell proceeds from metaphase, the activated CDC20 protein primes the APC to initiate Anaphase. We find that the APC activator, CDC20 is downregulated by the tumor suppressor protein p53 in response to various DNA damages. Previously, another SAC gene *Mad1L1* has also been reported to be downregulated by p53 under DNA damaged conditions (6). Thus, it appears that tumor suppressor protein p53 uses multiple genes as targets to control the SAC function. It is conceivable that p53 would do so because the deregulation of SAC is known to lead to chromosomal instability (22). In human cancer cell lines and primary tumors frequent mitotic abnormality due to overexpression/underexpression of SAC genes have been reported (32–34). Overexpression of CDC20 is known to initiate premature anaphase leading to aneuploidy in tumor cells (22). Furthermore, level of CDC20 is regulated in a cell cycle dependent manner (1,2). Thus, a tight regulation of CDC20 ensures proper checkpoint function which may be controlled by p53 under cellular stress.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Dr Hemanta Kumar Majumder (Indian Institute of Chemical Biology, India) and to Dr Utpal Sanyal (Chittaranjan National Cancer Research Institute, India) for providing the drug Etoposide and 5 Fluoro Uracil respectively. We thankfully acknowledge Dr Riccardo Dalla-Favera (Columbia University, NY, USA) for providing us the hTERT luciferase vector. We are also indebted to Dr Raghunath Chatterjee (Indian Institute of Chemical Biology, India) for his valuable input in our bioinformatic-based studies. The authors thank all members of Molecular & Human Genetics Division, IICB for support and encouragement during the study.

FUNDINGS

DBT grant [BT/PR/5524/Med/14/649/2004, BT/01/COE/05/04] and CSIR grant IAP 001, awarded to Dr Susanta Roychoudhury. Pre-doctoral fellowship from the Council of Scientific and Industrial Research (New Delhi, India) (to T.B.). The Open Access Publication charge was partially waived by Oxford University Press.

Conflict of interest statement. None declared.

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