E2 Ubiquitin-conjugating Enzyme, UBE2C Gene, Is Reciprocally Regulated by Wild-type and Gain-of-Function Mutant p53*

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Spindle assembly checkpoint governs proper chromosomal segregation during mitosis to ensure genomic stability. At the cellular level, this event is tightly regulated by UBE2C, an E2 ubiquitin-conjugating enzyme that donates ubiquitin to the anaphase-promoting complex/cyclosome. This, in turn, facilitates anaphase-onset by ubiquitin-mediated degradation of mitotic substrates. UBE2C is an important marker of chromosomal instability and has been associated with malignant growth. However, the mechanism of its regulation is largely unexplored. In this study, we report that UBE2C is transcriptionally activated by the gain-of-function (GOF) mutant p53, although it is transcriptionally repressed by wild-type p53. We showed that wild-type p53-mediated inhibition of UBE2C is p21-E2F4-dependent and GO mutant p53-mediated transactivation of UBE2C is NF-Y-dependent. We further explored that DNA damage-induced wild-type p53 leads to spindle assembly checkpoint arrest by repressing UBE2C, whereas mutant p53 causes premature anaphase exit by increasing UBE2C expression in the presence of 5-fluorouracil. Identification of UBE2C as a target of wild-type and GOF mutant p53 further highlights the contribution of p53 in regulation of spindle assembly checkpoint.

The tumor suppressor gene TP53 plays a pivotal role in the maintenance of cellular and genomic integrity by preventing accumulation of errors in the cell duplication process under stressed conditions (1). Therefore, it ensures the fidelity of the cell division as well as protects the cell from cancerous growth. Wild-type p53 gets activated and stabilized in response to a variety of stresses that in turn regulates the transcription of various genes involved in cell growth and survival (2, 3). Proper cell cycle progression is monitored by a set of checkpoints, G1/S, G2/M, and SAC,5 that takes a cell forward from one stage to another (4). These checkpoints sense defects in chromosomal status and delay the progression to the next stage, if necessary (5). Wild-type p53-p53-mediated control of G1/S and G2/M regulators like CMYC, CDC25C, CCNB1, Piki1, and BiRCS have been documented (6–10). However, little is known about the control of SAC by wild-type p53. In most cases, the knowledge is based on studies of global gene expression datasets where genes involved in mitotic arrest arise as putative transcriptional targets of wild-type p53 (11).

TP53 gene is mutated in almost half of the human cancers. These are mostly missense mutations in the TP53 gene leading to either DNA contact-defective or conformation-defective p53 mutants. These mutated forms of p53 are highly stable and expressed in human cancers (12). Extensive investigations have established that mutant p53 can contribute to malignancy by providing selective growth advantage to cancer cells and resistance to anticancer therapy (13, 14). The growing description of biochemical and biological functions of mutant p53 shows that it not only loses the tumor-suppressive functions of its wild-type counterpart but also acquires novel oncogenic gain-of-function (GOF) properties (12). It has been previously demonstrated that mutant p53 interacts with the CCAAT-binding factor NF-Y, and this complex serves to up-regulate NF-Y target genes such as CCNA, CCNB, CDK1, CDC25C, and EFNB2 to promote cell cycle progression and chemoresistance following drug treatment (4, 15). Surprisingly, wild-type p53 has been shown to interact with the same transcription factor NF-Y (16) and regulates many of the same target genes as described for mutant p53. However, the regulation of wild-type p53 is often exactly reciprocal to that mediated by GOF mutant p53 (4). It has been shown that in response to DNA damage, wild-type p53 and mutant p53 recruit different cofactors, mainly epigenetic modifiers to regulate differentially (4).

SAC ensures an equal distribution of chromosomes to daughter cells during mitosis, thereby maintaining chromo-

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5 The abbreviations used are: SAC, spindle assembly checkpoint; APC/C, anaphase-promoting complex/cyclosome; GOF, gain-of-function; 5-FU, 5-fluorouracil; MI, mitotic index; CDK, cyclin-dependent kinase.
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somal stability (17, 18). Improper SAC results in malignancies or birth defects (19). The regulation of SAC requires the concerted effort of a multitude of cell cycle proteins such as Bub1, BubR1, Bub3, Mad1, Mad2, APC/C, Cdc20, UBE2C, etc. (20–22). Central to the checkpoint control is the ubiquitin pathway consisting of an E3 ligase, the APC/C, E2 ubiquitin-conjugating enzyme UBE2C, and their mitotic substrates securin and cyclin-B1 (23). APC/C brings about mitotic exit by initiating anaphase onset through the proteolysis of these substrates by binding to an adaptor protein, Cdc20. This association is fostered by UBE2C, which ubiquitinates Cdc20 to facilitate its binding to APC/C leading to activation of the latter (24).

Recently, efforts are being made to explore the role of p53 in transcriptional regulation of SAC genes (21, 25). Interestingly, several microarray studies have helped to identify the entire repertoire of cell cycle genes regulated by p53 (11, 26). However, mechanistic insights into the regulation of SAC genes remain poorly understood. Using microarray analyses, at least two independent studies have identified UBE2C as a putative transcriptional repression target of p53 (11, 27). Besides being an important gene in the spindle assembly checkpoint pathway, UBE2C has also been well implicated in multiple cancers (28). It is highly expressed in several cancer cell lines and primary tumors of the lung, stomach, uterus, and bladder, etc. as compared with corresponding normal tissues (28–31). Aberrant levels of UBE2C in cancer cells led to compromised SAC (24).

Although UBE2C maintains a cell cycle-dependent expression pattern peaking at mitosis (32), its transcriptional regulation by wild-type and mutant p53 is not known.

In this study, we examine the dichotomy between wild-type and mutant p53 in relation to the newly uncovered p53 target UBE2C. We started by confirming that UBE2C is a bona fide target of wild-type and mutant p53 and that they control its expression in an opposite manner. We worked out the mechanistic details of the regulation. Furthermore, we also show wild-type p53 causes SAC arrest in response to DNA damage, whereas mutant p53 activates the SAC prematurely.

Experimental Procedures

Cell Culture—Human cancer cell lines, HCT116p53+/+ (CCL-247), SW480 (CCL-228), Saos-2 (HTB-85), C33A (HTB-31), SKBR3 (HTB-30), and HepG2 (HB-8065) were purchased from American Type Culture Collection (ATCC, Manassas, VA). CCL-247 derivatives, HCT116p53+/− and HCT116p21−/−, were the kind gifts from Prof. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD) (33). Pancreatic cancer cell line MIAPaCa2 was a kind gift from Prof. Chitra Mandal (CSIR-Indian Institute of Chemical Biology, Kolkata, India). HCT116p53+/−, SW480, C33A, MIAPaCa2, and HepG2 were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Carlsbad, CA), and HCT116p53+/−, Saos-2, SKBR3, and HCT116p21−/− were maintained in McCoy’s 5A modified medium (Gibco). All the media were supplemented with 10% fetal calf serum (v/v) (Gibco) and antibiotics (Gibco).

Plasmids and siRNAs Used—Different deletion constructs of the UBE2C promoter containing the −693/+39, −321/+39, or −141/+39-bp fragments across the transcription start site were prepared as described previously (34). The site-directed mutations of the CCAAT and CHR elements on the −141/−141/+39-bp UBE2C promoter were created using the site-directed mutagenesis kit (Stratagene, La Jolla, CA). All the clones were verified by sequencing using the Big-Dye terminator kit (Applied Biosystems, Foster City, CA) in the 3130XL Genetic Analyzer (Applied Biosystems). The wild-type p53 (pCMV-WTp53) expression plasmid and GOF mutant p53 (pCMV-R273H and pCMV-R175H) expression plasmids were the kind gifts from Prof. Bert Vogelstein (The Johns Hopkins University). The full-length UBE2C expression plasmid pCS2-UBE2C was kindly provided by Prof. Marc W. Kirschner (Harvard Medical School, Boston). The E2F4, NF-YA wild-type (NF-YA-WT), and the NF-YA dominant-negative mutant (NF-YA-MUT) expression plasmids were the kind gifts from Dr. Joseph R. Nevins (Durham, NC) and Dr. R. Mantovani (University of Milan, Milan, Italy), respectively. For siRNA transfection, 80 nm p53 siRNA (Ambion, Austin, TX, and Dharmacon, Lafayette, CO), 80 nm UBE2C siRNA (Santa Cruz Biotechnology, Santa Cruz, CA), and 80 nm of E2F4 siRNA (Santa Cruz Biotechnology, sc-29300) were used. Scrambled siRNA was purchased from Ambion.

siRNA Transduction—pGPZIP lentiviral short hairpin RNA (shRNA) for human TP53 and non-silencing control were purchased from Thermo Scientific (Rockford, IL) and were prepared according to the manufacturer’s protocol. SW480 cells were infected with lentiviral shRNA, and stable cells were selected for the experiments after puromycin (Gibco) selection.

Drug Treatments and Transfections—5-Fluorouracil (5-FU), doxorubicin, etoposide, anacardic acid, and proteasomal inhibitor MG115 were purchased from Sigma. Thymidine and Nocodazole were purchased from United States Biologicals (USB, Cleveland, OH). Transient transfections using various expression plasmids and reporter constructs were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol, and cells were harvested after 48 or 72 h. The total amount of DNA was held constant for all the transfection experiments using corresponding empty vectors. All the transfections were performed at least three times with independent plasmid preparations.

Reverse Transcription-PCR (RT-PCR) Analysis—Total RNA was isolated from different cell lines using TRIzol (Invitrogen) following the manufacturer’s protocol. Five micrograms of total RNA was treated with DNase-I (Promega, Madison, WI) in a total of 10 µl of reaction volume. 2 µl of this mixture was used for cDNA preparation using random hexamer (Invitrogen) and Moloney murine leukemia virus-RT (Promega). For semi-quantitative analysis, the cDNA was then PCR-amplified using the following primer sets: p53, forward 5′-CCGCAGTCAGATCCTAGCG-3′ and reverse 5′-AATCATCATATGTGGAGGTCGCTAATGAGG-3′; UBE2C, forward 5′-TGGTCTGCCCTGTATGAAATCATCCATTGCTTGG-3′ and reverse 5′-AAAAAGCTGTGGGGTTTTTCC-3′; GAPDH, forward 5′-ATGGGAAAGGTGAAGGTCGG-3′ and reverse 5′-GGGTCTAACGAGTCACTT-3′. Real time PCR was performed on the 7500 Fast Real Time PCR system (Applied Biosystems) using power SYBR Green PCR Master Mix (Applied Biosystems) with the same primers for UBE2C and GAPDH as mentioned above. The comparative threshold cycle method (ΔΔCT) was used to quantify the relative amount
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of UBE2C transcripts at different times of 5-FU treatment with respect to the untreated cells. GAPDH was used as an endogenous control.

Western Blotting Analysis—Western blotting analysis was carried out as described previously (35). The following antibodies were used in this study and were purchased from Santa Cruz Biotechnology (catalogue number, lot number, and the dilution used): mouse anti-p53 (sc-126, lot no. J1810, 1:100); goat anti-UBE2C (sc-47545, lot no. A0412, 1:100); rabbit anti-p21 (sc-397, lot no. F0608, 1:100); rabbit anti-E2F4 (sc-1082, lot no. C2411, 1:100); mouse anti-cyclin-B1 (sc-7393, lot no. D1813, 1:200); and rabbit anti-phosphorylated histone H3 (S10) (sc-8656-R, lot no. A2113, 1:200). Rabbit anti-acetyl-p53 (K382) (2525, lot no. 10, 1:1000) and rabbit anti-histone H3 (4499, lot no. 3, 1:1000) were purchased from Cell Signaling Technology. Mouse anti-β-actin (A2228, lot no. 112M4762V, 1:5000) was obtained from Sigma. Primary antibodies were detected with goat anti-rabbit (A0545, lot no. 022M4811, 1:80,000) or rabbit anti-mouse (A9044, lot no. 112M4816, 1:40,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752, 1:80,000) or rabbit anti-goat (A5420, lot no. 052M4752,
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counted among 100–200 cells each time. Mitotic index (MI) was calculated as a percentage of the ratio between p-H3-positive cells and DAPI-positive cells. MI was represented as a mean of three independent experiments.

**Determination of Chromosomal Abnormalities**—Synchronized SW480 cells were harvested at 10 h after the second thymidine release. Cells were then examined under a super-resolution confocal microscope (Leica TCS SP8). Abnormal chromosomes were counted among 40–50 cells each time from three independent experiments and plotted as percentage of mitotic cells with chromosomal abnormalities.

**Soft Agar Assay**—Each well of a 6-well plate was coated with 0.8% agar mix containing 1:1 low melting agarose (Sigma) and 2× DMEM complete medium. After the layer was solidified, ~10,000 cells were added to 0.4% agar containing DMEM complete medium and plated over the base agar layer. The plates were incubated in a 37°C CO2 incubator for 2–3 weeks before the colony appears. Colony images were taken using an Olympus IX71 microscope at ×20 magnification, and visible colonies were counted using ImageJ software (imagej.nih.gov).

**Statistical Analysis**—Statistically significant differences between the two groups were determined by Student’s t test using GraphPad QuickCalcs on line. Two-tailed p values were defined as p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***)**, and p ≤ 0.0001 (****)

**Results**

**Reciprocal Regulation of UBE2C by Wild-type and Mutant p53**—We observed a dose-dependent decline in the expression of UBE2C upon ectopic expression of wild-type p53 protein in HCT116p53−/− cells (Fig. 1A). Next, we treated HCT116p53+/+ cells with the DNA-damaging agent 5-FU and observed significant reduction in UBE2C mRNA expression in a time-dependent manner when compared with an untreated set (Fig. 1B). The above observation also held true in a more physiological context, where treatment of HCT116p53+/+ cells with three different DNA-damaging agents, 5-FU, doxorubicin, and etoposide, caused a decline in the UBE2C protein levels upon drug-induced accumulation of wild-type p53 (Fig. 1C). To confirm this decline is wild-type p53-dependent, we did a similar experiment in p53 null cells, HCT116p53−/−. As expected, there was no change observed in the mRNA and protein levels of UBE2C despite the DNA damage (Fig. 1F). It is known that wild-type p53 is rapidly expressed at the protein level within 4–8 h post-drug treatment in HCT116p53+/+ cells (21). Therefore, we wanted to evaluate the mRNA and protein levels of UBE2C at early time points following DNA damage. We observed inhibition of UBE2C following induction of wild-type p53 by 5-FU treatment in HCT116p53+/+ cells as early as 4–8 h both at the mRNA (data not shown) and protein levels (Fig. 1E). We next asked whether the decline in UBE2C protein levels by wild-type p53 was due to protein degradation. Treatment of HCT116p53+/+ cells with the proteasomal inhibitor MG-132 alone led to an accumulation of wild-type p53 because it utilizes the proteasomal degradation pathway for the maintenance of its levels within a normal cell (Fig. 1F). However, in the presence of a DNA damage signal wild-type p53 continued to repress UBE2C even in the presence of MG-132 (Fig. 1F). Therefore, these results suggest that UBE2C repression by wild-type p53 was not mediated by protein degradation. As a control experiment, when we tested the effect of p53 having missense mutation in its DNA binding domain, we observed an opposite effect. These mutant forms of p53 increases UBE2C expression in HCT116p53−/− cell lines in a dose-dependent manner (Fig. 1G). Contact-defective mutant p53 (R273H) renders its effect by increasing UBE2C expression in a dose-dependent manner although conformation-defective mutant p53 (R249S) failed to do the same. Another conformation-defective mutant p53 (R175H) modulates UBE2C expression similar to R273H mutant p53 albeit at a low level (Fig. 1G). Next, treatment of 5-FU in SW480 cell lines significantly up-regulates the UBE2C mRNA level in a time-dependent manner (Fig. 1B). Moreover, UBE2C expression was also found to increase gradually upon treatment with three different chemotherapeutics (5-FU, doxorubicin, and etoposide) in SW480 cells (R273H GOF p53 mutant) (Fig. 1H). Similar up-regulation of UBE2C was also observed in two other mutant p53 cell lines, C33A (R237C p53 mutant) and SKBR3 (R175H GOF p53 mutant), suggesting that this is not a cell type-specific phenomenon (data not shown). The increase in UBE2C levels is attributed to accumulation of the acetylated form of mutant p53 and not due to an increase in basal protein levels of mutant p53 in SW480 cells (Fig. 1H). This hypothesis was further supported by the observation that inhibiting acetylation using anacardic acid did not evoke drug-induced activation of UBE2C (Fig. 1F). Further confirmation of the effect of mutant p53 on UBE2C expression was obtained by knockdown experiments. UBE2C protein levels declined upon siRNA-mediated mutant p53 down-regulation in both SW480 and MIA PaCa2 cells (Fig. 1J). However, the same was not observed in scrambled siRNA-treated cells (Fig. 1J). Collectively, these observations suggest a direct correlation between UBE2C and mutant p53 expression in human cancer cell lines.

**Wild-type p53 Down-regulates and Mutant p53 Up-regulates UBE2C Transcription**—We next asked whether modulation of UBE2C expression is a transcriptional function of wild-type and mutant p53 proteins. To test our hypothesis, we cloned three UBE2C promoter DNA fragments into the pGL3 basic luciferase reporter vector (Fig. 2A). We observed a dose-dependent decrease in the activity of all three UBE2C promoter–luciferase constructs in HCT116p53−/− cells when wild-type p53 is ectopically expressed (Fig. 2B). Similar decrease in promoter activity was also observed for all the three UBE2C promoter constructs upon induction of endogenous p53 either by 5-FU or etoposide in HCT116p53+/+ cells (Fig. 2C) but not in its isogenic null counterpart, HCT116p53−/− cells (Fig. 2D). The Western blot analysis for p53 with the above luciferase lysates revealed similar levels of p53 induction in the drug-treated HCT116p53+/+ cells but not in HCT116p53−/− cells (Fig. 2, C and D). These results suggest that wild-type p53 represses UBE2C promoter activity. On the contrary, we observed a dose-dependent increase in all the UBE2C promoter activity in HCT116p53−/− cells when two different mutant p53 constructs pCMV-R175H and pCMV-R273H are ectopically expressed (Fig. 2E). Hence, we conclude wild-type and mutant p53 reciprocally regulate the UBE2C promoter.
Tumor Suppressor p21 Is Required for Wild-type p53-mediated UBE2C Suppression—The absence of any putative p53-binding site near the transcription start site of the UBE2C promoter (up to 1 kb from the transcription start site) led us to hypothesize an indirect mode of repression mechanism. It is known that indirect repression of some wild-type p53-responsive genes requires the activation of the cyclin-dependent kinase inhibitor p21 (36). p21 facilitates the formation of the Rb-E2F complex, which in turn represses several cell cycle genes (11). Therefore, we examined whether UBE2C expression could be inhibited by wild-type p53 in a p21-dependent manner. When HCT116p53+/+ and HepG2 cells were treated either with 5-FU or etoposide, the UBE2C expression declined in a dose-dependent manner along with a concomitant increase in p21 protein levels (Fig. 3A). However, to ascertain the role of p21 in wild-type p53-mediated UBE2C repression in a con-
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A, three different deletion constructs of the UBE2C promoter were cloned into pGL3 basic reporter luciferase vector. Resulting vectors were designated as pUBE2C-Luc(−693/+39), pUBE2C-Luc(−321/+39), and pUBE2C-Luc(−141/+39). B, transient transfection of three UBE2C reporter constructs (100 ng) along with 0, 100, 250, and 500 ng of pCMV-wild-type p53 vector was done in HCT116p53−/− cells followed by luciferase assay after 48 h. HCT116p53−/− cells (C) or HCT116p53−/− cells (D) were transiently transfected with any of the three UBE2C reporter constructs (100 ng) followed by either 10 μg/ml of 5-FU or 20 μg/ml of etoposide (ETOP) treatment for 48 h. Luciferase assay was performed post-treatment. The protein lysates from the above luciferase experiments were used for immunoblot analysis to check p53 induction. E, transient transfection of either R175H-mutant p53 (250 ng) or R273H-mutant p53 (250 ng) along with different deletion constructs of UBE2C promoter was done in HCT116p53−/− cells followed by luciferase assay after 48 h. All values in histograms were expressed as means ± S.D. of three independent experiments. * indicates p<0.05.

cclusive manner, we treated an isogenic p21 null cell line, HCT116p21−/−, either with 5-FU or etoposide. HCT116p21−/− cells showed no such alterations in the UBE2C protein levels in the presence of DNA damage, although wild-type p53 gets accumulated (Fig. 3B). It may be assumed that unaltered levels of UBE2C in HCT116p21−/− cells upon drug treatment could be due to 5-FU-mediated arrest at G2/M checkpoint, where this protein is known to be highly expressed. This may result in masking the effect of wild-type p53 upon UBE2C expression in these cell lines. However, the cell cycle analysis in HCT116p21−/− cells following 5-FU treatment refutes this possibility because there was no accumulation of cells at the G2/M phase in early time point (Fig. 3C). The same was also observed in HCT116p53−/− cells (Fig. 3C). The expression of UBE2C continues to remain unaltered following treatment with 5-FU in HCT116p21−/− cells even at early time points (4–8 h) despite wild-type p53 induction (Fig. 3D). Thus, it confirmed that the wild-type p53-mediated UBE2C repression was p21-dependent and not affected by the cell cycle. Moreover, there was no wild-type p53-mediated repression of pUBE2C-Luc(−141/+39) promoter activity upon DNA damage in HCT116p21−/− cells (Fig. 3E) unlike in HCT116p53−/− cells despite similar levels of induction of p53 in both the cells (Fig. 3F). These observations collectively suggest that wild-type p53 represses UBE2C in a p21-dependent manner upon DNA damage.

Wild-type and Mutant p53 Differentially Regulate UBE2C Promoter through CCAAT-CHR Module—Several studies have implicated the concerted action of p21, NF-Y, and E2F in mediating wild-type p53-dependent transcriptional repression, especially in the context of G2/M genes (37–39). Using bioinformatics tools like MatInspector, we found the presence of a highly conserved CCAAT-CCAAT-CHR module in the p53-responsive UBE2C promoter (Fig. 4A). p21 can modulate the activity of these elements in a p53-dependent manner (40). Therefore, we considered that these elements might contribute to UBE2C gene transcription. We generated a series of UBE2C promoter mutants by mutating either the CCAAT or CHR elements alone or in combination to test their role in UBE2C transcription (Fig. 4A). It is noteworthy that mutation of any of these elements in the CCAAT-CCAAT-CHR module resulted in decline of the basal UBE2C promoter activity (data not shown). Therefore, these elements may contribute to general UBE2C transcriptional activation. We next tested whether
these elements are truly functional in the context of UBE2C promoter activity. To understand whether the CCAAT element shows NF-Y-dependent UBE2C promoter activity, we co-transfected the UBE2C promoter with either NF-YA expression vector or dominant-negative NF-YA mutant (NF-YA–MUT) or both together in HCT116 p53/H11002/H11002 cells. We observed a significant increase of UBE2C promoter activity upon ectopic expression of wild-type NF-YA (NF-YA–WT) but not for the dominant-negative mutant alone or in combination (Fig. 4B).

Taken together, these results demonstrate the existence of a NF-Y trimeric complex and functional CCAAT site on the UBE2C promoter that controls UBE2C expression. We further checked for the cell cycle-dependent regulation of the putative CHR element in the UBE2C promoter. The FACS analysis of the serum-starved HCT116p53/−/− cells showed that the majority of the synchronized cells reached G2/M phase after 18 h of release from serum starvation, and by 30 h they were in the G1 phase (data not shown). The wild-type UBE2C promoter-driven luciferase expression in these synchronized cells revealed a gradual increase until 18 h coinciding with the G2/M peak and subsequently declined as cells reached G1 phase (Fig. 4C). However, similar cell cycle-dependent regulation was absent when the CHR element was mutated (Fig. 4C). Thus, we observed a deregulation of the cell cycle-dependent transcription of the UBE2C promoter upon mutation of the CHR element. Taken together, these results demonstrate the existence of the functional CHR site on the UBE2C promoter that regulates UBE2C expression in a cell cycle-dependent manner. Next, we tested the effect of wild-type p53 on the mutant UBE2C promoter activities either by expressing wild-type p53 in HCT116p53/−/− cells or inducing endogenous wild-type p53 by 5-FU in HCT116p53/−/− cells (Fig. 4, D and E). There was moderate release from repression upon transfection of the distal (−90 to −86) CCAAT site mutant compared with the wild-

FIGURE 3. p21-mediated repression of UBE2C. Immunoblot analysis was done in HCT116p53/−/−, HepG2 (A) and HCT116p21/−/− (B) cells after treatment with either 5-FU or etoposide at indicated doses for 48 h. All experiments were repeated at least three times. C, FACS analysis was done in HCT116p53/−/− and HCT116p21/−/− cells following treatment with 10 μg/ml 5-FU at the indicated times. The experiment was repeated three times. D, HCT116p21/−/− cells were treated with 10 μg/ml of 5-FU and immunoblot analysis was carried out at 0, 4, 8, 12, and 24 h of post-drug treatment. The experiment was repeated at least three times. Luciferase assay was performed in HCT116p21/−/− (E) and HCT116p53/−/− (F) cells following transient transfection of pUBE2C-Luc (−141/+39) construct (100 ng). Cells were treated with either 5-FU or etoposide at the indicated doses. Immunoblot analysis was done with the luciferase lysates to check for p53 induction. All values in histograms were expressed as means ± S.D. of three independent experiments. * indicates p ≤ 0.05.
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A. CCAAT and CHR elements are present within the UBE2C promoter (−141 to +39 nucleotides). The boxes indicate the putative inverted CCAAT boxes (−90 to −86 and −59 to −55) and inverted CHR (−31 to −25) element. The CCAAT and CHR elements on the UBE2C promoter have been mutated by site-directed mutagenesis. The crossed boxes refer to the mutated responsive elements.

B. Luciferase assay was done in HCT116 p53−/− cells following transfection of pUBE2C-Luc(−141/+39) with either wild-type NF-YA expression plasmid (NF-YA-WT) or dominant-negative NF-YA expression plasmid (NF-YA-MUT) or both NF-YA-WT and NF-YA-MUT plasmid together in the ratio of 1:2. C. Luciferase assay was done in HCT116p53−/− cells following transient transfection of either pUBE2C-Luc(−141/+39) or pUBE2C-Luc(−141/+39)CHR mutant reporter plasmids. Twenty four hours post-transfection cells were serum-starved for another 60 h. Lysates were prepared at indicated time points following release from G0/G1 arrest. All values in plots were expressed as means ± S.D. of three independent experiments. Luciferase assays was done in HCT116p53−/− cells (D) and HCT116p53−/− cells (E) following transient transfection with different site-directed mutants of UBE2C promoter (as indicated). pCMV-wild-type p53 vector was ectopically expressed in HCT116p53−/− cells, where 5-FU was used to induce endogenous p53 in HCT116p53−/− cells. Representative number indicates pUBE2C-Luc(−141/+39), pUBE2C-Luc(−141/+39)NFY1 mutant, pUBE2C-Luc(−141/+39)NFY2 mutant, pUBE2C-Luc(−141/+39)CHR mutant, pUBE2C-Luc(−141/+39)NFY1-CHR mutant, pUBE2C-Luc(−141/+39)NFY2-CHR mutant, and pUBE2C-Luc(−141/+39)NFY1-NFY2-CHR mutant, respectively.

F. Transient transfection of either pUBE2C-Luc(−141/+39) or pUBE2C-Luc(−141/+39)NFY1 mutant or pUBE2C-Luc(−141/+39)NFY2 mutant or pUBE2C-Luc(−141/+39)NFY1-CHR mutant was done in SW480 cells following treatment of 5-FU for 24 h. All values in histograms were expressed as means ± S.D. of three independent experiments. *, **, ***, **** indicates p < 0.05, p < 0.01, p < 0.001, and p < 0.0001, respectively.

type promoter construct both in cells expressing wild-type p53 and in cells where wild-type p53 is induced by 5-FU (Fig. 4, D and E, compare constructs 1 and 2). However, mutation of either the proximal CCAAT (−59 to −55) element or the CHR (−31 to −25) element alone led to a significant attenuation of repression of the UBE2C promoter activity by wild-type p53.
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(Fig. 4, D and E, compare constructs 3 and 4). It is noteworthy that the mutations of distal CCAAT and CHR together (construct 5) did not show any additional change of wild-type p53’s suppressive effect on the UBE2C promoter when compared with the CHR mutant alone (Fig. 4, D and E, compare constructs 4 and 5). However, the same was not true when both proximal CCAAT and CHR sites were mutated (Fig. 4, D and E, compare constructs 4 and 6), suggesting that the proximal CCAAT and CHR elements are more crucial in mediating p53 repression function (Fig. 4, D and E, compare constructs 5 and 6). However, mutations of both the distal (−90 to −86) and proximal (−59 to −55) CCAAT sites along with the CHR (−31 to −25) site as in construct 7 led to a complete loss of the wild-type p53-mediated repression (Fig. 4, D and E). This led us to believe that the two functional CCAAT elements along with CHR element are required for wild-type p53-mediated repression of UBE2C.

Having established that wild-type p53 represses the UBE2C promoter through CCAAT-CHR elements, we further checked the mechanism of transcriptional activation by mutant p53. It is known that indirect activation of some mutant p53-responsive genes upon DNA damage requires the NF-Y-responsive CCAAT module (4). Therefore, we considered that two CCAAT elements might contribute to mutant p53-mediated UBE2C gene transcription. Hence, we tested two UBE2C promoter mutants having mutation either in the distal CCAAT element (−90 to −86) or in the proximal CCAAT element (−59 to −55). First of all there was significant loss of promoter activity following mutation in any of the two elements (Fig. 4F). Second, in context of the proximal CCAAT site mutant promoter, moderate activation was observed compared with wild-type promoter in drug-treated SW480 cells (Fig. 4F). However, the distal CCAAT site mutant did not show any activation in drug-treated cells (Fig. 4F). Taken together, these results demonstrate that mutant p53 activates UBE2C expression indirectly by virtue of functional CCAAT elements.

E2F4 and NF-Y Are Two Key Transcription Factors Responsible for Wild-type and Mutant p53-mediated Regulation of UBE2C Promoter—We observed that wild-type p53-mediated repression was released by mutating CCAAT-CCAAT-CHR module. It prompted us to investigate the recruitment of corresponding transcription factors on the UBE2C promoter. It has been previously reported that CHR alone or in conjunction with CDE can modulate the transcription of G2/M genes (38, 41). The control of these genes via the CDE/CHR elements has been linked with transcriptional regulation by activating or repressing E2Fs (42–44). Activator E2Fs (in particular E2F1) are associated with the activation of genes involved in G1/S progression, whereas repressor E2Fs (in particular E2F4) bind to gene promoters showing the CCAAT-CDE/CHR DNA module that peaks at G2 or G2/M phase (27, 38, 39, 45). p21 has been shown to facilitate E2F4 recruitment to the CDC2 promoter upon DNA damage (46). All the evidence helped us to speculate E2F4 as a possible candidate in regulating UBE2C gene transcription. In fact, we observed a sharp decline in the UBE2C promoter activity upon ectopic expression of either E2F4 or wild-type p53 constructs in HCT116p53−/− cells (Fig. 5A). The repression of the UBE2C promoter was released even in the presence of wild-type p53 when E2F4 protein was knocked down from the cells (Fig. 5A). Furthermore, the decrease in UBE2C promoter activity upon ectopic expression of E2F4 was not observed in the presence of E2F4 siRNA (Fig. 5A). Collectively, our findings revealed that wild-type p53-mediated UBE2C repression is driven by E2F4. To understand whether E2F4 was recruited on the UBE2C promoter in a p53/p21-dependent manner, we performed ChIP assays in HCT116p53+/+, HCT116p53−/−, and HCT116p21−/− cells. We observed enhanced recruitment of E2F4 after DNA damage on the endogenous UBE2C promoter in HCT116p53+/+ cells, although it failed to be recruited on the UBE2C promoter in HCT116p53−/− and HCT116p21−/− cells (Fig. 5B). To check E2F4 recruitment on the exogenous UBE2C promoter, we performed transient ChIP assay in HCT116p53−/− cells having either ectopically expressed wild-type p53 or empty vector. We observed E2F4 was recruited on exogenous UBE2C promoter in a p53-dependent manner only when the CHR element was intact (Fig. 5C). The NF-Y trimeric complex consisting of NF-YA, NF-YB, and NF-YC binds to its responsive element upon phosphorylation by cyclin-CDK complex (39). However, when cyclin-CDK complex is inhibited by p21, it cannot bind to the responsive promoter. Thus, we examined the recruitment of NF-YA on the UBE2C promoter in HCT116p53+/+ cells treated or untreated with 5-FU by ChIP assay. There was a complete loss of NF-YA recruitment on the UBE2C promoter in 5-FU treated HCT116p53+/+ cells compared with untreated cells (Fig. 5B). To confirm this loss of NF-YA recruitment was p53- and p21-dependent, we performed a similar ChIP assay in HCT116p53−/− and HCT116p21−/− cells and showed that NF-YA is equally recruited to the UBE2C promoter in both 5FU-treated and -untreated cells (Fig. 5B). These results suggest that DNA damage-induced wild-type p53 activates p21, which in turn inhibits NF-Y phosphorylation by cyclin-CDK complex resulting in sequestration of the NF-Y complex from the UBE2C promoter. All these observations collectively suggest that both the E2F4 and the NF-Y participate in tuning the wild-type p53-mediated repression of UBE2C expression. We next investigated whether the GOF mutant p53 physically interacts with the UBE2C promoter to mediate its transactivation function. We performed a ChIP assay in HCT116p53+/− cells following transfection with two GOF p53 mutants, R175H or R273H. Mutant p53 was present in the transcription complex on the UBE2C promoter for both the mutants in contrast to the untransfected cells (Fig. 5D). Recruitment of endogenous mutant p53 to the UBE2C promoter was also observed in SW480 cells after 5-FU treatment (Fig. 5E). It has been previously reported that mutant p53-mediated recruitment of p300 leads to activation of several cell cycle genes (4). Interestingly, p300 was also selectively recruited on the UBE2C promoter by mutant p53 both upon transfection of the p53 mutants (R175H and R273H) in HCT116p53−/− cells (Fig. 5D) or upon drug treatment in SW480 cells (Fig. 5E). As the presence of p300, a histone acetyltransferase, correlates with presence of highly acetylated histones, we asked whether the recruitment of p300 affects the acetylation status of neighboring histones. We observed a striking increase in the promoter-bound acetylated lysine 9 of histone 3 (H3K9Ac) in a mutant p53-harboring cells after drug treatment (Fig. 5D). Surprisingly, we did not notice...
any change in the recruitment of H3K9Ac on UBE2C promoter when mutant p53 constructs were expressed in HCT116p53+/− cell lines (Fig. 5F). Therefore, our data suggest that DNA damage-induced recruitment of p300 to the UBE2C promoter by mutant p53 promotes histone acetylation and thus provide a molecular mechanism for transactivation of the UBE2C gene by p53 mutants. To investigate whether the NF-Y trimeric complex is recruited on the endogenous UBE2C promoter, ChIP assay was done in either non-silencing SW480 cells or TP53 knockdown SW480 cells following 5-FU treatment (10 μg/ml) for 24 h with anti-mutant p53, anti-NF-YA, and anti-NF-YB antibodies. ChIP assay was performed in SW480 cells that were transfected with either 80 nM scrambled siRNA or 80 nM NFYA siRNA for 72 h. Cells were either treated or untreated with 5-FU (10 μg/ml) for 24 h, and protein-DNA complexes were immunoprecipitated with anti-mutant p53, anti-NF-YA, and anti-NF-YB antibodies. H, luciferase assay was done in SW480 cells following transfection of different UBE2C promoter constructs along with either wild-type NF-YA expression plasmid (NF-YA-WT) or dominant-negative NF-YA expression plasmid (NF-YA-MUT). All the experiments were repeated at least three times. All values in histograms were expressed as means ± S.D. of three independent experiments. *, **, ***, **** indicates p ≤ 0.05, p ≤ 0.01, p ≤ 0.001, and p ≤ 0.0001, respectively.
moter in a mutant p53-dependent manner, we performed ChIP experiments in both non-silencing and p53 stable knocked down SW480 cells (Fig. 5F). We observed that recruitment of NF-YA and NF-YB on the UBE2C promoter requires the functional mutant p53 (Fig. 5F), and occupancy of the NF-Y complex was increased following DNA damage in non-silencing control cells (Fig. 5F). Conversely, we also showed abrogation of mutant p53 recruitment on the EFNB2 promoter following knockdown of NF-YA in SW480 cells (Fig. 5G). In accordance with a previous report (4), we also observed that knockdown of NF-YA did not abolish the recruitment of NF-YB on the EFNB2 promoter because NF-YB and NF-YC heterodimerization is a prerequisite for NF-YA association (Fig. 5G). The specificity of p53 recruitment was ascertained by the absence of mutant p53 on the 4th exon of the WDR36 gene, which served as a negative control for mutant p53 binding (data not shown). Similar to previous results, the drug-induced change in UBE2C promoter activity having functional CCAAT elements was observed in both WT NF-YA-expressed and DN NF-YA-expressed SW480 cells (Fig. 5H, compare construct 1). Mutation in the distal CCAAT element abrogates promoter activity, and even ectopic expression of WT NF-YA failed to rescue it (Fig. 5H, compare construct 2). On the contrary, mutation in the proximal CCAAT element showed a similar change in promoter activity on 5-FU treatment compared with WT UBE2C promoter. However, basal promoter activity is lower in the proximal CCAAT element-mutated promoter compared with the WT UBE2C promoter (Fig. 5H, compare constructs 1 and 3). In this case, again the expression of dominant-negative mutant NF-YA attenuated the mutated promoter activity of both treated and untreated cells but failed to do so in WT UBE2C promoter (Fig. 5H, compare DN NF-YA panel). Taken together, these observations clearly suggest that mutant p53 in association with NF-Y regulates UBE2C expression by interacting with the two CCAAT elements.

Wild-type p53-mediated UBE2C Repression Causes Mitotic Delay—The UBE2C is a critical regulator of the SAC and helps the cell to proceed from metaphase to anaphase. Its levels within the cells contribute to control of mitosis (23, 24). Therefore, we wanted to check whether the wild-type p53-mediated repression of UBE2C could actually trigger the SAC and halt the cell division. G$_1$/S-synchronized HCT116p53$^{+/+}$ cells were allowed to proceed toward G$_2$ phase after removal of the double thymidine block (Fig. 6A). We determined the expression of p53, UBE2C, and the mitotic marker, cyclin-B1, and phosphorylated histone H3 (p-H3) in the drug-treated and -untreated synchronized cells harvested at indicated time points post-etoposide treatment (Fig. 6B). Cyclin-B1 degradation and reduction of phosphorylation in histone H3 marks the transition from metaphase to anaphase. It has been already known that cyclin-B1 is a target of UBE2C (23). For vector-transfected cells, cyclin-B1 and p-H3 levels peaked at 8–10 h post-release, after which they began to decline, reflecting mitotic exit (Fig. 6B, left panel). In contrast, levels of both proteins were maximal at 8 h
post-release upon etoposide treatment in HCT116p53+/+ cells and degraded very minimally even at the 14-h time point implying that cells were still in mitosis (Fig. 6B, middle panel). Following DNA damage, however, we observed a gradual increase in the wild-type p53 levels with time along with concomitant decline in the UBE2C in the drug-treated synchronized HCT116p53+/+ cells (Fig. 6B, middle panel). Furthermore, specificity of wild-type p53-mediated mitotic regulation was proved by the observation that rapid degradation of cyclin-B1 and p-H3 levels were rescued by UBE2C expression even in the presence of etoposide-induced wild-type p53 (Fig. 6B, right panel). Next, we evaluated the MI of wild-type p53 cells by staining nucleus with DAPI and p-H3 protein with FITC. We observed that MI for etoposide-treated cells was highest at the 10-h time point (Fig. 6C, middle panel, and D) in comparison with untreated cells, which showed highest MI at 8 h post-release (Fig. 6C, left column, and D). Even at 10 h post-release, MI was significantly higher in drug-treated cells than for untreated cells (Fig. 6C, left and middle panels, and D). Rescue experiment showed MI similar to untreated cells (Fig. 6C, right panel, and D). These results suggest that DNA damage-induced wild-type p53 can trigger SAC and delay mitotic exit by transcriptionally repressing UBE2C.

DNA Damage-induced Mutant p53-mediated Up-regulation of UBE2C Leads to Early Anaphase Initiation—To examine the effect of mutant p53-mediated activation of UBE2C on spindle assembly checkpoint, we again performed cell synchronization experiments in SW480 cells arrested at G1/S boundary by double-thymidine block. Cells were then allowed to progress through the cell cycle in media containing nocodazole, and the levels of UBE2C, cyclin-B1, and p-H3 were monitored at regular intervals under different treatment conditions. 5-FU was added to these cells 6 h post-release from double thymidine block (Fig. 7A). Metaphase arrest mediated by nocodazole is

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**FIGURE 7. DNA damage-induced mutant p53 causing early anaphase exit.** A, schematic representation of the experimental strategy used for cell synchronization experiment with double thymidine block followed by nocodazole and 5-FU treatment in SW480 cells. B, Western blotting analysis was performed in G1/S-synchronized SW480 cells that were transfected either with scrambled siRNA (80 nM) or UBE2C siRNA (80 nM) for 72 h. Cell were further treated with 5-FU (10 μg/ml) at the indicated time point and blot was probed with anti-p53, anti-UBE2C, anti-cyclin-B1, anti-phospho-histone H3, anti-total histone H3, and anti-β-actin antibodies. C, Western blot analysis was performed in either G1/S-synchronized control SW480 ( scrambled shRNA) or TP53 knocked down SW480 cells following treatment of 5-FU (10 μg/ml) at indicated hours with anti-p53, anti-UBE2C, anti-cyclin-B1, anti-phospho-histone H3, anti-total histone H3, and anti-β-actin antibodies. All the Western blot experiments were repeated at least three times. D, G1/S-synchronized SW480 cells were released from the arrest and either incubated with 5-FU (10 μg/ml) or left untreated. In the rescue experiment UBE2C siRNA (80 nM) were transfected in SW480 cells for 72 h. At the indicated time points, cells were fixed and visualized under a fluorescence microscope. All the images are taken at ×20 magnification. The experiment was repeated three times. E, graph shows percentage of mitotic cells (MI) observed at indicated time point. On average 120–200 nuclei were analyzed by fluorescence microscopy. 5-FU-treated cells at 10- and 12-h time points show significant reduction (****, p < 0.0001, n = 3) of MI compared with either control or rescue panel. The results are averages of three independent experiments.
marked by accumulation of mitotic cyclin and p-H3. Indeed, cyclin-B1 is accumulated in the presence of nocodazole in G1/S-synchronized SW480 cells (Fig. 7B, left panel). However, cells bypassed the checkpoint control in 5-FU-treated condition, even in the presence of nocodazole, as seen by cyclin-B1 degradation and reduction in p-H3 level (Fig. 7B, middle panel). UBE2C levels were higher in 5-FU-treated cells than untreated (Fig. 7B, compare left and middle panels) thereby suggesting that slight alterations in the levels of UBE2C can make tumor cells insensitive to checkpoint control. Therefore, it seems that 5-FU can induce mutant p53-mediated accumulation of UBE2C and leads to early anaphase initiation marked by degradation of cyclin-B1 and p-H3. The supposition that this deregulation in cell cycle is due to altered levels of UBE2C was confirmed upon siRNA-mediated knockdown of UBE2C in the same cells. Despite an upstream inducer like 5-FU, forced decline in the levels of UBE2C helps to restore the cyclin-B1 and p-H3 levels (Fig. 7B, right panel). Taken together, these results suggested that DNA damage-induced mutant p53 can disrupt SAC control by expressing its regulator UBE2C that leads to early anaphase initiation. To further ascertain the role of mutant p53 in the mitotic checkpoint, we monitored the mitotic progression in synchronized stable p53 knocked down SW480 cells. Similar to previous findings, we noticed cyclin-B1 and p-H3 levels were stabilized in untreated control cells manifesting arrest at mitosis (Fig. 7C, 1st panel), whereas drug-treated control cells showed early anaphase onset by lowering the p-H3 level, degrading cyclin-B1, and the subsequent increase in UBE2C protein levels (Fig. 7C, 2nd panel). In contrast, p53 knocked down SW480 cells were arrested at SAC even in the presence of 5-FU because drug treatment did not increase UBE2C levels (Fig. 7C, last panel). Next, we scored the mitotic indices of SW480 cells treated with 5-FU based on the p-H3-FITC-positive signal. Nuclei were stained with DAPI. We observed that the MI was lower for cells treated with 5-FU as compared with untreated SW480 cells at the 12-h time point (Fig. 7, D, compare left and middle panels, and E). As shown previously, UBE2C knockdown was able to increase the MI to a significant extent (Fig. 7, D, compare middle and right panels, and E). Taken together, these results suggest that DNA damage-induced mutant p53 can disrupt SAC control by expressing its regulator UBE2C that leads to early anaphase initiation.

DNA Damage-induced Early Anaphase Entry Initiates Chromosomal Abnormalities in Mutant p53-bearing Cancer Cells—To understand whether p53 mutants exert their oncogenic gain-of-function activity by deregulating the expression of mitotic checkpoint components, we performed meta-analysis using the oncomine database (47). Eight out of 12 such tumor datasets classified on the basis of p53 status showed significant overexpression of the mitotic checkpoint regulator, UBE2C in mutant p53 tumors (Fig. 8A). Similarly, UBE2C protein is differentially expressed in wild-type and mutant p53-harboring cancer cell lines (Fig. 8B). Next, we hypothesized that drug-induced mutant p53 might cause chromosomal abnormalities as a consequence of the high expression of UBE2C and early anaphase exit. Addressing this in synchronized SW480 cells revealed that chromosomal abnormalities were indeed pronounced in UBE2C overexpressed cells at the late time point (10 h from double thymidine release) as compared with drug-untreated cells (Fig. 8C, left and middle panels). Knockdown of UBE2C expression restores chromosomal stability even in the presence of 5-FU (Fig. 8C, middle and right panels), suggesting that the drug causes chromosomal instability by up-regulating the UBE2C expression in mutant p53-harboring cell lines. Immunostaining of p-H3 in these abnormal chromosomes strengthened our hypothesis that drug-induced early mitotic exit causes chromosomal aberrations (Fig. 8, C, circles and white arrows, and D). We then evaluated the phenotypic relevance of high UBE2C expression in mutant p53-harboring cells by examining its effect on the tumorigenic property of SW480 cells. Surprisingly, the number of colonies on soft agar formed after a week by UBE2C knockdown was significantly less than those formed by scrambled siRNA-transfected cells (Fig. 8E). Collectively, our data suggest that UBE2C induces chromosomal abnormalities, and knockdown of UBE2C reduces the tumorigenic property in mutant p53-harboring cancer cells.

Discussion

Maintenance of genomic stability is a prerequisite for the cells to undergo several rounds of successful division throughout their lifetime. This requires tight temporal and spatial coordination of cell cycle events and proper functioning of the checkpoints. This is brought about by the regulated expression of several cell cycle proteins and their interactions. Wild-type p53, a crucial mediator of genomic stability, prevents cell from further advancement at these checkpoints in the context of DNA damage through transcriptional regulation of several of these checkpoint proteins. Unlike the G1/S and G2/M checkpoints, the role of wild-type p53 in transcriptional regulation of SAC genes is not fully explored. It is pertinent to understand the connectivity between the two because both cellular responses to DNA damage and spindle assembly checkpoint maintain genomic integrity by delaying cell cycle progression in the presence of DNA or spindle damage, respectively. We have previously reported the direct regulation of a crucial SAC checkpoint gene, CDC20, by wild-type p53 upon DNA damage (21). In our attempts to further explore the regulation of the transcriptional potential of wild-type p53 in SAC control, we show that UBE2C, an E2 ubiquitin-conjugating enzyme whose activity is central to metaphase to anaphase transition, was a transcriptional target of wild-type p53. UBE2C was found to be repressed at the mRNA and protein levels both upon ectopic expression of wild-type p53 or its induction by DNA damage. Interestingly, this repression of UBE2C by wild-type p53 was observed at very early times of p53 induction suggesting that the effect was not secondary to normal cell cycle-regulated expression of UBE2C. On the contrary, we identified UBE2C also as a novel target of GOF p53 mutants. Like many other wild-type p53-repressed cell cycle targets, UBE2C was found to be an indirect repression target of wild-type p53. The possibility of direct recruitment of p53 to its response element was ruled out by the observation that p53 continued to repress the UBE2C minimal promoter, which is devoid of putative p53-binding sites. The CDE/CHR promoter elements are important cis-regulatory sequences of G1/M cell cycle genes (38). Bioinformatic analysis of the p53-responsive minimal promoter region identified an inverted
CHR element in the UBE2C promoter that is identical to the consensus 5'-TTTGAA-3' sequence (38). The functionality of this putative CHR element was established from the fact that it showed maximum activity at the G2/M phase of the cell cycle, which declined as cells were approaching G0/G1 phase. Moreover, this cell cycle-regulated expression of UBE2C was lost upon mutation of the CHR element. Notably, we did not find any CDE element adjacent to the putative CHR element in the UBE2C promoter suggesting that it is a class II CDE/CHR promoter (38). The class I promoter contains both functional CDE and CHR elements (38). The CDE/CHR-regulated genes are usually accompanied by another positive regulatory element.
CCAAT (38). Generally, 2—3 copies of CCAAT elements with a spacing of 31—33 nt are present close to the CDE/CHR elements (38). In the case of the UBE2C promoter, we identified two CCAAT elements with a spacing of 31 nucleotides. The sequence of the CCAAT and the CHR elements and the distance between them were highly conserved across several species, therefore hinting at its possible role in UBE2C transcriptional control. It is known that the hetero-trimeric transcription factor, NFY, binds to this element and positively regulates promoter activity. We also observed that the UBE2C promoter was activated by ectopic expression of NF-YA, and mutation of any of the CCAAT elements led to a large drop in UBE2C promoter activity. Thus, these results suggest that the CCAAT elements of the UBE2C promoter are functional. Importantly, many CDE/CHR promoters are repressed by the wild-type p53 (38). In addition, the wild-type p53-mediated control on the expression of these genes is regulated by concerted effort of CDE/CHR elements along with CCAAT-boxes (38). We show that the presence of this module is also critical to the p53 response with respect to the UBE2C gene. The p53-dependent UBE2C repression was much hindered in the absence of the intact CHR or proximal CCAAT element and to a lesser extent for the distally mutated CCAAT element, although it was completely lost when the entire CCAAT-CCAAT-CHR module was mutated. Indirect repression by wild-type p53 of the cell cycle targets mostly involves p53-mediated activation of the cyclin-dependent kinase inhibitor, p21 (36). We observed a similar decline in UBE2C levels in a p53/p21-dependent manner. There were no changes in the level of UBE2C in the absence of p21 even in the presence of upstream p53 signaling. This phenomenon was observed at both early and late time points of p53 induction and is also independent of the cell cycle phase. Tumor suppressor p21 facilitates the formation of RB-E2F complexes that act to repress E2F target genes. Interestingly, a large body of evidence suggests cooperation between wild-type p53 and E2F in the control of several cell cycle targets (48). In general, there are two classes of E2Fs, activator E2Fs (E2F1, E2F2, and E2F3) and repressor E2Fs (E2F4 and E2F5), classified on the basis of their effect on gene expression (48). In most cases, activator E2Fs bring about cellular proliferation, whereas repressor E2Fs bring about exit from the cell cycle and differentiation. Also, it is seen that p53-E2F cross-talk where activator E2Fs repress p53 activity and repressor E2Fs function downstream of p53 bring about cell cycle arrest (48, 49). Because we observed complete elimination of UBE2C repression in the absence of p21 despite intact upstream p53 signaling, we speculated the involvement of downstream repressor E2Fs (especially E2F4) in mediating p53 response upon UBE2C gene expression. Also, E2F4 has been shown to bind to CCAAT-CHR modules in G2/M gene promoters (38, 39, 45). We report the involvement of E2F4 in the repression of UBE2C. This is supported by the observation that ectopic expression of E2F4 in HCT116p53+/− cells resulted in repression of UBE2C promoter activity. Besides, we also observed E2F4 recruitment to the UBE2C promoter in wild-type p53 expressing HCT116p53+/− cells in the presence of DNA damage; however, the same was not true for HCT116p53−/− and HCT116p21−/− cells under the same conditions. We further observed that the E2F4 recruitment to the UBE2C promoter occurred in a CHR-dependent manner and was lost when CHR was mutated. Therefore, E2F4 cross-talks between the p53/p21-mediated DNA damage response and UBE2C gene expression; however, the RB family protein, which conjugates with E2F4 to mediate this effect, needs to be explored.

Besides E2F, p21 can also inhibit cyclin-CDK-dependent phosphorylation of another transcription factor, NFY, and thereby hinder its recruitment to gene promoters (39, 50). Upon DNA damage, we observed a decline in the recruitment of NFY to the UBE2C promoter in a p53/p21-dependent manner. It was reported that NFY and E2F components integrate to mediate the p53 response in the case of several human cell cycle gene promoters, especially the G1/M genes (39). We showed that the presence of two NFY-dependent CCAAT boxes and an E2F4-bound CHR element in tandem on the UBE2C promoter was critical in mediating the p53 response with respect to the UBE2C gene. Apparently, several proteins have been implicated to bind to CDE/CHR elements upon induction of wild-type p53. For instance, a recent report by Mannfeldt et al. (51) demonstrated that in the presence of p53 the DREAM complex, which is known to occupy some CDE/CHR promoters, switches from containing activating B-Myb to repressing E2F4/p130. We observed similar recruitment of the E2F4 molecule in a p53-dependent manner, whereas NFY was removed simultaneously. We speculate that the combined effects of removal of an activator, NFY, and recruitment of a repressor molecule, E2F4, are central to the wild-type p53-mediated repression of UBE2C. Although the control of UBE2C by wild-type p53 highlights exquisite sophistication involving multiple regulators, it would be interesting to understand what are the intermittent players in bringing about this switch in recruitment process in the presence of wild-type p53. Notably, UBE2C, a critical regulator of SAC, belongs to chromosomal instability cluster, and its deregulated expression correlates with high levels of genomic instability (52). UBE2C expression is a common event in human cancer.

**FIGURE 8. UBE2C induces chromosomal instability.** A, difference in the transcript levels of UBE2C in mutant p53 versus wild-type p53 tumors was performed using the database Oncomine. Box plot analysis of the above study showed the distribution of normalized expression values of UBE2C in wild-type and mutant p53 tumors. B, Western blot experiment showing high expression of UBE2C in mutant p53-harboring cancer cell lines compared with wild-type p53 or TP53 null cell lines. The experiment was repeated twice. C, Chromosomal abnormalities are enhanced upon 5-FU treatment in SW480 cells. Cells were transiently transfected with either scrambled siRNA (80 nm) or UBE2C siRNA (80 nm) and synchronized. Six hours post double-thymidine treatment, respective cells were treated with 5-FU (10 μg/ml). Immunostaining was done with antibody against p-H3, and nuclei were stained with DAPI at 10 h from second thymidine release. Cells were visualized under a super-resolution confocal microscope. Representative images are shown, and the defects are indicated by circles. All the images are taken at either ×63 or ×63 (with additional ×3.18 digital zoom) magnification. Scale bar, 10 μm. The experiment was repeated at least three times. D, graph shows percentage of mitotic cells with chromosomal abnormalities observed at 10 h post-double thymidine release. On average, 40–50 nuclei were analyzed by super-resolution confocal microscopy. 5-FU-treated cells show a significant high percentage of abnormal chromosome-containing cells compared with either untreated or rescue panel. **p ≤ 0.01. The results are averages of three independent experiments. E, colony-forming ability of SW480 cells on soft agar was monitored for 7–14 days following transfection with either scrambled siRNA (80 nm) or UBE2C siRNA (80 nm). The number of colonies was counted and plotted in GraphPad Prism. The graph represents the average number of colonies from three independent experiments. p value is shown in the graph.
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cancers. It is therefore expected that a tight regulation of this gene by wild-type p53 is employed to ensure proper SAC functioning and thereby to maintain chromosomal integrity under the stressed condition. We indeed observed that wild-type p53 was able to halt a G1/S-synchronized population of cells at mitosis with a concomitant decline in UBE2C levels and rise in cyclin-B1 levels. On the other side, GOF mutant p53 mediated increased expression of UBE2C leading to impaired spindle assembly checkpoint by facilitating premature anaphase. Therefore, our study suggests that wild-type p53 may also control spindle assembly checkpoint through transcriptional regulation of UBE2C. Loss of such a control would favor accelerated growth and enhanced chemoresistance in cancer cells where p53 is absent or mutated.

Author Contributions—S. R. conceived and coordinated the study and wrote the paper. S. B. and S. K. A. designed the study, performed the experiments, and wrote the paper. K. S. R. revised the manuscript and performed the experiments. A. D. analyzed the data and performed the soft agar experiment. S. N. designed and constructed vectors of the UBE2C promoter and analyzed microscopic data. All authors reviewed the results and approved the final version of the manuscript.

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