Gain of function of mutant p53: The mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation

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Summary

This article investigates the mechanistic aspects of mutant p53 "gain of function" in response to DNA damage. We show that mutant forms of p53 protein interact with NF-Y. The expression of cyclin A, cyclin B1, cdk1, and cdc25C, as well as the cdk1-associated kinase activities, is upregulated after DNA damage, provoking a mutant p53/NF-Y-dependent increase in DNA synthesis. Mutant p53 binds NF-Y target promoters and, upon DNA damage, recruits p300, leading to histone acetylation. The recruitment of mutant p53 to the CCAAT sites is severely impaired upon abrogation of NF-YA expression. Endogenous NF-Y, mutant p53, and p300 proteins form a triple complex upon DNA damage. We demonstrate that aberrant transcriptional regulation underlies the ability of mutant p53 proteins to act as oncogenic factors.

Introduction

The human *p53* gene is mutated in about 50% of human cancers (Beroud and Soussi, 1998; Hainaut and Hollstein, 2000). The p53 protein contains three functional domains: an N-terminal transactivation domain (TAD), a central DNA binding core domain (DBD), and a C-terminal oligomerization domain (OLD) (Levine, 1997). The integrity of these domains is required for p53 activities such as cell cycle arrest, apoptosis, senescence, and differentiation (Harris and Levine, 2005). Unlike other tumor suppressor genes, whose inactivation occurs mainly by deletions, *p53* is a frequent target of missense mutations that mainly reside in the region coding for the DNA binding domain. The resulting proteins, whose half-life is greatly prolonged, are full-length with a single amino acid change and are unable to recognize wild-type p53 (wtp53) consensus DNA binding sites (Prives and Hall, 1999). Most of the p53 mutations can be ascribed to two main classes: DNA contact-defective and conformational mutants (Levine, 1997; Prives and Hall, 1999). The biological significance of p53 mutations ranges from the mere loss of function to gain of function. Many in vitro and in vivo studies have clearly shown that some p53 mutants can acquire new functions, thereby contributing actively to the maintenance, the spreading,

and the increased resistance to conventional anticancer treatments of mutant p53 tumors (Dittmer et al., 1993; Blandino et al., 1999; Sigal and Rotter, 2000). At the molecular level, gain-of-function mutant p53 proteins can exert their activities either through the binding, the sequestration, and the inactivation of tumor suppressor proteins or through the transcriptional modulation of target genes (Di Como et al., 2002; Strano et al., 2000, 2002; Gaiddon et al., 2001).

wtp53 has been shown to interact with a number of transcription factors (E2F1, SP1, NF-Y, YY1, TBP, TAFs), giving rise to macromolecular complexes that modulate the transcription of genes whose promoters lack p53 binding sites. These promoters are often inhibited by wtp53 in specific phases of the cell cycle after DNA damage (Lu and Levine, 1995; Farmer et al., 1996; Manni et al., 2001; St Clair et al., 2004; Imbriano et al., 2005).

NF-Y is a heterotrimeric transcription factor with a high binding affinity for the CCAAT consensus motif that is present in 30% of eukaryotic promoters, and it consists of three subunits, NF-YA, -B, and -C, all required for CCAAT binding (Mantovani, 1998). NF-Y modulates, at least in part, the activity of the CCAAT box-containing promoters of the *E2F1*, *cyclin A*, *cyclin B1*, *cyclin B2*, *cdk1*, *cdc25C*, *chk2*, *topo IIα*, *cdc25C*, and *MDR-1* genes

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S I G N I F I C A N C E

Approximately half of all human tumors express mutant p53 proteins. Our study demonstrates the oncogenic cooperation of two major regulators of the cell cycle, mutant p53 and NF-Y, in proliferating cells and upon DNA damage. This cooperation allows cells to override cellular failsafe programs, thus permitting tumor progression. We provide evidence supporting the hypothesis that mutant p53, through its ability to interact with a variety of transcription factors, takes part in the control of key regulatory activities during the cell cycle. The identification of specific molecular mechanisms through which mutant p53 exerts its "gain-of-function" activities provides the opportunity to target transcriptionally competent mutant p53 complexes in cancer cells.
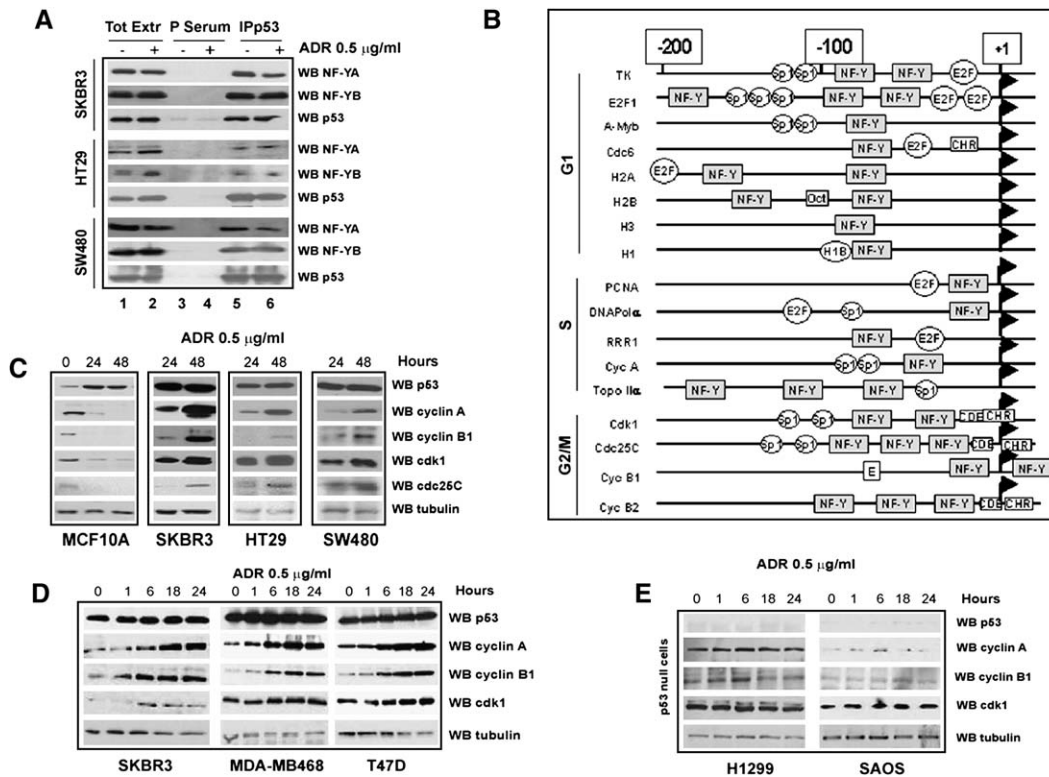
Figure 1. The protein complex mutant p53/NF-Y upregulates cell cycle NF-Y target genes in response to DNA damage

A: Cellular extracts (1 mg) from SKBR3, HT29, and SW480 cells, treated or not with 0.5 μg/ml ADR for 48 hr, were immunoprecipitated with αp53 or sheep serum as control. Twenty micrograms of SKBR3, 40 μg of HT29, and 40 μg of SW480 cellular extracts and half of the related immunoprecipitates were subject to Western blotting using αp53, NF-YA, and NF-YB antibodies.

B: List of some cell cycle genes regulated by NF-Y.

C: MCF10A, SKBR3, HT29, and SW480 cells were incubated with 0.5 μg/ml of ADR for 24 and 48 hr. Eight micrograms per sample (MCF10A) and 40 μg/sample (SKBR3, HT29, and SW480) of protein extracts were subjected to Western blotting using αp53, cyclin A, cyclin B1, cdk1, cdc25C, and tubulin antibodies.

D: SKBR3, MDA-MB468, and T47D breast cancer cell lines were incubated with 0.5 μg/ml of ADR for 1, 6, 18, and 24 hr. Cellular extracts (40 μg/sample) were subject to Western blotting using the same antibodies described in **C**.

E: The same experiment described in **C** and **D** was performed on H1299 and SAOS p53 null cell lines.

during the different phases of the cell cycle and in response to DNA damage (Huet et al., 1996; Van Ginkel et al., 1997; Bolognese et al., 1999; Farina et al., 1999; Yun et al., 1999; Adachi et al., 2000; Hu et al., 2000). Interestingly, the wtp53-dependent transcriptional inhibition of these genes upon DNA damage requires the integrity of the CCAAT boxes and a functional NF-Y complex (Manni et al., 2001; St. Clair et al.,2004; Imbriano et al., 2005).

Here, we show that diverse mutant p53 proteins interact in vivo with NF-Y. The mutant p53/NF-Y protein complex (mutp53/NF-Y) regulates, at the transcriptional level, NF-Y target genes involved in cell cycle control. Indeed, the expression of the *cyclin A*, *cyclin B1*, *cyclin B2*, *cdk1*, and *cdc25c* genes is upregulated in response to DNA damage in cells harboring endogenous mutant p53 proteins. This upregulation requires the presence within the target promoters of an intact CCAAT box. The functional consequence of this upregulation is increased DNA synthesis, which is strictly dependent on the existence of the protein complex mutp53/NF-Y. We further show the existence of a transcriptional competent complex including mutp53, NF-Y, and p300 on the promoters of NF-Y cell cycle target genes. The recruitment of p300 is severely impaired in cells whose endogenous expression of mutant p53 was specifically knocked down. Of note, the recruitment of mutant p53 to the

CCAAT sites of NF-Y target promoters is severely impaired upon abrogation of NF-YA expression. Our observations reveal a crosstalk between mutant forms of p53 protein and NF-Y that takes part in the aberrant regulation of the cell cycle in response to DNA-damaging agents.

Results

Mutant p53 proteins interact in vivo with NF-Y
To investigate whether mutant forms of p53 protein and NF-Y interact in vivo, we performed coimmunoprecipitation experiments, employing cell extracts derived from tumor cells (SKBR3, HT29, and SW480) harboring endogenous mutant p53His175, p53His273, and p53His273/Ser309 proteins, respectively. As shown in Figure 1A (lanes 5 and 6), we found the presence of protein complexes between mutant p53 and NF-YA and NF-YB. Immunostaining analysis revealed that mutant p53 and NF-YA localize in the nucleus of SKBR3 cells. RNAi knockdown of mutant p53His175 did not affect the localization of NF-YA (Figure S1A in the Supplemental Data available with this article online). Adriamycin (ADR) treatment does not impact on the endogenous levels of mutant p53 and NF-Y, nor on the formation of the complex (Figure 1A, lanes 1–6). Reciprocal protein complexes involving mutant p53, NF-YA, and NF-YB
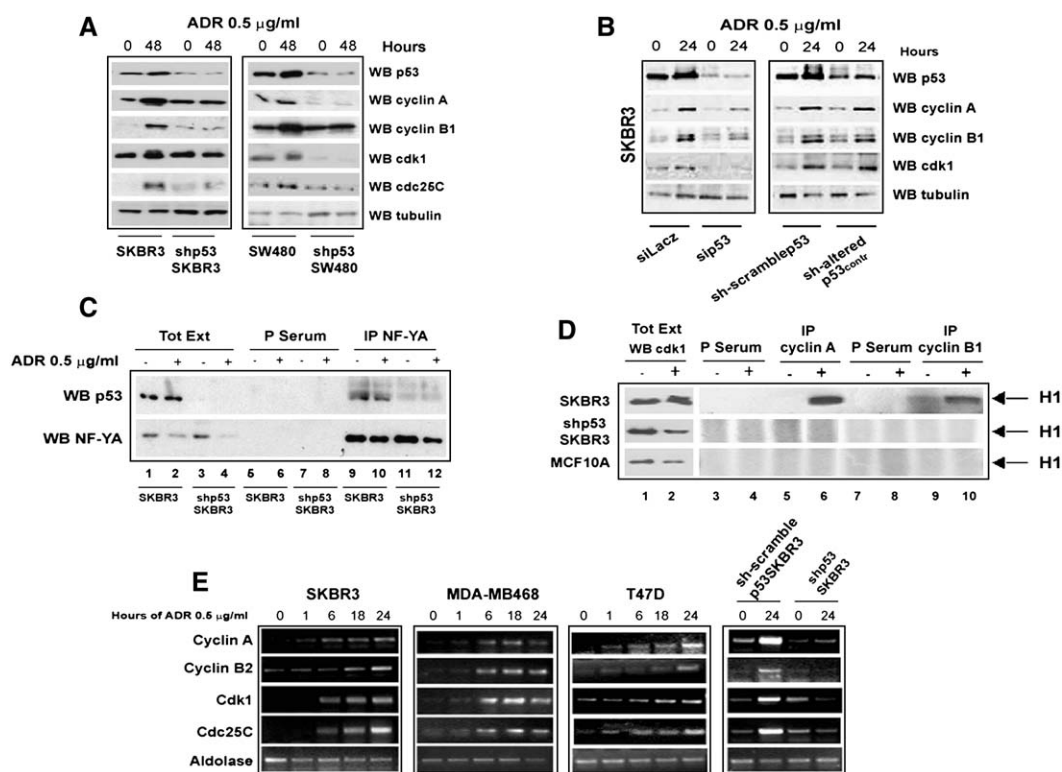
Figure 2. Expression of mutant p53 is necessary for transcriptional activation of NF-Y target genes

A: SKBR3, SW480, shp53-SKBR3, and shp53-SW480 cells were treated with 0.5 µg/ml of ADR for 48 hr. Forty micrograms per sample were subjected to Western blot analysis using αp53, cyclin A, cyclin B1, cdk1, cdc25C, and tubulin antibodies.

B: SKBR3 cells transiently transfected with siLacZ or sip53 oligonucleotides (see Experimental Procedures) were treated and assessed as in **A** (left panel). SKBR3 transfected with shscramblep53 vector or pSuper-altered control p53 was analyzed as described above (right panel).

C: SKBR3 and shp53-SKBR3 were treated as in **A**. Cell extracts (1 mg) were immunoprecipitated with mouse preimmune serum or 50 µl of a specific monoclonal αNF-YA serum. Western blotting was performed with 20 µg/sample of total extracts, and 500 µg of the related immunoprecipitates were probed with sheep αp53 serum and rabbit αNF-YA antibody.

D: Cell extracts (1 mg) of ADR-treated MCF10A, SKBR3, and shp53-SKBR3 cells were immunoprecipitated with polyclonal αcyclin A or cyclin B1 antibodies and preimmune sera as control. Left panel shows 20 µg of cellular extracts subjected to Western blotting with αcdk1 antibody. Immunoprecipitated kinase activity was assessed using 1 µg of H1 histone and 10 µM ^{32}P-γ-ATP as substrates. One of three separate experiments is shown (right panel).

E: RT-PCR amplification of cyclin A, cyclin B1, cdk1, cdc25C, and aldolase on total cellular RNA extracted from the cell lines indicated in the figure.

were found in cells whose exogenous expression of mutant p53His175 (H-175#41) or wtp53 (H-wtp53#23) was under the control of ponasterone A (Figure S1B) (Strano et al., 2000). No protein complexes were detected in cell lysates immunoprecipitated with preimmune serum (Figure 1A, lanes 3 and 4; Figure S1B, lanes 8–10). Taken together, these results demonstrate the existence of complexes containing mutant p53 and NF-Y proteins under physiological conditions. Since NF-YB and NF-YC heterodimerization is a prerequisite for NF-YA association, our results further indicate that both mutant and wtp53 proteins associate with a transcriptional competent NF-Y trimeric complex.

Mutant p53 proteins upregulate NF-Y target genes after DNA damage

A number of genes controlling different phases of the cell cycle are regulated at the transcriptional level by NF-Y (Figure 1B) (Mantovani, 1998; Elkon et al., 2003). It has been previously reported that the expression of some cell cycle-related NF-Y target genes is inhibited by wtp53 in response to DNA damage (Manni et al., 2001; Imbriano et al., 2005). To verify whether the mutp53/NF-Y complex modulates the expression of such

NF-Y target genes, we assessed the protein levels of cyclin A, cyclin B1, cdc25, and cdk1 in ADR-treated SKBR3, HT29, SW480, MDA-MB468, and T47D mutant p53 expressing cells (Figures 1C and 1D). We found that, irrespective of the type of p53 mutation, cyclin A, cyclin B1, cdc25, and cdk1 protein levels were upregulated in response to ADR treatment (Figures 1C and 1D). This upregulation starts between 1 and 6 hr after the treatment (Figure 1D). Conversely, the expression of these genes was downregulated in ADR-treated human primary breast epithelial cells (MCF10A) carrying endogenous wtp53 protein (Figure 1C). These findings agree with those showing that ADR-induced p53 accumulation in MCF10A cells is critical for the cellular response to DNA damage (Kohn et al., 2002). Of note, we did not find any modulation of cyclin A, cyclin B1, cdc25, and cdk1 protein levels in p53 null cells, such as H1299 and Saos-2 cells, in response to DNA damage (Figure 1E). To further confirm the role of mutant p53 in the upregulation of the previously analyzed NF-Y target genes, we assessed their expression in SKBR3 and SW480 cells whose mutant p53 expression had been knocked down (Figures 2A and 2B). Knockdown was achieved by stable transfection of a shp53 expression plasmid (Figure 2A), or by p53-siRNA (small interfering RNA)

oligonucleotide transduction (Figure 2B). As shown in Figures 2A and 2B, reduction of mutant p53 severely impaired the upregulation of cyclin A. cyclin B1, cdk1, and cdc25C in response to ADR treatment. This was not observed in cells transduced with control oligo (LacZ) or with plasmids expressing a scrambled p53 shRNA or a triple point mutated p53 shRNA (Figure 2B; Zalcenstein et al., 2006). Altogether, our findings indicate that the status of p53 plays a role in the modulation of cell cycle-related NF-Y target genes in response to DNA damage.

Next, we investigated whether the increased protein levels of cyclin B1, cyclin A, and cdk1 resulted in the activation of the corresponding protein kinases. Indeed, the upregulation of cyclin B1 and cdk1 was accompanied by an upward mobility shift, which suggests phosphorylation and activation of these proteins (Figure 1C). As shown in Figure 2D (lanes 3–10, upper panel), kinase assays performed on the indicated immunoprecipitates revealed that both cyclin A/cdk1 and cyclin B1/cdk1 kinase activities were upregulated after DNA damage in SKBR3 cells, indicating that the upward mobility shift of cdk1 is due to an activating phosphorylation. An intact mutp53/NF-Y protein complex (Figure 2C) is necessary for the kinase activity associated with cyclin B1 and cyclin A complexes, as shown by the lack of such activity in cells whose mutant p53 expression had been knocked down (Figure 2D, lane 3–10, lower panel). No increase in the activity of cyclin A/cdk1 or cyclin B1/cdk1 kinases was observed in MCF10A cells (Figure 2D, lanes 3–10, middle panel).

To study whether the regulation of NF-Y target genes occurs at the transcriptional level, we assessed the behavior of their mRNAs in response to ADR treatment. We found that cyclin A, cyclin B2, cdc2, and cdc25C mRNAs are already upregulated between 1 and 6 hr after the addition of ADR (Figure 2D). Knockdown of mutant p53 expression severely impairs this upregulation (Figure 2C).

Altogether, these data indicate that mutant p53 is necessary for the upregulation of cell cycle-related NF-Y target gene expression. Notably, the activity of important cyclin/cdk complexes involved in control of the G2/M phase of the cell cycle in response to DNA damage is strictly related to the presence of mutant p53.

Transcriptional activation of NF-Y target promoters by mutant p53 proteins requires CCAAT box integrity

To further dissect the transcriptional control of mutant p53 on the NF-Y target promoters, we performed transactivation assays. First, we found that a ponasterone-inducible mutant p53His175 (H-175#41) (Strano et al., 2000) transactivates the exogenously expressed luciferase gene driven by a 1.1 kb fragment of the murine *cyclin B2* promoter (pCCAAT-B2LUC), which contains three CCAAT boxes (Figure 3A). This activity was not revealed in control cells treated with ponasterone A (Figure 3A). Second, H1299 cells were transiently cotransfected with expression plasmids encoding either mutant p53His175 or wtp53 together with the cyclin B2 promoter reporter employed in Figure 3A. We found that the activity of the cyclin B2 promoter was upregulated in the presence of mutant p53His175 (Figure 3B). In agreement with our previous findings, the activity of the cyclin B2 promoter was downregulated by wtp53 (Manni et al., 2001; Imbriano et al., 2005). These activities were lost in the context of a cyclin B2 promoter carrying three mutated CCAAT boxes (pmutCCAAT-B2LUC) (Figure 3B). Similar results were

obtained using the cyclin A and cdc25C promoters (Figures 3C and 3D).

To further evaluate the role of NF-Y in the mutant p53His175-dependent upregulation of NF-Y target gene expression, we used a dominant-negative NF-Y mutant (YA13 m29). This mutant bears a triple amino acid substitution in the NF-YA DNA binding domain, which allows trimer formation but prevents the binding to CCAAT boxes (Mantovani, 1998). As shown in Figure 3E, the exogenous expression of YA13m29 protein impaired the opposing transcriptional effects of mutp53 His175 and wtp53 on cyclin B2 promoter activity.

To investigate whether endogenous mutant p53His175 protein regulates NF-Y target promoters in response to ADR treatment, we analyzed mutant p53 transcriptional activity in SKBR3 cells harboring an exogenous cyclin B2 promoter (Figure 3F). We found that cyclin B2 promoter activity was enhanced by the addition of ADR (Figure 3F). This activity requires the presence of an active mutant p53 and the integrity of the CCAAT boxes of cyclin B2 promoter (Figure 3F). Indeed, knockdown of mutant p53 expression abolished the transcriptional activation of the cyclin B2 promoter upon ADR treatment (Figure 3F). In contrast, cyclin B2 promoter activity was decreased in wtp53-carrying MCF10A cells (Figure 3G).

To verify whether the transcriptional effects exerted by mutp53 His175 on NF-Y target promoters were also recapitulated by other types of human tumor-derived p53 mutants, we performed a transactivation assay employing mutant p53His273 and p53Gly281 proteins (Figure 3H). To this end, H1299 cells were cotransfected with expression plasmids encoding the above p53 mutants in combination with luciferase reporter plasmids driven by the cyclin B2 or cdc25c promoters. We found that activity of both promoters was enhanced by the concomitant expression of mutant p53His273 and p53 Gly281 (Figure 3H).

To find out whether the transactivation domain of mutant p53His175 takes part in the transcriptional activation of NF-Y target promoters, we performed transactivation assays employing the mutant p53His175/22,23, harboring point mutations in two critical residues within the N-terminal transcriptional activation domain. We found that this mutant bound to NF-YA as efficiently as mutant p53His175 (Figure S2A). Coexpression of this mutant with NF-YA resulted in the activation of the cyclin B2 promoter to an extent very similar to that seen with mutant p53His175/NF-Y protein complex (Figure S2B). Unlike the mutp53/NF-Y complex, the wtp53/NF-Y protein complex required an intact transactivation domain to induce transcriptional repression of NF-Y target genes (Figure S2B).

Altogether, these results demonstrate that the mutp53/NF-Y protein complex is capable of transcriptionally regulating NF-Y target promoters in response to ADR. This activity requires the presence of a transcriptionally competent NF-Y complex and the integrity of the CCAAT boxes of the target promoters.

Mutant p53 protein binds NF-Y target promoters in vivo

To assess whether mutant p53 protein can be recruited in vivo onto NF-Y target promoters, we carried out chromatin immunoprecipitation (ChIP) experiments in SKBR3 cells (Figure 4A, lanes 2 and 3). We found that mutant p53 is predominantly bound to *cyclin A*, *cyclin B1*, *cyclin B2*, *cdk1*, and *cdc25C* promoter sequences containing CCAAT boxes in chromatin immunoprecipitates derived from cells treated with ADR. A similar occupancy pattern was evident for NF-Y (Figure 4A, left panel). In
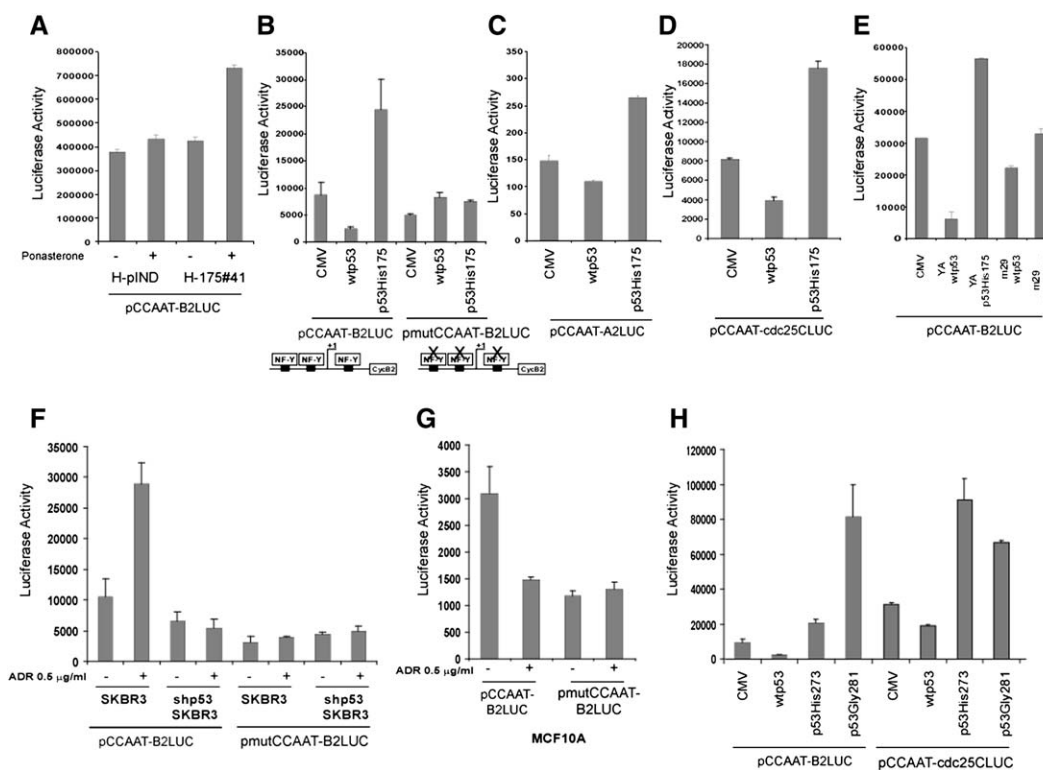
Figure 3. The integrity of CCAAT boxes is necessary for mutant p53 transcriptional activation of NF-Y target promoters

A: H-pIND#1 and H-175#41 cells were transfected with pCCAAT-B2-LUC (3 μg). p53 expression was induced by addition of ponasterone A (2.5 μM) for 24 hr.
B: H1299 cells were cotransfected with pCCAAT-B2LUC (3 μg) or pmutCCAAT-B2LUC (3 μg) and with pcDNA3-wtp53 (wtp53; 5 μg) or pcDNA3-p53His175 (p53[175]; 5 μg) vectors. pcDNA vector (5 μg) was used as basal control of CMV promoter activity.
C and D: The same experiments described in **B** were performed on H1299 cotransfected with pCCAAT-A2LUC and pCCAAT-cdc25CLUC promoters.
E: H1299 cells were transiently cotransfected as in **A** plus vectors encoding NF-YA (5 μg) or the dominant-negative m29NF-YA (5 μg).
F and G: SKBR3, shp53-SKBR3, and MCF10A cells were transiently transfected with pCCAAT-B2LUC (3 μg) or pmutCCAAT-B2LUC (3 μg) vectors. Sixteen hours after transfection, cells were treated with 0.5 μg/ml of ADR for 48 hr.
H: H1299 cells were cotransfected with pCCAAT-B2LUC (3 μg) or pCCAAT-cdc25CLUC (3 μg) vectors and with pcDNA3-wtp53 (5 μg), pcDNA3-p53His273 (p53[273]; 5 μg), or pcDNA3-p53His281 (p53[281]; 5 μg) vectors. Luciferase activity of all the above shown experiments was normalized for β-gal activity (cells were cotransfected with pCDNA3-β-gal vector) and for protein amount. The shown data represent the mean ± SD of triplicate determinations from three separate experiments.

contrast, when using primers corresponding to sequences of the *cyclin B1* gene that do not contain CCAAT boxes, we did not find any specific in vivo recruitment (Figure S3A).

It has been previously showed that p300 binds and activates NF-Y target promoters. As the presence of this HAT on NF-Y target promoters correlates with the presence of highly acetylated histones (Caretti et al., 2003; Salsi et al., 2003), we asked whether the recruitment of p300 affects the acetylation/methylation status of neighboring histones (Figure 4A, lanes 6–10). Interestingly, p300 was selectively recruited to the indicated promoter regions only after ADR treatment (Figure 4A, lane 6). In agreement with the p300 recruitment, we found that ADR treatment induces a striking increase in the global acetylation of promoter bound histone 4 and lysine 9 of histone 3, albeit to different extents (Figure 4A, lanes 7 and 9). Unlike what we observed with the *cyclin A*, *cyclin B1*, and *cyclin B2* promoters, methylation of lysine 20 of histone 4 at the *cdk1* and *cdc25C* promoters was clearly reduced in response to ADR treatment (Figure 4A, lane 8).

To assess the role of mutant p53 in the recruitment of p300, we analyzed the previously described pattern of in vivo occupancy in SKBR3 cells whose endogenous mutant p53 had been specifically knocked down (Figure 4B). As shown in Figure 4B (lanes 2–4), while the presence of NF-Y on the

analyzed promoters did not vary, p300 was not recruited onto the *cyclin A*, *cyclin B1*, *cyclin B2*, *cdk1*, and *cdc25c* promoters in response to ADR treatment. The lack of p300 recruitment resulted in the marked reduction of acetylation accompanied by methylation of neighboring histones in the indicated promoter regions in ADR-treated cells (Figure 4B, lanes 5–9).

Next, we aimed to analyze the kinetics of in vivo occupancy of mutant p53, NF-Y, and p300 on NF-Y target promoters. To this end, crosslinked chromatin derived from SKBR3 cells harvested at different time points after ADR treatment was immunoprecipitated with antibodies specific for the indicated proteins (Figure 4C). We observed that NF-Y is constantly and ADR-independently present on the *cyclin B2* promoter (Figure 4C, left panel). The amount of mutant p53 bound to the *cyclin B2* promoter is maximal between 1 hr and 6 hr after ADR addition, and p300 is recruited already at 1 hr and peaks at 18 hr. As shown in Figure 4C, the kinetics of p300 recruitments is comparable with that of histone 4 acetylation. Conversely, HDCA1 recruitment and H3 methylation on the *cyclin B2* promoter, which are clearly evident at 0 hr and 1 hr, drop down markedly at 6 hr and 18 hr after ADR treatment. Similar findings were obtained in the analysis of the in vivo occupancy of *cdk1* promoter regions (Figure 4C, right panel). We and others have previously shown
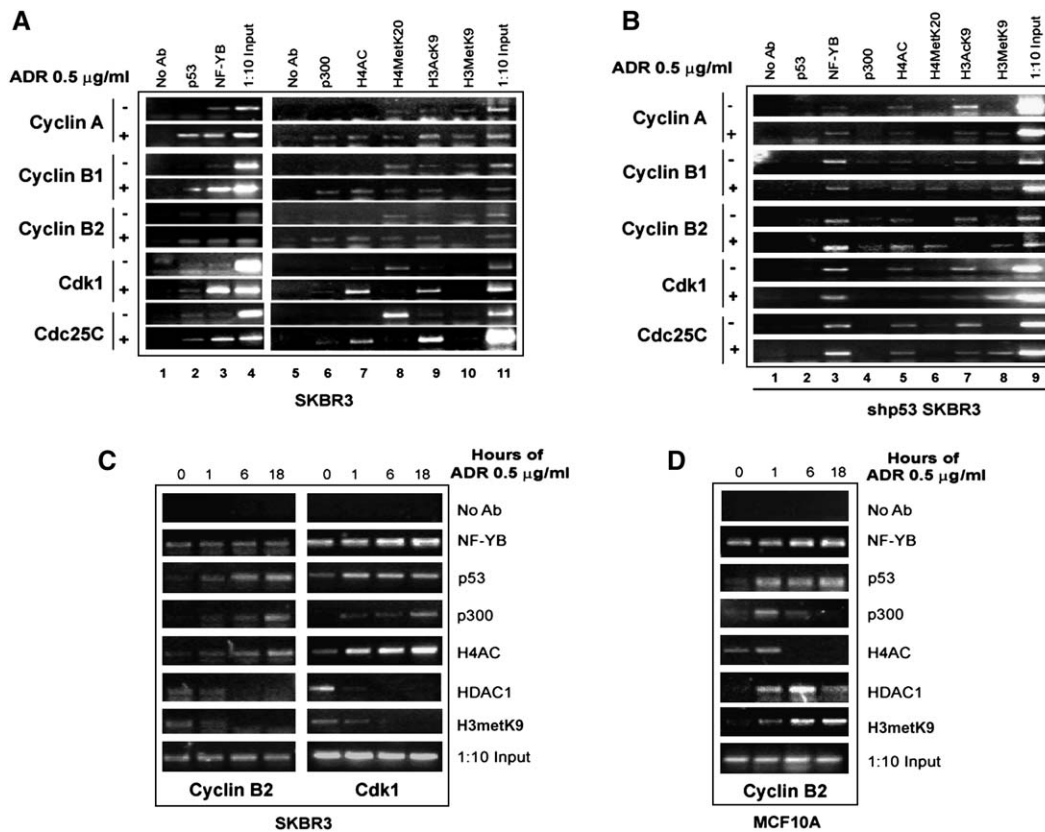
Figure 4. Mutant p53 binds NF-Y target promoters in vivo

A and B: ChIP was performed on SKBR3 and shp53-SKBR3 cells before and after ADR treatment (0.5 µg/ml for 48 hr) with the indicated antibodies. No antibody was used as a control (No Ab). PCR analysis was performed on the endogenous promoters of *Cyclin A2, Cyclin B1, Cyclin B2, Cdk1,* and *Cdc25C* genes.
C and D: ChIP was performed on SKBR3 and MCF10A cells treated for 1, 6, and 18 hr with 0.5 µg/ml ADR. PCR analysis was performed on *Cyclin B2* and *cdk1* promoters.

that wtp53 represses NF-Y target promoters in response to DNA damage (Manni et al., 2001; Imbriano et al., 2005). Therefore, we analyzed the previously described pattern of in vivo occupancy of the *cyclin B2* promoter in normal wtp53-positive breast epithelial cells (MCF10A), treated with ADR (Figure 4D). We observed that, while the amount of wtp53 bound to the *cyclin B2* promoter increases with time, that of NF-Y was slightly augmented (Figure 4D). The recruitment of p300 and the related H4 acetylation markedly decreased from 1 hr to 6 hr and 18 hr after the addition of ADR (Figure 4C). Of note, the in vivo occupancy of HDAC1 and lysine 9 methylation of H3 on the *cyclin B2* promoter became clearly detectable only in the presence of ADR (Figure 4D).

Altogether, these data demonstrate that mutant p53 binds in vivo to NF-Y target promoters. They also indicate that the binding of mutant p53 to those promoters is increased in response to ADR treatment and is necessary for the selective recruitment of p300. The switch between the recruitment of HADC1, which promotes histones deacetylation, and that of p300, which results in an increased H4 and H3 acetylation, provides a molecular explanation for the transcriptional activation of NF-Y target genes in response to DNA damage.

p300 binds to the mutant p53/NF-Y protein complex
The transcriptional crosstalk between mutant p53, NF-Y, and p300 highlights the possibility that a triple complex might be present in tumor cells. To investigate this issue, we transiently overexpressed NF-YA and p300 in H1299 cells expressing ponasterone-inducible mutant p53His175. We found that reciprocal protein complexes involving NF-YA, mutp53, and p300 were present in ponasterone-treated cells (Figure 5A, upper panels). Furthermore, mutant p53 and NF-Y are necessary for the binding of p300 (Figure 5A, lanes 1 and 4). Of note, the presence of p300 in the protein complex resulted in the acetylation of both NF-YA and mutant p53His175 (Figure 5B, lanes 2 and 4). To evaluate in a more physiological context the existence of mutant p53/NF-Y/p300 protein complexes, we performed coprecipitation experiments in SKBR3 cells. Interestingly, this complex was found predominantly in cells treated with ADR (Figure 5C, lanes 5 and 6). We also found that the presence of a protein complex involving mutant p53 and HDAC1, unlike that containing p300, can be detected predominantly in the absence of ADR (Figure 5C, lanes 3–6). Conversely, wtp53 interacted preferentially with HDAC1 and not p300 in ADR-treated MCF10 cells (Figure 5D). To evaluate the contribution of p300 to the mutp53-induced transcriptional control of NF-Y target promoters, we assessed the ability of a p300 acetylase-defective mutant to cooperate with mutant p53 in transactivating the cyclin B2 promoter (pCCAAT-B2LUC) (Figure 5E). We found that transient coexpression of the p300LYRR mutant strongly impaired the transcriptional activation of the cyclin B2 promoter by the mutp53/NF-Y protein
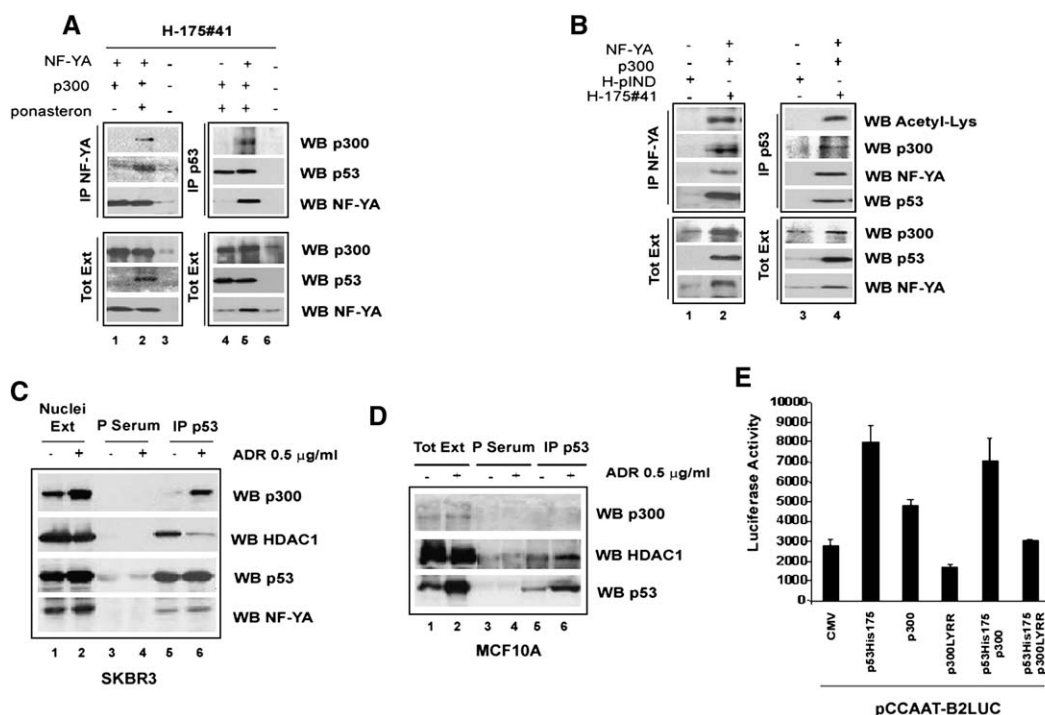
Figure 5. p300 binds to the protein complex mutant p53/NF-Y

A: H-175#41 mutp53His175 ponasterone-inducible cells were transfected with vectors for NF-YA and p300 proteins. Immunoprecipitations of related cell extracts were performed with αNF-YA and αp53 antibodies and preimmune serum. Cell extracts (60 μg) and immunoprecipitated samples (500 μg) were subjected to Western blotting with the indicated antibodies.

B: Cell extracts (80 μg) and immunoprecipitations (500 μg) as in **A** were subjected to Western blotting performed with αAcetyl-Lys, p300, p53, and NF-YA antibodies.

C: SKBR3 nuclear extracts treated or not for 24 hr with 0.5 μg/ml ADR were immunoprecipitated (500 μg) with polyclonal αp53 antibody or its preimmune sheep serum as control. Nuclear extracts (30 μg) and 250 μg of immunoprecipitated samples were probed with αp300, HDAC1, p53, and NF-YA antibodies.

D: Total cell extracts derived from MCF10A cells were processed and analyzed as in **C**.

E: H1299 cells were cotransfected with pCCAAT-B2LUC (3 μg) and pcDNA3-p53His175 (m175; 5 μg), or pcDNA3-p300 (p300; 5 μg), or pcDNA3-p300LYRR (p300LYRR; 5 μg) vectors or CMV vector as control. All data represent the mean ± SD of triplicate determinations from three separate experiments. Luciferase activity was normalized to β-gal activity in all experiments and to protein amount.

complex, when compared to that promoted by wild-type p300 (Figure 5E).

These results indicate that the physical binding to and the acetylase activity of p300 are critical for the transcriptional activity of the mutp53/NF-Y protein complex.

NF-YA is necessary for the recruitment of mutant p53 to the CCAAT sites

In an attempt to provide molecular insights into the involvement of the mutp53/NF-Y protein complex in the regulation of cell cycle genes, we analyzed the in vivo recruitment of NF-Y, mutant p53, p300, and HDAC1 onto the *cyclin B2* promoter. We found that, while NF-Y and mutant p53 are present on that promoter independently of DNA damage, p300 was specifically recruited upon ADR treatment. Notably, HDAC1 recruitment was inversely correlated to that of p300, occurring only in the absence of ADR (Figure 6A, lower panels). By stable transfection of pmutCCAAT-B2LUC promoter, we assessed within the same cell population the requirement for CCAAT integrity in order to assemble a transcriptional active complex containing NF-Y, mutant p53, p300, or HDAC1 onto the *cyclin B2* promoter (Figure 6A, upper and middle panels). Indeed, we found that none of the above-mentioned proteins was recruited to the exogenously expressed pmutCCAAT-B2LUC promoter in the presence or absence of

ADR. Interestingly, the presence of both mutant p53 and NF-Y on regulatory regions containing intact CCAAT boxes seems to drive the selective recruitment of an acetylase (p300) or a deacetylase (HDAC1), whose activities determine the activation or repression of target genes.

To directly assess the role of NF-YA in the recruitment of mutant p53 to the CCAAT sites, we performed ChIP experiments in SKBR3 cells, whose endogenous NF-YA expression was silenced through specific siRNA oligos (Figure 6B). As shown in Figure 6C, the binding of mutant p53 to the CCAAT sites is largely dependent on the presence of NF-YA protein. These findings pair with those of a gel shift analysis showing that both the DNA binding domain of wtp53 and the core domain of mutant p53His175 (Figure S4A) do not bind to CCAAT sites (Figure S4B).

The protein complex mutant p53/NF-Y plays a role in ADR-induced S phase accumulation

It has been previously reported that conformational and DNA contact-defective p53 mutants can increase cellular resistance to chemotherapy or contribute to genome instability (Gualberto et al., 1998; Li et al., 1998; Blandino et al., 1999; Murphy et al., 2000; El-Hizawi et al., 2002). To explore the molecular basis of these effects, we evaluated the response of SKBR3 and
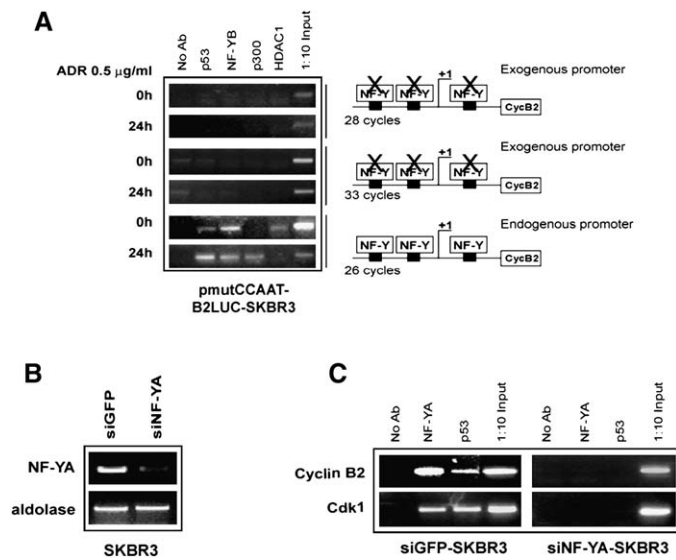
Figure 6. NF-YA is necessary for mutant p53 binding to CCAAT sites

A: Chromatin immunoprecipitation assays were performed on pmutCCAAT-B2LUC-SKBR3 cells before and after 24 hr of 0.5 µg/ml ADR treatment, with αNF-YB, p53, p300, and HDAC1 antibodies. No antibody was used as a control (No Ab). PCR analysis was performed on the same immunoprecipitated DNA samples using specific primers amplifying endogenous human *Cyclin B2* promoter, and the luciferase fragment of exogenous mutated *Cyclin B2* promoter.

B: cDNA derived from siGFP-SKBR3 and siNF-YA-SKBR3 cells were subjected to PCR to analyze NF-YA expression. Amplification of aldolase was used as control.

C: Chromatin immunoprecipitation experiments were performed on siGFP-SKBR3 and siNF-YA-SKBR3 cells. *Cyclin B2* and *cdk1* promoters were amplified by PCR (28 cycles).

shp53-SKBR3 cells to different amounts of ADR. As shown in Table 1, the number of dead cells did not vary significantly in SKBR3 cells, even at the higher ADR concentration used. Conversely, shp53-SKBR3 cells showed pronounced cell death (Table 1). These results indicate that mutant p53 contributes to chemoresistance of SKBR3 cells to ADR.

To investigate the role of the mutp53/NF-Y complex in cell cycle progression after DNA damage, we focused on SKBR3, whose expression of NF-YA (siNF-YA-SKBR3) and mutant p53 (shp53-SKBR3) was independently knocked down (Figures 7A–7C). By using nonapoptotic amounts of ADR, we found that siGFP-SKBR3 cells accumulate in S phase, as measured by bromodeoxyuridine (BrdU) incorporation in response to ADR treatment (Figure 7A). siNF-YA-SKBR3 cells showed a reduced BrdU incorporation compared to that of siGFP-SKBR3 cells (Figure 7A). Of note, the ADR-induced accumulation in S phase siNF-YA-SKBR3 cells was abolished (Figure 7A). In

agreement with these findings, we found that the basal level of cyclin B1, cdk1, and cdc25C expression was reduced, and ADR-induced upregulation of these genes was severely impaired in siNF-YA-SKBR3 cells (Figure 7B).

Further support for the role of the mutp53/NF-Y protein complex in the S phase accumulation in response to DNA damage was provided by the observations that shp53-SKBR3 and shp53-SW480 cells, unlike their respective control cells, did not show any increase in DNA synthesis after ADR treatment (Figure 7C). This is coupled with the impairment of NF-Y target gene upregulation in cells whose mutant p53 expression had been specifically interfered with (Figures 2A and 2B).

Altogether, these results demonstrate that the protein complex mutp53/NF-Y plays an important role in cell cycle modifications due to nonapoptotic amounts of DNA damage.

Discussion

In this report, we provide insights into the molecular mechanisms that underlie aberrant cell cycle responses of gain-of-function mutant p53-carrying tumor cells to anticancer drug treatment. We demonstrate that human tumor-derived p53 mutants interact physically with the transcription factor NF-Y. The net biological output of such protein complexes is the ability to increase DNA synthesis in response to DNA damage.

Here, we show that mutp53/NF-Y complexes lead to an aberrant upregulation of the expression of NF-Y cell cycle target genes after DNA damage. Notably, this leads to an aberrant activation of cyclin/cdk1 kinase complexes. We also demonstrate that upregulation of the expression of NF-Y target genes after DNA damage occurs, at least in part, at the transcriptional level. By analyzing cells whose endogenous expression of both mutant p53 and NF-Y was selectively inhibited, we verified that the upregulation of NF-Y target promoters was severely impaired. The functional dissection of the mutp53/NF-Y protein complex has allowed us to provide further molecular insights into the gain of function of mutant p53. Our findings show that the intact transactivation domain of mutant p53 does not play a major role in the transcriptional activation of NF-Y target promoters (Figure S2B). Furthermore, we found that mutant p53 is recruited in vivo onto a region of these promoters that is also bound by NF-Y. Those promoter regions also recruit p300, whose acetylase activity might represent the key event that turns on the transcriptional activity of the mutp53/NF-Y protein complex. This is further supported by the results showing that the recruitment of p300 leads to a global increase of histone acetylation and a decrease of histone methylation, which have been shown to play a critical role in the control of gene transcription (Kalkhoven, 2004). Acetylated forms of mutant p53 and NF-YA are present in the resulting complex, suggesting that

Table 1. Analysis of nuclei by trypan blue exclusion

Dead cells (% ± SD)	ADR 0.5 µg/ml			ADR 1 µg/ml		ADR 2.5 µg/ml	
	0 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
SKBR3	14.7 ± 1.3	15.5 ± 2.1	18.5 ± 1.2	16.3 ± 2.3	20.2 ± 2.4	16.6 ± 4.1	16.1 ± 2.6
shp53-SKBR3	20.1 ± 2.4	19.4 ± 1.8	24.5 ± 3.1	22.9 ± 2.5	28.4 ± 1.7	43.6 ± 3.1	40.3 ± 5.6

SKBR3 and shp53-SKBR3 cells were treated for 24 hr and 48 hr with adriamycin (ADR) at a final concentration of 0.5, 1, and 2.5 mg/ml, and death was evaluated counting alive cells with trypan blue. Approximately 300 cells were counted from representative microscope fields of each sample. Slides from the three independent experiments were analyzed.
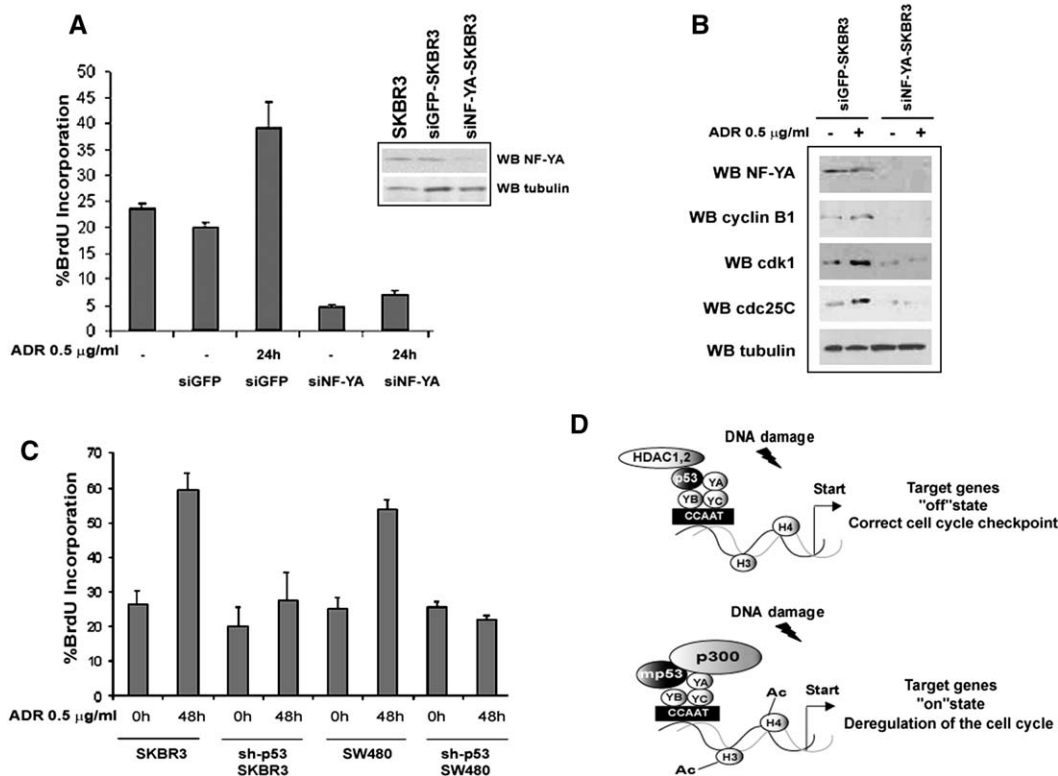
Figure 7. The protein complex mutant p53/NF-Y plays a role in ADR-induced S phase accumulation

A: SKBR3, siGFP-SKBR3, and siNF-YA-SKBR3 cells were incubated or not for 24 hr with 0.5 µg/ml ADR. Knockdown expression of NF-YA is shown in the right panel. Cells were then incubated with BrdU for 1 hr, and its incorporation was detected with mouse anti-BrdU antibody (Roche) and cyanin 2-conjugated IgGs (Calbiochem) as secondary antibody. BrdU incorporation is presented as the percentage of positive nuclei to total cell number. The data represent the mean ± SD of triplicate counts. shscramble-SKBR3, shp53-SKBR3, shscramble-SW480, and shp53-SW480 cells were incubated or not for 48 hr in the presence of 0.5 µg/ml ADR. Cells were then incubated with BrdU for 1 hr, and its incorporation was detected with mouse BrdU antibody (Roche) and cyanin 2-conjugated IgGs (Calbiochem) as secondary antibody. BrdU incorporation is presented as the percentage of positive nuclei to total cell number. The data represent the mean ± SD of triplicate counts.

B: SKBR3 cells were transfected with siGFP and siNF-YA oligonucleotide cells and incubated or not for 24 hr in the presence of 0.5 µg/ml ADR. Western blot analysis was performed with 50 µg of whole extracts, and αNF-YA, p53, cyclin B1, cdk1, cdc25C, and tubulin antibodies were used.

C: shscramble-SKBR3, shp53-SKBR3, shscramble-SW480, and shp53-SW480 cells were incubated or not with 0.5 µg/ml ADR and then processed and evaluated as in **A**.

D: Model proposing the molecular mechanism underlying the transcriptional control of cell cycle-related genes by mut-p53/NF-Y or wtp53/NF-Y protein complexes.

p300 not only leads to local histone acetylation, but also might regulate their acetylation status. Of note, the presence of mutant p53 on those promoter regions is critical for the recruitment of p300 (Figure 4B). Altogether, our findings allow us to propose a model (Figure 7D) in which NF-Y brings p53 onto CCAAT boxes containing promoters in response to DNA damage. The status of p53 might dictate the identity of the additional members of the transcriptional competent complex. Indeed, in the presence of mutant p53 the acetylase p300 is preferentially recruited, while wtp53 containing complexes binds to HDACs. The fine balance of this selective recruitment, whose spatial and temporal coordinates need to be further investigated, might have a great impact on the cell cycle events and consequently on the response of a tumor cell to conventional anticancer treatment. Interestingly, immunostaining experiments conducted in a panel of 39 rectal cancer samples of resected patients pretreated with adjuvant chemotherapy reveal that the expression of cyclin A and cdk1 is strongly increased in those samples whose p53 staining is highly positive, indicating overexpressed mutant p53 (data not shown).

Recent studies have indicated that NF-Y could serve as a common transcription factor for an increasing number of cell cycle control genes (Elkon et al., 2003). This suggests that other genes involved in cell cycle progression and known to be targets of NF-Y could also be transcriptional targets of gain-of-function mutant p53 proteins through a similar molecular mechanism. Further support to this hypothesis might be provided by microarray experiments that have identified 91 genes upregulated by mutant p53His175 (G. Fontemaggi and G.B., unpublished data). Interestingly, an in silico analysis reveals that about 68% of these genes (62 genes) contain CCAAT boxes within their regulatory regions (S.D.A. and F. Goeman, unpublished data). A rather speculative hypothesis might suggest that the repertoire of potential target genes of gain-of-function mutant p53 proteins is as broad as the spectrum of their diverse biological activities described in many in vitro and in vivo studies (Sigal and Rotter, 2000).

The search for molecular mechanisms underlying gain of function of mutant p53 has highlighted two potential scenarios. Mutant p53 can function as a transcription factor, whose

specific DNA binding consensus is still unknown, and consequently modulate sets of genes that mediate its protumorigenic activities (Dittmer et al., 1993; Blandino et al., 1999). Since mutant p53 proteins are quite abundant in tumor cells, it is reasonable to speculate that they can physically interact with many other proteins, including some with antitumoral functions. Indeed, mutant p53 interacts with p73 and p63 and strongly impairs their transcriptional activities and their antitumoral effects (Strano et al., 2000, 2002).

Here, we provide evidence that a combination of protein-protein interactions and transcriptional activation of target genes sustains gain-of-function activity of human tumor-derived p53 mutants.

The gain-of-function activity exerted by the protein complex mutp53/NF-Y seems independent of the particular type of p53 mutations. Two of the p53 mutants studied here (p53His175 and p53His273) are among the first three most frequent p53 mutations found in human cancers. They are also prototypes of the two main classes of p53 mutants: conformational and DNA contact-defective. Thus, mutant p53, irrespective of the type of missense mutation, might become associated with transcriptional competent complexes, including other transcription factors such as NF-Y, acetylases, and/or deacetylases, in order to elicit its ''gain-of-function'' activity.

Altogether, our data indicate that the deregulated growth control of tumor cells might depend on the excessive expression of cell cycle genes, which are repressed by wtp53 and become aberrantly activated in the presence of mutant p53, thereby allowing cells to escape from cell proliferation control and fall into malignant transformation.

Experimental procedures

Cell culture and treatments
H1299, H-pIND#1, H-wtp53#23, and H-75#41 cells were cultured and induced as previously reported (Strano et al., 2000; Fontemaggi et al., 2002). SKBR3, shscramblep53-SKBR3, shp53-SKBR3, MDA-MB468, and T47D breast cancer cell lines; SW480, shp53-SW480, and HT29 colon cancer cell lines; and the SAOS p53 null osteosarcoma cell line all were cultured in DMEM with 10% FCS. MCF10A primary breast epithelial cells (gift from O. Segatto) were cultured in Ham's F-12 medium with 5% horse serum, 0.5 μg/ml hidrocortisone, 10 μg/ml insulin, and 20 ng/ml EGF. The cells were treated with 0.5 μg/ml of ADR for 1, 6, 18, and 24 hr.

Plasmids and transfections
The following plasmids were used in transfection experiments: pcDNA3-wtp53, pcDNA3-p53His175, pcDNA3-p53His273, pcDNA3-p53Gly281 (Strano et al., 2000), pCCAAT-B2LUC, pmutCCAAT-B2LUC (Bolognese et al., 1999), pCCAAT-A2LUC (Schulze et al., 1995), pCCAAT-cdc25CLUC (Manni et al., 2001), Δ4YA13 vector (NF-YA) and Δ4YA13m29 dominant-negative vector (m29) (Mantovani, 1998), pcDNA3-p300, pcDNA3-p300LYRR (gift from M. Levrero; Thompson et al., 2004), pcDNA-p53wt[22,23], pcDNA-p53His175[22,23], and pBABE-PURO. H1299 cells were transiently transfected as previously shown (Strano et al., 2000). SKBR3, shp53-SKBR3, SW480, shp53-SW480, and MCF10A cells were transfected with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen).

SKBR3 cells transfected with pmutCCAAT-B2LUC and pBABE-PURO (10:1 ratio) were selected in 2 μg/ml puromycin (Sigma) 48 hr after transfection and then used for ChIP experiments.

RNA interference
pSuper vectors encoding shRNA specific for p53 and targeting p53 in the aa 259–264 sequence (gift from R. Agami), the corresponding scramblep53, and control shRNA derived from pSuper-p53 by insertion of three base-paired mismatches in the p53-specific sequence (altered p53Super control; gift from M. Oren) were constructed according to Brummelkamp et al. (2002) and Zalcenstein et al. (2006).

siRNA oligonucleotides targeting p53 in the aa 245–251 sequence (gift from M. Oren) were used for transient transfection.

Oligonucleotides for siNF-YA were synthesized by MWG-Biotech. The targeted sequence was 5′-TGGGACATTGATGATCACA-3′. This sequence spans from nucleotide 1291 in human NF-YA transcript variant 1 (mRNA) and from nucleotide 1204 in human NF-YA transcript variant 2 (mRNA). The sequence of siGFP employed as nonsilencing control was 5′-GGCTACGTCCAGGAGCGCACC-3′. The targeting sequence for lacZ-siRNA was 5′-GTGACCAGCGAATACCTGT-3′, which is directed to the 1915–1933 region of the bacterial galactosidase gene. Transfections of siRNAs and shRNAs were performed using Lipofectamine 2000 reagents (Invitrogen).

Cell extracts and Western blotting
Cell extracts were prepared as previously described (Imbriano et al., 2005). Solubilized proteins (20–50 μg) were resolved on 10% or 12% SDS-polyacrylamide gel electrophoresis. Western blotting was performed using the following primary antibodies: mouse monoclonal αp53 (DO1); rabbit polyclonal αNF-YA and YB (Rockland); rabbit polyclonal αcyclin A, αcyclin B1, αcdk1, αcdc25C, and αp300 (Santa Cruz); mouse monoclonal αtubulin (Calbiochem); and mouse monoclonal αacetyl-Lys (Upstate Cell). Secondary antibody used were goat anti-mouse and goat anti-rabbit, conjugated to horseradish peroxidase (Amersham). Immunostained bands were detected by the chemiluminescent method (Pierce).

Immunoprecipitations
The following antibodies were used for immunoprecipitations: 3 μl of sheep serum αp53 Ab7 antibody (Oncogene Science); 50 μl of mouse monoclonal αNF-YA antibody (gift of R. Mantovani); 1 μg rabbit αcyclin A or B1 (Santa Cruz) antibodies; and sheep serum, rabbit serum, and mouse serum as control. Precleared extracts were incubated with protein A/G-Sepharose beads (Pierce) in lysis buffer containing 0.05% BSA and antibodies, under constant shaking at 4°C for 2 hr. After incubation, Sepharose bead bound immunocomplexes were rinsed with lysis buffer and eluted in 50 μl of SDS-sample buffer for Western blotting or washed twice with the appropriate kinase buffer for immunokinase assays.

Immunokinase assays
Immunoprecipitated cell extracts were rinsed with kinase buffer (50 mM HEPES [pH 7.5], 5 mM β-glicerophosphate, 5 mM MnCl$_2$, 5 mM NaF, 0.1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Kinase reactions were carried out in 50 μl for 20 min at 37°C in kinase buffer supplemented with 10 μM ^{32}P-γ-ATP (0.2 μCi/μl), 10 μM ATP, 1 μg cAMP-dependent protein kinase inhibitor, and 1 μg full-length H1 histone (SIGMA, Type III-S, H-5505). Reactions stopped in SDS-sample buffer were separated on SDS-PAGE, and the dried gel was exposed to autoradiography.

BrdU incorporation assay
BrdU incorporation was visualized by immunostaining. SKBR3 cells, shp53-SKBR3 and shp53-SW480 cells, shscramblep53-SKBR3 and shscramblep53-SW480 cells, and siGFP-SKBR3 and siNF-YA-SKBR3 cells were treated with 0.5 μg/ml ADR for 24 hr or 48 hr. Twenty-three hours or 47 hr after stimulation with ADR, 20 μM BrdU (Roche) was added to the cultures for an additional incubation of 1 hr. Mouse monoclonal αBrdU antibody (Roche) diluted 1:20 in 0.1% BSA/PBS was used. BrdU incorporation is presented as the percentage of positive nuclei to total cell number of three independent experiments.

Transactivation assay
H1299, SKBR3, shp53-SKBR3, and MCF10A cells (1.5 × 10^5) were transiently transfected with expression plasmids, reporter constructs, and 0.5 μg of CMV β-galactosidase plasmid as an internal control for transfection efficiency. In the case of SKBR3, shp53-SKBR3, and MCF10A cells, precipitates were removed and cells were treated with 0.5 μg/ml ADR for 48 hr. LUC activity was assayed on whole-cell extract, as described (Manni et al., 2001). The luciferase values were normalized to β-galactosidase activity and protein contents.

RNA extraction and RT-PCR

Total RNA was extracted using the Trizol Reagent (Gibco BRL) and following the manufacturer's instructions. The first strand cDNA was synthesized according to the manufacturer's instructions (M-MLV RT kit; Invitrogen). PCR was performed with HOT-MASTER Taq (Eppendorf) using 2 µl of cDNA reaction, and the conditions were as follows: 94°C, 4 min; 28 cycles of 94°C, 30 s; 58°C, 40 s; 72°C, 40 s; and 72°C, 7 min. PCR products were run on a 2% agarose gel and visualized with ethidium bromide. The sequences of oligonucleotide primers were as follows: hCycA, 5′-AGCAGCCTGCAAACTGCAA AGTTG-3′ (forward), 5′-TGGTGGGTTGAGGAGAGAAACAC-3′ (reverse); hCycB2, 5′-GGCTGGTACAAGTCCACTCC-3′ (forward), 5′-GAAGCCAAGA GCAGAGCAGT-3′ (reverse); hCdk1, 5′-CCTTGCCAGAGCTTTTGGAATACC-3′ (forward), 5′-GACATGGGATGCTAGGCTTCCTGG-3′ (reverse); hCdc25C, 5′-GTATCTGGGAGGACACATCCAGGG-3′ (forward), 5′-CAAGTTGGTAGC CTGTTGGTTTG-3′ (reverse). The housekeeping aldolase A mRNA, used as an external standard, was amplified from the same cDNA reaction mixture using the following specific primers: 5′-CGCAGAAGGGGTCCTGGTGA-3′ (forward), 5′-CAGCTCCTTCTTCTGCTCCGGGGT-3′ (reverse).

ChIP assay

ChIP assay was performed as described (Gurtner et al., 2003). The chromatin solution was incubated overnight at 4°C with mild shaking with the following antibodies: 3 µl of sheep serum αp53 Ab7 (Calbiochem); 5 µl of rabbit polyclonal αNF-YB (gift of R. Mantovani); 1 µg of rabbit polyclonal αp300 (Santa Cruz); 6 µl of rabbit serum αH4Ac, αH4MetK20, αH3AcK9, and αH3MetK9 (Upstate); and 5 µl of rabbit polyclonal αHDAC1 (Sigma). Before use, protein G was blocked with 1 µg/µl sheared herring sperm DNA and 1 µg/µl BSA for 3 hr at 4°C and then incubated with chromatin and antibodies for 2 hr at 4°C. PCR was performed with HOT-MASTER Taq (Eppendorf) using 2 µl of immunoprecipitated DNA, and the conditions were as follows: 94°C, 4 min; 28 cycles of 94°C, 30 s; 58°C, 40 s; 72°C, 40 s; and 72°C, 7 min. PCR products were run on a 2% agarose gel and visualized with ethidium bromide. The primer sequences of the human promoters used in the PCR reactions are as follows: luciferase, 5′-TTGCTCTCCAGCGGTTCCAT-3′ (forward), 5′-CAG CCACTCCGGTCTGCGACA-3′ (reverse); hCycA2, 5′-GAGTCAGCCTTCGGA CAGCC-3′ (forward), 5′-CCAGAGATGCAGCGAGCAGC-3′ (reverse); hCycB1, 5′-GGCTTCCTCTTCACCAGGCA-3′ (forward), 5′-CGCGATCGCCCTGGA AAC-3′ (reverse); hCycB2, 5′-CAGAGGCGTCCTACGTCTGC-3′ (forward), 5′-TGCGCACGGGTCGCTGTTCT-3′ (reverse); hCdc25C, 5′-GAATGGACA TCACTAGTAAGGCGCG-3′ (forward), 5′-GCAGGCGTTGACCATTCAAACCT TC-3′ (reverse); hCdk1, 5′-GAACTGTGCCAATGCTGGGA (forward), 5′-GCA GTTTCAAACTCACCGCG-3′ (reverse).

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and four supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/3/191/DC1/.

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