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Dissection of the sequence-specific DNA binding and exonuclease activities reveals a superactive yet apoptotically impaired mutant p53 protein

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Abstract

Both sequence-specific DNA binding and exonuclease activities have been mapped to the central conserved core domain of p53. To gain more information about these two activities a series of mutants were generated that changed core domain histidine residues. Of these mutants, only one, H115N p53, showed markedly reduced exonuclease activity (ca. 15% of wild-type). Surprisingly, purified H115N p53 protein was found to be significantly more potent than wild-type p53 in binding to DNA by several criteria including gel mobility shift assay, filter binding and DNase I footprinting. Interestingly as well, non-specific DNA binding by the core domain of H115N p53 is superior to that of wild-type p53. To study H115N p53 in vivo, clones of H1299 cells expressing tetracycline regulated wild-type or H115N p53 were generated. H115N was both more potent than wild-type p53 in inducing p53 target genes such as *p21* and *PIG3* and was also more effective in arresting cells in G_1. Unexpectedly, in contrast to wild-type p53, H115N p53 was markedly impaired in causing apoptosis when cells were subjected to DNA damage. Our results indicate that the exonuclease activity and transcriptional activation functions of p53 can be separated. They also extend previous findings showing that cell cycle arrest and apoptosis are separable functions of p53. Finally, these experiments confirm that DNA binding and exonuclease activities are distinct features of the p53 core domain.

Keywords

p53; apoptosis; exonuclease activity; DNA binding; transcription; cell cycle

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Introduction

The p53 tumor suppressor protein is central to the DNA damage signal response pathway that results in cell cycle arrest or apoptosis.[1–4] Upon DNA damage to cells, p53 protein accumulates and becomes activated by post-transcriptional modifications followed by induction of its down-stream target genes involved in eliciting those two responses.[5–7] To date hundreds of p53 target genes have been identified either individually or as the result of different gene expression screens. More than 50% of human cancer cells contain missense mutant forms of p53 protein underlying the key roles of this protein in tumor suppression. The great majority (97%) of mutations in such tumor derived p53 variants are located within its central core domain, a region that is highly conserved in p53 proteins among different species and also in related p53 family members, p63 and p73 (The database is available at http://www.iarc.fr/P53). The core domain binds specifically to DNA containing 2 closely spaced copies of the p53 consensus sequence 5'-RRRCATGYYY-3' and tumor-derived mutation in this region usually leads to defective DNA binding reviewed in [8] and [9]. p53 is thus a sequence-specific transcriptional regulator of genes that facilitate the stress response circuitry in cells.

In addition to the regulation of genes involved in cell cycle and apoptosis, p53 has also been implicated in DNA repair. First, p53 can affect DNA repair processes through its ability to transactivate genes involved in these processes such as *p48*,[10] *GADD45*,[11] and *R2*.12 Second, a number of studies indicate that p53 can directly participate in DNA repair processes such as nucleotide excision repair (NER)1 or base excision repair (BER) as well as regulate recombination.[13–15] Several features of the p53 protein support its possible roles in DNA repair. p53 can directly interact with DNA repair related cellular factors including the transcription factor IIH (TFIIH) protein complex,16 single-stranded DNA binding protein RPA,17 DNA polymerase beta,18 AP endonuclease (APE),19 Rad 51,20,21 and mammalian homologs of the RecQ helicase family, BLN22,[23] and Wrn proteins.[23,24] Moreover, the p53 C-terminal domain can interact with various forms of damaged DNA in a sequence independent manner.[25–29] Finally, the central core domain contains an intrinsic 3'→5' exonuclease activity.[30–35]

It is intriguing that the central core domain contains two independent biochemical activities i.e., sequence-specific DNA binding and non-sequence-specific 3'→5' exonuclease activity. This poses the question as to whether sequence specific DNA binding and exonuclease activities reflect similar properties of the core domain (e.g., the ability to bind tightly to DNA) or whether they are separable. Some tumor derived hot-spot mutants are defective in both activities30 although it was later reported that these two activities are differentially regulated either by interaction with an antibody recognizing the C-terminus of p53 or post-translational modification of the p53 C-terminus.31 We initiated this project to determine whether the DNA binding surface of the core domain and the exonuclease active site may be separable. Since His residues are frequently located within the active site of exonucleases (see Discussion for more details), we generated seven His/Asn point mutants within the core domain and compared their sequence-specific DNA binding and 3'→5' exonuclease activity with wild-type p53. Our results showed that one of these mutants H115N was unique in being severely impaired in exonuclease activity. Remarkably, however, H115N p53 was more active than wild-type p53 in binding to DNA in vitro. Further characterization of H115N revealed that, when expressed at physiological levels in vivo, this mutant produces different effects in cells than does wild-type p53. Although H115N p53 can induce cell cycle arrest more efficiently than wild-type p53, its ability to produce apoptosis in DNA damaged cells is markedly impaired. We discuss the significance of these observations.

Results

Comparison of exonuclease and sequence-specific DNA binding activity of wild type and seven His/Asn mutants

To confirm and extend previous observations that p53 possesses an intrinsic exonuclease activity, we compared the activity of bacterially expressed p53 that has been purified through three chromatographic columns with that of commercially obtained bacterial exonuclease III. The substrate was a heteroduplex consisting of a 5' end-labeled 25-mer annealed to a 5' end-labeled 45-mer. As shown in Figure 1A, the exonuclease activity of p53 protein (0.5 to 1.0 pmol) was comparable to that of 1×10^{-4} to 1×10^{-5} pmol of exonuclease III. Therefore, under these conditions, p53 displayed far weaker exonuclease activity than exonuclease III. In each case the 25 base oligonucleotide was significantly more susceptible to digestion than the 45 base olignucleotide suggesting that both exonucleases prefer recessive 3' ends as substrates.

Since His residues are located within the active sites of other known exonucleases (see Discussion section) we generated seven His/Asn point mutants (relative positions shown in Fig. 1B below diagram). Histidine mutants were restricted to those within the p53 core domain (residues 100–300) since the exonuclease activity of p53 was previously localized to this domain.[30] The two remaining core histidine residues (His178 and His296) were not mutated to Asn since each was adjacent to another histidine residue (His179 or His297) that was chosen to be mutated. Wild-type and seven His mutant p53 proteins were purified from *E. coli* using Ni-NTA agarose column chromatography and then analyzed by SDS-PAGE (Fig. 1C). The exonuclease activities of these proteins were then measured by an immuno-exonuclease assay as described in Experimental Procedures. The enzymatic activities of mutant p53 proteins were compared to that of wild-type p53 measured by this assay (Table 1). Exonuclease activities of H168N, H179N and H297N p53 proteins were approximately comparable to that of wild-type p53, while H193N, H214N and H233N mutant p53 proteins actually showed significantly increased exonuclease activity. Only one mutant, H115N p53, showed just 15% of wild-type exonuclease activity in this assay. The reduced exonuclease activity of H115N p53 relative to wild-type p53 was confirmed when these proteins were extensively purified through three different columns as described in Experimental Procedures (Fig. 1D) and then tested as soluble proteins (Fig. 1E).

We then examined the sequence-specific DNA binding activity of the seven His/Asn mutants (H115N, H168N, H179N, H214N, H233N and H297N) as well as wild-type p53 by purifying each protein and performing gel shift assays using a [^{32}P] labeled oligo-nucleotide duplex containing the GADD45 p53 binding site. The results of this experiment are also summarized in Table 1. While mutation of p53 to H168N, H233N and H297N resulted in little change in DNA binding activity when compared to wild type p53, the H179N, H193N and H214N mutants each had significantly reduced DNA binding activity. Interestingly, however, H115N showed markedly enhanced DNA binding activity when compared to wild-type p53 protein. Therefore the reduced exonuclease enzymatic activity of H115N is not the result of global or partial unfolding as is the case with some of the hot-spot conformational p53 mutants such as R273H p53.[42] Our results with these p53 His substitution mutants thus indicate that the DNA binding and exonuclease activities of the p53 core domain are genetically separable.

H115N is more active than wild type in sequence-specific DNA binding as measured by several different assays

Since many tumor derived mutations located within the core domain of p53 disable sequence specific DNA binding it was of interest that H115N p53 showed superior binding than wild-type p53. Nevertheless, results with one type of assay for p53 DNA binding are not always confirmed when a different assay is used.[43] For example, the p53 monoclonal antibody PAb421

stimulates p53 binding to a DNA fragment when assessed by gel mobility shift assays using short oligonucleotides[44–46] but not by DNase I footprinting[40] and acetylation of p53 C-terminal residues was reported to stimulate p53 binding to short but not long DNA.[47] Accordingly, we wished to determine whether or not the superior DNA binding by H115N p53 could be confirmed by different assays measuring protein-DNA interactions (Fig. 2). To ensure that comparable amounts of proteins were used in the different assays, the amounts of wild type and H115N mutant proteins used in the assays were assessed by immunoblotting purified proteins using a mixture of three antibodies DO-1, 1801 and 421 (Fig. 2A). Consistent with the results summarized in Table 1 as measured by EMSA, the purified H115N mutant p53 was more potent than wild-type p53 in binding to a labeled 32 base pair oligonucleotide containing the p53 binding within the p21 promoter over a range of protein concentrations (0.2–0.6 ng/μL; Fig. 2B). Note that this assay was performed using p53 bound to the C-terminal antibody PAb 421 that greatly stimulates p53 DNA binding under these conditions. This suggests that increased binding by H115N does not involve relief of repression by the C-terminus. Additionally, as measured by a filter binding assay, H115N p53 bound the same [^{32}P] labeled p21 site containing 32-mer oligonucleotide 2–3 fold better than wild-type p53 in the absence of PAb 421 (Fig. 2C). Furthermore, after performing a DNase I footprinting assay with a 405 base pair DNA fragment from the p21 promoter containing the 5' p53 binding site, it was clear that the H115N mutant protein was more effective that wild type p53 in protecting its site within this longer fragment (Fig. 2D). Thus, using 3 different assays employing DNAs of different length, DNA binding by H115N p53 is superior to that of wild-type p53. Combining data in Table 1 and Figure 2 H115N is better than wild-type p53 in binding to two different sites (p21 and GADD45). On average H115N p53 binds two- to four-fold better than wild-type p53, although on occasion it has bound as much as 8-fold more efficiently than wild-type p53 (data not shown).

H115N p53 non-specific DNA binding is superior to that of wild-type p53

We previously showed that the p53 C-terminus positively affects the ability of the core DNA binding domain to interact with its specific binding site within a mini-circular but not a linear DNA molecule.[39] Our results demonstrated that the p53 C-terminus can provide a positive structure-specific component of DNA binding by p53. Although our results in Figure 2 indicate that H115N does not involve relief of negative regulation of core DNA binding, it was of interest to determine whether differences in the abilities of wild-type and H115N p53 proteins in binding to circular DNA would be observed. Gel shift assays were performed using both linear and circular 66 BP DNAs containing wild-type and mutated forms of the p53 binding site within the GADD45 promoter (Fig. 3A). In fact, as seen with other assays the p53 H115N mutant interacted with circular DNA with a wild-type GADD45 site about two- to four-fold more efficiently than wild-type p53. This indicates that in this assay the functional relationship between the C-terminus and core DNA binding domains is preserved in the H115N mutant. Surprisingly, however, H115N p53 also bound to the *mutant* GADD45 circular probe about two- to four-fold more efficiently than wild-type p53, even though we had previously shown that binding this probe requires only the intact p53 C-terminus.[39] Since the H115N mutation is in the core DNA binding domain this result suggests that the H115N mutation affects the ability of the core of the protein to interact with DNA in a sequence-nonspecific fashion. To confirm that H115N p53's superior binding involves both a sequence-nonspecific and structure-nonspecific interaction with DNA, we examined the relative abilities of wild-type and H115N p53 proteins to bind to a linear DNA fragment containing a mutant GADD45 site (Fig. 3B). Here the amount of p53 used in the EMSA was increased in order to be able to detect binding to the mutant linear DNA. Again we observed the same two- to four-fold increase in DNA binding ability to mutant linear probe. Based on these results we conclude that the underlying mechanism of increased DNA binding by p53 H115N is an increase in its core domain non-sequence specific interaction with DNA.

H115N is more active than wild type p53 in inducing p53 target genes in vivo and inducing cell cycle arrest

To extend our in vitro results, we went on to examine the response to wild-type and H115N mutant forms of p53 in H1299 cells that were engineered to express tetracycline-dependent versions of these genes. In these cells, p53 proteins were tagged with the HA epitope at the N-terminus. By varying the concentrations of tetracycline in medium of cell cultures (0–2,500 ng/mL) the levels of HA-p53 proteins in cells expressed ranged from high to low (Fig. 4). The protein levels of two well validated down-stream p53 target genes, p21 and PIG3, were analyzed before and after p53 protein was induced (Fig. 4A). At lower levels of expressed p53 protein induction of both genes was more pronounced with H115N than with wild-type p53. To exclude the possibility of clonal variation two separate H1299 clones expressing either wild-type (WT-1 and WT-2) or H115N p53 (H-1 and H-2) proteins were also analyzed and again at limiting concentrations H115N was more effective in inducing PIG3 (Fig. 4B, upper) and p21 (not shown). To confirm that the increased ability of H115N p53 to induce p53 targets was due to increased target gene transcription RT-PCR analysis of *p21* mRNA was performed on the same samples as shown in Figure 4B. Here again, there were discernibly greater quantities of *p21* mRNA induced by both H115N expressing clones when compared to the wild-type p53 expressing clones of H1299 cells and such differences were more pronounced at lower levels of p53 proteins (Fig. 4B, lower). Transcriptional activities of WT and H115N p53 were also quantified and compared by q-PCR. Specific concentration of tetracycline were chosen for both cell lines, in which similar levels of p53 expressions were detected (Fig. 4C). Transcriptional level of *p21* gene was higher in H115N expressing cells than WT by a factor of two- to three-fold fold (Fig. 4D). These data, taken together, indicate that the increased DNA binding by H115N p53 correlates with increased transcriptional activity when this mutant is expressed in cells. These data were not entirely consistent with the results of experiments examining the DNA binding properties of H115N p53 in vitro which shows that H115N p53 binds to DNA better than wild-type p53 at all concentrations tested. By contrast, results in Figure 4 show that the differential induction of two p53 target genes by mutant and wild-type p53 were most apparent at lower levels of expressed p53 protein. Thus we cannot exclude that H115N p53's superior ability to activate transcription is the result of properties other than or in addition to its increased affinity for DNA. Nevertheless, the following experiment provides the possibility that there may be one or more target genes that are better induced by H115N p53 even at higher levels of protein.

In H1299 cells engineered to express tetracycline regulated ("tet-off ") p53, removal of tetracycline from the medium leads to cell cycle arrest in both G_1 and G_2.[41,48–51] We examined the cell cycle profiles of H1299 cells after induction of the two forms of p53 at various levels as described in Figure 4, by FACS analysis (Fig. 5). To be able to present all the relevant data we show only values for S phase cells. Without induction of p53 proteins, both H1299 derivative cell lines showed similar cell cycle profiles (Suppl. Figs. 1 and 2) and similar proportion (30–35%) of S phase cells (Fig. 5). However, upon induction of p53 protein, at each comparable level of p53 protein expressed, cells expressing H115N p53 displayed a significantly greater reduction in S-phase than did wild-type p53 expressing cells. These results were quite dramatic. The two clones of wild-type p53 showed a reduction in S phase extending from 35% to 15% as a function of amount of p53 expressed, while the two clones of H115N underwent a greater S phase reduction at each level of p53 protein such that there were approximately 3-fold greater % of cells in S phase in the wild-type p53 expressing H1299 cells when p53 was fully induced. Since the levels of p53 protein in the different cells are very similar (Fig. 4A), this was not due to overexpression of mutant vs. wild-type p53 protein. Thus

Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/AhnCC8-10-Sup.pdf

the increased DNA binding by H115N p53 seen in vitro is correlated with increased cell cycle arrest in vivo. Our results also show that marked reduction in exonuclease activity does not negatively affect the ability of p53 to cause cell cycle arrest.

Wild-type p53 is superior to H115N p53 in eliciting DNA damage facilitated apoptosis

H1299 cells engineered to express inducible p53 undergo only a modest extent of apoptosis upon induction of p53.51 Treatment of these cells with DNA damaging agents, however, markedly augments the apoptotic response to p53 without altering the protein levels of p53 in cells.41,[51] To determine whether differences in such DNA damage-facilitated apoptosis between wild-type and H115N p53 could be observed, H1229 cells were either not induced (−) or induced to express either wild-type or H115N p53 protein at maximal (++) or lower (+) levels prior to treatment with NCS for the times indicated (Fig. 6A). The extent of apoptosis was measured by determining the % of cells with sub-G_1 content after FACS analysis of propidium mainiodide stained cells as previously described.41,[51] Surprisingly, despite its ability to produce a more profound cell cycle arrest, H115N p53 was markedly impaired in being able to elicit apoptosis. After 24 hours of NCS treatment, 11% of cells maximally expressing wild-type p53 (++) underwent apoptosis while only 2% of cells maximally expressing H115N (++) did so. Further, when treatment with NCS was extended to 48 hours, 22% of wild-type p53-expressing cells became apoptotic, and yet again, less than 3% of cells expressing H115N had subG_1 DNA content. The increase in apoptotic subG_1 cells after NCS treatment was due to p53 expression since cells with lower levels of p53 expression (+) had a smaller percentage of apoptotic cells and only 1–2% of cells with no p53 (−) underwent apoptosis. When the second clone of H1299 cells expressing H115N p53 was examined for its ability to undergo DNA damage facilitated apoptosis, it too was defective in this respect (data not shown). Therefore, while cells expressing H115N p53 can undergo a more profound cell cycle arrest, they are impaired in producing the apoptotic response in H1299 cells.

It was also important both to measure apoptosis by another assay as well as to confirm that the second clone of H115N p53 was also defective in producing apoptosis. Here we measured caspase-3 activity (Fig. 6B) in cell expressing either wild-type p53 (W-1) or the two clones of H1299 cells expressing H115N p53 (H-1 and H-2). In these experiments the background of the assay was quite high. Nevertheless we were able to show as before that H115N in the H-1 clone is severely defective in eliciting caspase-3 activity. Moreover there was also a significant reduction in caspase-3 activity with in H-2 cells (the second H115N expressing clone) after NCS treatment of cells although the decrease was not as drastic. Taken together H115N p53 is both better at causing cell cycle arrest and less effective in producing apoptosis in H1299 cells when compared to wild-type p53.

Discussion

Our initial interest was to generate mutant p53 that is devoid of exonuclease activity while the DNA binding activity was maintained intact. While the significance of p53 mutations rendering the transactivation function of the protein inactive has been well studied, the impact of deficiency of exonuclease activity in p53 is not known. This might be partly due to the observation that tumor derived hot-spot mutants not only fail to function as transcriptional activators but also were reported to be deficient in exonuclease activity.[30] p53 hot spot mutants were categorized into two classes; structural and functional mutants.[52] Since representative members of both classes were defective in exonuclease activity it is likely that both structural integrity of the protein and DNA binding activity are essential for each of these two biochemical activities.

We first compared the exonuclease activity of bacterial exonuclease III and p53. The enzymatic activity of p53 was dramatically lower than that of Exo III with a turnover rate of 0.046 s^{-1}.

We have not determined the turnover rate of p53 exonuclease isolated from eukaryotic cells and it remains possible that it would be higher than bacterially expressed p53. After confirming that bacterial p53 is active as an exonuclease (albeit with very low activity), we generated seven core domain His/Asn point mutants (H115N, H168N, H179N, H193N, H214N, H233N and H297N). The rationale behind this was that other nucleases such as DNase I,53 Exo III54 and apurinic/apyrimidinic endonuclesae (HAP1)55 use a His-Asp catalytic dyad at the active site. Consistent with this, mutation of His or Asp within the catalytic site of HAP1 results in significant loss of enzymatic activity.56,57 We chose Asn for substitution of His residues because it is considered to be the most conservative mutation. Asn is less bulky than His, but partly maintains hydrogen bonding donor and acceptor properties. Of the His mutants generated only H115N, showed reduced enzymatic activity. Although this suggests that H115 may be a part of the catalytic dyad, the reduction of its activity, while pronounced (i.e., by a factor of 5–6) is less defective than other exonucleases, which show a virtual ablation (i.e., by a factor of 100) of their enzymatic activity upon mutation of catalytic residues.56,57 Therefore we cannot conclusively state that H115 is the active site residue for the p53 exonuclease. The less severe reduction in enzymatic activity of H115N could result from a conformational change close to the active site. Indeed, it is possible that p53 does not utilize a His/Asp catalytic dyad for its exonuclease activity. It is important to stress that the reduced exonuclease activity of H115N was not a result of general unfolding that is a characteristic of the conformationally altered tumor derived hot-spot mutants mentioned above since H115N p53 is superior in DNA binding activity in vitro and cell cycle arrest in vivo when compared to wild-type p53. Furthermore, our data indicate that markedly reduced exonuclease activity does not negatively affect the ability of p53 to cause cell cycle arrest.

It was not only unexpected that H115N displays supra-wild-type DNA binding, but the suggested mechanism was also unanticipated. The fact that the protein shows superior core non-specific binding is unprecedented. H115N is located at the boundary of the L1 loop defined from the original structure.[52] The structure-functional role of the L1 loop containing H115 has not been well studied but it is known to be a “cold-spot” for mutation in human tumors.[58] Interestingly as well, yeast based screens have revealed mutants in this region that display either altered transactivation specificity of target promoter reporters or altered DNA binding activity.[59–65] Based on the crystal structure of the core domain of p53 complexed with DNA the H115 residue does not directly contact DNA.[52] NMR analyses of the p53 core domain with and without DNA suggest that the L1 loop forms a dimerization interface upon DNA binding.[66] Thus, it is possible that H115N mutant is more effective in cooperative DNA binding as a tetramer. The crystal structure of the mouse core domain without bound DNA revealed that this loop may undergo a rearranged position upon DNA binding, and further, that its position without DNA would actually disfavor contact with DNA.[67] Perhaps mutation of H115 allows for greater ease of assuming the favorable conformation for DNA binding. Our data comparing binding of p53 proteins to linear and circular DNA with mutated p53 binding site show as well that the core possesses measurable non-specific DNA binding ability and such non-specific binding is increased by mutation of H115. Thus the function of H115 and perhaps other L1 loop residues may be to reduce non-specific interactions of the core with DNA.

We found that H115N is more potent than wild-type p53 in inducing expression of p21, PIG3 and HDM2 (not shown) in H1299 cells which suggests that its enhanced DNA binding activity can be translated to concomitantly increased induction of target genes. However, it should be mentioned that we failed to observe enhanced sequence-specific DNA binding activity of or transactivation by H115N over wild-type p53 when the proteins were overexpressed in mammalian cells by transient transfection (data not shown). This is most likely due to the fact that such transiently expressed proteins are often vastly overproduced when compared to the amounts normally present in cells even after induction by DNA damage. Indeed, even when wild-type and H115N p53 proteins were fully expressed in H1299 cells by complete removal

of tetracycline from the culture medium as shown in Figure 5, there was little difference in the induction of the targets that were measured including p21 and PIG3 (Fig. 4) or Mdm2 (not shown). It is therefore intriguing that at the highest levels of the two forms of p53, while p21 levels were similar, H115N expressing cells produced a more severe cell cycle arrest than wild-type p53 cells. This suggests the interesting possibility that there may be additional targets that can also cause cell cycle arrest whose expression may require either higher levels of wild-type p53 or more active forms of p53. Surprisingly, H115N p53 was significantly less effective in DNA damage facilitated apoptosis. p21 has been shown in numerous studies to protect cells from apoptosis.[68–70] While it is possible that the superior ability of H115N p53 to induce p21 at lower levels of increased protein is responsible for its defect in initiating apoptosis, we consider this to be quite unlikely. Most relevantly, under conditions such that H115N induced equivalent levels of p21 as WT p53, there was still a selective impairment in apoptosis induced by the mutant p53. Another possible explanation is that the superior transactivation activity of H115N allows for the induction of an anti-apoptotic factor(s) that is normally not induced by wild-type protein. If so this would imply that it is not advantageous for p53 to have very high DNA binding.

H115N p53 is not the first example of a p53 core domain variant that is selectively defective in arrest or apoptosis. For example human p53 mutants such as R143A[71] and R175P[72–74] are able to induce cell cycle arrest and a number of candidate targets such as p21 but are defective in causing apoptosis and in activating some pro-apoptotic p53 targets. Interestingly, another human p53 L1 loop mutant S121F produces essentially the opposite phenotype in displaying superior ability to induce apoptosis and yet is impaired in causing cell cycle arrest when compared to wild-type p53.[61] Thus future analysis of L1 loop mutants may eventually provide important insight into the individual roles of p53-mediated apoptosis or arrest in tumor suppression.

Experimental Procedures

Site-specific mutagenesis, protein expression and purification

Single mutations (His→Asn) at H115, H168, H179, H193, H214, H233 and H297 were introduced to p53 cDNA using a previously published method.[36] Briefly, *E. coli*, strain CJ236 (*ung*$^{-1}$*dut*$^{-1}$) was transformed with pRSET-p53,[37] and infected with T4 helper phage (Promega, Madison, WI) to produce single-stranded DNA containing uracil. Double-stranded DNA was synthesized with synthetic mutagenic primers (Invitrogen, Carlsbad, CA), T4 DNA polymerase and DNA ligase (New England BioLabs, Beverly, MA). Mutations were screened by transforming *E. coli*. DH5α with double-stranded DNA and sequencing the entire coding region of purified plasmids from selected clones. Proteins were expressed in BL21(DE3) at 22°C for 2.5 h. The N-terminally His-tagged p53 proteins were first purified on a Ni-NTA agarose column (Qiagen, Valencica, CA). For further purification, the proteins were subjected to heparin, and then Superose 6 liquid column chromatography.[18]

Exonuclease assays

DNA oligonucleotides to be used as substrates were purchased from Invitrogen (Carlsbad, CA). The sequences of the oligomers are as follows: 5'-GCC TCG CAG CCG TCC AAC CAA CTC A-3', and 5'-GGA CGG CAT TGG ATC GAG ATT GAG TTG GTT GGA CGG CTG CGA GGC-3'. These sequences do not share any homology to the cognate p53 binding sequence. The DNA oligomers were further purified by electrophoresis in 16% (w/v) polyacrylamide/8 M urea gels. Duplex substrates were prepared by purifying annealed DNA from 10% (w/v) polyacrylamide gels and quantitated as described in Ahn et al.[38] DNA substrates were labeled at the 5'-end with ^{32}P by incubating with T4 polynucleotide kinase and [γ-^{32}P] ATP (4,500 Ci/mmol). The 5'-radiolabelled DNA was separated from unreacted

[γ-^{32}P] ATP on a G-25 microspin column (0.8 mL). Typically, 2–4 times molar excess of tetrameric p53 was incubated with DNA substrates in an exonuclease activity assay buffer containing 50 mM TrisHCl (pH 8.0), and 10 mM $MgCl_2$ at 30°C for 30 min. The reaction was quenched with gel loading buffer, denatured at 85°C for 5 min and run on an 18% (w/v) polyacrylamide/8 M urea gel. Product formation was analyzed by PhosphoImager (Molecular Dynamics). For exonuclease III (New England BioLabs, Beverly, MA), 1–1 $\times 10^{-7}$ pmol of protein was incubated with 0.25 pmol of substrate as described above. For immunoprecipitation-exonuclease assays, each p53 protein (200 ng) purified from Ni-NTA column was immunoprecipitated with 20 μL of mAb1801 conjugated to Protein A-Agarose beads (Pharmacia LKB, Piscataway, NJ) in an exonuclease buffer. After washing beads with the exonuclease buffer 5 times, the exonuclease activities of p53 proteins bound to mAb1801 were measured as described above.

Electromobility shift assays (EMSA)

p53 proteins were incubated with or without PAb 421 in 20 μL EMSA buffer containing 20 mM HEPES (pH 7.9), 25 mM KCl, 0.5 mM EDTA (pH 8.0), 10% glycerol, 2 mM $MgCl_2$, 0.025% NP-40, 50 ng dI/dC DNA and 2.5 ng of ^{32}P-labeled oligonucleotide containing the GADD45 site or p21 site. The sequences of the oligomers are as follows: 5'-TAG AGC GAA CAT GTC TAA GCA TGC TGG CGT CG-3' and 5'-CGA CGC CAG CAT GCT TAG ACA TGT TCG CTC TA-3' are the GADD45 site.

5'-TAG AGC GAA CAT GTC CCA ACA TGT TGG CGT CG-3' and 5'-CGA CGC CAA CAT GTT GGG ACA TGT TCG CTC TA-3' are the p21 site. The duplex forms of DNA were prepared as described above.

For EMSA using 66 bp linear and circular DNA, 20-μL reaction mixtures contained 20 mM HEPES (pH 7.9), 25 mM KCl, 0.5 mM EDTA (pH 8.0), 10% glycerol, 2 mM $MgCl_2$, 2 mM spermidine, 0.025% NP-40, 60 nmol of unlabeled 66 bp blunt-ended mutant GADD45, and 0.15 nmol (or 3 ng) of the indicated ^{32}P-labeled oligonucleotide. Sixty-six-base-pair oligonucleotides containing the p53 binding site from the GADD45 promoter had the following sequences: wild-type, 5'-AGC TGA TAT CGA ATT CTC GAG CAG AAC ATG TCT AAG CAT GCT GGG CTC GAG AAT TCC TGC AGC GCT-3' and mutant, 5'-AGC TGA TAT CGA ATT CTC GAG CAG AAA ATT TCT AAG AAT TCT GGG CTC GAG AAT TCC TGC AGC GCT-3'. 66 bp circular probes containing wild-type and mutant GADD45 binding sites of identical sequence were generated as described previously.[39] Probes were labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. After 30 min of incubation at room temperature, the reaction mixtures were subjected to electrophoresis on a 4% native polyacrylamide gel (30:1 acrylamide/bisacrylamide) containing 0.5x Tris-borate-EDTA buffer at 165 V for 1.5 h at room temperature. DNA-protein complexes were quantified by phosphorimaging with ImageQuant software (Molecular Dynamics).

Filter binding assays

p53 protein samples (0–60 ng) were incubated in EMSA buffer with 2.5 ng of ^{32}P-labeled oligonucleotide containing the p21 site and 125 ng of unlabeled competitor DNA (mutant GADD45 site) for 20 min at RT. The sequences of oligomers (mutant GADD45 site) are as follows: 5'-TAG AGC GAA AAT TTC TAA GAA TTC TGG CGT CG-3' and 5'-CGA CGC CAG AAT TCT TAG AAA TTT TCG CTC TA-3'. Reaction mixtures were layered onto and filtered through 25 mm nitrocellulose filters (Protran, Schleicher & Schuell, Keene, NH) under vacuum. Filters were washed 3 times with 5 mL of 25 mM Hepes buffer pH 7.9, dried and counted by liquid scintillation.

DNase I footprinting assay

The assay was performed essentially as described in Cain et al.[40] Briefly, a 405-bp HindIII-ScaI fragment containing the 5' p53 response element from the p21 promoter (5'-GAA CAT GTC CCA ACA TGT TG-3') was labeled with ^{32}P via a fill-in reaction using the large fragment Klenow of *E. coli* DNA polymerase I (New England BioLabs, Beverly, MA). Each reaction mixture contained DNA probe (100,000 cpm), 32.5 mM HEPES, pH 7.9, 6.25 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM KCl, 100 μg/mL bovine serum albumin, 0.05 mM EDTA, 5% glycerol, 0.025% Nonidet P-40, 0.5 mM spermidine, 5 ng/mL poly(dG·dC). Reaction mixtures (50 μL) containing indicating amounts of proteins were incubated for 40 min at room temperature, following which 50 μL of ice-cold DNase I digestion buffer (5 mM $CaCl_2$/10 mM $MgCl_2$) with 45 ng Dnase I (Worthington, Lakewood, NJ) was added to each mixture on ice. Reactions were terminated by the addition of 90 μL of Stop Solution (1% SDS, 20 mM EDTA, 200 mM KCl and 250 μg/mL yeast tRNA) after 2.5 min. DNA samples were then deproteinized by phenol/chloroform extraction, and loaded onto an 8% (w/v) polyacrylamide/8 M urea gel. Electrophoresis was performed at room temperature for 1 h at 25 mA after which the gels were dried and autoradiographed.

Cell culture condition and generation of HA-p53 inducible cell lines

H1299 cells (ATCC) were grown in RPMI medium supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO_2. Cell lines expressing inducible HA-p53 wild type or HA-H115N mutant p53 were generated as described in Chen et al.[41] Cells were selected and maintained with 5 μg/mL of puromycin (Invitrogen, Carlsbad, CA), 400 μg/mL of G418 (Invitrogen), and 2.5 μg/mL of tetracycline (Sigma, St. Louis, MO). Individual clones were screened for inducible expression of the p53 protein after removal of tetracycline by immunoblot analysis using anti-HA antibody. Clones, p6 and p15 were found to express wild-type p53 (W-1 and W-2, respectively for ease of reference) in a tetracycline dependent manner. Clones, H-6 and H-14 (H-1 and H-2, respectively for ease of reference) express H115N mutant p53 in a tetracycline dependent manner.

Cell cycle analysis

For cell cycle analysis, approximately 2×10^5 cells were seeded per 60-mm culture dish without or with tetracycline as indicated after washing the plates 3 times with medium lacking tetracycline. Twelve hours later medium was removed from cultures, and replaced with fresh medium with the appropriate amount of tetracycline (0–2.5 μg/mL). Twenty four hours later the cells were treated with a radiomimetic compound neocarzinostatin (NCS; 100 ng/mL, Kayaku Co., Tokyo, Japan). After an additional twenty four hours, the cells were trypsinized and then cells and media were pooled and centrifuged at 1,500 r.p.m for 5 min. The pellet was resuspended in 0.3 mL phosphate-buffered saline (PBS: 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, pH 7.5) and then fixed with 5 mL of ice-cold methanol for at least 1 h at −20°C. The fixed cells were then centrifuged and resuspended in 2 mL of cold PBS. After incubation at 4°C for at least 30 min, cells were centrifuged and resuspended in 0.5 mL of PBS solution containing RNase (50 μg/mL, Sigma) and propidium iodide (PI) (60 μg/mL, Sigma). After incubation at 20°C for 30 min, the stained cells were analyzed in a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). The percentages of cells in various phases of the cell cycle were determined using the ModFit LT program (Becton Dickinson, Franklin Lakes, NJ).

Immunoblotting

Cells were collected from 60 mm plates by trypsinization followed by centrifugation at 1,000 rpm. The cells were lysed with a buffer containing 50 mM HEPES, pH 7.8, 150 mM KCl, 10 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgSO_4$, 1 mM DTT, 1% NP-40, 0.25 mM PMSF, 60 nM

okadaic acid, 240 pM Cypermethrin, 1 mM NaF, 100 μM $NaVO_4$ and 20% glycerol. Extracts were cleared by centrifugation at 13,000 rpm for 30 min at 4°C. Extracts were separated using 8 or 12% SDS-PAGE gels and then transferred to nitrocellulose. Anti-HA antibody (Covance, Princeton, NJ) was used to detect p53 and anti-actin antibodies (Sigma) were used for loading control. The p21 monoclonal antibody, WAF1 (Ab-1), was obtained from Calbiochem (San Diego, CA). The PIG3 antibody was a generous gift from D Hill (Oncogene Research Products, Cambridge, MA). In the indicated experiments anti-mouse or anti-rabbit secondary antibodies conjugated to IRDye 800 or 680 (Licor) were incubated with membranes and detected by the Odyssey fluorescence system (Licor).

RT-PCR detection of p21 mRNA expression in H1299 cells

Total mRNA was isolated from H1299 cells expressing tetracycline regulated p53 after withdrawal of tetracycline using a RNeasy protection mini kit according to the protocol provided by the manufacturer (Qiagen, Valencia, CA). Shuichi Ohkubo kindly provided us with all primers for RT-PCR. To amplify cDNAs we used as forward primer, 5'-CTC TAA GGT TGG GCA GGG TG-3' and as reverse primer, 3'-GAA GAA GGG TAG CTG GGG CTC-5' that anneal to the p21 coding region. In a parallel experiment, for an internal control forward primer, 5'-CGA GAT CCC TCC AAA ATC AAG-3', and reverse primer 5'-ATC CAC ACT CTT CTG GGT GG-3' were used to amplify GAPDH mRNA. The PCR reactions were performed at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min for 25 cycle for GAPDH and 30 cycles for p21 using 1 μg of total cDNA. The PCR products were run on a 1.7% agarose gel and stained with ethidium bromide for visualization.

For qRT-PCR analysis RNA was harvested using a QIAGEN RNeasy Mini kit and first-strand cDNA synthesis was performed with the Superscript III Supermix for qRT-PCR kit (Invitrogen) according to the manufacturers' specifications. A "No RT" reaction, in which RNA was subjected to the conditions of cDNA synthesis without reverse transcriptase enzyme, was included as a negative control in all RT-PCR experiments to confirm the purity of RNA samples. Samples were analyzed by quantitative Real-Time PCR on an ABI 7300 Real-time PCR instrument. PCR reaction mixtures contained 2 ng of cDNA, 1X SYBR Green Mix (Applied Biosystems), and 100 nM primers. Values were normalized to those of the *hprt1* housekeeping gene. All primer sequences are available upon request.

Caspase-3 activity assays

Cells were collected from 100 mm plates and lysates were prepared as described above. Activity of caspase-3 in lysates was measured by using a ApoAlert™ Caspase colorimetric assay kit (BD Biosciences Clontech, San Jose, CA) according to the manufacturer's protocol. Briefly, cell lysates (100 μg) were incubated with a caspase-3 substrate (DEVD-pNA; 50 μM) at 37°C for 1 h. Samples were read at 405 nm in a microplate reader (Bio-Rad, Hercules, CA).

Abbreviations

NER	nucleotide excision repair
BER	base excision repair
HA-p53	N-terminally HA-tagged p53 protein
EMSA	electromobility shift assays
PBS	phosphate-buffered saline
SDS-PAGE	sodium dodacyl sulfate polyacrylamide gel electrophoresis
NCS	neocarzinostatin

FACS	fluorescent activated cell sorter

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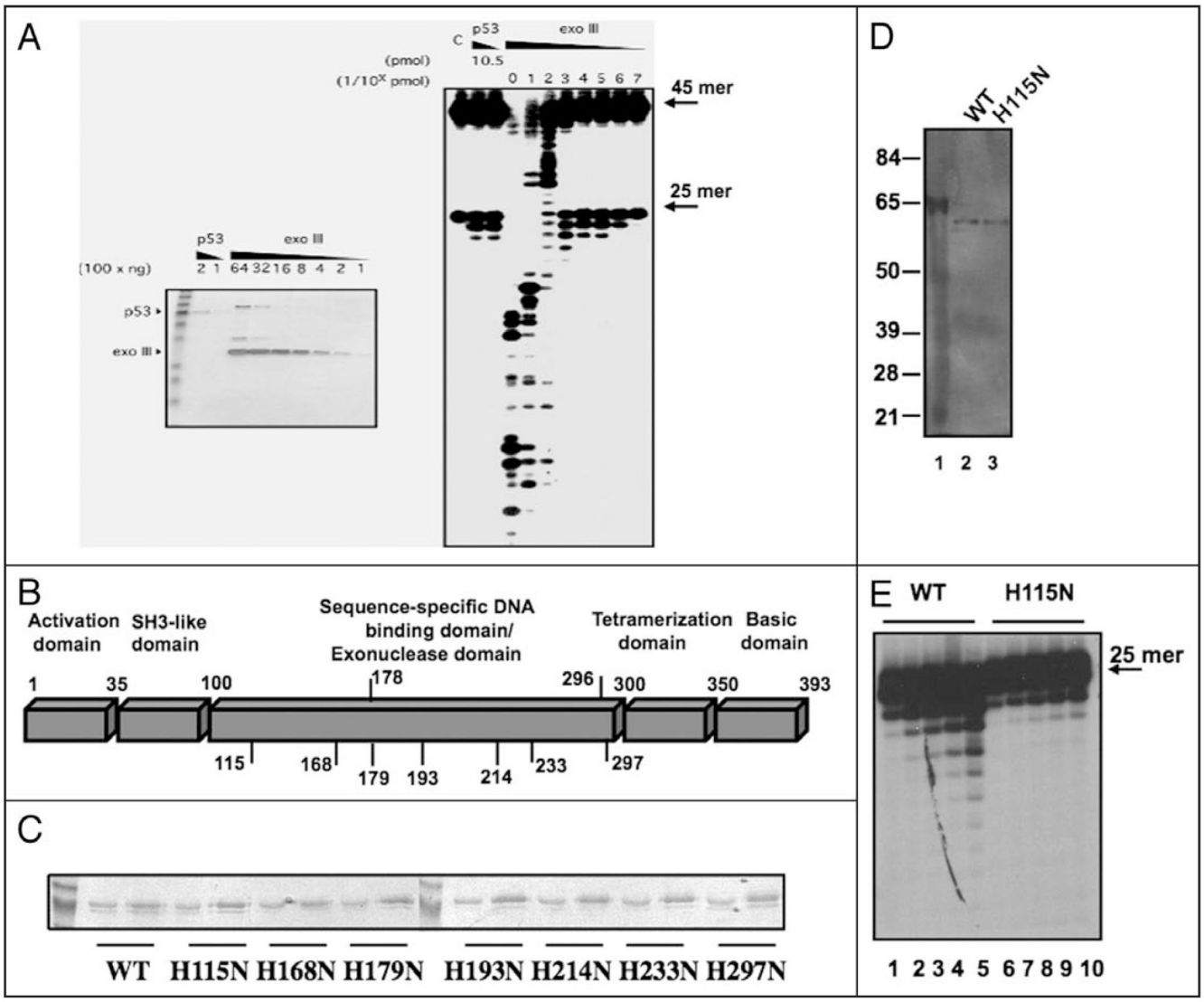

Figure 1. Exonuclease activities of wild-type and H115N mutant p53 proteins

(A) Comparison of exonuclease activity of wild-type p53 and exonuclease III. p53 (0.1, 0.2 μg) and exonuclease III (0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μg) were separated by 8% SDS-PAGE and visualized by Coomassie Blue staining (left). 2.5 pmol of 5'-end labeled DNA duplex with a recessed 3'-end was incubated with either p53 (5, 10 pmol) or Exonuclease III (1×10^{-7}–1 pmol) in an exonuclease activity assay buffer at 30°C for 30 min. The products formed were separated by 18% acrylamide/8 M urea gel and visualized by autoradiography (right). (B) Schematics of functional domains of p53 and His residues in the core domain. Functional domains of p53 are indicated above diagram. The positions of the 9 core His residues are indicated. H115, H168, H179, H214, H233 and H297 (below diagram) were mutated to Asn by site-directed mutagenesis. (C) Wild-type and seven His/Asn mutants were purified from *E. coli* using Ni-NTA agarose columns. Two different levels (200 and 400 ng) of proteins for each p53 variant were visualized by Coomassie Blue staining of an 8% SDS polyacrylamide gel. (D) Wild-type and H115N p53 proteins were purified from *E. coli* by using three subsequent Ni-NTA, Heparin and Superose 6 column chromatography steps. Wild type and H115N p53 proteins (50 ng) were visualized by 8% SDS-PAGE and silver-staining. (E) Wild-type and H115N proteins (10 pmol each) were incubated with 2.5 pmol of 5'-end labeled DNA

duplex with a recessed 3'end in an exonuclease assay buffer at 30°C for 10 min (lanes 1 and 6), 20 min (lanes 2 and 7), 30 min (lanes 3 and 8), 40 min (lanes 4 and 9) and 60 min (lanes 5 and 10). Reactions were stopped and the products formed were analyzed as described in (A).

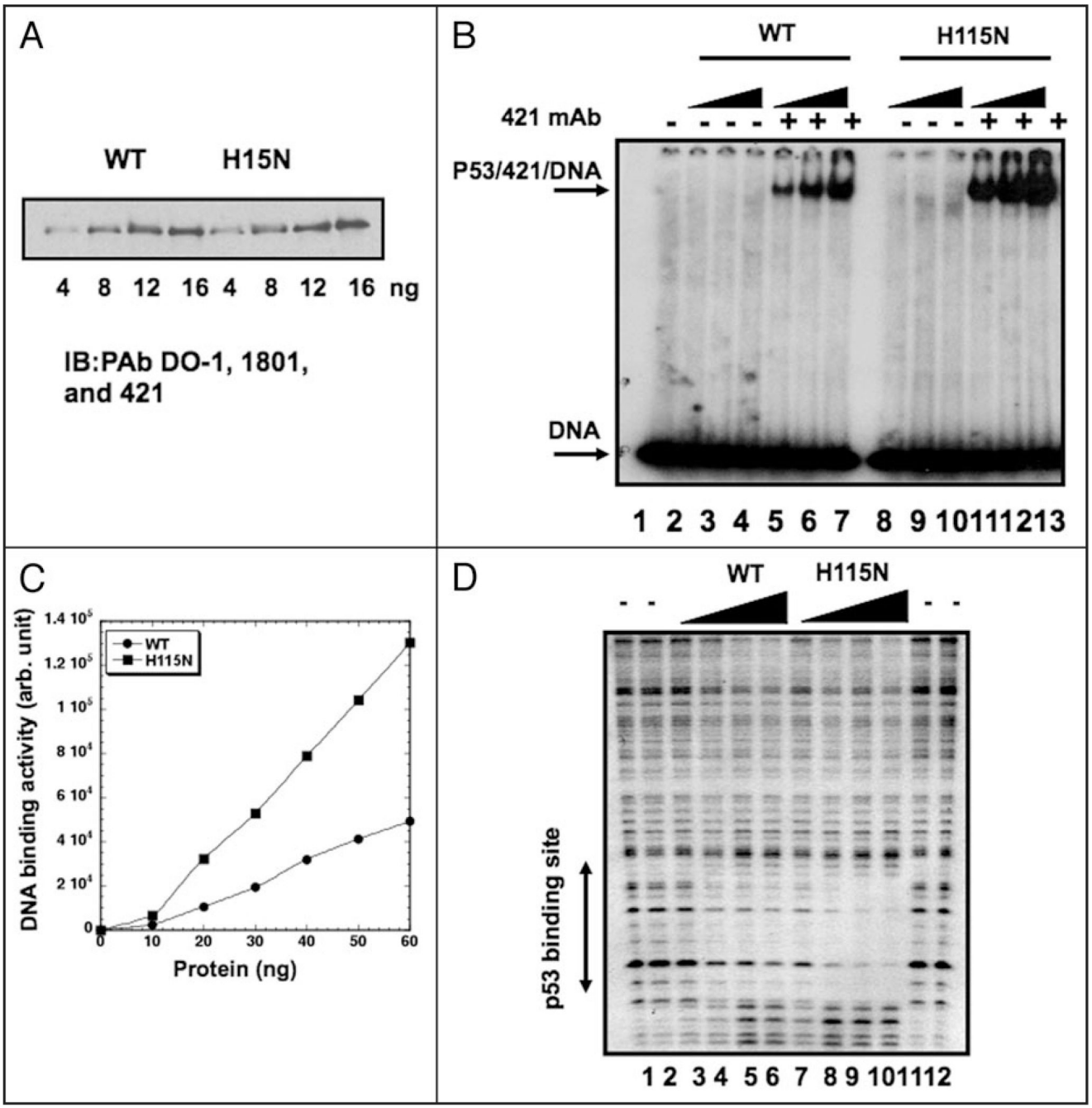

Figure 2. DNA binding by H115N p53 is greater than that of wild-type p53 as measured by EMSA, filter binding assay, and DNase I footprinting

(A) Wild-type p53 and H115N proteins were purified from bacteria by NI-NTA, Heparin and gel-filtration column chromatography as described in Experimental Procedures. The relative amounts of p53 proteins (4, 8, 12 and 16 ng) used in the following assays were detected by immunoblot analysis using mixture of p53 monoclonal antibodies PAb DO-1, 1801 and 421. (B) Wild-type (lanes 2–7) and H115N mutant (lanes 8–13) proteins (4, 8 and 12 ng each) were incubated with a 32 BP DNA duplex containing the 5' p21 promoter p53 binding site (2.5 ng) with PAb 421 (50 ng). DNA was resolved on a 4% polyacrylamide gel, dried and visualized by autoradiography. (C) Either wild-type (solid circles) or H115N (solid squares) p53 protein

(each at 0, 10, 20, 30, 40, 50 and 60 ng) was incubated with [^{32}P] labeled DNA duplex (2.5 ng) containing the p21 site and 125 ng of competitor DNA oligonucleotide containing a mutant GADD45 site and then filtered through nitrocellulose membrane. Labeled DNA bound to p53 protein was quantitated by liquid scintilation. (D) Wild-type p53 (100, 300, 500, 700 ng; lanes 3–6) or the same quantities of H115N p53 (lanes 7–10) were incubated with a 5'-end labeled 405 bp HindIII-ScaI fragment (approximately 25 ng) from the p21 promoter containing the 5' p53 binding site and subjected to DNAse I footprinting. Control digestion with DNase I was also performed (lanes 1 and 2, 11 and 12). The p53 binding site is indicated.

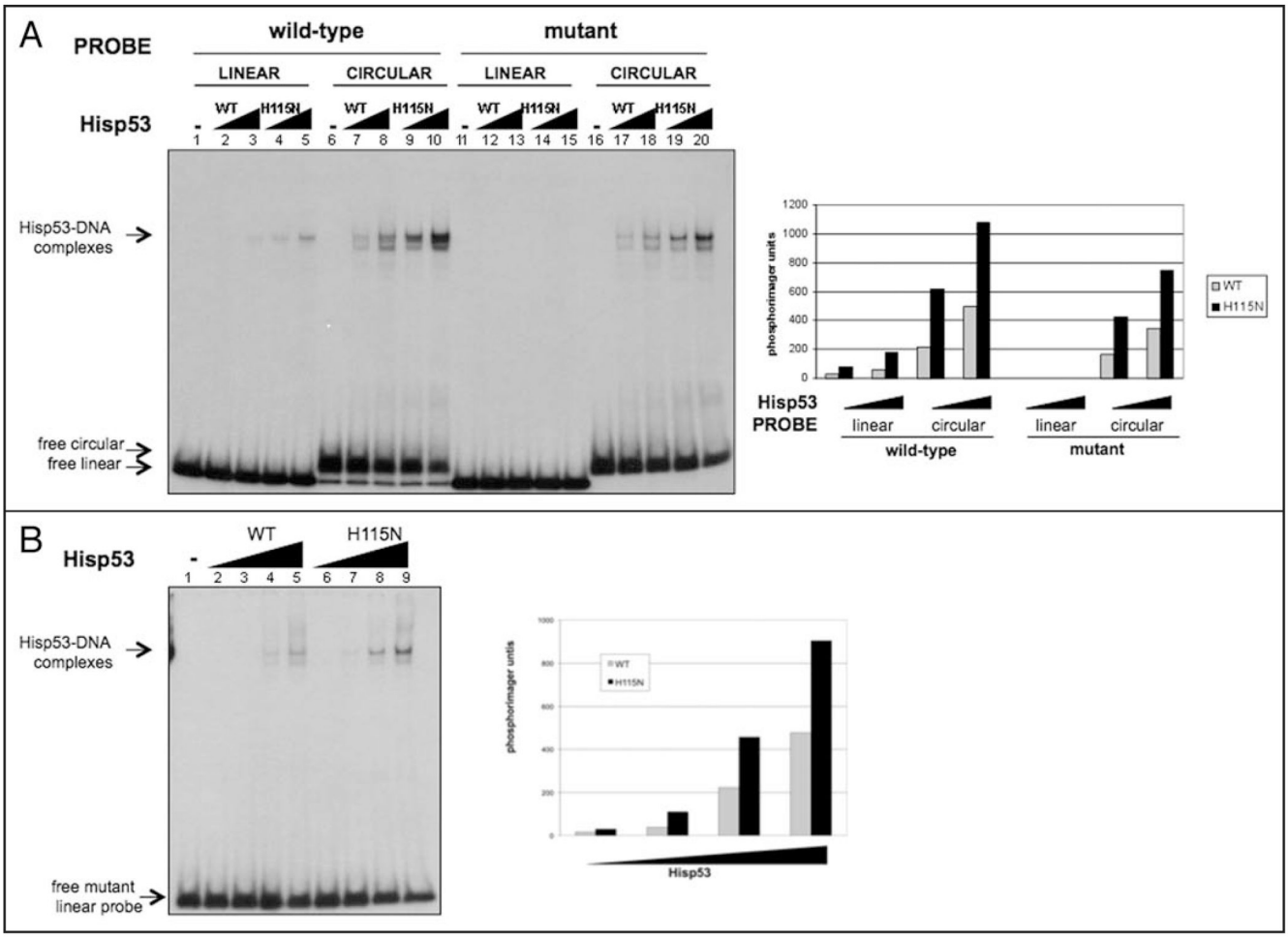

Figure 3. H115N p53 interacts with DNA more efficiently than wild-type p53 in a sequence- and structure-independent manner

(A) H115N p53 binds both unstructured (linear) and structured (circular) DNA more efficiently than wild-type p53. Wild-type p53 or H115N p53 (0.8 and 1.6 ng) were bound to either linear or circular 66 BP DNA fragments containing either wild-type or mutated GADD45 p53 binding sites as indicated followed by EMSA. (B) H115N p53 interacts with mutant binding site containing linear DNA more efficiently. Wild-type and H115N p53 protein levels (5, 10, 25 and 50 ng) were higher than in (A) in order to detect binding to a linear 66 bp oligonucleotide containing a mutated GADD45 binding site. p53: DNA complexes were quantified by Phosphorimaging and results are graphically represented at the bottom or right of EMSA autoradiograms in (A and B) (wild-type; open bars; and H15N; sold bars).

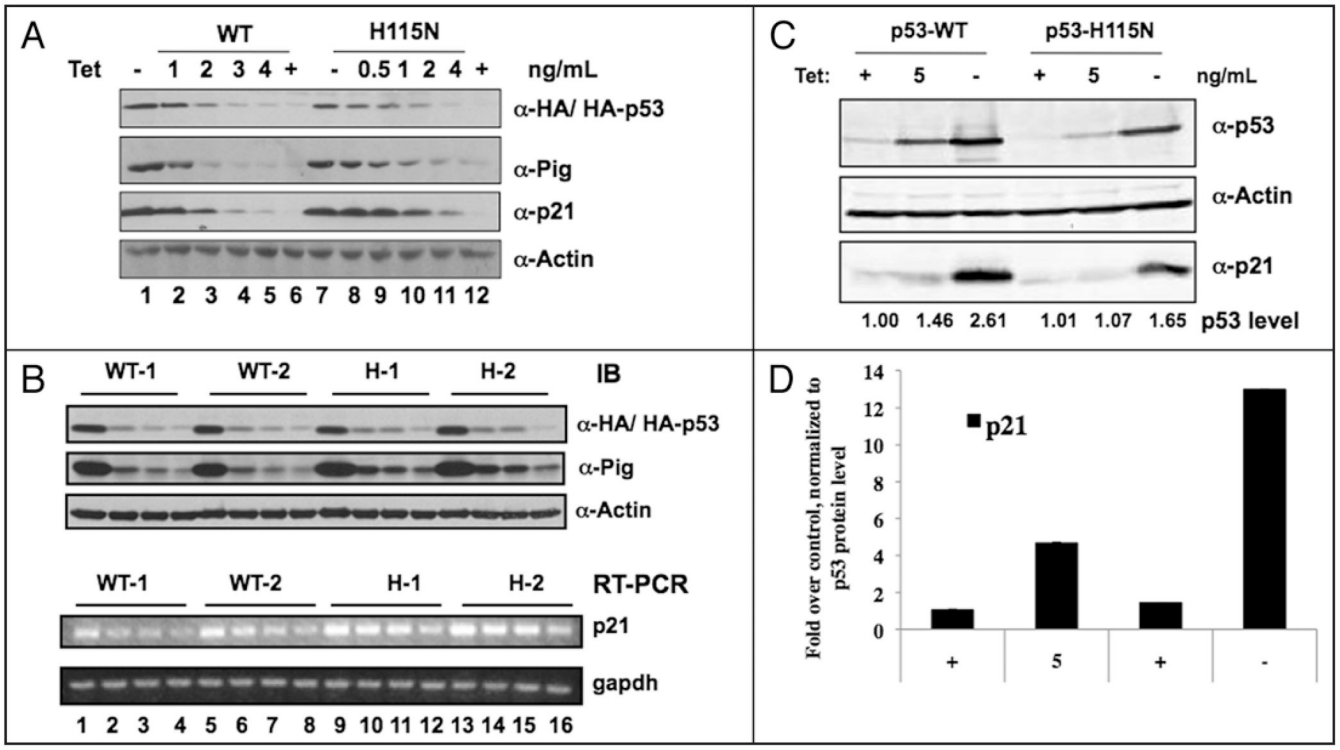

Figure 4. Induction of down-stream target genes and cell cycle arrest by wild-type p53 and H115N in H1299 cells

(A) Cultures maintained in medium containing 2,500 ng/mL tetracycline were switched to medium containing various tetracycline concentration as indicated above panel for 24 hours prior to cell lysis. Cell extracts (25 μg total protein) were resolved by SDS PAGE and subjected to immunoblotting for p53 (α-HA), PIG3 (α-Pig), p21 (α-p21) and as control actin (α-Actin). (B) Top (IB). Two separate clones of H1299 cells expressing wild-type p53 (W-1; used in A, and W-2; the second clone) or H115N p53 (H1; shown in A, and H2; the other clone) were maintained in medium containing tetracycline (2,500 ng/mL) prior to switching to medium containing 0 (lanes 1, 5, 9 and 13), 4 (lanes 2, 6, 10 and 14), 6 (lanes 3, 7, 11 and 15) or 8 (lanes 4, 8, 12 and 16) ng/mL tetracycline for 24 hours prior to lysis and immunoblotting for PIG3 and actin as in (A) but using 100 μg of total cell extracts. Botton (RT-PCR). Parallel cultures were lysed, and RNA extracted and levels of p21 and GAPDH mRNA were determined by RT-PCR as described in Experimental Procedures. (C) Tetracycline levels were regulated as indicated to express equivalent amounts of wild-type or mutant p53 protein as assayed by immunoblotting. Actin levels were visualized as a loading control. p53 levels were quantified using the Odyssey system (Licor). (D) Increase in p53-H115N transcriptional activity as quantified by q-PCR. RNA and cDNA were made from samples in (C) expressing roughly the same p53 levels (WT p53: 5 ng/uL tet (C, lane 2); H115N no tet (C, lane 6)) and subjected to q-PCR for the *p21* gene. Samples in which no p53 was expressed ("+ tet") were used as a control. Values from the PCR were normalized to the p53 levels quantified by the Odyssey system in (C).

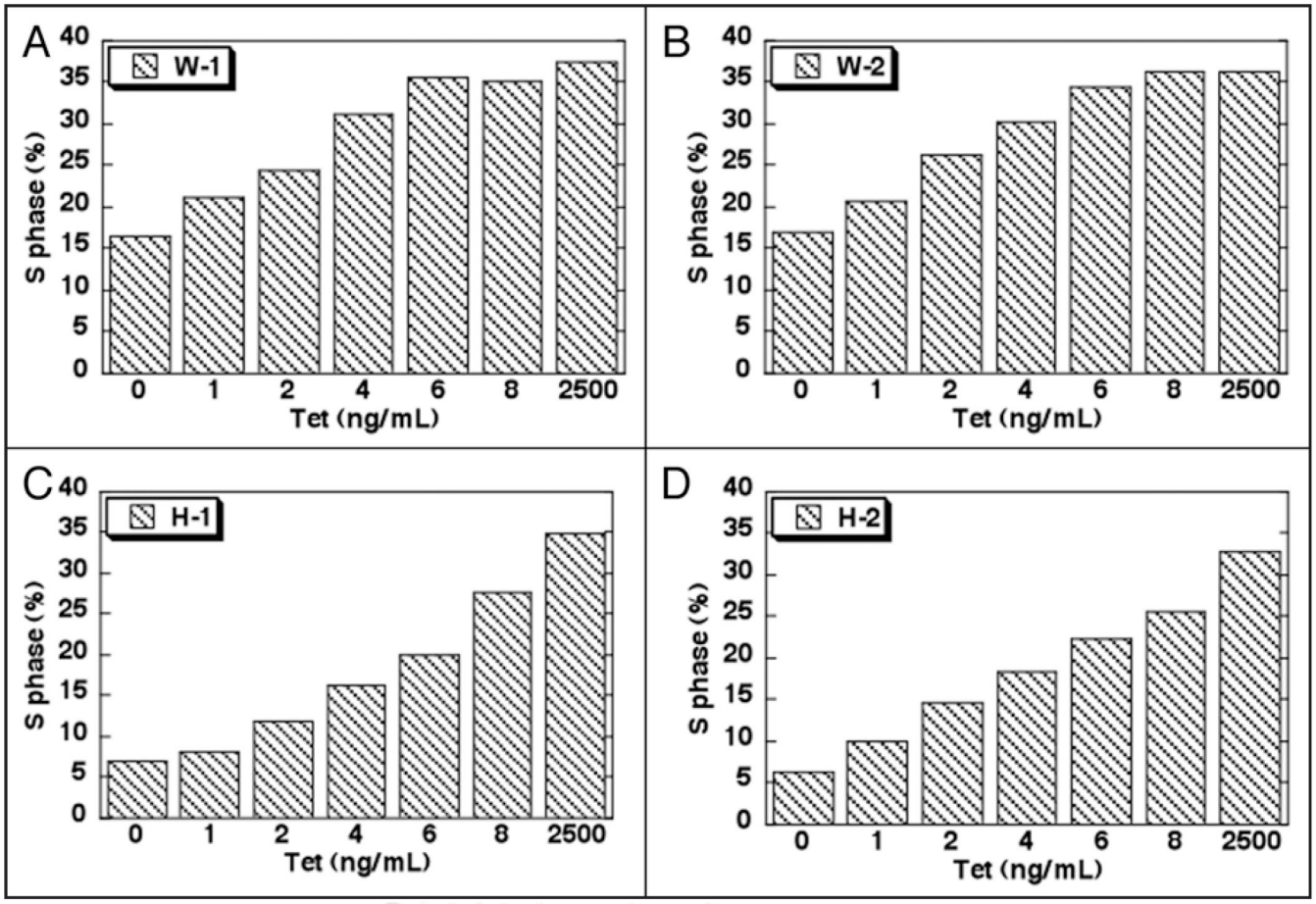

Figure 5. H115N is more potent in inducing cell cycle arrest than wild-type p53

H1299 clones expressing wild-type p53 (A; W-1, and B; W-2) or H115N mutant p53 (C; H-1 and D; H-2) as in Figure 4B were maintained in cultures containing 2,500 ng/mL tetracycline and then switched to medium containing the indicated amounts of tetracycline (0, 1, 2, 4, 6, 8, 2500 ng/mL) for 24 hours prior to analysis by FACS as described in Experimental Procedures. Shown are percent of cells in S phase determined for each culture. A representative cell cycle profile is shown in Supplemental Figures 1 and 2. These plots are representative data of three independent experiments.

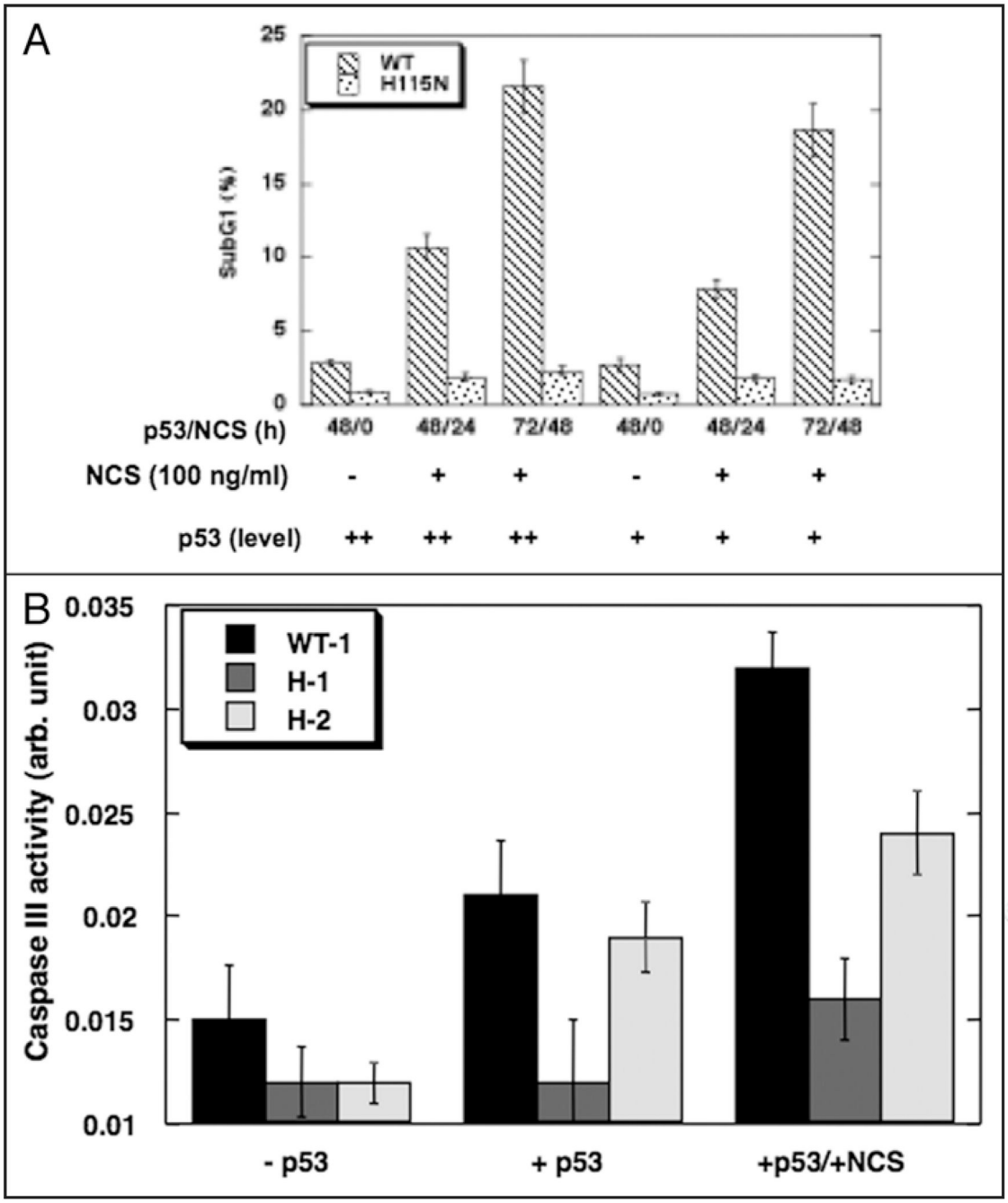

Figure 6. H115N fails to induce DNA damage facilitated apoptosis

(A) H1299 cells were maintained in medium containing 2,500 ng/mL tetracycline and then switched to medium containing 0 ng (++) or 1 ng/mL tetracycline to express low (+) or high (++) levels of wild-type p53 or H115N p53 indicated by striped or dotted bars respectively. After 24 hours cells were either untreated (−) or treated (+) with NCS (100 ng/mL) for an additional 24 h or 48 h before they were subjected to cell cycle analysis by FACS. These treatments are expressed as time during which cells expressed p53/time cells were either untreated (0) or treated (for 24 or 48 hours) with NCS prior to FACS analysis. Apoptotic cell populations were expressed as percent of cells with sub-G_1 content as determined by FACS analysis. (B) Wild-type p53 (WT-1, solid bars) or H115N (H-1; meshed bars, and H2; dotted

bars) was expressed in H1299 cells for 24 h by removing tetracycline from the medium. Cells expressing p53 proteins were treated (+p53/+NCS) with NCS (100 ng/mL) or untreated (+p53) for additional 24 h before they were collected for Caspase III assays. As a negative control, cells without p53 (−p53) were also collected and the activity of Caspase III in the lysate was measured.

Table 1

DNA binding and exonuclease activities of wild-type p53 and core His p53 mutants

p53	DNA binding activity (%)[a]	Exonuclease activity (%)[b]
WT	100	100
H115N	300	15
H168N	70	100
H179N	5	100
H193N	25	670
H214N	10	500
H233N	120	270
H297N	130	130

[a] DNA binding activities of p53 proteins were measured by performing EMSA with 4 ng of protein as described in Experimental Procedures. The relative DNA binding activities of His mutants are presented as %value of wild-type activities.

[b] Exonuclease activities of p53 proteins were measured by immunoprecipitation-exonuclease assays as described Experimental Procedures. Each p53 protein (200 ng) was immunoprecipitated with PAb1801 and the exonuclease activity of p53/antibody complex was measured. The relative exonuclease activities of His mutants are presented as % value of that of wild-type p53.