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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) <p>The p53 tumor suppressor protein is a sequence-specific DNA binding transcription factor that induces cell cycle arrest or apoptosis in response to DNA damage. We had previously demonstrated that ionizing radiation (IR) leads to association of p53 with 14-3-3 in breast cancer cells and hypothesized that this association activates the tumor suppressor function of p53. To test this hypothesis, we had proposed to determine whether the interaction of p53 with 14-3-3 affects:</p> <ol style="list-style-type: none"> 1. the sequence-specific DNA binding activity of p53 (months 1-12); 2. the cell cycle arrest and/or apoptotic functions of p53 (months 13-24); and 3. p53 intracellular localization and half-life (months 25-36). <p>During the first year of funding we showed that the interaction of p53 with 14-3-3 proteins does not affect p53 sequence-specific DNA binding activity in vivo (Task 1).</p> <p>During the second year of funding we showed that the interaction of p53 with 14-3-3 is critical for the ability of p53 to induce cell cycle arrest and established that 14-3-3 proteins regulate the transcriptional activity of p53 (Task 2).</p> <p>Overall, the results support the initial hypothesis that the interaction of p53 with 14-3-3 activates the function of p53.</p>				
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INTRODUCTION

The p53 tumor suppressor protein, a transcription factor for genes that induce cell cycle arrest or apoptosis, is a critical factor for the response of mammalian cells to DNA damage (Maltzman and Czyzyk, 1984; Kastan et al., 1991; Kuerbitz et al., 1992). DNA damage leads to increased p53 protein levels and increased p53 functional activity (Maltzman and Czyzyk, 1984; Kastan et al., 1991; Haapajarvi et al., 1997). The mechanism by which DNA damage increases p53 protein levels involves dissociation of p53 from Mdm2 (Shieh et al., 1997; Unger et al., 1999; Chehab et al., 1999), a protein that targets p53 for degradation (Haupt et al., 1997; Kubbutat et al., 1997; Midgley and Lane, 1997); whereas the mechanism by which DNA damage increases the functional activity of p53 has not been established. DNA damage leads to dephosphorylation of Ser376 of p53, which in turn leads to binding of p53 to 14-3-3 proteins (Waterman et al., 1998). The subject of the USARMRC-funded research is to establish whether the binding of p53 to 14-3-3 proteins underlies the increase in p53 functional activity after DNA damage. Because DNA damaging agents are used to treat breast cancer and because their efficacy depends on their ability to activate p53 (Fisher, 1994), understanding the molecular mechanism by which p53 function is upregulated after DNA damage will help the development of novel therapies to treat this disease.

BODY

The Tasks outlined in the approved Statement of Work for the entire research funding period are:

1. Determine whether the interaction of p53 with 14-3-3 affects the sequence-specific DNA binding activity of p53 (months 1-12);
2. Determine whether the interaction of p53 with 14-3-3 modulates the cell cycle arrest and/or apoptotic functions of p53 (months 13-24); and
3. Determine whether the interaction of p53 with 14-3-3 affects p53 intracellular localization and half-life (months 25-36).

During the first year of funding we addressed Task 1. We showed that the association of p53 with 14-3-3 proteins does not affect the sequence-specific DNA binding activity of p53 isolated from cells. This result was unexpected, because 14-3-3 enhances the DNA binding activity of recombinant p53 expressed in bacteria. Nevertheless, we are now confident that 14-3-3 proteins do not enhance the DNA binding activity of p53 in vivo, because in another study we did that looks at the effects of acetylation on p53 DNA binding we obtained similar results, i.e. that acetylation of Lys382 of p53 enhances the DNA binding activity of p53 expressed in bacteria, but did not affect DNA binding of p53 in vivo.

During the second year of funding we addressed Task 2. We showed that the interaction of p53 with 14-3-3 proteins is required for p53 to induce cell cycle arrest. These experiments are described in detail in the accompanying manuscript (Stavridi et al., 2001), which has been submitted for publication to the journal "Cancer Research". The results pertaining to the cell cycle arrest properties of wild-type p53 and the p53 mutants that fail to interact with 14-3-3 proteins are shown in Figure 5 of the manuscript (Stavridi et al., 2001). We also wanted to study apoptosis, but unfortunately in our experimental system wild-type p53 did not induce apoptosis. Nevertheless, we do not consider that our inability to study apoptosis compromises the study, because it is now well accepted that p53 mutants that are defective in cell cycle arrest are also defective in apoptosis. The reverse does not hold true. That is, p53 mutants that are defective in apoptosis may not necessarily be defective for cell cycle arrest. Thus, if we had not observed a cell cycle arrest defect, it would have been important to examine the apoptotic function of the p53 mutants.

Our initial hypothesis was that the association of p53 with 14-3-3 proteins is important for p53 function. This hypothesis is confirmed by our findings showing that p53 mutants that do not interact with 14-3-3 proteins are defective in cell cycle arrest. However, we anticipated that the mechanism by which 14-3-3 proteins enhance p53 function would involve enhancement of the sequence-specific DNA binding activity of p53. As described above (Task 1), this is not so. We therefore performed experiments to address the mechanism. We found that 14-3-3 proteins enhance the transcriptional activity of p53. The experiments are described in the appended manuscript and the results that pertain to p53 transcriptional activity are shown in Figure 4 of the manuscript (Stavridi et al., 2001).

Having completed Tasks 1 and 2, we are well positioned to address Task 3 in the final year of support and expect to complete this Task, as originally proposed.

KEY RESEARCH ACCOMPLISHMENTS

We have constructed a panel of p53 mutants which are defective in their ability to interact with 14-3-3 proteins.

We have expressed these p53 mutants in a variety of cancer cells lines of breast and non-breast origin and studied their DNA binding properties and their ability to activate transcription and induce cell cycle arrest in response to DNA damage.

REPORTABLE OUTCOMES

We submitted a manuscript describing our findings to the journal "Cancer Research". A copy of this manuscript, which lists the support provided by the DOD, is appended.

We have developed plasmids directing the expression of mutant p53 proteins that do not associate with 14-3-3 proteins in breast cancer cell lines.

CONCLUSIONS

Our key conclusions can be summarized as follows:

1. The interaction of p53 with 14-3-3 proteins does not affect the sequence-specific DNA binding activity of p53 when assayed using cell extracts.
2. The interaction of p53 with 14-3-3 proteins is required for the ability of p53 to induce cell cycle arrest in G1 in response to DNA damage.
3. The interaction of p53 with 14-3-3 proteins is required for the ability of p53 to activate transcription of its target genes.

The second and third conclusions support our hypothesis that the interaction of p53 with 14-3-3 proteins is important for the functional activity of p53. These findings are therefore important, because they suggest that modulating the interaction of p53 with 14-3-3 proteins is a viable mechanism to regulate p53 activity in patients with breast cancer. Augmenting p53 function in patients with breast cancer could have a therapeutic effect equivalent to the one currently obtained using DNA damaging agents, but without the toxicity of the latter.

REFERENCES

- Chehab,N.H., Malikzay,A., Stavridi,E.S. and Halazonetis,T.D. (1999) Phosphorylation of Ser20 mediates stabilization of human p53 in response to DNA damage. *Proc. Natl. Acad. Sci. USA*, 96, 13777–13782.
- Fisher, D.E. (1994) Apoptosis in cancer therapy: crossing the threshold. *Cell*, 78, 539–542.
- Haapajarvi,T., Pitkanen,K., Tsubari,M. and Laiho,M. (1997) p53 transactivation and protein accumulation are independently regulated by UV light in different phases of the cell cycle. *Mol. Cell. Biol.*, 17, 3074–3080.
- Haupt,Y., Maya,R., Kazaz,A. and Oren,M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature*, 387, 296–299.
- Kastan,M.B., Onyekwere,O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, 51, 6304–6311.
- Kubbutat,M.H., Jones,S.N. and Vousden,K.H. (1997) Regulation of p53 stability by Mdm2. *Nature*, 387, 299–303.
- Kuerbitz,S.J., Plunkett,B.S., Walsh,W.V. and Kastan,M.B. (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl Acad. Sci. USA*, 89, 7491–7495.
- Maltzman,W. and Czyzyk,L. (1984) UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol. Cell. Biol.*, 4, 1689–1694.
- Midgley,C.A. and Lane,D.P. (1997) p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. *Oncogene*, 15, 1179–1189.
- Shieh,S.Y., Ikeda,M., Taya,Y. and Prives,C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, 91, 325–334.
- Stavridi,E.S., Chehab,N.H., Malikzay,A. and Halazonetis,T.D. (2001) The ionizing radiation-induced association of p53 with 14–3–3 proteins facilitates the ability of p53 to activate transcription and induce cell cycle arrest. *Cancer Research*, submitted.
- Unger,T., Juven-Gershon,T., Moallem,E., Berger,M., Sionov,R.V., Lozano,G., Oren,M. and Haupt,Y. (1999) Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J.*, 18, 1805–1814.
- Waterman,M.J., Stavridi,E.S., Waterman,J.L. and Halazonetis,T.D. (1998) ATM-dependent activation of p53 involves dephosphorylation and association with 14–3–3 proteins. *Nature Genet.*, 19, 175–178.

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The ionizing radiation-induced association of p53 with 14-3-3 proteins facilitates the ability of p53 to activate transcription and induce cell cycle arrest¹

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Abstract

The p53 tumor suppressor protein is an important participant in the response of mammalian cells to ionizing radiation (IR). Activation of p53 by IR involves an increase in p53 protein levels and functional activity. The increased functional activity is attributed to IR-induced post-translational modifications, but experimental evidence linking specific post-translational modifications to functional activation of p53 is mostly lacking. One of the IR-induced p53 modifications is dephosphorylation of Ser376, which leads to association of p53 with 14-3-3 proteins. To establish the significance of this interaction, we examined the function of mutant p53 proteins that do not interact with 14-3-3 proteins *in vivo*. These mutant p53 proteins retained sequence-specific DNA binding activity and transactivated reporter plasmids as efficiently as wild-type p53. However, they were defective in activation of transcription of the endogenous p21/waf1/cip1 gene and induction of G1 arrest. Thus, dephosphorylation of Ser376 and the association of p53 with 14-3-3 proteins contribute to functional activation of p53 in cells exposed to IR.

Introduction

The integrity of the genome is subject to constant challenge by DNA damaging agents. The response of cells to ionizing radiation (IR) is of significant importance, because IR induces DNA double-strand breaks (DSBs), which are considered to be one of the most difficult lesions to repair. IR activates a DNA damage checkpoint response, which facilitates DNA repair by activating expression of a ribonucleotide reductase subunit and by inducing cell cycle arrest. Under certain circumstances, such as when DNA damage is deemed to be unreparable, the DNA damage checkpoint induces programmed cell death (Zhou and Elledge, 2000).

The p53 tumor suppressor protein is a critical component of the DNA damage checkpoint machinery (Vogelstein et al., 2000). p53 is a transcription factor for genes that facilitate DNA repair or induce cell cycle arrest or apoptosis in response to DNA damage. The p53-regulated genes that induce cell cycle arrest include p21/cip1/waf1, whose protein product inhibits the cyclin-dependent kinases cdk2 and cdc2, and 14-3-3 sigma, a 14-3-3 isoform, whose protein product induces arrest in G2 by inhibiting accumulation of cyclin B1 and cdc2 in the nucleus (El-Deiry et al., 1993; Hermeking et al., 1997; Chan et al., 1999).

The p53-regulated genes that induce apoptosis include bax, fas/apo-1, killer/dr5, and probably many others (Miyashita and Reed, 1995; Owen-Schaub et al., 1995; Wu et al., 1997; Brodsky et al., 2000), whereas DNA repair is facilitated by increased expression of a DNA damage-inducible isoform of the small subunit of ribonucleotide reductase (Tanaka et al., 2000).

The mechanisms by which DNA damaging agents activate p53 are being intensely studied. It appears that most DNA damaging agents, including IR, lead to post-translational modifications of p53, which have been shown to or are presumed to regulate p53 function. Some of these modifications are induced by most, if not all, DNA damaging agents, while others are induced by specific DNA damaging agents. One of the modifications induced by most DNA damaging agents is phosphorylation of p53 on Ser20 (Chehab et al., 1999; Unger et al., 1999a; Shieh et al., 1999). This modification leads to increased p53 protein levels by inducing dissociation of p53 from Mdm2 (Chehab et al. 1999; 2000; Hirao et al., 2000), a protein that targets p53 for degradation through the ubiquitin pathway (Haupt et al. 1997; Kubbutat et al. 1997; Midgley and Lane 1997). Other modifications induced in response to DNA damage include phosphorylation of Ser6, Ser9, Ser15, Ser33, Ser37, Ser46, Ser392, dephosphorylation of Ser376 and acetylation of Lys320, Lys373 and Lys382 (Giaccia and Kastan, 1998; Vogelstein et al., 2000). The functional significance of most of these modifications is unclear. The exceptions include phosphorylation of Ser15 and Ser46, which appear to contribute to transactivation of p53-target genes and induction of cell cycle arrest and apoptosis (Dumaz and Meek, 1999; Unger et al., 1999b; Chao et al., 2000; Oda et al., 2000).

One of the modifications, whose functional significance is unclear, is dephosphorylation of Ser376 of p53. This modification creates a binding site for 14-3-3 proteins and leads to association of p53 with 14-3-3 (Waterman et al., 1998). In vitro, 14-3-3 proteins enhance the sequence-specific DNA binding activity of p53, but in vivo the functional significance of this interaction is not known. In addition, it would be important to define which 14-3-3 isoforms bind to p53. Specifically, if 14-3-3 sigma binds to p53 and enhances its activity, then

p53 activation by IR would involve a positive-feedback loop, since p53 activates transcription of the gene encoding 14-3-3 sigma (Hermeking et al., 1997). Here, we address the functional significance of the interaction of p53 with 14-3-3 proteins and explore which of the 14-3-3 isoforms interact with p53 in response to DNA damage.

Materials and Methods

Interaction of p53 with 14-3-3 in vitro. Expressed sequence tag (EST) clones containing the seven known human 14-3-3 genes were obtained from Research Genetics (Huntsville, AL). The coding sequences were subcloned in the pGEX4T1 expression vector and the corresponding GST/14-3-3 fusion proteins were expressed in *E. coli* and bound to glutathione sepharose 4B beads (Pharmacia, Piscataway, NJ) in 1X IP buffer (25 mM Hepes [pH 7.4], 100 mM NaCl, 5 mM MgCl₂, 100 mM EDTA, 200 ng/ml BSA, 0.1% Tween 20). Unbound proteins were washed off and the beads were incubated with ³⁵S-labeled in vitro translated p53 (Waterman et al., 1996) for 1 hr at 4°C. The beads were then washed 3 times with 1x IP buffer and the ³⁵S-labeled proteins bound to the beads were subjected to SDS-PAGE and visualized by autoradiography.

Interaction of p53 with 14-3-3 in vivo. U2OS osteosarcoma cells were either mock-irradiated or exposed to 9 Gy IR or 50 J/m² UV light. Whole cell extracts were prepared 2 hr after exposure to IR or 16 hr after exposure to UV light by lysis in 1x extraction buffer (50 mM Tris [pH 8], 120 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.4 mg/ml Pefabloc SC, 2 mg/ml pepstatin, 1 μM wortmannin, 0.1 μM

staurosporine, 15 mM NaF, 1 mM sodium vanadate). 14-3-3 was precipitated using isoform-specific antibodies or antibody K19, which recognizes all 14-3-3 isoforms (Santa Cruz Biotech, Santa Cruz, CA). Coprecipitated p53 was detected by immunoblotting with antibody DO7 (Calbiochem, San Diego, CA). The interaction of HA-tagged p53IND proteins with endogenous 14-3-3 was performed as described above using U2OS cells transiently transfected with 2.5 μ g of plasmids encoding the various p53IND proteins and 27.5 μ g of pBC12/PLseap carrier plasmid (Chehab et al., 1999). Coprecipitated HA-tagged p53IND was detected with antibody Y11 (Santa Cruz Biotech, Santa Cruz, CA), which recognizes the HA tag.

DNA binding assay. U2OS cells were transfected with 2.5 μ g of plasmids encoding the various p53IND proteins and 27.5 μ g of pBC12/PLseap carrier plasmid (Chehab et al., 1999). 24 hr after transfection, the cells were exposed to 9 Gy IR or were mock-irradiated and a further 1 hr later the cells were lysed using 1x extraction buffer. Oligonucleotides BCV4A and TT3 (Waterman et al., 1995) with biotin tags at their 5-prime ends were coupled to streptavidin-agarose beads and incubated with the cell lysates for 1 hr at 4°C in 1x extraction buffer containing a single-stranded oligonucleotide, as non-specific competitor DNA (Waterman et al., 1995). After washing the beads, bound HA-tagged p53 was detected by immunoblotting with antibody Y11.

Transcription activation assays. Saos2 cells were transfected by calcium phosphate precipitation with 2.5 μ g of a plasmid expressing various p53 proteins with C-terminal amino acid substitutions targeting Ser376, Thr377 and Ser378 and 27.5 μ g of pBC12/PLseap carrier plasmid (Chehab et al., 1999). 24 hr after transfection, the cells were exposed to 9 Gy IR and a further 24 hr later the cells

were washed twice with PBS and lysed by scraping in 0.5 mls 2X RIPA buffer (40 mM Tris [pH 7.4], 2 mM EDTA, 300 mM NaCl, 20 mM KCl, 2% NP-40, 0.2% Triton-X and 0.2% SDS). p21/cip1/waf1 protein levels were monitored by immunoblotting using a specific monoclonal antibody (Calbiochem, San Diego, CA). Alternatively, 1 μ g of the plasmids expressing the various p53 mutants were transfected with 29 μ g of the p53-specific reporter plasmid pEp21-TK-SEAP. Alkaline phosphatase activity was determined 48 hr later, as previously described (Liu et al., 1999).

Cell cycle arrest. U2OS osteosarcoma cells were transfected by calcium phosphate precipitation with 2.5 μ g of a plasmid expressing various p53IND proteins, 5 μ g of a plasmid expressing a dominant negative p53 mutant (p53Trp248), 1 μ g of a plasmid expressing GFP (as a marker) and 24 μ g of pBC12/PLseap carrier plasmid (Chehab et al., 1999; 2000). 24 hr after transfection, the cells were exposed to 5 Gy IR or were mock-irradiated. The cells were trypsinized 12 hr later, washed once with PBS supplemented with 1% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY), resuspended in 200 μ l of 0.4% paraformaldehyde in PBS and incubated for 12 minutes at 37°C and subsequently for 10 min on ice. The fixed cells were overlaid with 1800 μ l of cold (-20°C) methanol with gentle vortexing. After a 10 min incubation on ice, the cells were washed in 1X PBS-TF (PBS with 0.1% Tween20 and 2% FBS) and incubated in 1 ml PBS-TF containing 20 μ l RNase (GIBCO BRL, Grand Island, NY) and 10 μ l propidium iodide (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 37°C. Flow cytometry analysis was performed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Results

14-3-3 isoform specificity

We previously showed binding of p53 to 14-3-3 proteins after irradiation, but we did not explore which 14-3-3 isoforms bind to p53. We therefore addressed this question here both in vitro and in vivo. The in vitro studies examined the seven known human genes encoding 14-3-3 isoforms. We generated plasmid vectors that allowed each 14-3-3 gene product to be expressed as a glutathione-S-transferase (GST) fusion protein; the GST/14-3-3 fusion proteins were immobilized on glutathione beads and examined for their ability to capture full-length wild-type p53. As a negative control, we used a p53 protein bearing three amino acid substitutions (Ser376 to Ala, Thr377 to Ala and Ser378 to Ala) targeting the 14-3-3 binding site in p53. ³⁵S-labeled wild-type p53 translated in vitro was captured specifically by the GST/14-3-3 gamma isoform, whereas the negative control p53 protein was not captured by any of the GST/14-3-3 fusion proteins (Fig. 1A).

In vivo, the interaction between p53 and 14-3-3 isoforms was examined by coimmunoprecipitation analysis of the endogenous wild-type p53 and 14-3-3 proteins. We used commercially available 14-3-3 isoform-specific antibodies, whose specificity was verified by immunoblotting GST/14-3-3 isoforms expressed in *E. coli* (Fig. 1B). p53 and 14-3-3 interact in cells exposed to IR, but not in non-irradiated cells or in cells exposed to UV light (Chehab et al., 2000). We therefore prepared lysates from U2OS osteosarcoma cells that were either exposed to IR or UV light or were mock-irradiated. The gamma, epsilon and tau isoforms exhibited an IR-specific interaction with wild-type p53, whereas no interaction could be

detected with the sigma and zeta isoforms (Fig. 1C). Thus, both in vivo and in vitro p53 interacts with specific 14-3-3 isoforms.

Functional significance of the p53/14-3-3 interaction

To examine the functional significance of the p53/14-3-3 interaction, we studied a panel of p53 mutants with substitutions targeting the 14-3-3 binding site within the C-terminus of p53. The panel consisted of p53 mutants with single substitutions of Ser376 to Ala (A376), Thr377 to Ala (A377) or Ser378 to Ala (A378) and a p53 mutant with all these three amino acid substitutions (A376-8). These mutants were selected because the interaction between p53 and 14-3-3 in vitro and in vivo is regulated by the phosphorylation states of serines 376 and 378 (Waterman et al., 1998).

The functional properties of the C-terminal p53 mutants were first examined in U2OS osteosarcoma cells, which provide a very good experimental system to study p53 activation in response to DNA damage and the effect of activated p53 on cell cycle progression (Waterman et al., 1998; Chehab et al., 1999; 2000). Because these cells express wild-type p53, the C-terminal p53 mutants were modified in two ways (Fig. 2A): first, an N-terminal hemagglutinin (HA) tag was inserted to distinguish them from endogenous p53; and second, the tetramerization domain was modified by introducing seven amino acid substitutions. The modified domain, hereafter referred to as IND (independent), allows the C-terminal p53 mutants to form tetramers, but prevents hetero-oligomerization with endogenous p53 (Stavridi et al., 1999).

The p53 C-terminal mutants were expressed transiently in U2OS cells and their level of expression and interaction with endogenous 14-3-3 proteins was characterized by immunoblotting and coimmunoprecipitation assays, respectively. Transfection with 0.1 µg plasmid DNA encoding HA-tagged p53IND with a wild-type C-terminus (p53INDwt) or the C-terminal substitutions that are the focus of this study (p53INDA376, A377, A378, A376-8) leads to low levels of p53 protein expression, which increase significantly after exposure of the cells to IR or UV light (Chehab et al., 1999 and data not shown). The DNA damage-induced p53 stabilization was a handicap for this study, which focuses on regulation of p53 functional activity. We found that transfecting the cells with 2.5 µg plasmid DNA led to higher levels of p53 protein, which did not increase further in response to DNA damage (Fig. 2B). Thus, under these conditions we could study the effects of DNA damage on p53 functional activity independent of its effects on p53 protein levels.

The ectopically-expressed p53INDwt protein behaved similarly to endogenous wild-type p53 in that it interacted with 14-3-3 proteins only in cells exposed to IR (Fig. 2C). The C-terminal substitutions had no effect on protein expression levels (Fig. 2D), but, as expected, some of the substitutions interfered with the ability of p53 to interact with 14-3-3 proteins. Specifically, substitution of Ser376 abrogated the interaction, substitution of Thr377 had no effect and substitution of Ser378 weakened, but did not abrogate, the interaction of p53 with 14-3-3 (Fig. 2E). Thus, the panel of p53 mutants bearing these substitutions could be used to explore the role of the p53/14-3-3 interaction on the ability of p53 to bind DNA, activate transcription and induce cell cycle arrest.

Sequence-specific DNA binding was examined by transfecting the panel of p53 C-terminal mutants in U2OS cells, preparing cell lysates and analyzing the DNA binding activities of the ectopically-expressed p53 proteins in these lysates. The lysates were prepared 1 hr after exposure of the cells to IR or from mock-irradiated cells. The HA-tagged p53IND proteins were examined for their ability to bind to beads coated with oligonucleotides containing the specific p53 DNA binding site or a non-specific DNA site. p53INDwt bound to beads coated with the specific oligonucleotide, but not to beads coated with the non-specific oligonucleotide, establishing the sequence-specificity of the assay (Fig. 3A). Exposure of the cells to IR did not affect the sequence-specific DNA binding activity. Furthermore, the p53IND proteins with mutant 14-3-3 binding sites bound the specific DNA as efficiently as p53INDwt (Fig. 3B). Thus, the association of p53 with 14-3-3 proteins did not affect the sequence-specific DNA binding activity of p53 in this assay.

We subsequently examined the ability of the p53 C-terminal mutants to activate expression of the endogenous p21/cip1/waf1 gene. For these studies, expression of the endogenous wild-type p53 protein in U2OS cells complicated the analysis, since only a subset of the transfected cells actually expressed the p53 C-terminal mutants and both the endogenous and ectopically-expressed p53 could contribute to p21/cip1/waf1 expression. We therefore decided to pursue analysis of the transcriptional activity of the p53 C-terminal mutants in Saos2 osteosarcoma cells, which do not express endogenous p53. The pattern of interaction of these mutants with endogenous 14-3-3 in irradiated Saos2 cells was essentially the same as described above for U2OS cells (Fig. 4A). Interestingly, the ability of the mutants to activate transcription of the endogenous p21/cip1/waf1 gene correlated with their ability to interact with 14-3-3 proteins indicating that

in this assay the association of p53 with 14-3-3 proteins enhances the transcriptional activity of p53 (Fig. 4B). Interestingly, however, all p53 C-terminal mutants activated transcription from a reporter plasmid (Fig. 4C). Thus, it appears that the interaction of p53 with 14-3-3 is needed for p53 to activate transcription from chromatin templates, but not from naked DNA.

The ability of the p53 C-terminal mutants to induce cell cycle arrest was studied in U2OS osteosarcoma cells, which are better characterized than Saos2 cells. The research design involved co-expressing in transiently-transfected cells green fluorescent protein (GFP) to mark the efficiently-transfected cells; a dominant negative tumor-derived p53 mutant to inactivate endogenous wild-type p53 and one of the p53IND proteins described above. Parental (non-transfected) U2OS cells arrested predominantly in G2 after exposure to IR, whereas cells expressing p53INDwt showed a significant fraction of cells arresting in G1 (Fig. 5). The C-terminal mutants that did not interact with 14-3-3 proteins were unable to induce G1 arrest; p53INDA378, which interacted weakly with 14-3-3 proteins, induced partial G1 arrest; and p53INDA377, which interacted strongly with 14-3-3 proteins, induced G1 arrest as efficiently as p53INDwt (Fig. 5). Thus, the ability of the mutants to induce cell cycle arrest correlated with their ability to induce expression of p21/cip1/waf1.

Discussion

The first question that we addressed in this study is that of 14-3-3 isoform specificity in the interaction between p53 and 14-3-3. There are seven distinct genes encoding 14-3-3 isoforms in mammals and these are highly related to each

other at the amino acid sequence level. Yet, despite the high similarity, the isoforms differ significantly in their affinity for specific ligands (Rosenquist et al., 2000). Our analysis further illustrates that 14-3-3 isoforms have distinct ligand specificities. In vitro the gamma isoform displayed significantly higher affinity for p53 than the other isoforms. In vivo, we observed an interaction of p53 with 14-3-3 gamma, but also with isoforms epsilon and tau. The difference between the in vitro and in vivo results may be due to differences in p53 or 14-3-3 post-translational modifications, differences in the relative abundances of the 14-3-3 isoforms in vivo and/or hetero-dimerization of 14-3-3 in vivo. Differences in relative abundance may be important, as a highly abundant 14-3-3 isoform that binds weakly to p53 may coprecipitate as much p53 as a less abundant isoform that has higher affinity. Hetero-dimerization of 14-3-3 isoforms in vivo can also explain the observed differences, since the in vitro experiments involve only 14-3-3 homodimers, whereas in vivo different 14-3-3 isoforms can form heterodimers (A. Aitken, personal communication).

Interestingly, we did not observe a significant interaction between p53 and 14-3-3 sigma. If these two proteins interacted, then we would have the potential for a positive feedback loop leading to p53 activation, since p53 induces expression of the 14-3-3 sigma gene (Hermeking et al., 1997) and binding of 14-3-3 to p53 enhances its functional activity.

The second question that we wanted to address is whether the interaction between p53 and 14-3-3 proteins is functionally significant. Analysis of the function of p53 mutants that are defective in their ability to interact with 14-3-3 indicates that 14-3-3 enhances p53 function. Furthermore, the results suggest that the effect was not all-or-none, since the p53 mutants that failed to interact with

14-3-3 were active in some functional assays, but not others. Since p53 interacts with 14-3-3 in response to IR, but not in response to other DNA damaging agents, this could be a mechanism for p53 to have different activities depending on the nature of the DNA damaging agent.

The mechanism by which 14-3-3 proteins enhance p53 function remains elusive. In our DNA binding assay using cell lysates as a source of p53 protein, 14-3-3 proteins did not affect the sequence-specific DNA binding activity of p53. This result contradicts our previous finding, in which recombinant p53 expressed in *E. coli* showed increased affinity for sequence-specific DNA in the presence of 14-3-3 (Waterman et al., 1998). The discrepancy might reflect differences in the p53 protein concentration or in the post-translational modifications of p53. More careful analysis of the DNA binding activity of p53 is warranted, including analysis of p53 binding to endogenous promoters using chromatin-immunoprecipitation assays. There are several examples of 14-3-3 proteins regulating the intracellular localization of the proteins with which they interact (Chan et al., 1999; Lopez-Girona et al., 1999; Zeng and Piwnicka-Worms, 1999). However, 14-3-3 proteins did not regulate the intracellular localization of p53, since wild-type p53 and the p53 mutants that failed to interact with 14-3-3 were all localized in the nucleus (data not shown). The ability of 14-3-3 proteins to regulate p53-dependent expression of the endogenous p21/cip1/waf1 gene and cell cycle arrest without affecting expression of reporter plasmids suggests that 14-3-3 proteins may affect the interaction of p53 with transcription factors that regulate chromatin structure. Consistent with this model, in plant cells 14-3-3 proteins facilitate interaction of sequence-specific DNA binding transcription factors with the basal transcription machinery (Pan et al., 1999). Further analysis

of the p53 mutants described in this study may help elucidate the mechanisms by which p53 exerts its tumor suppressor effect.

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References

- Brody M.H., Nordstrom W., Tsang G., Kwan E., Rubin G.M., and Abrams J.M. 2000. Drosophila p53 binds a damage response element at the reaper locus. *Cell* 101: 103-113.
- Chan T.A., Hermeking H., Lengauer C., Kinzler K.W., and Vogelstein B. 1999. 14-3-3 sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 401: 616-620.
- Chao C., Saito S., Anderson C.W., Appella E., and Xu Y. 2000. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. *Proc. Natl. Acad. Sci. USA* 97: 11936-11941.
- Chehab N.H., Malikzay A., Appel M., and Halazonetis T.D. 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G1 by stabilizing p53. *Genes Dev.* 14: 278-288.
- Chehab N.H., Malikzay A., Stavridi E.S., and Halazonetis T.D. 1999. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. *Proc. Natl. Acad. Sci.* 96: 13777-13782.
- Dumaz N. and Meek D.W. 1999. Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. *EMBO J.* 18: 7002-7010.
- El-Deiry W.S., Tokino T., Velculescu V.E., Levy D.B., Parsons R., Trent J.M., Lin D., Mercer W.E., Kinzler K.W., and Vogelstein B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817-825.
- Giaccia A.J. and Kastan M.B. 1998. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.* 12: 2973-2983.
- Haupt Y., Maya R., Kazaz A., and Oren M. 1997. Mdm2 promotes the rapid degradation of p53. *Nature* 387: 296-299.

Hermeking H., Lengauer C., Polyak K., He T.C., Zhang L., Thiagalingam S., Kinzler K.W., and Vogelstein B. 1997. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol. Cell* 1: 3-11.

Hirao A., Kong Y.Y., Matsuoka S., Wakeham A., Ruland J., Yoshida H., Liu D., Elledge S.J., and Mak T.W. 2000. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* 287: 1824-1827.

Kubbutat M.H., Jones S.N., and Vousden K.H. 1997. Regulation of p53 stability by Mdm2. *Nature* 387: 299-303.

Liu L., Scolnick D.M., Trievel R.C., Zhang H.B., Marmorstein R., Halazonetis T.D., and Berger S.L. 1999. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. *Mol. Cell. Biol.* 19: 1202-1209.

Lopez-Girona A., Furnari B., Mondesert O., and Russell P. 1999. Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* 397: 172-175.

Midgley C.A., and Lane D.P. 1997. p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. *Oncogene* 15: 1179-1189.

Miyashita T., and Reed J.C. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80: 293-299.

Oda K., Arakawa H., Tanaka T., Matsuda K., Tanikawa C., Mori T., Nishimori H., Tamai K., Tokino T., Nakamura Y., and Taya Y. 2000. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 102: 849-862.

Owen-Schaub L.B., Zhang W., Cusack J.C., Angelo L.S., Santee S.M., Fujiwara T., Roth J.A., Deisseroth A.B., Zhang W.W., Kruzel E., and Radinsky R. 1995. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol. Cell. Biol.* 15: 3032-3040.

Pan S., Sehnke P.C., Ferl R.J., Gurley W.B. 1999. Specific interactions with TBP and TFIIB in vitro suggest that 14-3-3 proteins may participate in the regulation of transcription when part of a DNA binding complex. *Plant Cell* 11: 1591-1602.

Rosenquist M., Sehnke P., Ferl R.J., Sommarin M., and Larsson C. 2000. Evolution of the 14-3-3 protein family: does the large number of isoforms in multicellular organisms reflect functional specificity? *J. Mol. Evol.* 51: 446-458.

Shieh S.Y., Taya Y., and Prives C. 1999. DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. *EMBO J.* 18: 1815-1823.

Stavridi E.S., Chehab N.H., Caruso L.C., and Halazonetis T.D. 1999. Change in oligomerization specificity of the p53 tetramerization domain by hydrophobic amino acid substitutions. *Protein Sci.* 8: 1773-1779.

Tanaka H., Arakawa H., Yamaguchi T., Shiraishi K., Fukuda S., Matsui K., Takei Y., and Nakamura Y. 2000. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* 404: 42-49.

Unger T., Juven-Gershon T., Moallem E., Berger M., Sionov R.V., Lozano G., Oren M., and Haupt Y. 1999a. Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J.* 18: 1805-1814.

Unger T., Sionov R.V., Moallem E., Yee C.L., Howley P.M., Oren M., and Haupt Y. 1999b. Mutations in serines 15 and 20 of human p53 impair its apoptotic activity. *Oncogene* 18: 3205-3212.

Vogelstein B., Lane D., and Levine A.J. 2000. Surfing the p53 network. *Nature* 408: 307-310.

Waterman J.L., Shenk J.L., and Halazonetis T.D. 1995. The dihedral symmetry of the p53 tetramerization domain mandates a conformational switch upon DNA binding. *EMBO J.* 14: 512-519.

Waterman M.J., Waterman J.L., and Halazonetis T.D. 1996. An engineered four-stranded coiled coil substitutes for the tetramerization domain of wild-type p53 and alleviates transdominant inhibition by tumor-derived p53 mutants. *Cancer Res.* 56: 158-163.

Waterman M.J., Stavridi E.S., Waterman J.L., and Halazonetis T.D. 1998. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nature Genet.* 19: 175-178.

Wu G.S., Burns T.F., McDonald E.R., 3rd, Jiang W., Meng R., Krantz I.D., Kao G., Gan D.D., Zhou J.Y., Muschel R., Hamilton S.R., Spinner N.B., Markowitz S., Wu G., and El-Deiry W.S. 1997. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet.* 17: 141-143.

Zeng Y., and Piwnica-Worms H. 1999. DNA damage and replication checkpoints in fission yeast require nuclear exclusion of the Cdc25 phosphatase via 14-3-3 binding. *Mol. Cell. Biol.* 19: 7410-7419.

Zhou B.B., and Elledge S.J. 2000. The DNA damage response: putting checkpoints in perspective. *Nature* 408: 433-439.

Figure Legends.

Figure 1. Interaction of p53 with specific 14-3-3 isoforms. A. Interaction of GST/14-3-3 isoforms with 35S-labeled *in vitro* translated wild-type p53 or p53 with alanines at positions 376-8. The latter protein serves as a negative control, since it does not bind to 14-3-3 proteins. B. Coimmunoprecipitation of p53 with specific 14-3-3 isoforms. Lysates prepared from non-irradiated cells (NR) or cells exposed to IR or UV light were immunoprecipitated (IP) with 14-3-3 isoform-specific antibodies or antibody K19, which recognizes all 14-3-3 isoforms. Coprecipitated p53 was detected by immunoblotting (IB). As a control, buffer only (Cells -) was used instead of the cell lysates. C. Specificity of 14-3-3 isoform-specific antibodies. GST/14-3-3 isoforms were immunoblotted with the isoform-specific antibodies or with antibody K19.

Figure 2. Interaction of p53 C-terminal mutants with 14-3-3 proteins in U2OS cells. A. Diagrammatic representations of wild-type p53 (p53wt) and p53IND. HA, hemagglutinin tag; Tx, transactivation domain; DNA-B, sequence-specific DNA binding domain; 4, native tetramerization domain; IND, independent tetramerization domain; R, C-terminal regulatory region, which contains the binding site for 14-3-3 proteins. B. Protein levels of p53INDwt in mock-irradiated (0 Gy) and irradiated (5 Gy) U2OS cells. C. Interaction between p53INDwt and endogenous 14-3-3 in mock-irradiated and irradiated U2OS cells. D. Protein levels of p53INDwt and C-terminal p53IND mutants in irradiated (5 Gy) U2OS cells. Note that the C-terminal mutants with Ala at position 376, unlike p53INDwt and the rest of the mutants, do not migrate as doublets. The basis for this difference in electrophoretic migration is not understood. E. Interaction between p53INDwt

and C-terminal p53IND mutants with endogenous 14-3-3 in irradiated U2OS cells.

Figure 3. Sequence-specific DNA binding of wild-type and mutant p53 proteins expressed in U2OS cells by transient transfection. A. Cell lysates containing HA-tagged p53INDwt prepared from non-irradiated (0 Gy) or irradiated (5 Gy) cells were incubated with beads coated with oligonucleotides containing the specific (Sp) p53 binding site or a non-specific (NS) site. Bound p53 was detected by immunoblotting. B. Cell lysates containing HA-tagged p53IND proteins with wild-type or mutant C-termini were incubated with beads coated with oligonucleotides containing the specific (Sp) p53 binding site. Bound p53 was detected by immunoblotting.

Figure 4. Transcriptional activities of wild-type p53 and p53 C-terminal mutants in Saos2 cells. A. Interaction of p53 C-terminal mutants with endogenous 14-3-3 proteins in irradiated Saos2 cells. B. Transactivation of the endogenous p21/cip1/waf1 gene, as indicated by determining the levels of p21/waf1/cip1 protein in cells transiently transfected with the indicated p53 proteins. C. Transcriptional activities of the same p53 mutants using a reporter plasmid that contains the p53 binding site present in the p21/waf1/cip1 gene. The tumor-derived p53 mutant p53W248 serves as a negative control.

Figure 5. Cell cycle arrest activities of wild-type p53 and p53 C-terminal mutants in U2OS cells. The cells were either not-transfected or transfected with plasmids encoding the indicated proteins. All transfected cells express a tumor-derived p53 mutant (p53W248) and some transfected cells additionally express a p53IND

protein, as indicated. The cells were either mock-irradiated (0 Gy) or exposed to 5 Gy IR.

Figure 1
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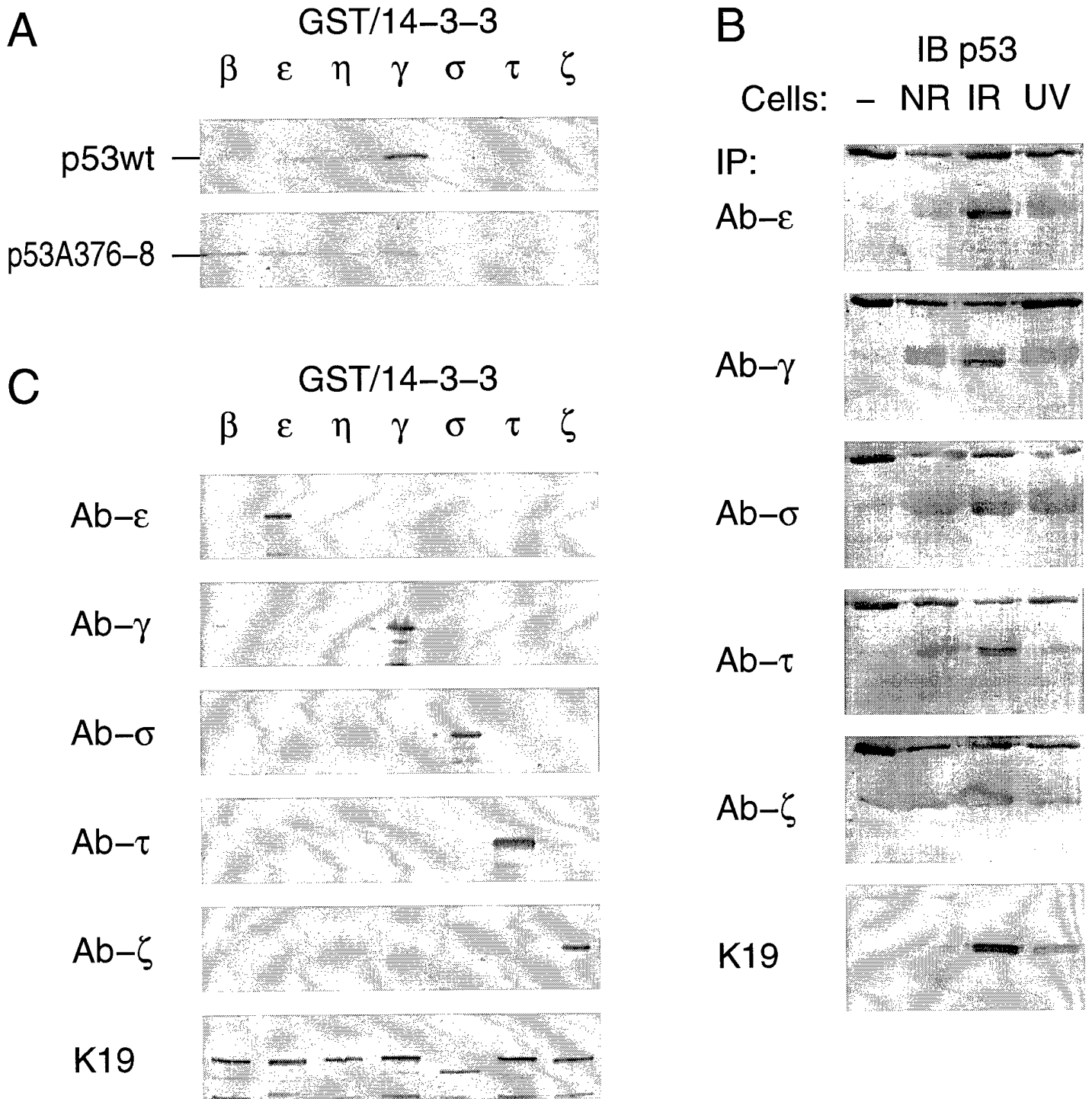


Figure 2
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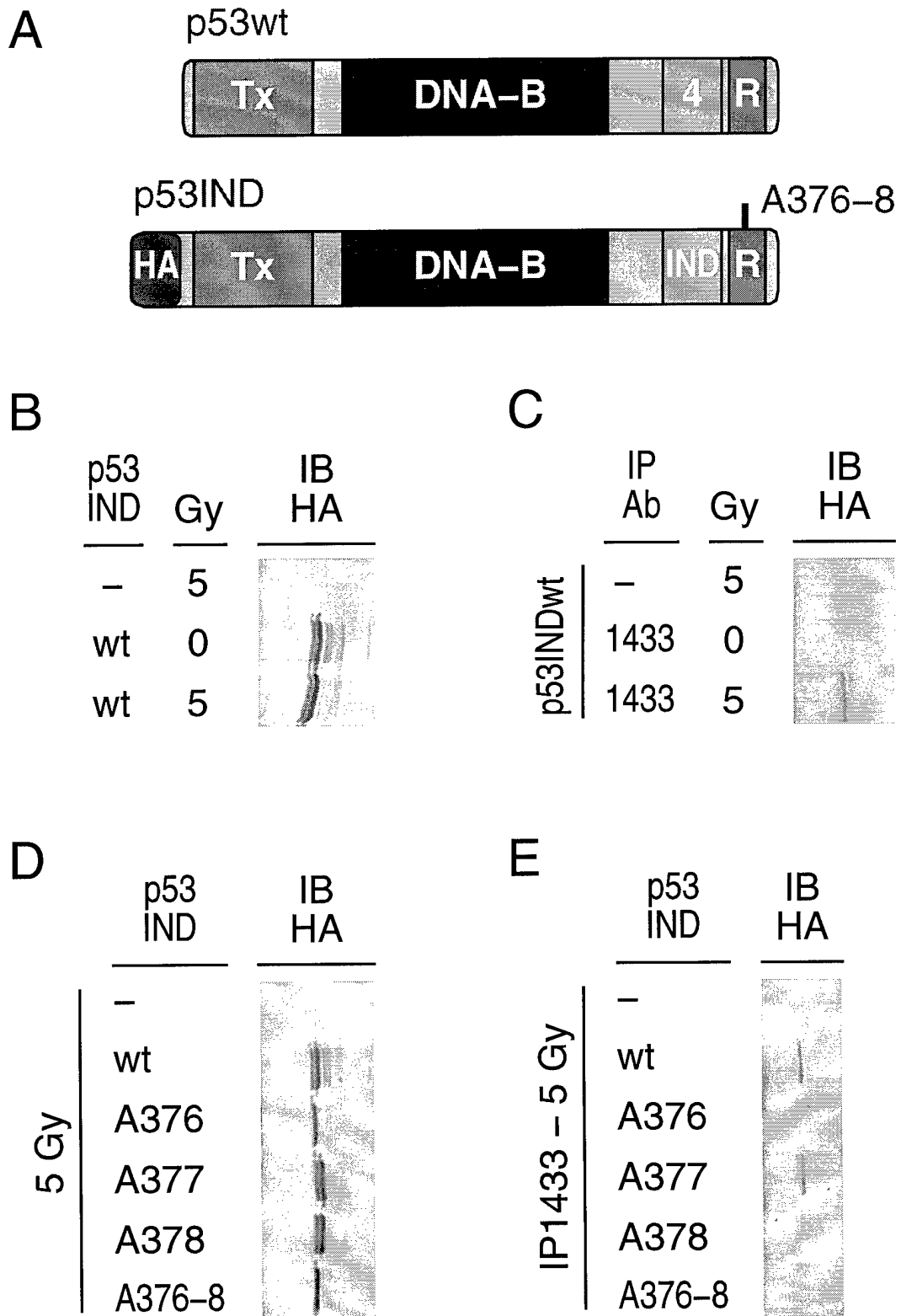


Figure 3
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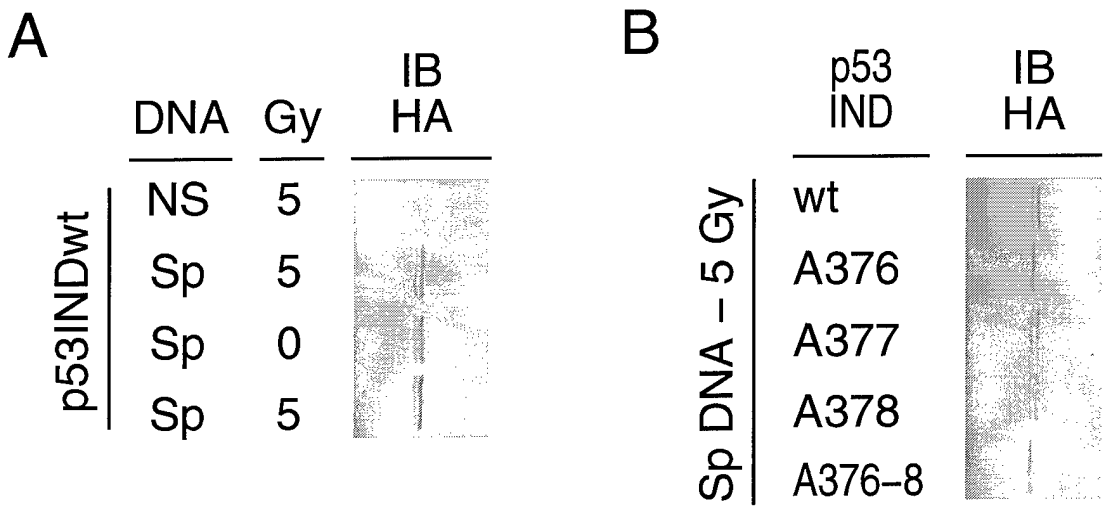


Figure 4
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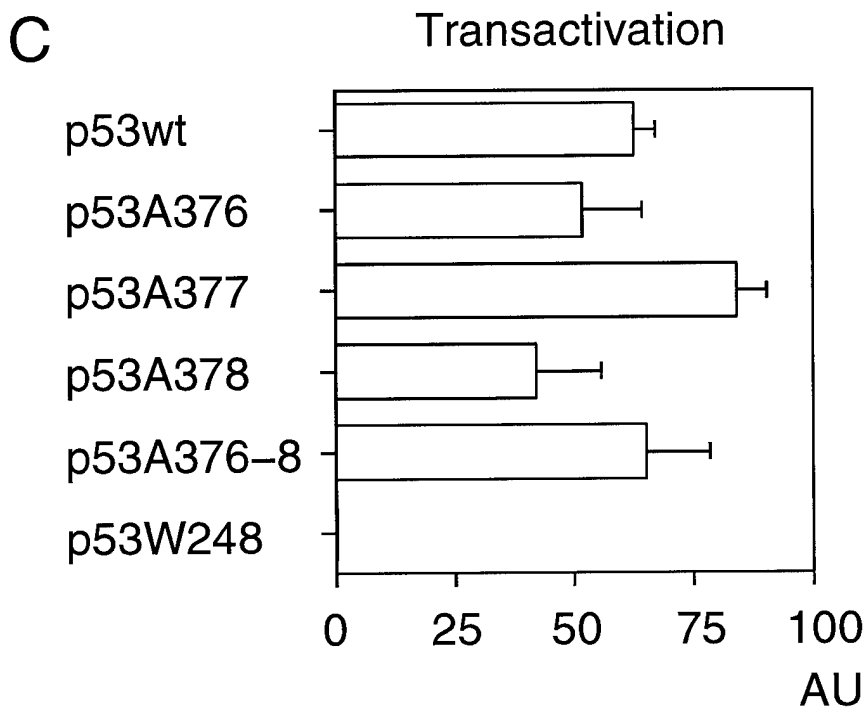
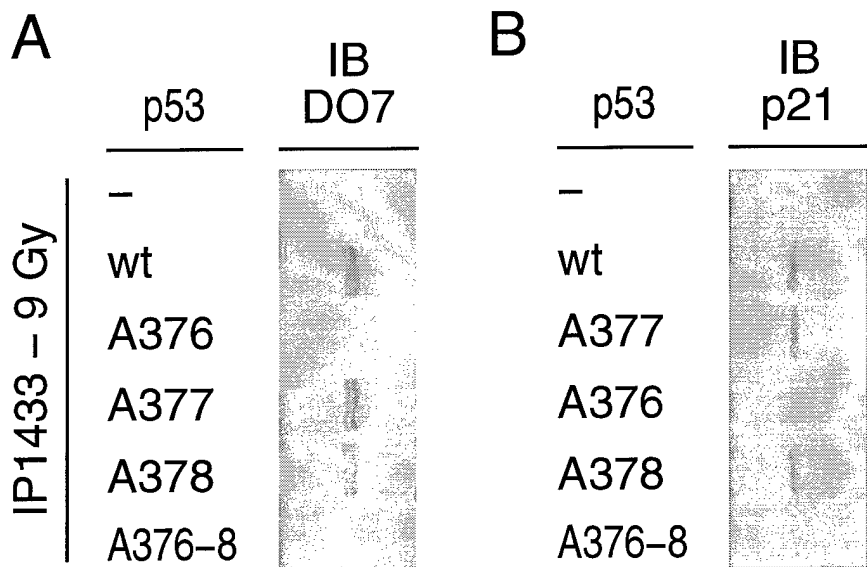


Figure 5
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