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p53 and p73 display common and distinct requirements for sequence specific binding to DNA

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ABSTRACT

Although p53 and p73 share considerable homology in their DNA-binding domains, there have been few studies examining their relative interactions with DNA as purified proteins. Comparing p53 and p73β proteins, our data show that zinc chelation by EDTA is significantly more detrimental to the ability of p73β than of p53 to bind DNA, most likely due to the greater effect that the loss of zinc has on the conformation of the DNA-binding domain of p73. Furthermore, prebinding to DNA strongly protects p73β but not p53 from chelation by EDTA suggesting that DNA renders the core domain of p73 less accessible to its environment. Further exploring these biochemical differences, a five-base sub-sequence was identified in the p53 consensus binding site that confers a greater DNA-binding stability on p73β than on full-length p53 *in vitro*. Surprisingly, p53 lacking its C-terminal non-specific DNA-binding domain (p53Δ30) demonstrates the same sequence discrimination as does p73β. *In vivo*, both p53 and p73β exhibit higher transactivation of a reporter with a binding site containing this sub-sequence, suggesting that lower *in vitro* dissociation translates to higher *in vivo* transactivation of sub-sequence-containing sites.

INTRODUCTION

The p53 gene family consists of the well studied tumor suppressor p53, as well as p73 and p63 (1–3). All three share significant sequence homology and can act as transcription factors initiating cell-cycle arrest or apoptosis under certain conditions. The three proteins are thought to have evolved from a common ancestor, possibly similar to the *Caenorhabditis elegans* p53 ortholog CEP-1 (4–6), with p53 being the most recently evolved family member (3). Each p53 family gene can be expressed as multiple isoforms generated both by differential promoter usage and alternative splicing (7,8).

The p53 protein is organized into several functional domains (9). The N-terminal region (amino acids 1–100) contains two transactivation sub-domains (amino acids 20–40 and 40–60) followed by a proline-rich region (amino acids 60–90) that plays a selective role in transactivation and apoptosis. The central region comprising a protease-resistant core domain (amino acids 100–300) has sequence-specific DNA-binding activity and sustains the vast majority of missense mutations that occur in many forms of human cancer. The core domain is joined to the tetramerization domain (amino acids 325–355) by a 25 amino acid linker region that contains a nuclear localization signal. Finally, at the extreme C-terminus is a highly basic region (amino acids 363–393) which can associate in a non-sequence dependent manner with various forms of both DNA and RNA. The transactivation and tetramerization domains and, to a much greater extent, the sequence-specific DNA-binding domain are conserved between the p53 family members. There is no region within any of the known p73 and p63 isoforms, however, that resembles the p53 basic C-terminus.

Interaction with DNA is crucial to the role of p53 as a transcription factor. The consensus sequence bound by p53 contains two PuPuPuCWWGPyPyPy 10 bp half-sites separated by 0–13 bases (10). Following induction by DNA-damaging agents, hypoxia and other stresses, p53 binds DNA as a tetramer; the two dimers each bind to one 10mer of the bipartite consensus sequence (11–14). Recent analyses derived from crystal structures show that each p53 dimer is then further stabilized on DNA by protein–protein interactions (15,16). *In vivo*, an exact p53 consensus site is rarely found (17) and p53 can tolerate some variation in its binding site, both in sequence and, to a lesser extent, spacing between the 10 base half-sites. The bases within the CWWG component of the consensus sequence, however, are the least frequently changed *in vivo*. p53 does exhibit a difference in binding affinity and transactivation of different target gene promoters *in vivo*, most interestingly between pro-apoptotic and non-apoptotic genes (18–21). Furthermore, frequencies of nucleotides found at each position in known p53-binding sites have been assessed, but the correlation between

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sequence of and p53 transcriptional activity from various promoters is still unclear (22–24).

Proper conformation of the p53 core DNA-binding domain is necessary for DNA binding and transactivation of target genes. Many p53 tumor derived mutations within the core disrupt the domain conformation and diminish or abrogate sequence-specific DNA binding (25). The p53 core domain structure consists of a beta sandwich that supports two large loops and a loop–sheet–helix motif (26,27). Loops 2 and 3 are held together in part by a zinc atom, and, together with the loop–sheet–helix motif, form the DNA-binding surface of p53. Three cysteines (Cys176, Cys238 and Cys242) and one histidine (His179) in the DNA-binding domain of p53 coordinate one molecule of zinc (26). Zinc coordination is necessary for proper folding of the p53 core domain *in vitro* and in cultured mammalian cells and disruption of this interaction greatly reduces or abrogates p53 DNA binding and transactivation of target genes (28–30).

Understanding the role(s) of the second sequence-non-specific DNA-interacting domain of p53 located in its extreme C-terminus has been challenging. Biochemical experiments using long oligonucleotides or circular DNA indicate that the C-terminus is a positive regulator of p53 sequence-specific DNA binding, presumably by allowing p53 to locate specific binding sites more efficiently (31,32). Further, this region confers on p53 the ability to diffuse linearly along DNA (31). On short oligonucleotides, however, experiments indicate that the C-terminus negatively regulates DNA binding, a phenomenon possibly explained by its rapid diffusion from the ends of the short DNA which would increases the dissociation rate of p53 (33).

p73 was first identified by Kaghad *et al*. (34) and Jost *et al*. (35) in 1997. Since then, much has been discovered about this protein but many questions still remain unanswered (3,36–40). The *p73* gene contains 14 exons, which, through splicing, can produce at least six TA full-length isoforms that differ in their C-terminus (generated by alternative splicing) and the corresponding ΔN isoforms (generated by transcription from an internal promoter). The domain organization of the p73 protein is similar to that of p53: the TA isoforms contain an N-terminal transactivation domain (20–30% homology to p53), a core DNA-binding domain (65% homology), and a tetramerization domain (35–45% homology). The ΔN isoforms of p73 lack the transactivation domain and are thought to act in a dominant-negative fashion, both for TAp73 and p53 (41–43) although in at least one setting they can display the ability to activate transcription (44). *In vivo*, p73α and β are the most commonly found forms, both in the TA and ΔN variety. Of these, p73β has a higher affinity for DNA *in vitro*, and TAp73β is the most potent isoform for transactivation and induction of growth suppression and apoptosis (2,3,36,45–47). Like p53, p73 is presumed to bind DNA as a tetramer, and its different isoforms can form heterotetramers, allowing for another level of regulation (48,49).

p73 has been shown to transactivate a number of p53 target genes, and induce cell-cycle arrest and apoptosis (1). Interestingly, while p73 and p53 have a number target genes in common, p73 also exhibits promoter selectivity and has a number of unique target genes that are distinct from p53 (50–53). Unlike p53, p73-mediated apoptosis does not require its extreme C-terminal domain and deleting the p73 C-terminus does not affect the *in vivo* or *in vitro* DNA-binding activities of the protein (31,45,47,54).

While p53 structure and its interaction with DNA have been explored in great detail, little is known about p73 in this regard and very few papers have reported results on the DNA-binding properties of purified p73 proteins. In this work, we have determined a purification strategy for producing active p73β and characterized similarities and differences between p53 and p73β in their interaction with DNA.

MATERIALS AND METHODS

Tissue culture and antibodies

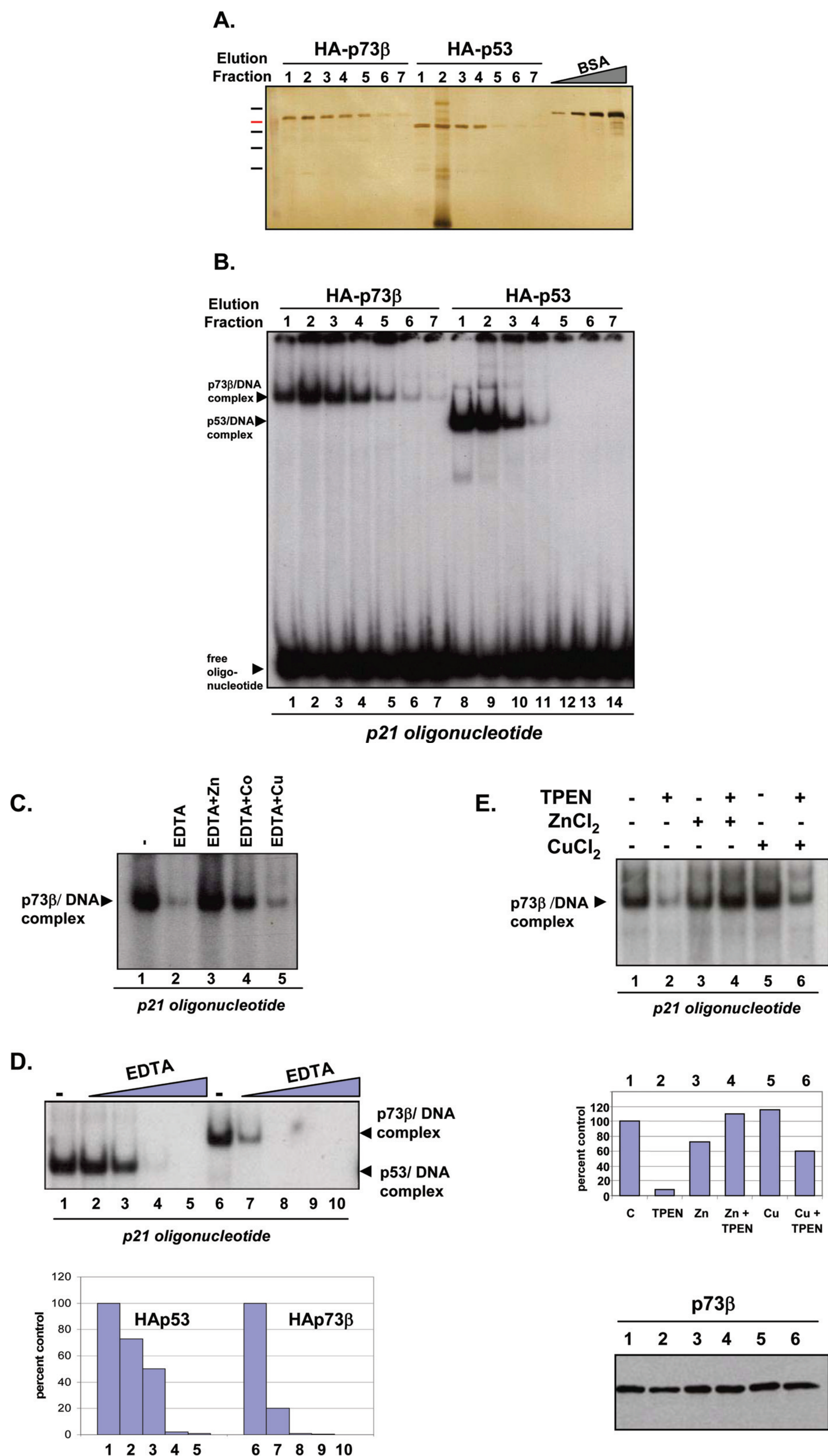
SF9 cells used for production of proteins expressed from recombinant baculoviruses were grown in TC100 medium, supplemented with 10% FBS. H1299 human lung epithelial cells (p53 null) were grown in DME medium supplemented with 10% FBS. H1299 cells that express tetracycline regulated HA-tagged p73β (H24-p73β-22 HA-tagged) were previously described (50) and were a generous gift from Dr Xinbin Chen (University of Alabama). They were grown in DME medium supplemented with 10% FBS, 2 μg/ml puromycin, 250 μg/ml G418 and 5 μg/ml tetracycline. To induce protein expression cells were plated without tetracycline, washed three times with medium lacking tetracycline, and 24 h later were treated with *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) and/or metals as described in Figure 1 for 2 h prior to lysis in buffer described below in the purification protocol.

For electrophoretic mobility shift assays (EMSAs) examining p53 DNA binding, we used affinity purified monoclonal p53 antibodies DO-1 and 421 and for p73, an affinity purified anti TAp73 N-terminal polyclonal antibody (TAp73N) (55) and anti-p73 Ab-2 (ER15) (Calbiochem). Anti-HA (Covance), anti-Flag (Sigma) and anti-His (Santa Cruz) antibodies were used to detect the recombinant p53 and p73 proteins by western blotting.

Purification of p53 and p73

SF9 insect cells were infected with baculoviruses expressing HA-tagged versions of p53 and p73. After 48 h, the cells were collected and incubated for 30 min in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% aprotinin, 0.35 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 100 μM benzamidine, 300 μg/ml leupeptine, 10 μg/ml bacitracin and 100 μg/ml α-macroglobulin) on ice and centrifuged to remove debris. The supernatant was incubated with an anti-HA monoclonal antibody (12CA5) cross-linked to Protein A Sepharose beads while rocking for 3 h at 4°C. Beads (300 μl) were collected by centrifugation at 1400 *g* and washed twice with 10 ml buffer containing 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1% NP-40, 10% glycerol and 1 mM DTT, twice with 10 ml of the same buffer lacking NP-40, followed by washing in 1 ml of a high salt buffer (20 mM Tris–HCl, pH 8.5, 10% glycerol, 250 mM NaCl and 1 mM DTT). p73 or p53 proteins were then eluted at 30°C with buffer (20 mM Tris–HCl, pH 8.5, 10% glycerol, 500 mM NaCl and 1 mM DTT) containing 100 μg/ml HA

peptide (YPYDVPDYA) purchased from SynPep (Dublin, CA). Consecutive fractions were taken every 2 min by centrifugation. To determine preparation yield, the protein was then incubated in Protein Sample (PS) buffer (final concentration: 10% glycerol, 0.7 M 2-mercaptoethanol, 4% SDS, 167 mM Tris, pH 6.8, and 0.3 mg/ml Bromophenol Blue) for 10 min at 100°C and analyzed by SDS–PAGE and silver staining.



Electrophoretic mobility shift assay

p53 or p73 protein was preincubated for 10 min in 1× EMSA buffer (12.5 mM Tris–HCl, pH 6.8, 25 mM KCl, 10% glycerol, 0.05% Triton-X, 0.5 mg/ml BSA, 1 mM DTT and 150 ng mutant *p21* oligonucleotide) and then incubated with 5 ng oligonucleotide, which was radioactively labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, for another 20 min unless otherwise stated. The samples were run on a 4% polyacrylamide gel at 200 V. The gel was transferred to blotting paper, dried and exposed to KODAK film.

For dissociation experiments, protein was incubated with labeled oligonucleotide for 20 min. 50-fold excess of competitor was then added for the indicated time and the reaction mixture was loaded onto a running gel.

For experiments with extracts of H1299 cells, the same buffers and conditions were used as above, except the experiments were performed in the presence of 300 ng of mutant *p21* oligonucleotide.

The following oligonucleotides were used (boldface—p53-binding site in which lower case signifies deviation from consensus sequence):

p21, 5′-AGCTAGTAGAGC**GAACATGTCCcAACATG TTg**GCGTGCTGCAGC; *p21*, 5′-mut AGCTAGTAGAGC-**GAAtATaTCCcAAtATaTTg**GCGTGCTGCAGC; *GADD45*, AGCTAGTAGAGC**GAACATGTCTAAGCATGCTg**GC-GTGCTGCAGC; *MDM2*, AGCTAGTAGAGC**GGtCAAG-TTgGGACAcGTCC**GCGTGCTGCAGC; *14-3-3-sigma*, AG-CTAGTAGAGC**tAGCATtagCCCAGACATGTCC**GCGT-GCTGCAGC; PIG3, AGCTAGTAGAGC**cAGCTTGCCC-AccCATGCTC**GCGTGCTGCAGC; *KILLER/DR5*, AGCT-AGTAGAGC**GGGCATGTCCGGGCAAGaCg**GCGTGC-TGCAGC; PUMA, AGCTAGTAGAGC**ctGCAAGTCCtG-ACTTGTCC**GCGTGCTGCAGC; PCNA, AGCTAGTAGA-GC**GAACA AGTCCGGGCATaTgT**GCGTGCTGCAGC; PPC, AGCTAGTAGAGC**tGGCATGTCCcGACTTGTTa**-GCGTGCTGCAGC; PPN(s), AGCTAGTAGAGC**tGGCAT-GCTTcGGCTTGCTa**GCGTGCTGCAGC; PPN(o), AGC-TAGTAGAGC**tGGCATGaggcGTCTTGATa**GCGTGCTG-CAGC.

Cloning and mutagenesis

Mutant p53 C242S and p73β C262S were made using the Stratagene Quickchange mutagenesis kit and the corresponding baculoviruses were generated using the BactoBac kit (Invitrogen).

Site-specific protein modification

mPEG-MAL 5000 was purchased from Nektar Therapeutics. A stock solution (20 mM) was prepared in water. For modification, purified protein (20 ng) was preincubated with 50 mM EDTA for 30 min on ice and then further incubated with 1 mM mPEG-MAL in 20 mM Tris–HCl (pH 6.8) on ice for 1 h.

Luciferase assay

The PPC and PPN(s) binding sites were cloned into the pGL3-OFLuc vector upstream of a minimal *c-fos* promoter at position 58 (vector kindly provided by R. Prywes). These constructs were then co-transfected with pcDNA3 plasmids expressing Flag-tagged p53 or p73β (gift of T. Tanaka) into H1299 cells using Lipofectamine 2000 transfection reagent (Invitrogen) and the luciferase activity was assessed using the manufacturer's protocol (Promega). Renilla luciferase was used as transfection control.

DNA-binding immunoassay

HA-p53, HA-p73β, His-p53C242S and His-p73β262S baculovirus infected or uninfected SF9 cells were lysed as described above, and incubated for 2 h at 4°C with anti-HA antibody (MAb 12CA5) cross-linked Protein A–Sepharose or nickel-NTA Agarose beads as appropriate. The matrices were washed three times with lysis buffer, and then incubated for 20 min with γ-^{32}P-labeled *p21* oligonucleotide, as described for EMSA above. They were then washed three times with EMSA buffer and the *p21* oligonucleotide bound was measured in a LKB Wallace liquid scintillation counter.

RESULTS

Purified p53 and p73β are differentially affected by the zinc chelator EDTA

We purified baculovirally expressed HA-tagged versions of p53 and p73β from SF9 cells and analyzed their *in vitro* DNA binding by EMSA. Initial purifications of these two proteins were performed using a protocol previously described for p53 in this laboratory using buffers which contain 1 mM EDTA (56). These protocols yielded active p53 but inactive p73β (data not shown). As both proteins share homologous zinc-coordinating residues within their DNA-binding domains, we postulated that DNA binding of p73β

Figure 1. p73β–DNA binding is more affected by zinc chelation than that of p53. (**A** and **B**) Purified p53 and p73β can bind DNA with similar affinity. Baculovirally expressed HA-tagged p53 and p73β were affinity purified in the absence of EDTA using 12CA5 crosslinked beads, fractions 1–7 were eluted with HA peptide as described in Methods and aliquots were subjected to SDS–PAGE and silver staining using 50, 100, 200 and 500 ng BSA as a standard protein (A). Migration of molecular weight standard proteins is indicated at left. The red line at left indicates the 63 kDa protein weight marker, the two lines below it indicate 49 and 37 kDa markers respectively, and the line above is the 81 kDa marker. DNA binding of HA-p73β (lanes 1–7) and p53 (lanes 8–14) proteins (10 ng) to an oligonucleotide containing the *p21* 5′ p53-binding site (5 ng) was measured using EMSA (B). (**C**) Zinc, cobalt, but not copper reverse the effect of EDTA on p73β–DNA binding. HA-tagged p73β was purified with buffers containing either no metals or EDTA, or EDTA (1 mM), or EDTA with zinc (both 1 mM), EDTA with copper (both 1 mM), or EDTA with cobalt (both 1 mM) as in A. DNA binding to a *p21* oligonucleotide was visualized by EMSA as in (B). (**D**) p73β–DNA binding activity is inhibited at much lower concentrations of EDTA than that of p53. HA-tagged p53 and p73β (purified in the absence of zinc or chelator) were preincubated for 30 min with increasing concentrations of EDTA [12.5, 25, 50 and 100 mM (pH 6.8)] and DNA binding of p53 (lanes 1–5) and p73β (lanes 6–10) to a *p21* oligonucleotide was assessed by EMSA as in (B). Graph shows quantification of results by phosphorimaging. The results are plotted as percentage of control incubated in the absence of EDTA (lanes 1 and 6). (**E**) Zinc reverses the TPEN-induced inhibition of p73β–DNA binding *in vivo*. HA-tagged p73β expression was induced in H1299 cells by removal of tetracycline. After 24 h, cells were treated for 2 h with 40 μM TPEN (lane 2), 160 μM $ZnCl_2$ (lane 3), 160 μM $CuCl_2$, or a combination of TPEN and $ZnCl_2$ (lane 4) or TPEN and $CuCl_2$ (lane 6) at the same concentrations for 2 h and then lysed. An EMSA was performed using whole cell extracts with a *p21* 5′ binding site containing oligonucleotide (upper panel). Quantification of the EMSA DNA-binding results is in the middle panel with DNA-binding normalized for protein expression and plotted as percent of untreated control. The p73β protein expression levels (detected by HA antibody) in the corresponding cultures treated as described above are in the bottom panel.

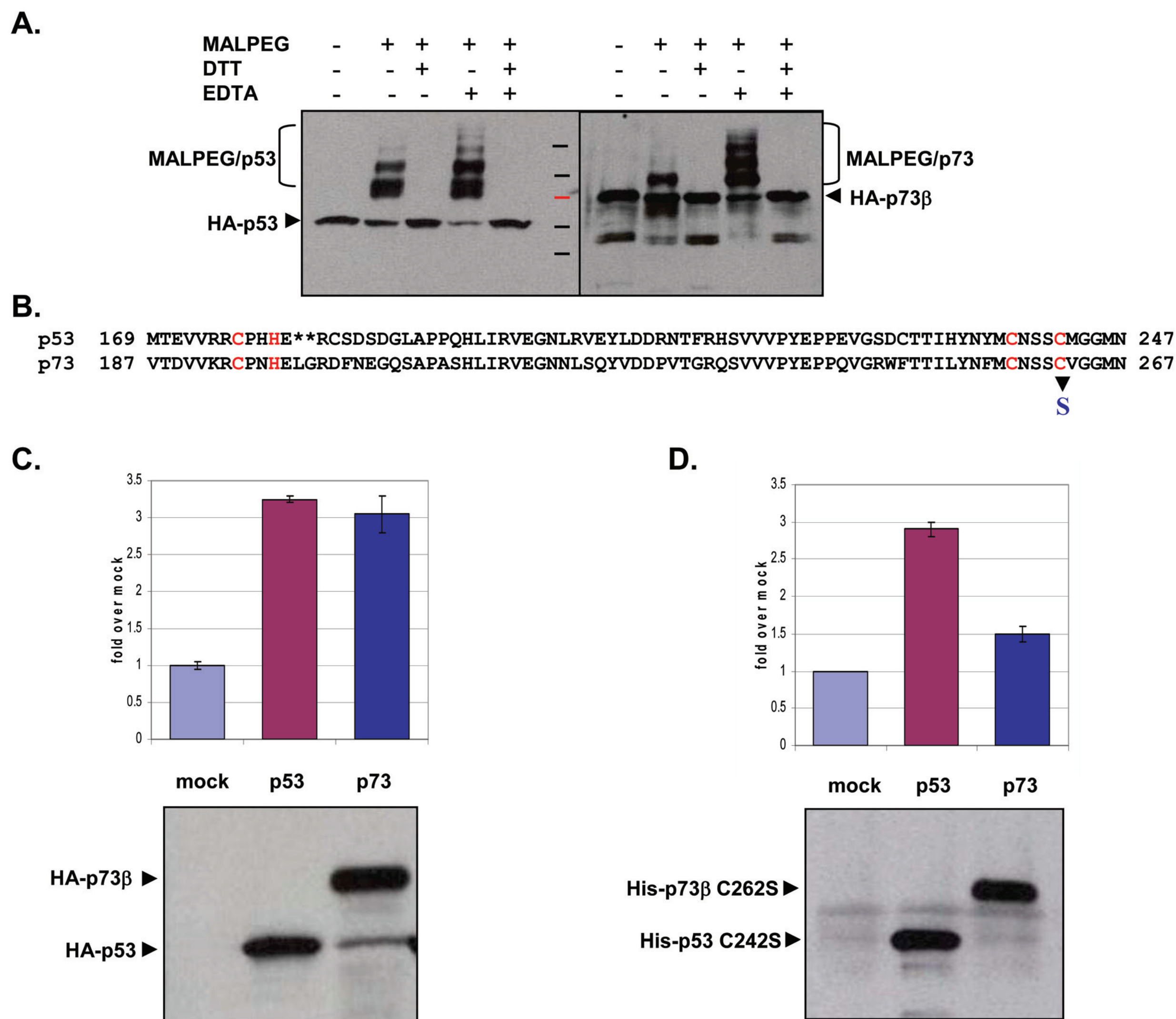


Figure 2. Differential roles of cysteine residues in core domains of p53 and p73. (**A**) The conformation of p73β is more significantly affected by zinc chelation than that of p53. Purified HA-tagged p53 and p73β (20 ng) proteins were incubated with MAL-PEG (1 mM) in the presence or absence of 50 mM EDTA as indicated. Proteins were visualized by SDS–PAGE. More slowly migrating p53 and p73 species arising from MAL-PEG tagging are indicated (MAL-PEG/p53 and MAL-PEG/p73). Migration of molecular weight standard proteins is indicated in center lane. The red line indicates the 63 kDa protein weight marker, the two below it are 49 and 37 and the two above are 81 and 115 kDa respectively (B–D). A zinc-coordinating mutant of p73β cannot bind DNA. (**B**) A sequence alignment of the zinc-coordinating regions of p53 and p73. The zinc-coordinating cysteines and histidine are highlighted in red and the substitution made in the p53C242S and p73βC262S is highlighted in blue. (**C**) No difference is observed in the DNA binding of wild-type p53 and p73β using an immunobinding assay. Wild-type HA-tagged p53 and p73β proteins were baculovirally expressed in SF9 cells at 23°C. Upper panel: The DNA-binding activity of the two proteins immobilized on 12CA5 crosslinked to Protein A Sepharose was assessed by a DNA immunobinding assay, with lysate from uninfected cells used as a control (mock). The immobilized protein was incubated with 5 ng of a γ-^{32}P-labeled *p21* oligonucleotide, the matrix was washed, and the amount of bound DNA assessed by scintillation counting. Background levels of radioactivity of matrix that was incubated with oligonucleotide alone were subtracted from the mock and HA-p53 and HA-p73β infected samples, and the fold increase in radioactivity over the mock samples was plotted. Lower panel: The proteins immobilized on beads were heated in PSB and subjected to SDS–PAGE followed by immunoblotting with anti-HA antibody. (**D**) p53(C242S) but not p73β(C262S) retains DNA-binding activity. His-tagged p53(C242S) and p73β(C262S) mutant proteins were baculovirally expressed in SF9 cells at 23°C. Upper panel: The DNA-binding activity of the two proteins immobilized on Ni-T-Agarose matrix was assessed by a DNA immunobinding assay using a *p21* oligonucleotide as in (C). Lower panel: The amount of protein present on the matrix was visualized by immunoblotting with anti-His antibody.

is affected by zinc chelation and repeated the purification in the absence of EDTA. This did yield p73β protein that was able to bind DNA (Figure 1). The quantification by silver staining of consecutive fractions of proteins from a representative purification using BSA as standard is shown in Figure 1A and their DNA binding, as assessed by performing an EMSA, using a radioactively labeled oligonucleotide containing the *p21* 5′ p53 binding site/response element (RE) is shown in Figure 1B. While there was some variation in DNA binding between different preparations of the two proteins, overall there were no consistent differences in steady-state DNA binding between p53 and p73β.

To confirm that the observed effect of EDTA on p73β was due to the metal chelating properties of EDTA, we added zinc, copper and cobalt in equimolar ratios to EDTA to the purification buffers (all at 1 mM). The addition of zinc and (to a lesser extent) cobalt, but not copper, rescued p73β DNA binding, presumably by saturation of EDTA (Figure 1C).

To further characterize the effect of EDTA on p53 and p73β DNA binding, we incubated the two proteins (purified without chelator) with increasing amounts of EDTA (0, 12.5, 25, 50 and 100 mM). An almost 4-fold higher amount of EDTA was needed to disrupt p53 binding to a *p21* 5′ RE-containing oligonucleotide as compared to p73β (Figure 1D).

To determine if zinc chelation has an effect on p73 DNA binding *in vivo*, we followed a protocol previously described for p53 (29), using the intracellular zinc chelator, TPEN, a membrane permeable metal chelator with a high specificity for zinc (57). Ectopic p73β expression was induced in H1299 cells (50) which were then treated either with TPEN alone, or in the presence of zinc or copper. The cells were lysed after 2 h, and p73 DNA binding was measured by EMSA in cell extracts (Figure 1E). The DNA-binding activity of p73 was significantly impaired in the presence of TPEN (compare lanes 1 and 2), and, as seen with purified protein, this inhibition was completely reversed by simultaneous addition of zinc (compare lanes 3 and 4) but only partially reversed by addition of copper (lanes 5 and 6).

Altered roles and exposure of cysteine residues in p53 and p73 core domains

A previous report has shown that treatment of cells with the metal chelator TPEN disrupts p53 conformation as assessed by immunoprecipitating with an antibody MAb 240 (29) that recognizes p53 in a partially unfolded mutant conformation (26,58). Since p73β DNA binding was more affected by EDTA than that of p53, we hypothesized that the structure of p73β is more affected by chelation of its zinc atom than is that of p53 under our conditions. All p53 cysteine residues and all but one (located at the extreme C-terminus) p73β cysteine residues are located in their respective core domains. Since no conformation-specific antibody currently exists for p73β, we decided to assess differential exposure of cysteines in the two proteins by tagging the thiol groups of the cysteines with maleimide-polyethylene glycol (MAL-PEG) (59,60). If the cysteines are buried within the protein, MAL-PEG should not be able to access them. However, if the protein conformation is altered and these residues are exposed following EDTA chelation, they will be accessible to MAL-PEG and tagged. Furthermore, since the bond formed between a thiol and a maleimide is a disulfide bond, the tagging can be assessed by denaturing SDS–PAGE to observe the appearance of slower-migrating species.

HA-tagged p53 and p73β were either untreated or treated with 50 mM EDTA for 20 min and then incubated for 1 h with 1 mM MAL-PEG on ice. After denaturation at 100°C in protein sample buffer, SDS–PAGE was performed, and the proteins were visualized by immunoblotting with anti-HA antibody. Slower-migrating species of both p53 and p73β were present following MAL-PEG tagging without EDTA, indicating that some core cysteines in each protein are accessible to this reagent (Figure 2A). Strikingly, while there were only minor additional species detected when p53 was pretreated with EDTA, much more prominent slower-migrating species were present when p73β was preincubated with EDTA before MAL-PEG. These data therefore indicate that zinc chelation by EDTA more profoundly changes the conformation of p73β than of p53.

Since the structure of p53, as measured by cysteine exposure, did not seem to be grossly disturbed by zinc chelation *in vitro*, we hypothesized that under some conditions p53 but not p73β should be able to bind DNA in the absence of a key residue required for coordinating zinc. In fact, it was previously shown that mutating zinc-coordinating cysteines of murine p53 significantly decreases, but does not completely abrogate the DNA-binding activity of the protein when it is expressed at 20°C (61). We generated baculoviruses expressing His-tagged versions of the zinc-coordinating mutant human p53(C242S) and the equivalent residue in p73β(C262S) (Figure 2B). These respective sites were chosen because in p53 this mutation had been shown to have the least effect on DNA binding (61). Note that when we used MAL-PEG to assess EDTA-induced unfolding, the two mutant proteins did not show a change in the appearance of tagged species, suggesting weak zinc coordination activity (data not shown). Since these mutants were more easily denatured during the process of purification from insect cells, we employed a DNA-binding immunoassay in which lysates of insect cells infected with baculoviruses expressing wild-type or mutant p53 or p73β proteins were immunoprecipitated with the appropriate antibody immediately followed by incubation with a radioactively labeled *p21* oligonucleotide.

Wild-type versions of both proteins bound approximately similar amounts of the *p21* oligonucleotide (Figure 2C). While the p53(C242S) mutant has reduced DNA-binding activity compared to wild-type protein (∼20-fold reduction, data not shown) it did retain some DNA-binding activity (∼3-fold over background) when incubated with a *p21*-RE-containing oligonucleotide. In contrast, the p73β(C262S) mutant exhibited minimal DNA-binding activity (1.5-fold over background, Figure 2D). This suggested that DNA binding of p73β is more significantly affected by the inability to coordinate zinc than that of p53. Since wild-type p53 and p73β proteins exhibit a roughly equivalent DNA-binding activity on a per mole basis, we conclude that the difference in DNA binding of the mutants is due to the differential effect of the cysteine mutation.

DNA binding protects p73 from EDTA chelation

To further elucidate these differences, we compared the effects of EDTA on p53 and p73β after they were bound to DNA. We used oligonucleotides containing p53 binding sites from the *p21* and *KILLER/DR5* promoters. Purified HA-tagged p53 and p73β were either incubated with EDTA after which DNA was added, or preincubated with DNA and then exposed to EDTA. DNA binding was then assessed by EMSA (Figure 3). In agreement with our previous results (see Figure 1D) preincubating with EDTA significantly reduced the DNA-binding activity of both proteins although different concentrations of EDTA were used in each case to assure that some DNA binding was still detectable (40 mM for p53, 12.5 mM for p73β). Using the *p21* oligonucleotide, preincubating p53 with DNA only modestly reversed the inhibition by EDTA (∼2-fold) while p73β DNA binding was dramatically rescued after such preincubation (∼16-fold). Similar effects were shown over a range of EDTA concentrations and overall p73β dissociation rate was not affected under these conditions (data not shown).

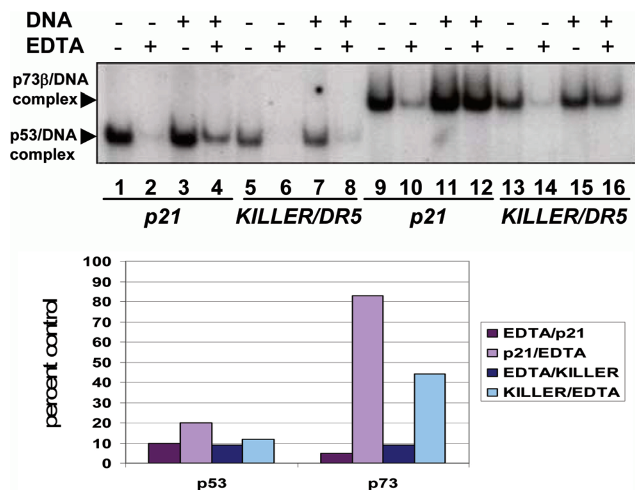


Figure 3. DNA prebinding protects p73β but not p53 from treatment with EDTA. Purified HA-tagged p53 (lanes 1–8) and p73β (lanes 9–16) proteins (10 ng) were incubated with EDTA (40 mM for p53 and 12.5 mM for p73) for 20 min and then oligonucleotides (*p21* or *KILLER/DR5* as indicated) were added (lanes 1, 2, 5, 6, 9, 10, 13 and 14) for 20 min. Alternately p73β and p53 were incubated first with oligonucleotides for 20 min followed by addition of EDTA at the same respective concentrations and incubation for another 20 min (lanes 3, 4, 7, 8, 11, 12, 15 and 16). DNA binding was quantified by phosphorimaging, and the results plotted as percentage-binding retained in each EDTA treated sample versus its untreated control.

Interestingly, the protection of p73β binding to the *KILLER/DR5* site (∼5-fold) was significantly less than to the *p21* site.

Taken together, these data suggest a stronger need for the zinc atom for the DNA-binding active conformation of p73β than p53, which supports our speculation of differential folding of their respective DNA-binding domains.

p53 and p73 bind DNA in a sequence-specific manner but exhibit different sequence-specific stability

The differential protection of p73β on the *p21* and *KILLER/DR5* REs (Figure 2B), the putative difference in the folding of DNA-binding domains of p53 and p73β, as well as previously reported effects of mutation on p53 sequence specificity (62,63), led us to further investigate differences in DNA binding between the two proteins. It is well documented that a C-terminal specific p53 antibody (MAb 421) increases the binding of p53 to short oligonucleotides containing p53-binding sites (33). We were first interested in whether an antibody that interacts with the C-terminus of p73β might have a similar stimulatory effect. To test this, p53 and p73β were each incubated with antibodies that recognize their respective C- and N-termini (MAbs 421 and DO-1 for p53 and Mab ER15 and PAb TAp73N for p73) and EMSAs were performed comparing binding of the proteins to oligonucleotides containing either *p21* or *PIG3* p53 binding sites (Figure 4A). As predicted, while the N-terminal antibody MAb DO-1 did not affect p53 binding to either site, the C-terminal MAb 421 markedly increased p53 DNA binding under the assay conditions used. In contrast, neither the TAp73 N-terminal polyclonal antibody (TAp73N) nor the C-terminal antibody (ER15) affected p73β DNA binding to the *p21* oligonucleotide, although there was a modest increase in binding to the *PIG3* oligonucleotide with both antibodies. Nevertheless, the effects with the p53 C-terminal antibody were much more significant with both sites, indicating a difference in the function of the C-terminus between the two proteins. This is consistent with the fact that there is no region in p73 isoforms that resembles the highly basic p53 C-terminus.

To confirm that sequence-specific DNA binding was being observed, a competitor curve was performed, using unlabeled oligonucleotides containing either the wild-type 5′ RE p53-binding site in *p21* (p21wt) or a sequence in which key nucleotides in this site were mutated (p21mut) to compete for binding to a radioactively labeled oligonucleotide containing the *p21* 5′ RE. As expected, both p53 and p73β DNA binding was competed by p21wt but not p21mut oligonucleotides. Surprisingly, however, p73β was dramatically more stable on the *p21* DNA site than was p53 (Figure 4B).

To determine if the lower dissociation rate of p73β is specific to the *p21* site, competitor EMSAs were performed using oligonucleotides containing p53 binding sites from several p53 target genes including *p21*, *GADD45*, *MDM2*, *14-3-3-sigma*, *PCNA*, *PIG3*, *KILLER/DR5* and *PUMA*. We used sequences from both pro-apoptotic and non-apoptotic target genes, since some difference in transactivation by p53 have been reported for these two classes of genes. Interestingly, p53 had roughly similar dissociation rates from all promoters used (Figure 4C), while p73β exhibited dramatically lower dissociation rates specifically from two of the sites tested: *p21* and *PUMA* (Figure 4D). Note that although p73α binds more weakly to DNA than p73β (45) we obtained essentially similar results in that purified p73α protein dissociated more slowly from the *p21* than the *PIG3* binding site-containing oligonucleotides (data not shown). Moreover, purified ΔNp63β protein was also more stably associated with the *p21* than the *KILLER/DR5* site (Supplementary Figure 1).

Five bases in the p53 consensus sequence confer stable binding exclusively on p73β

Comparing the sequences of the *p21* and *PUMA* binding sites revealed that they share five bases that are not together present in any of the other p53 sites examined (Figure 5A). In order to determine if these bases are necessary for the decreased dissociation rate, we designed three oligonucleotides that each conform generally to the p53 consensus site but in which either the five bases common to *p21* and *PUMA* were present, with the rest scrambled so as to still conform to the p53 consensus site (*p21-PUMA* consensus; PPC) or in which these five bases were changed either to the same [purine → purine; PPN(s)] or the opposite [i.e. purine → pyrimidine, PPN(o)] base. PPN(s) conforms to the general consensus binding sequence to the same extent as PPC, while the PPN(o) site deviates from consensus by another five bases. The sequences of these sites are shown in Figure 5B. When a competitor EMSA was then performed with these oligonucleotides, there were no significant differences in the dissociation rates of p53 from either of the above artificial sites, which were similar to its dissociation from the *p21* site. Importantly, the dissociation of p73β was markedly increased from the PPN as compared to the PPC sequence, which approximated that of the *PUMA* and *p21* binding sequences. This shows that the five-base sub-sequence that

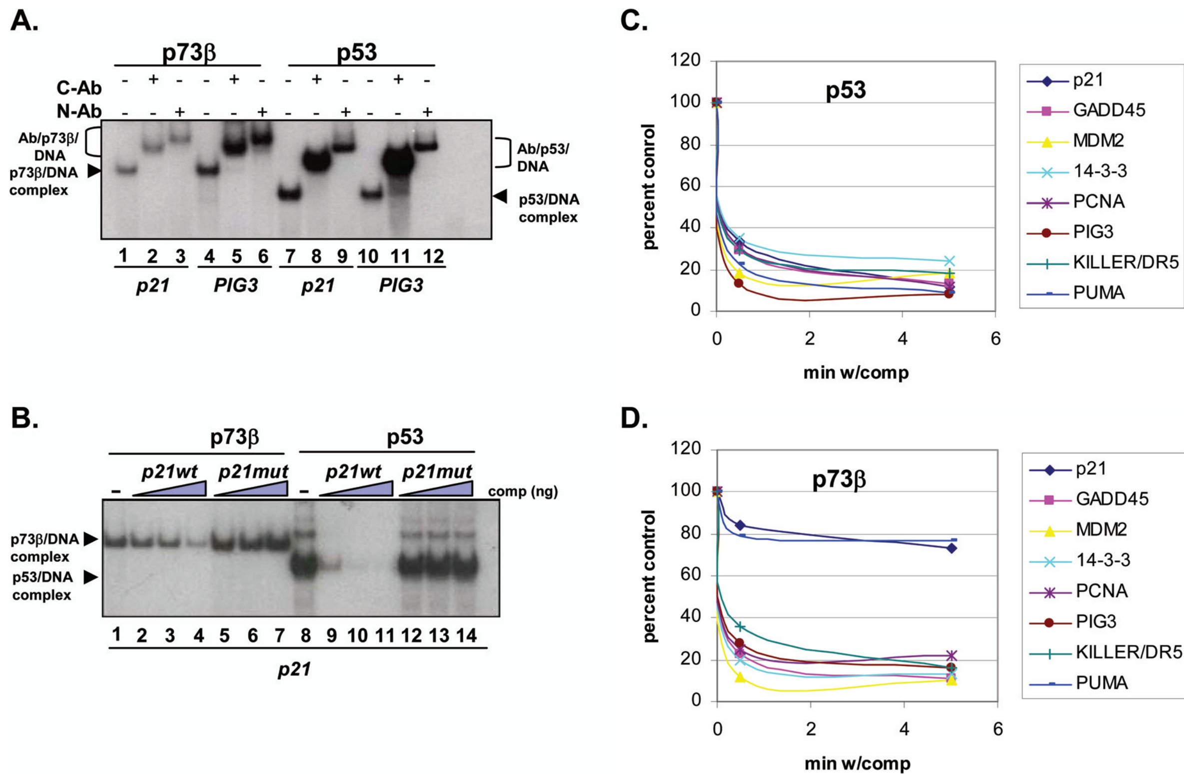


Figure 4. p73β but not p53 dissociates very slowly from p21 and PUMA-binding sites. (**A**) EMSAs with HA-tagged p73β (lanes 1–6) and p53 (lanes 7–12) proteins (10 ng) were performed in which DNA–protein mixtures were either untreated (lanes 1, 4, 7 and 10) or treated with antibodies to the C- (MAb ER15, lanes 2 and 5) and N- (TAp73N, lanes 3 and 6) termini of p73β or C- (MAb 421, lanes 8 and 11) and N- (MAb DO-1, lanes 9 and 12) termini of p53. The *p21 5′* (lanes 1–3 and 7–9) and *PIG3* (lanes 4–6 and 10–12)-binding site containing oligonucleotides were used. (**B**) The sequence-specificity of HA-tagged p73β (lanes 1–7) and p53 (lanes 8–14) (10 ng)–DNA binding was assessed by performing EMSAs in which binding to a γ-^{32}P-labeled oligonucleotide containing the *p21 5′* binding site sequence was competed by adding excess unlabeled oligonucleotides with wild-type *p21*-binding site sequence (p21wt) or mutated *p21 5′* binding site sequence (p21mut) at increasing fold molar excess (25, 50 and 100). (**C** and **D**) Competitor EMSAs were performed with HA-tagged p53 and p73β proteins using p53-binding sites from non-apoptotic (*p21*, *GADD45*, *MDM2*, *14-3-3-sigma* and *PCNA*) and pro-apoptotic (*PIG3*, *KILLER/DR5* and *PUMA*) genes. In each case a 50-fold excess of the corresponding unlabeled oligonucleotide was used as competitor. The results are plotted as amount of binding retained as percent of control.

is unique to *p21* and *PUMA* is in fact responsible for the reduced dissociation of p73β. It is also interesting to note that while the dissociation rate of p53 from PPC, PPN(s) or PPN(o) did not vary, its steady-state binding to PPC was greater than to PPN(s), and binding was even more reduced to the PPN(o) sequence. This result correlates well with results of transactivation by p53 *in vivo* (see below).

Deletion of the C-terminus allows p53 to discriminate between PPC and PPN sites

As mentioned previously, p53 has a second sequence-non-specific DNA-binding domain in its C-terminus that can linearly diffuse and dissociate from ends of DNA (31). Furthermore, it has been shown that the dissociation rate of the C-terminus is up to 1000-fold higher than that of the core DNA-binding domain (64). Based on this, we hypothesized that the difference between p53 and p73β could be due to the activity of the C-terminus, which might increase the dissociation rate under the conditions of the assay in which relatively short oligonucleotides (44 bp) were tested. If this were the case, the C-terminus would mask any differences in the ability of the core domain to dissociate from its binding sites in DNA. To test this, we used a version of p53 protein in which the C-terminal 30 amino acids have been deleted (HA-tagged p53Δ30), rendering the protein unable to diffuse linearly along DNA (31). Indeed, when a similar analysis was performed, p53Δ30 exhibited much lower dissociation from the PPC versus the PPN site-containing oligonucleotides (Figure 6). Thus, the core DNA-binding domains of p73β and p53 are similar in their relative dissociations from the PPC and the PPN sites *in vitro*. Furthermore, the p53 C-terminus can overcome the slower dissociation from these sites, presumably through its sliding activity and rapid dissociation from the oligonucleotide ends.

PPC and PPN minimal promoters are differentially transactivated by p53 and p73β

To explore the difference between sites that do or do not contain the five-base *p21-PUMA* consensus sub-sequence in the context of a cell, we used a luciferase reporter assay to investigate the contribution of different p53-binding sites to the abilities of p53 and p73β to activate transcription. pGL3-OFLuc vectors containing the PPC and PPN(s) binding sites were co-transfected into H1299 cells along with Flag-tagged versions of p53 and p73β (Figure 7). Both p53 and p73β transactivated the PPC promoter to a greater extent

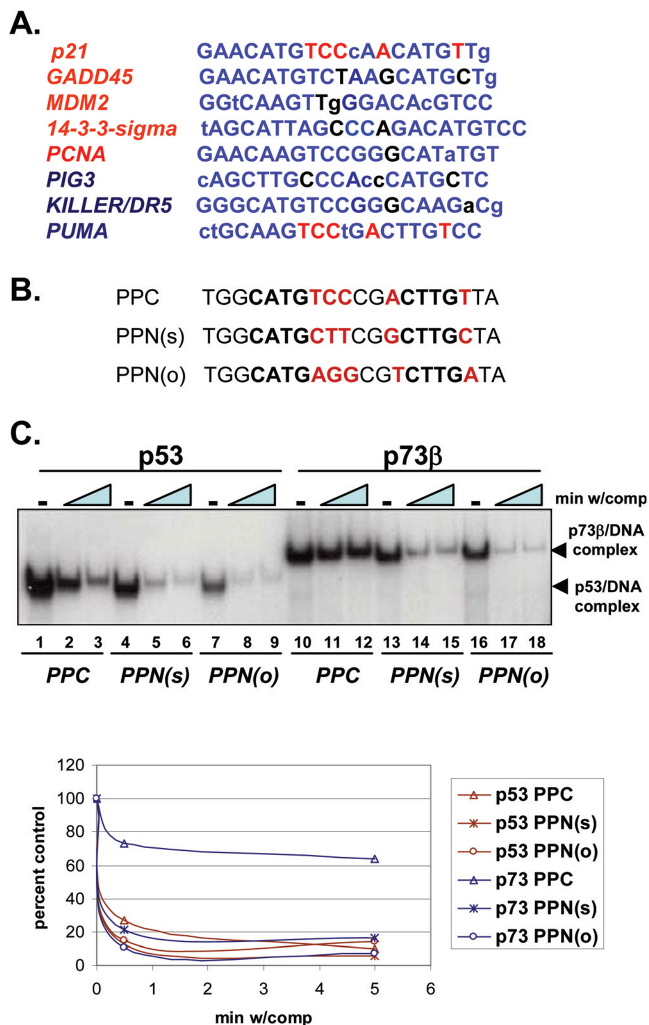


Figure 5. A five-base sub-sequence in the canonical p53-binding site is required for the lower dissociation rate of p73β. (**A**) Sequence alignment of the binding sites used in Figure 4C and D reveal five conserved bases in the *p21*- and *PUMA*-binding sites. The five bases conserved between *p21* and *PUMA* are in red, the bases that deviate from this consensus in the other six binding sites are in black. Bases that deviate from p53 consensus are in lower case. (**B**) A *p21-PUMA* consensus (PPC) and two non-consensus sequences (PPNs) were generated. The PPC sequence is a combination of *p21* and *PUMA* sequence, the PPN(s) is the same sequence with the five common *p21*/*PUMA* nucleotides mutated purine to purine and pyrimidine to pyrimidine, and the PPN(o) has the 5 nt mutated purine to partner pyrimidine and vice versa. Invariable CWWG is in bold. Changed bases are in red bold. (**C**) The dissociation rate of p73β is increased to that of p53 by changing five bases in the *p21-PUMA* consensus binding site. Competitor EMSAs with HA-tagged p53 (lanes 1–9) and p73β (lanes 10–18) proteins were performed as in Figure 4 using the PPC and two PPN sequences. The proteins were preincubated with radioactively labeled oligonucleotide, followed by a 0.5, 1 and 5 min incubation with a 50-fold excess of self competitor. DNA binding was quantified by phosphorimaging and the values plotted as percent of control. Note that reaction mixtures were loaded onto a running gel at different time points after the start of the experiment.

than the PPN promoter, although the difference was more pronounced for p53 (4- and 15-fold) than p73β (2-fold). This result suggested that the five-base sub-sequence in the binding site plays a role in the level of transactivation. The stronger effect on p53 was surprising in that from the DNA-binding assays one would have predicted the opposite result. This finding is consistent, however, with the fact that, as shown in Figure 5C, there was a much bigger difference in net p53 binding to the PPC versus PPN sites than observed with p73β. Furthermore, p53Δ30 exhibited a transactivation pattern similar to that of full-length p53 (data not shown) further supporting core DNA-binding domain sequence specificity. Taken together, there is a good correspondence between the *in vitro* dissociation data and *in vivo* transactivation results for both p53 and p73β. Furthermore, the p53 C-terminus plays a critical role *in vitro* but not *in vivo* in this sequence selectivity.

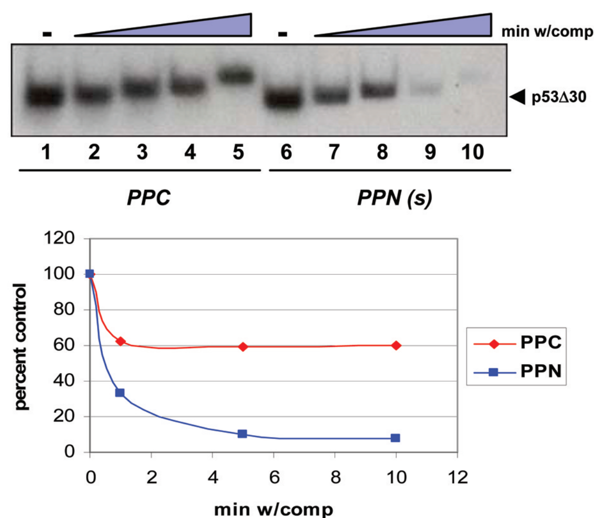


Figure 6. The DNA-binding domain of p53 exhibits similar sequence selectivity as that of p73β. Baculovirally expressed, purified HA-tagged p53Δ30 protein (5 ng) was preincubated with radioactively labeled PPC or PPN oligonucleotides as indicated, followed by a 0.5, 1, 5 and 10 min incubation with a 50-fold excess of unlabeled corresponding oligonucleotide as competitor. Reaction mixtures were loaded onto a running gel at different time points after the start of the experiment. DNA binding to PPC or PPN sites as indicated was quantified by phosphorimaging and the values plotted as percent of control.

DISCUSSION

Our studies have revealed several differences between purified preparations of p53 and p73β proteins in their interaction with DNA. First, p73β is apparently significantly more dependent on zinc coordination for binding DNA than is p53 based on its relatively greater sensitivity to EDTA, reactivity with MAL-PEG and virtually complete loss of activity caused by mutation of a zinc-coordinating cysteine. Second, DNA binding offers a much greater protection to p73β than p53 from zinc chelation. Finally, while full-length p53 and p73β proteins differ in their preference for binding to p53-binding sites with a specific sub-sequence that we have identified, the core sequence-specific DNA-binding domains of the two proteins exhibit similar sequence preference both *in vitro* and *in vivo*.

Our data suggest that there is a difference in the structure of the DNA-binding domains of p53 and p73, especially with respect to their zinc-coordinating residues. Our initial experiments revealed a more significant effect of the metal chelator EDTA on p73β than p53, an effect reversed by

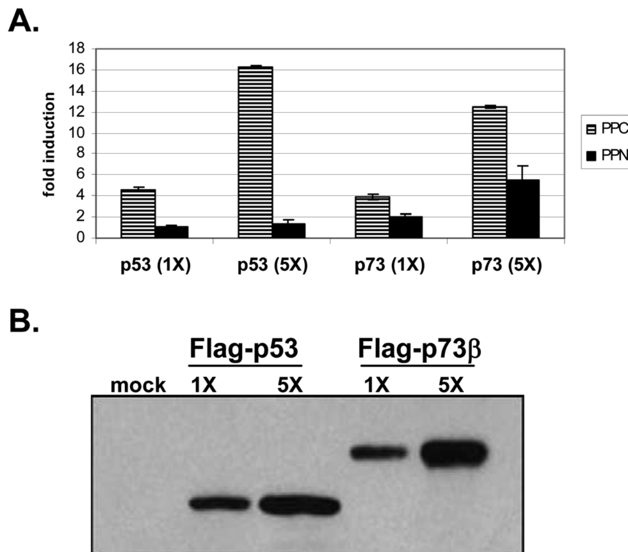


Figure 7. Both p53 and p73β can discriminate between PPC and PPN sites *in vivo*. (**A**) p53 and p73β transactivate a PPN reporter to a lower extent than a PPC reporter. Plasmids (8 and 40 pg, represented as 1X and 5X, respectively) expressing either Flag-tagged p53 or p73β proteins were cotransfected with pGL3-OF Luc reporter vectors containing either PPC or PPN(s)-binding sites as indicated along with a pRL (Renilla) control vector into H1299 cells. The cells were lysed, 24 h after transfection, and luciferase activity measured. The luciferase activity was normalized to Renilla and the induction was plotted versus the appropriate pGL3 reporter alone. A representative experiment performed in triplicate is presented. (**B**) Constructs expressing Flag- tagged p53 and p73β proteins were transfected into H1299 cells (8 and 40 ng, represented as ×1 and ×5, respectively) as in (A) and proteins were detected by immunoblotting with an anti-Flag antibody.

addition of zinc. The thiol tagging data provided insight into this observation by showing that, in contrast to p53, the structure of the p73β DNA-binding domain is significantly changed after zinc chelation by EDTA. More cysteines become accessible to maleimide tagging, indicating that the structure takes on an unfolded conformation in the absence of the zinc atom. This is not seen with p53.

Recent publications describe the interaction of p53 dimers across DNA (15,16). Data from crystal structures of p53 core domain bound to DNA suggest that several p53 residues in dimers bound to the same half-site interact with each other forming an interface that further stabilizes the protein on DNA. Of these, neither the two charged residues (His178 and Arg181) nor the one hydrophobic residue (Met243) are conserved in p73, where they are replaced by non-charged and non-hydrophobic residues (Asn196, Leu201 and Val263). This might provide a further explanation for our observation of differential zinc dependence of p53 and p73β such that if p53 has an additional means to stabilize its interaction with DNA, structural perturbations are likely to affect p53 DNA binding to a lesser extent than that of p73. Our data showed that a mutation of a zinc-coordinating residue in p53 and p73β leads to an inactive protein in the case of p73β but not p53 and is consistent with p73β DNA binding being more affected by zinc chelation. In the absence of zinc, the DNA-binding domain of p73 may undergo a greater conformational change not compensated for by dimer interaction across the DNA, thereby resulting in a complete loss of DNA binding.

DNA protection from EDTA demonstrates another possible structural difference between p53 and p73β. It is possible that p73β is protected from zinc chelation by being prebound to DNA because the zinc-coordinating residues of p73 are relatively more buried within the protein when it is bound to oligonucleotide. It was reported that Tyr236 and Thr253 in the DNA-binding domain of p53 destabilize the structure and these residues are not conserved in p73 (65). These unpaired hydrogen bond donors/acceptors may allow for greater solvent exposure of the zinc-coordinating region of p53 which could in turn explain why p53 is not well protected from EDTA by DNA. We speculate that in the case of p73, this region is not exposed to solvent when the protein is bound to DNA.

Our findings of differential sensitivity of p73β and p53 to EDTA might be important when using chelating chemotherapy in tumors [e.g. reduction of copper levels to inhibit angiogenesis (66)] that lack functional p53 but have wild-type p73. In this scenario, the chemotherapeutic drug might render p73 non-functional, and potentially cause the cell to be even less capable of undergoing apoptosis.

These differences in the DNA-binding domain structure of p53 and p73 prompted us to examine whether the two proteins might display dissimilarities in preferred DNA binding sites. Several previous studies have examined DNA binding by p73 proteins (1,41,42,45,67–70), although currently a single report directly compares *in vitro* DNA binding of purified p53 and p73 proteins, and demonstrates that baculovirally expressed Flag-tagged p53 and p73 exhibit similar sequence-specific affinity for a consensus binding site as assessed by EMSA (67). However, the presence of both p53 and p73-specific promoters in the genome (53), along with the recent discovery of a novel p73/p63 but not p53 responsive promoter, consisting of a canonical p53 RE and a second, GC-rich RE (71) raised the possibility of differences in DNA sequence selectivity between p53 and p73. Indeed, our initial results suggested this to be the case when we found that p73β dissociates much more slowly from *p21* and *PUMA* binding sites, a phenomenon not observed with full-length p53. It was therefore unexpected that deletion of the p53 C-terminus produces a protein which behaves similarly to p73β in its discrimination of sequences from which it dissociates. That similar results were obtained for the third family member, p63 shows that this sequence discrimination is a property of the DNA-binding domains of all three p53 family members.

In fact, our conclusion that the core domains of p53 and p73β are similar in slowed dissociation from sites with a unique sub-sequence are consistent with the observation that in the cell, both p53 and p73β exhibit relatively higher transactivation from a minimal promoter containing this sub-sequence. While both p53 and p73β transactivate PPC and PPN site containing reporters in transfection assays to a different extent, the difference is more pronounced in the case of p53. Although this is difficult to explain at present it could be due to some structural difference between the DNA-binding domains of the two proteins, though we cannot rule that they recruit different transcriptional co-factors.

That p73β binding resembles that of p53Δ30 but not full-length p53 further supports the finding that p73 isoforms

do not have a sequence nonspecific DNA-binding domain similar to that located in the C-terminus of p53. Interestingly, when a four-base spacer was inserted between the two PPC half-sites, the dissociation rate of p53Δ30 increased to that observed on a PPN site, suggesting the importance of spacing as well as sequence (data not shown).

There is some evidence suggesting that *p21* and *PUMA* are stronger transactivation sites and are sites which can be uniquely transactivated by certain p53 mutants. For example, there is greater p53 binding, as seen by ChIP, to the *p21* and *PUMA* than *PIG3* and *AIP1* promoters (19) and a greater histone acetylation at the *p21* and *PUMA* than *MDM2* promoters (72). Furthermore, several p53 mutants have been shown to transactivate the p21 promoter, but not the *Bax* and *PIG3* promoters in yeast (73). In addition, Nicholls *et al*. (74) have shown that wild-type/hotspot mutant hetero-tetramers show almost wild-type activity on *p21* out of all binding sites used except for an artificial full consensus sequence (*PUMA* was not used in their experiment). The sub-sequence we have here described could confer more stable binding, thus allowing even the p53 mutants to retain some DNA binding on *p21* and *PUMA* promoters.

Recent crystal structure of p53 core domains bound to different oligonucleotide sequences from Kitayner *et al*. (15) suggests that an A preceding and a T following the invariable CWWG core of the binding site (which is one feature of the sub-sequence we identified) allows Lys120, Ala276 and Arg280 to mediate a stronger p53/DNA interaction. These residues are conserved in p73 (Lys138, Ala322 and Arg325) further supporting the preferential interaction with some DNA-binding sites by p53 and p73β. These data in conjunction with our findings suggest a conservation of RE selectivity as well as DNA-interacting residues between p53 and p73. Hopefully the differences and similarities between p53 and p73 that we have observed can be elucidated when the atomic structure of the p73 core domain is solved.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Levrero,M., De Laurenzi,V., Costanzo,A., Gong,J., Wang,J.Y. and Melino,G. (2000) The p53/p63/p73 family of transcription factors: overlapping and distinct functions. *J. Cell Sci*., **113**, 1661–1670.
2. Moll,U.M., Erster,S. and Zaika,A. (2001) p53, p63 and p73—solos, alliances and feuds among family members. *Biochim. Biophys. Acta*., **1552**, 47–59.
3. Yang,A., Kaghad,M., Caput,D. and McKeon,F. (2002) On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet*., **18**, 90–95.
4. Derry,W.B., Putzke,A.P. and Rothman,J.H. (2001) *Caenorhabditis elegans* p53: role in apoptosis, meiosis, and stress resistance. *Science*, **294**, 591–595.
5. Schumacher,B., Hofmann,K., Boulton,S. and Gartner,A. (2001) The *C.elegans* homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. *Curr. Biol*., **11**, 1722–1727.
6. Mendoza,L., Orozco,E., Rodriguez,M.A., Garcia-Rivera,G., Sanchez,T., Garcia,E. and Gariglio,P. (2003) Ehp53, an Entamoeba histolytica protein, ancestor of the mammalian tumour suppressor p53. *Microbiology*, **149**, 885–893.
7. Murray-Zmijewski,F., Lane,D.P. and Bourdon,J.C. (2006) p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death Differ*., **13**, 962–972.
8. Scoumanne,A., Harms,K.L. and Chen,X. (2005) Structural basis for gene activation by p53 family members. *Cancer Biol. Ther*., **4**, 1178–1185.
9. Laptenko,O. and Prives,C. (2006) Transcriptional regulation by p53: one protein, many possibilities. *Cell Death Differ*., **13**, 951–961.
10. el-Deiry,W.S., Kern,S.E., Pietenpol,J.A., Kinzler,K.W. and Vogelstein,B. (1992) Definition of a consensus binding site for p53. *Nature Genet*., **1**, 45–49.
11. Halazonetis,T.D. and Kandil,A.N. (1993) Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. *EMBO J*., **12**, 5057–5064.
12. Prives,C. and Hall,P.A. (1999) The p53 pathway. *J. Pathol*., **187**, 112–126.
13. McLure,K.G. and Lee,P.W. (1998) How p53 binds DNA as a tetramer. *EMBO J*., **17**, 3342–3350.
14. Friedman,P.N., Chen,X., Bargonetti,J. and Prives,C. (1993) The p53 protein is an unusually shaped tetramer that binds directly to DNA. *Proc. Natl Acad. Sci. USA*, **90**, 3319–3323.
15. Kitayner,M., Rozenberg,H., Kessler,N., Rabinovich,D., Shaulov,L., Haran,T.E. and Shakked,Z. (2006) Structural basis of DNA recognition by p53 tetramers. *Mol. Cell*, **22**, 741–753.
16. Ho,W.C., Fitzgerald,M.X. and Marmorstein,R. (2006) Structure of the p53 Core Domain Dimer Bound to DNA. *J. Biol. Chem*., **281**, 20494–20502.
17. Kim,E., Albrechtsen,N. and Deppert,W. (1997) DNA-conformation is an important determinant of sequence-specific DNA binding by tumor suppressor p53. *Oncogene*, **15**, 857–869.
18. Szak,S.T., Mays,D. and Pietenpol,J.A. (2001) Kinetics of p53 binding to promoter sites *in vivo*. *Mol. Cell. Biol*., **21**, 3375–3386.
19. Kaeser,M.D. and Iggo,R.D. (2002) Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity *in vivo*. *Proc. Natl Acad. Sci. USA*, **99**, 95–100.
20. Thornborrow,E.C. and Manfredi,J.J. (1999) One mechanism for cell type-specific regulation of the bax promoter by the tumor suppressor p53 is dictated by the p53 response element. *J. Biol. Chem*., **274**, 33747–33756.
21. Qian,H., Wang,T., Naumovski,L., Lopez,C.D. and Brachmann,R.K. (2002) Groups of p53 target genes involved in specific p53 downstream effects cluster into different classes of DNA binding sites. *Oncogene*, **21**, 7901–7911.
22. Inga,A., Storici,F., Darden,T.A. and Resnick,M.A. (2002) Differential transactivation by the p53 transcription factor is highly dependent on p53 level and promoter target sequence. *Mol. Cell. Biol*., **22**, 8612–8625.
23. Hoh,J., Jin,S., Parrado,T., Edington,J., Levine,A.J. and Ott,J. (2002) The p53MH algorithm and its application in detecting p53-responsive genes. *Proc. Natl Acad. Sci. USA*, **99**, 8467–8472.
24. Wei,C.L., Wu,Q., Vega,V.B., Chiu,K.P., Ng,P., Zhang,T., Shahab,A., Yong,H.C., Fu,Y., Weng,Z. *et al*. (2006) A global map of p53 transcription-factor binding sites in the human genome. *Cell*, **124**, 207–219.
25. Olivier,M., Eeles,R., Hollstein,M., Khan,M.A., Harris,C.C. and Hainaut,P. (2002) The IARC TP53 database: new online mutation

analysis and recommendations to users. *Hum. Mutat.*, **19**, 607–614.
26. Cho,Y., Gorina,S., Jeffrey,P.D. and Pavletich,N.P. (1994) Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science*, **265**, 346–355.
27. Pavletich,N.P., Chambers,K.A. and Pabo,C.O. (1993) The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. *Genes Dev.*, **7**, 2556–2564.
28. Hainaut,P. and Milner,J. (1993) A structural role for metal ions in the 'wild-type' conformation of the tumor suppressor protein p53. *Cancer Res.*, **53**, 1739–1742.
29. Verhaegh,G.W., Parat,M.O., Richard,M.J. and Hainaut,P. (1998) Modulation of p53 protein conformation and DNA-binding activity by intracellular chelation of zinc. *Mol. Carcinog.*, **21**, 205–214.
30. Meplan,C., Richard,M.J. and Hainaut,P. (2000) Metalloregulation of the tumor suppressor protein p53: zinc mediates the renaturation of p53 after exposure to metal chelators *in vitro* and in intact cells. *Oncogene*, **19**, 5227–5236.
31. McKinney,K., Mattia,M., Gottifredi,V. and Prives,C. (2004) p53 linear diffusion along DNA requires its C terminus. *Mol. Cell*, **16**, 413–424.
32. Espinosa,J.M. and Emerson,B.M. (2001) Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Mol. Cell*, **8**, 57–69.
33. Ahn,J. and Prives,C. (2001) The C-terminus of p53: the more you learn the less you know. *Nature Struct. Biol.*, **8**, 730–732.
34. Kaghad,M., Bonnet,H., Yang,A., Creancier,L., Biscan,J.C., Valent,A., Minty,A., Chalon,P., Lelias,J.M., Dumont,X. *et al.* (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell*, **90**, 809–819.
35. Jost,C.A., Marin,M.C. and Kaelin,W.G.,Jr (1997) p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature*, **389**, 191–194.
36. Irwin,M.S. and Kaelin,W.G. (2001) p53 family update: p73 and p63 develop their own identities. *Cell Growth Differ.*, **12**, 337–349.
37. Ozaki,T. and Nakagawara,A. (2005) p73, a sophisticated p53 family member in the cancer world. *Cancer Sci.*, **96**, 729–737.
38. Oberst,A., Rossi,M., Salomoni,P., Pandolfi,P.P., Oren,M., Melino,G. and Bernassola,F. (2005) Regulation of the p73 protein stability and degradation. *Biochem. Biophys. Res. Commun.*, **331**, 707–712.
39. Moll,U.M. and Slade,N. (2004) p63 and p73: roles in development and tumor formation. *Mol. Cancer Res.*, **2**, 371–386.
40. Melino,G., Lu,X., Gasco,M., Crook,T. and Knight,R.A. (2003) Functional regulation of p73 and p63: development and cancer. *Trends Biochem. Sci.*, **28**, 663–670.
41. Stiewe,T., Theseling,C.C. and Putzer,B.M. (2002) Transactivation-deficient Delta TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis. *J. Biol. Chem.*, **277**, 14177–14185.
42. Nakagawa,T., Takahashi,M., Ozaki,T., Watanabe Ki,K., Todo,S., Mizuguchi,H., Hayakawa,T. and Nakagawara,A. (2002) Autoinhibitory regulation of p73 by Delta Np73 to modulate cell survival and death through a p73-specific target element within the Delta Np73 promoter. *Mol. Cell. Biol.*, **22**, 2575–2585.
43. Grob,T.J., Novak,U., Maisse,C., Barcaroli,D., Luthi,A.U., Pirnia,F., Hugli,B., Graber,H.U., De Laurenzi,V., Fey,M.F. *et al.* (2001) Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. *Cell Death Differ.*, **8**, 1213–1223.
44. Liu,G., Nozell,S., Xiao,H. and Chen,X. (2004) DeltaNp73beta is active in transactivation and growth suppression. *Mol. Cell. Biol.*, **24**, 487–501.
45. Ozaki,T., Naka,M., Takada,N., Tada,M., Sakiyama,S. and Nakagawara,A. (1999) Deletion of the COOH-terminal region of p73alpha enhances both its transactivation function and DNA-binding activity but inhibits induction of apoptosis in mammalian cells. *Cancer Res.*, **59**, 5902–5907.
46. Chen,X. (1999) The p53 family: same response, different signals? *Mol. Med. Today*, **5**, 387–392.
47. Ueda,Y., Hijikata,M., Takagi,S., Chiba,T. and Shimotohno,K. (1999) New p73 variants with altered C-terminal structures have varied transcriptional activities. *Oncogene*, **18**, 4993–4998.
48. Alarcon-Vargas,D., Fuchs,S.Y., Deb,S. and Ronai,Z. (2000) p73 transcriptional activity increases upon cooperation between its spliced forms. *Oncogene*, **19**, 831–835.
49. Arrowsmith,C.H. (1999) Structure and function in the p53 family. *Cell Death Differ.*, **6**, 1169–1173.
50. Zhu,J., Jiang,J., Zhou,W. and Chen,X. (1998) The potential tumor suppressor p73 differentially regulates cellular p53 target genes. *Cancer Res.*, **58**, 5061–5065.
51. Lee,C.W. and La Thangue,N.B. (1999) Promoter specificity and stability control of the p53-related protein p73. *Oncogene*, **18**, 4171–4181.
52. Fontemaggi,G., Kela,I., Amariglio,N., Rechavi,G., Krishnamurthy,J., Strano,S., Sacchi,A., Givol,D. and Blandino,G. (2002) Identification of direct p73 target genes combining DNA microarray and chromatin immunoprecipitation analyses. *J. Biol. Chem.*, **277**, 43359–43368.
53. Harms,K., Nozell,S. and Chen,X. (2004) The common and distinct target genes of the p53 family transcription factors. *Cell. Mol. Life Sci.*, **61**, 822–842.
54. Nozell,S., Wu,Y., McNaughton,K., Liu,G., Willis,A., Paik,J.C. and Chen,X. (2003) Characterization of p73 functional domains necessary for transactivation and growth suppression. *Oncogene*, **22**, 4333–4347.
55. Urist,M., Tanaka,T., Poyurovsky,M.V. and Prives,C. (2004) p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. *Genes Dev.*, **18**, 3041–3054.
56. Jayaraman,L., Freulich,E. and Prives,C. (1997) Functional dissection of p53 tumor suppressor protein. *Meth. Enzymol.*, **283**, 245–256.
57. Arslan,P., Di Virgilio,F., Beltrame,M., Tsien,R.Y. and Pozzan,T. (1985) Cytosolic Ca2+ homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca2?. *J. Biol. Chem.*, **260**, 2719–2727.
58. Stephen,C.W. and Lane,D.P. (1992) Mutant conformation of p53. Precise epitope mapping using a filamentous phage epitope library. *J. Mol. Biol.*, **225**, 577–583.
59. Malpica,R., Franco,B., Rodriguez,C., Kwon,O. and Georgellis,D. (2004) Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. *Proc. Natl Acad. Sci. USA*, **101**, 13318–13323.
60. Wu,H.H., Thomas,J.A. and Momand,J. (2000) p53 protein oxidation in cultured cells in response to pyrrolidine dithiocarbamate: a novel method for relating the amount of p53 oxidation *in vivo* to the regulation of p53-responsive genes. *Biochem. J.*, **351**, 87–93.
61. Rainwater,R., Parks,D., Anderson,M.E., Tegtmeyer,P. and Mann,K. (1995) Role of cysteine residues in regulation of p53 function. *Mol. Cell. Biol.*, **15**, 3892–3903.
62. Freeman,J., Schmidt,S., Scharer,E. and Iggo,R. (1994) Mutation of conserved domain II alters the sequence specificity of DNA binding by the p53 protein. *EMBO J.*, **13**, 5393–5400.
63. Zupnick,A. and Prives,C. (2006) Mutational analysis of the p53 core domain L1 loop. *J. Biol. Chem.*, **281**, 20464–20473.
64. Weinberg,R.L., Freund,S.M., Veprintsev,D.B., Bycroft,M. and Fersht,A.R. (2004) Regulation of DNA binding of p53 by its C-terminal domain. *J. Mol. Biol.*, **342**, 801–811.
65. Canadillas,J.M., Tidow,H., Freund,S.M., Rutherford,T.J., Ang,H.C. and Fersht,A.R. (2006) Solution structure of p53 core domain: structural basis for its instability. *Proc. Natl Acad. Sci. USA*, **103**, 2109–2114.
66. Nasulewicz,A., Mazur,A. and Opolski,A. (2004) Role of copper in tumour angiogenesis--clinical implications. *J. Trace Elem. Med. Biol.*, **18**, 1–8.
67. Mondal,N. and Parvin,J.D. (2005) The tumor suppressor protein p53 functions similarly to p63 and p73 in activating transcription *in vitro*. *Cancer Biol. Ther.*, **4**, 414–418.
68. Kartasheva,N.N., Contente,A., Lenz-Stoppler,C., Roth,J. and Dobbelstein,M. (2002) p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene*, **21**, 4715–4727.
69. Zeng,X., Lee,H., Zhang,Q. and Lu,H. (2001) p300 does not require its acetylase activity to stimulate p73 function. *J. Biol. Chem.*, **276**, 48–52.
70. Stros,M., Ozaki,T., Bacikova,A., Kageyama,H. and Nakagawara,A. (2002) HMGB1 and HMGB2 cell-specifically down-regulate the p53- and p73-dependent sequence-specific transactivation from the human Bax gene promoter. *J. Biol. Chem.*, **277**, 7157–7164.
71. Osada,M., Park,H.L., Nagakawa,Y., Begum,S., Yamashita,K., Wu,G., Kim,M.S., Trink,B. and Sidransky,D. (2006) A novel response element

confers p63- and p73-specific activation of the WNT4 promoter. *Biochem. Biophys. Res. Commun.*, **339**, 1120–1128.
72. Kaeser,M.D. and Iggo,R.D. (2004) Promoter-specific p53-dependent histone acetylation following DNA damage. *Oncogene*, **23**, 4007–4013.
73. Campomenosi,P., Monti,P., Aprile,A., Abbondandolo,A., Frebourg,T., Gold,B., Crook,T., Inga,A., Resnick,M.A., Iggo,R. *et al.* (2001) p53 mutants can often transactivate promoters containing a p21 but not Bax or PIG3 responsive elements. *Oncogene*, **20**, 3573–3579.
74. Nicholls,C.D., McLure,K.G., Shields,M.A. and Lee,P.W. (2002) Biogenesis of p53 involves cotranslational dimerization of monomers and posttranslational dimerization of dimers. Implications on the dominant negative effect. *J. Biol. Chem.*, **277**, 12937–12945.