Chemosensitivity linked to p73 function

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Summary

Most chemotherapeutic agents induce DNA damage, leading to p53 accumulation and apoptosis. The factors that determine chemosensitivity in p53-defective tumor cells are poorly understood. We found that the p53 family member p73 is induced by a wide variety of chemotherapeutic drugs. Blocking p73 function with a dominant-negative mutant, siRNA, or homologous recombination led to chemoresistance of human tumor cells and engineered transformed cells, irrespective of p53 status. Mutant p53 can inactivate p73 and downregulation of mutant p53 enhanced chemosensitivity. These findings indicate that p73 is a determinant of chemotherapeutic efficacy in humans.

Introduction

The tumor suppressor protein p53 plays a critical role in apoptosis induced by DNA-damaging agents, including many anticancer drugs (Johnstone et al., 2002; Ko and Prives, 1996). Unfortunately, at least 50% of human cancers lack functional p53. The p53 family members, p73 and p63, share significant sequence homology with p53 but, unlike p53, give rise to multiple protein isoforms due to alternative promoter utilization and alternative mRNA splicing (Irwin and Kaelin, 2001; Melino et al., 2002). The TA isoforms of p63 and p73 (such as p73α and p73β) contain an N-terminal transactivation domain that is lacking in the corresponding ΔN isoforms. The TA isoforms can transactivate p53 target genes and induce apoptosis, whereas the ΔN isoforms are dominant inhibitors of p53-responsive gene expression. Therefore, p73 and p63 can have both proapoptotic and antiapoptotic effects. The antiapoptotic isoforms are predicted to block the function of all three p53 family members (Ishimoto et al., 2002; Pozniak et al., 2000; Stiewe et al., 2002; Zaika et al., 2002). Accordingly, overproduction of antiapoptotic p73 isoforms can block chemotherapy-induced apoptosis in tumor cells that retain wild-type p53 (Vossio et al., 2002; Zaika et al., 2002).

It was initially reported that p73 is not induced by DNA damage (Kaghad et al., 1997). Subsequently, it was shown that p73 is induced by *cis*-platinum and adriamycin, implying that p73 responds to at least a subset of DNA-damaging drugs (Agami et al., 1999; Costanzo et al., 2002; Gong et al., 1999; Yuan et al., 1999). In the present report, we found that TAp73 is induced by a wide variety of chemotherapeutic agents and that blocking TAp73 function leads to enhanced chemoresistance. Earlier studies showed that overproduction of certain p53 mutants can (1) block chemotherapy-induced apoptosis and (2) block p73 function (Blandino et al., 1999; Di Como et al., 1999; Gaiddon et al., 2001; Li et al., 1998; Marin et al., 2000). Here we demonstrate that downregulation of endogenous mutant p53 enhances chemosensitivity in p53-defective tumor cells. Collectively, these results support a model wherein modulation of chemosensitivity by mutant p53 is due, at least in part, to perturbation of p73 function.

Results

We found that p73α and, to a lesser extent, p73β, are induced by a wide variety of chemotherapeutic agents in diverse tumor cell lines (Figure 1A and M.S.I. and W.G.K., unpublished data). In some cases, p73 induction was only revealed after titration experiments that examined the influence of drug dose and duration, perhaps accounting for the earlier conclusion that p73 was not responsive to DNA damage (Figure 1 and M.S.I. and W.G.K., unpublished data). For certain chemotherapeutic agents, p73 induction was observed at low, but not high, drug concentra-

SIGNIFICANCE

p53 mediates DNA damage-induced apoptosis, but the efficacy of DNA-damaging chemotherapeutic agents is not restricted to p53 (+/+) tumor cells. Moreover, p53 status cannot account for preferential killing of tumor cells by chemotherapeutic agents relative to normal (p53+/+) cells. p73 is a p53 paralog that encodes proapoptotic (TA forms) and antiapoptotic (ΔN forms) isoforms. TAp73 levels are higher in tumor cells relative to normal cells and mutant p53 inhibits TAp73. We found that TA p73 is induced by many chemotherapeutic agents and that inhibiting TA p73 increased chemoresistance. Conversely, downregulation of mutant p53 enhanced chemosensitivity. These results suggest that p73 contributes to chemotherapy-induced apoptosis and support a model wherein mutant p53 induces chemoresistance, at least partly, through neutralization of p73.

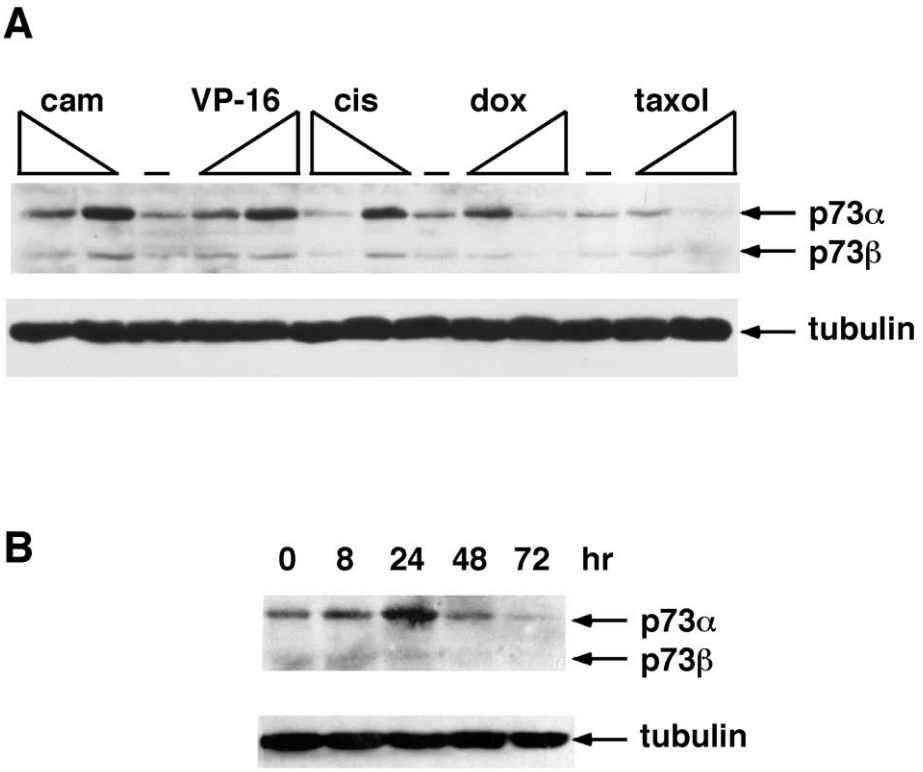

Figure 1. TA-p73 is induced by chemotherapy

A: Anti-p73 and anti-tubulin immunoblots of SW480 cells treated for 24 hr with camptothecin (0.3–1 μM), etoposide (VP-16) (0.5–5 μM), cisplatinum (2–10 μg/ml), doxorubicin (2–10 μM), taxol (25–100 nM), or DMSO ("-") (0.1%). Similar results were seen in HCT-116, T98G, and U20S cells (data not shown).

B: Anti-p73 and anti-tubulin immunoblots of SW480 cells treated with camptothecin (0.3 μM) for the indicated periods (hr).

tions (Figure 1A). The accumulation of p73 following chemotherapy treatment has been shown, when studied, to be due to increased transcription and protein stabilization (Agami et al., 1999; Chen et al., 2001; Costanzo et al., 2002; Gong et al., 1999; Yuan et al., 1999).

To ask whether accumulation of p73 contributes to chemotherapy-induced apoptosis, SW480 colon carcinoma cells were infected with recombinant adenoviruses encoding GFP and either a dominant-negative p73 (p73DD) or a point mutant derivative thereof [p73DD (L371P)]. p73DD, but not p73DD (L371P), binds to p73 and prevents it from activating p53-responsive promoters (Irwin et al., 2000). Likewise, p73DD, but not p73DD (L371P), protected against etoposide (VP-16)-induced cell death as shown by an increase in adherent, viable appearing, GFP-positive cells (Figure 2A). In contrast, no difference was observed in untreated cells expressing either p73DD or p73DD (L371P) (Figure 2A).

Next we sought to quantitate the effect of p73DD on chemotherapy-induced apoptosis in genetically defined transformed human cells. Parallel cultures of primary human embryonic kidney cells (HEK) transformed with the SV40 early region, H-Ras, and the telomerase catalytic subunit (hTERT) were infected with retroviruses encoding p73DD or p73DDL371P (Hahn et al., 1999) (Figure 2B). The SV40 early region encodes large T antigen, which inactivates both p53 and the retinoblastoma protein (pRB). After selection polyclonal, early passage cells were treated with chemotherapeutic agents. Corroborating our previous results, expression of p73 DD inhibited chemotherapy-induced apoptosis (Figures 2C and 2D). These effects were specific as they were not observed in cells infected with a retrovirus encoding p73DD L371P or with an empty virus and were not due to alterations in cell cycle distribution as determined by fluorescence-activated cell sorting of propidium iodide-stained cells (data not shown). Similar results were obtained with human foreskin fibroblasts transformed in a similar manner to these HEK cells (data not shown).

p73 DD blocks the function of both p73 and p63 (Irwin et al., 2000). To more clearly define the role of TA-p73 in chemotherapy-induced apoptosis, double-stranded RNA oligonucleotides (siRNA) (Elbashir et al., 2001) homologous to 5 prime unique TA-p73 sequences that are missing in ΔNp73 were used to inhibit the accumulation of p73 in tumor cells. Transfection of p73 siRNA, but not unrelated or scrambled siRNA, led to decreased TA-p73α and TA-p73β in multiple cell lines without affecting the levels of lamin or p53 (Figure 3A and data not shown). p73 siRNA, but not control siRNAs, also inhibited the accumulation of TA-p73 in response to DNA damage and conferred protection against a variety of chemotherapeutic agents in both SW480 (p53R273H/p53P309S) colon cancer cells (IARC TP53 Database; Rodrigues et al., 1990) and U2OS (p53 wild-type) osteosarcoma cells (Figures 3B, 4A and 4B, and data not shown). Notably, taxol did not increase p73 levels in SW480 cells under any condition tested (in contrast to U2OS cells) (Figure 1A and M.S.I. and W.G.K., unpublished data), and p73 siRNA did not protect SW480 cells from taxol-induced killing (Figure 4A). It is tempting to speculate that the more pronounced effects of p73 siRNA in SW480 cells relative to U2OS cells is due to the fact that the former have mutant p53 and the latter wild-type p53. However, a formal comparison between these two cell lines is not possible because of many other, potentially confounding, differences.

Likewise, *p73*$^{-/-}$ MEFs transformed with the SV40 large T

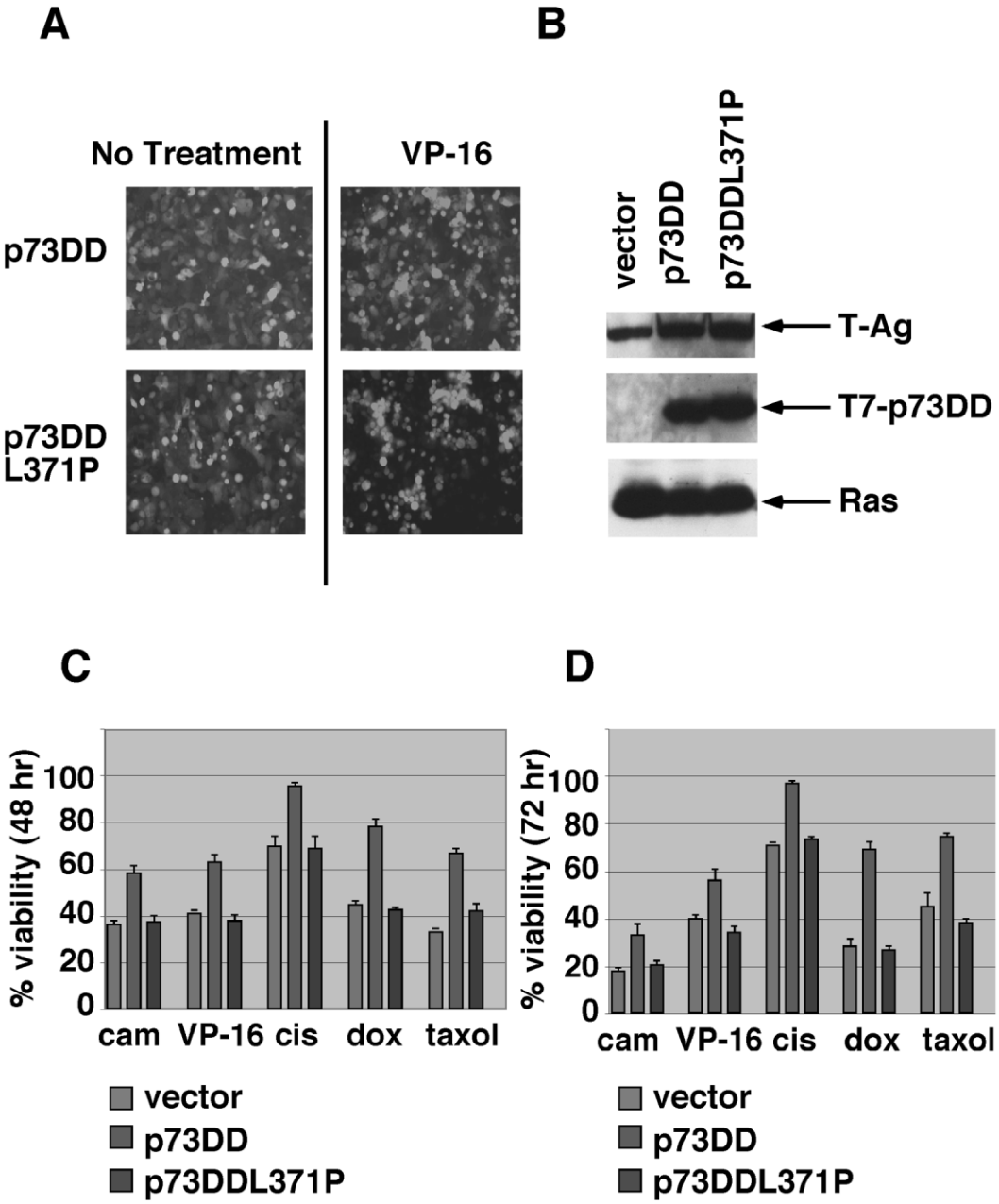

Figure 2. Dominant-negative p73 inhibits chemotherapy-induced cell death

A: SW480 cells infected with a bicistronic adenoviral vector encoding GFP and p73DD (top) or GFP and p73DDL371P (bottom) were treated for 3 days with etoposide (5 μM) (right panel) or diluent only (right panel). Shown are representative fields.

B: Anti-T, anti-T7, and anti-Ras immunoblots of primary human kidney epithelial cells infected with retroviruses encoding SV40 early region, hTERT, and H-Ras and then infected to produce T7 epitope-tagged p73DD or p73DD L371P.

C and **D:** Early passage (p4–8) transformed human kidney cells described in (**B**) were treated with camptothecin (1 μM), etoposide (5 μM), cisplatinum (2 μg/ml), doxorubicin (2 μM), or taxol (100 nM). XTT assays were performed 48 hr (**C**) and 72 hr (**D**) later in triplicate. Error bars equal one standard error.

antigen (T) + H-Ras were more resistant to chemotherapy-induced killing than wild-type MEFs transformed with these oncoproteins, although the effects were less dramatic than observed with human tumor cell lines (Figure 4C). Whether this is due to species or cell of origin differences is unknown.

Long-term viability assays often more accurately reflect the in vivo chemosensitivity profiles of tumors (Brown and Wouters, 1999). Thus, we performed clonagenic assays using SW480 cells transfected with p73 siRNA. Chemotherapy-treated cells transfected with p73 siRNA formed more colonies than cells transfected with buffer alone or scrambled p73 siRNA (Figure 4D). Importantly, neither the wild-type nor scrambled p73 siRNA affected colony formation by untreated cells (data not shown). Similarly, chemotherapy-treated HEK cells expressing p73DD formed more colonies than those expressing p73DDL371P (data not shown).

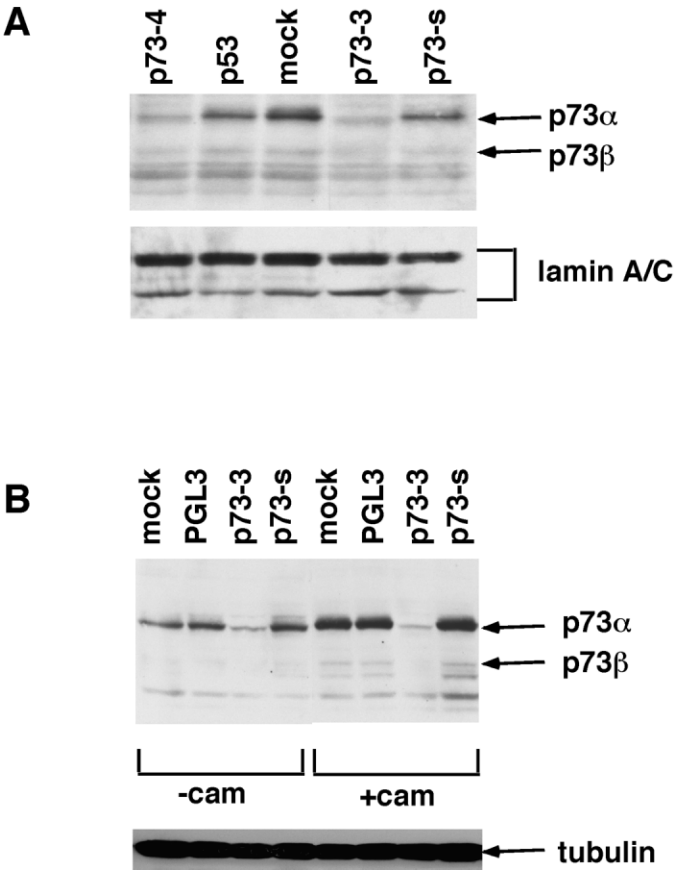

Figure 3. p73 siRNA inhibits TA-p73 induction by chemotherapy

A: Anti-p73 and anti-tubulin immunoblots of SW480 cells transfected with siRNA directed against TA-p73 (pairs p73-4, p73-3) or p53. In parallel, cells were transfected with annealing buffer only (mock) or scrambled p73 siRNA (p73-s).

B: Anti-p73 immunoblot of SW480 transfected as in (**A**). PGL3 = luciferase siRNA. 48 hr after transfection, cells were treated for 24 hr with DMSO or camptothecin (300 nM). Similar results were seen after treatment with etoposide (data not shown).

As expected, the increased viability of chemotherapy-treated cells transfected with p73 siRNA was due to a decreased apoptosis. Caspase activation and TUNEL positivity were significantly diminished in SW480 cells transfected with p73 siRNA relative to cells transfected with control siRNA (Figures 5A–5C).

The experimental overproduction of certain "gain-of-function" p53 mutants, in both wild-type and p53 null tumor cells, leads to increased chemoresistance (Blandino et al., 1999; Li et al., 1998). Likewise, some p53 mutants bind to and inactivate p73 (Di Como et al., 1999; Gaiddon et al., 2001; Marin et al., 2000, Bensaad et al., 2003). Our findings imply that these two phenomena might be linked. As anticipated, we detected mutant p53 bound to p73 in SW480 cell extracts (Figure 6A). Note that since the former is in vast excess of the latter (Marin et al., 2000 and data not shown), only a small fraction of the total pool of mutant p53 is bound to p73. On the other hand, when tested before in other cell types, the majority of p73 was bound to mutant p53 (Marin et al., 2000). To ask whether endogenous mutant p53 affects chemosensitivity, SW480 cells were treated with p53 siRNA. Transfection of p53 siRNA, but not control siRNA, inhibited mutant p53 accumulation (Figure 6B) and, in contrast to p73 siRNA, enhanced the effectiveness of chemotherapy (Figure 6C). This demonstrates that downmodulation of endogenous, mutant p53 levels in a human tumor cell translates into increased cell killing by chemotherapeutic agents. Similar results were obtained in C33A cervical carcinoma cells, which also contain mutant p53 (Figure 6D and data not shown). The effects in C33A cells were less striking than in SW480 cells, however, which likely reflects the less efficient knockdown of mutant p53 we achieved in these cells after siRNA treatment, as determined by immunoblot analysis (data not shown).

Discussion

P53 plays a central role in the apoptotic response to DNA-damaging agents. Nonetheless, the effectiveness of DNA-damaging anticancer drugs is not restricted to tumors that retain wild-type p53. Furthermore, the clinical utility of DNA-damaging chemotherapeutic agents implies that normal tissues, which retain wild-type p53, are less susceptible to apoptosis than tumor cells, which typically lack functional p53. Our findings shed light on these paradoxes by revealing a role for p73 in chemotherapy-induced apoptosis. p73 is rarely mutated in cancer. Moreover, a variety of oncogenes activate p73, leading to increased p73 levels in cancer cells relative to normal cells (Cai et al., 2000; Irwin et al., 2000; Mai et al., 1998; Stiewe and Putzer, 2000; Zaika et al., 1999; Zaika et al., 2001). A recent study showed that the induction of apoptosis by p53 required the presence of p73 (Flores et al., 2002). However, our findings clearly show that p73 can induce apoptosis in tumor cells that lack functional p53.

Our findings, together with the knowledge that mutant p53 can block p73 function, also provide a potential mechanistic basis for enhanced chemoresistance following experimental overproduction of mutant p53 in tumor cells, including cells lacking wild-type p53. Importantly, downregulation of endogenous mutant p53 levels enhanced chemosensitivity, thereby supporting a role for this phenomenon in nonmanipulated tumor cells. Additional studies will be required to rigorously establish the degree to which this enhanced chemosensitivity is due to p73.

The interaction of mutant p53 with p73 depends upon the nature of the p53 mutation as well the status of codon 72 in p53, which can encode Arg (R) or Pro (P) due to a common polymorphism in the human population (Marin et al., 2000). In this regard, both the p53 alleles in SW480 cells encode for Arg at codon 72 as determined by a PCR-based assay (data not shown; Harris et al., 1986). p53 Arg72 mutants bind to p73 as well or better than the corresponding Pro72 isoforms, perhaps accounting for the preferential mutation and retention of the R allele in tumors arising in R/P germline heterozygotes (Marin et al., 2000; Brooks et al., 2000; Soulitzis et al., 2002). In the accompanying manuscript, Crook and coworkers likewise report that p53 Arg72 mutants are more potent inhibitors of chemotherapy-induced apoptosis than the corresponding Pro72 isoforms and provide evidence that p53 codon 72 status influences clinical response to chemotherapy in man (Bergamaschi et al., 2003 [this issue of *Cancer Cell*]).

Our results suggest that the response to chemotherapy is influenced not only by p53, but instead by the status of a network that contains p53, p73, and perhaps the closely related protein p63. Therapeutic modulation of p73 and mutant p53 levels might

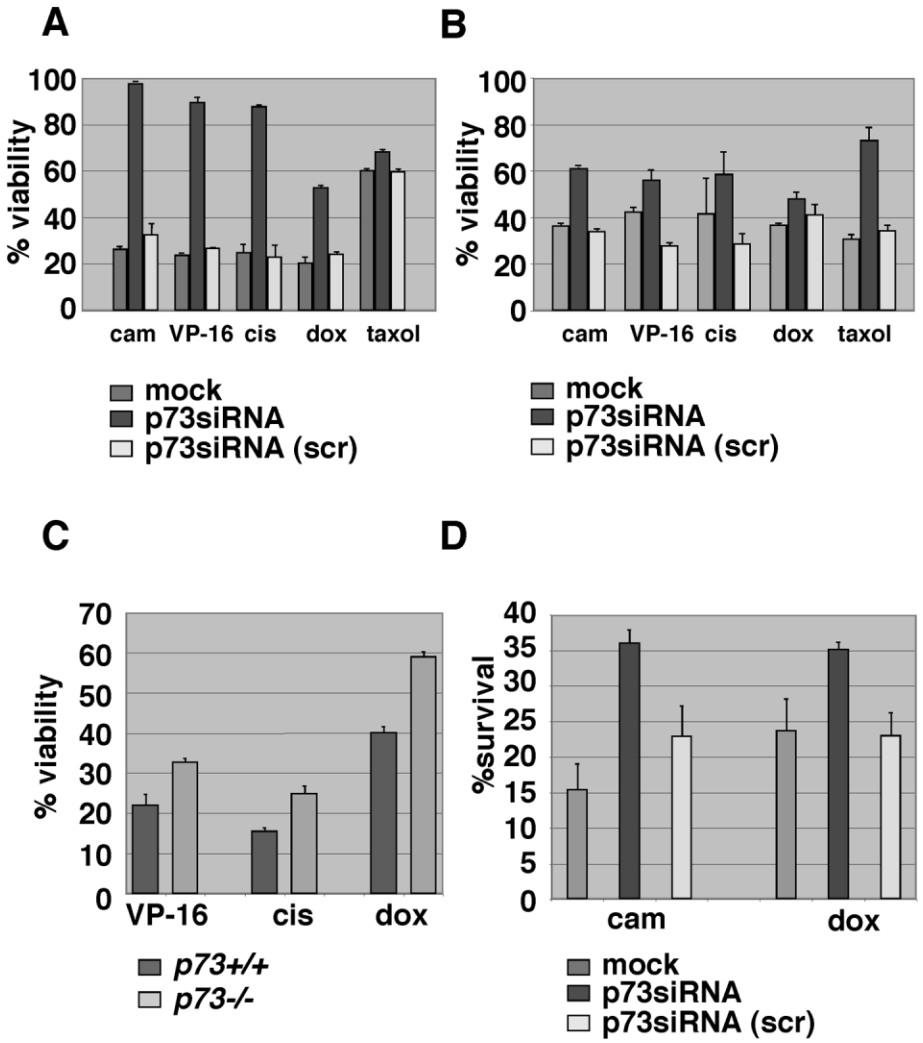

Figure 4. Genetic disruption of TA-p73 enhances chemoresistance

A and **B:** SW480 (**A**) and USOS (**B**) cells were transfected with the indicated siRNAs and subsequently treated with campothecin (1 μM), etoposide (5 μM), cisplatinum (2 μg/ml), doxorubicin (2 μM), or taxol (100 nM) for 72 hr prior to XTT analysis. Similar results were seen at 48 hr (data not shown).

C: Polyclonal populations of early passage $p73^{-/-}$ and $p73^{+/+}$ MEFS transfected with plasmids encoding SV40 T antigen (T) and H-Ras were treated with the indicated drugs for 72 hr prior to XTT analysis.

D: SW480 cells transfected with p73 siRNA, annealing buffer only, or scrambled p73 siRNA (p73-s) were plated in triplicate into methylcellulose/DMEM media containing doxorubicin, camptothecin, or diluent. The percentage of colonies surviving in camptothecin (left) or doxorubicin (right) relative to diluent alone is indicated.

be used to target the large percentage of human tumors that harbor p53 mutations. Intriguing in this regard are reports of small molecules that can induce mutant p53 to adopt a wild-type conformation (Bykov et al., 2002; Foster et al., 1999). Strategies to manipulate this network of proteins, including other small molecules and adaptations of siRNA technology (McCaffrey et al., 2002), may thus prove useful in enhancing the response to traditional chemotherapeutic drugs or in overcoming chemoresistance in human cancers.

Experimental procedures

Cell culture and chemotherapy treatment

SW480, U2OS, T98G, HCT-116, C33A human cancer cell lines and mouse embryo fibroblasts (MEFs) were grown in DMEM supplemented with 10% FBS at 37°C in the presence of 10% CO2. Human kidney epithelial (HEK) cells were grown in MEM-α with 10% FBS. Camptothecin, etoposide, cisplatinum, doxorubicin, and taxol were dissolved according to the manufacturer's instructions (Sigma) before addition to cell culture media.

Antibodies, western immunoblots, and immunoprecipitations

Immunoblot analysis was performed essentially as described before (Irwin et al., 2000) with anti-p73 (ER-15; Marin et al., 2000), anti-p53 (DO-1, Oncogene Research), anti-Lamin (Cell Signaling Technology), anti-tubulin (clone B1.2.5, Sigma), anti-Ras (C20, Santa Cruz Biotechnology), anti-T7 (Novagen), or anti-T (Pab419; Harlow et al., 1981). Each lane contained comparable amounts of protein as determined by the Bradford Method.

Immunoprecipitations were performed essentially as described by Marin et al. (2000), using 1.5 mg of cell extract and either anti-p73 (mixture of ER-15 and ER-13), anti-p53 (mixture of PAb1801 and PAb 421), or control anti-T (Pab419) monoclonal antibodies. ER-13 and ER-15 are specific for p73 and do not crossreact with p53 (Marin et al., 2000). Bound p53 was detected by immunoblot assay with an anti-p53 (DO-1) primary antibody and a horseradish peroxidase (HRP)-conjugated secondary antibody against mouse immunoglobulin light chain (Pharmingen, clone 187.1).

Generation of adenoviral plasmids

Bicistronic adenoviral vectors (pAdCMV) encoding GFP and p73DD were made using the AdEasy Vector System (Quantum Biotechnologies). The p73DD cDNAs from pcDNA3-T7-p73DD and pcDNA3-T7-p73DD (L371P) (Irwin et al., 2000) were excised with HindIII and XbaI and ligated into pAD-Track-CMV cut with these two enzymes. The resulting plasmids were used to transform *E. Coli* strain BJ5183 in the presence of pAdEasy-1 to make pAd GFP-T7-p73DD and pAd GFP-T7-p73DD (L371P), respectively. Tissue culture supernatants from QBI-293A cells transfected with pAd GFP-T7-p73DD or pAd GFP-T7-p73DD (L371P) were harvested and viral particles were purified by cesium chloride centrifugation. Equivalent amounts of infectious particles were used to infect SW480 cells.

Generation of HEK cell lines

HEK cells were sequentially infected with retroviruses encoding the SV40 early region, hTERT, and G12V H-Ras as described before (Hahn et al.,

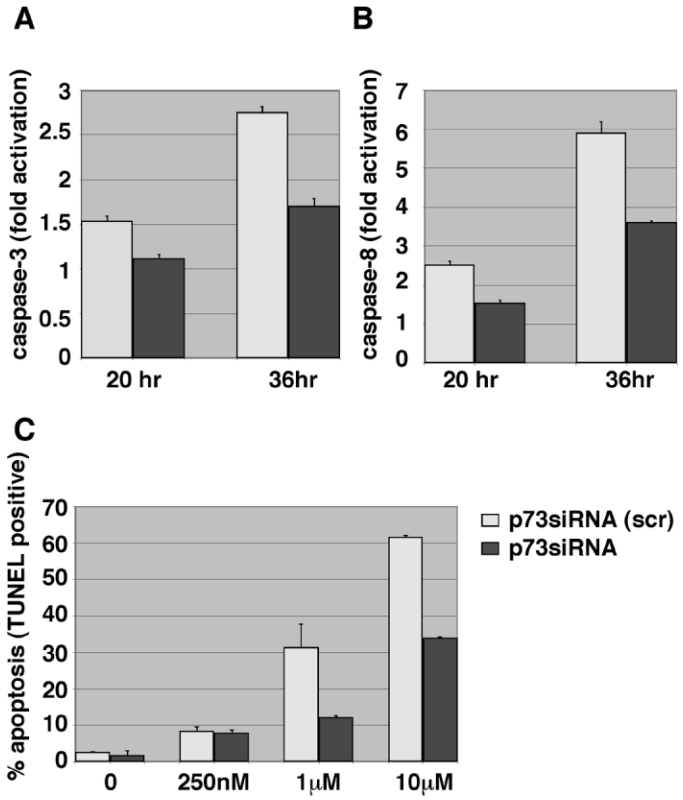

Figure 5. Genetic disruption of TA-p73 inhibits chemotherapy-induced apoptosis

A and **B:** SW480 cells transfected with the indicated siRNAs were treated with camptothecin (1 μM). Caspase-3 (**A**) and caspase 8 (**B**) activation assays were performed 20 and 36 hr later. Fold activation represents the caspase activity measured in camptothecin-treated cells relative to mock-treated cells harvested at the same time points.

C: SW480 cells transfected with the indicated siRNAs were treated with increasing concentrations of camptothecin or DMSO (= 0 μM). TUNEL staining was performed 48 hr later. Qualitatively similar results were obtained at 36 hr (data not shown).

1999). The p73DD cDNAs from pcDNA3-T7-p73DD and pcDNA3-T7-p73DD (L371P) (Irwin et al., 2000) were excised with HindIII and EcoRI (after 5′ fill-in with Klenow) and ligated into pBABE-zeo digested with SnaBI and EcoRI to make pBABE-zeo-T7-p73DD and pBABE-zeo-T7-p73DD (L371P). Tissue culture supernatants derived from 293T packaging cells transfected with either of these two plasmids were used to infect HEK cells. Successfully infected cells were selected by growth in Zeomycin (500 μg/ml).

Generation of MEF cell lines

Early passage (p4–6) *p73(−/−)* and *p73 (+/+)* MEFs obtained from matched littermates (a kind gift of E. Flores and T. Jacks) (Flores et al., 2002; Yang et al., 2000) were transfected with pT24-Ras and pSG5-T using Lipofectamine Plus. Transformed foci were harvested 10–14 days later and propagated as polyclonal pools.

siRNA transfections

The following double-stranded RNA 21 base pair oligonucleotides were used:

P73-3: 5′CGGAUUCCAGCAUGGACGUdTdT 3′ and 5′ dTdTGCCU AAGGUCGUACCUGCA 3′

P73-s: 5′ UAGCCACCACUGACGACCUdTdT 3′ and 5′dTdTAUCG GUGGUGACUGCUGGA 3′

P53: 5′ CUACUUCCUGAAAACAACGdTdT 3′ and 5′ dTdT GAUGAAG GACUUUUGUUGC 3′

The PGL3 sequence has been previously described (Elbashir et al., 2001). All siRNA sequences were subjected to BLAST search to confirm the absence of homology to any additional known coding sequences in the human genome. Oligonucleotides were deprotected and annealed according to the manufacturer's instructions (Dharmacon). Cells were transfected with siRNA at approximately 20%–30% confluency using Oligofectamine (Invitrogen). The final concentration of siRNA was 30–100 nM, but was comparable between each sample in a given experiment. For XTT assays, cells were allowed to recover for 24 hr prior to transfer into 96-well plates. 24 hr later, cells were treated with the indicated drugs. For TUNEL and caspase activation assays, cells were allowed to recover for 48 hr prior to treatment with chemotherapy.

XTT viability assays

XTT assays (sodium 3′–[1-(phenylamino-carbonyl)-3,4,-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) were performed in triplicate according to the manufacturer's instructions in 96-well plates (Roche) 24, 48, and 72 hr later. The colorimetric readout in this assay reflects the number of metabolically active mitochondria, and hence viable cells, in a given well. Values were normalized relative to cells not treated with chemotherapy. Data shown are representative of three independent experiments.

Clonagenic assays

SW480 cells were transfected with siRNA as described above. 48 hr later, 2×10^4 cells were plated in triplicate in methylcellulose-DMEM media with 30% FCS containing DMSO, doxorubicin (0.02 μM), or camptothecin (5 nM). Surviving colonies were counted 10–14 days later.

Apoptosis assays

For caspase activation assays, siRNA-transfected cells were harvested at the indicated time points following chemotherapy treatment and prepared according to the manufacturer's instructions (Caspase-3 Fluorometric and Caspase-8 Fluorometric Assays, R&D Systems). One hundred micrograms of each cell lysate was used in each reaction. Results were read using a fluorescent plate reader at 400 nm (light excitation) and 505 nm (emission). Caspase activation is expressed as fold increase relative to untreated cells. Data shown are representative of two independent experiments.

For TUNEL assays, cells were fixed in 4% paraformaldehyde at the indicated times following chemotherapy treatment. Cells were prepared and stained according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Fluorescein, Roche). TUNEL-positive cells were detected by flow cytometry. Data shown are representative of three independent experiments.

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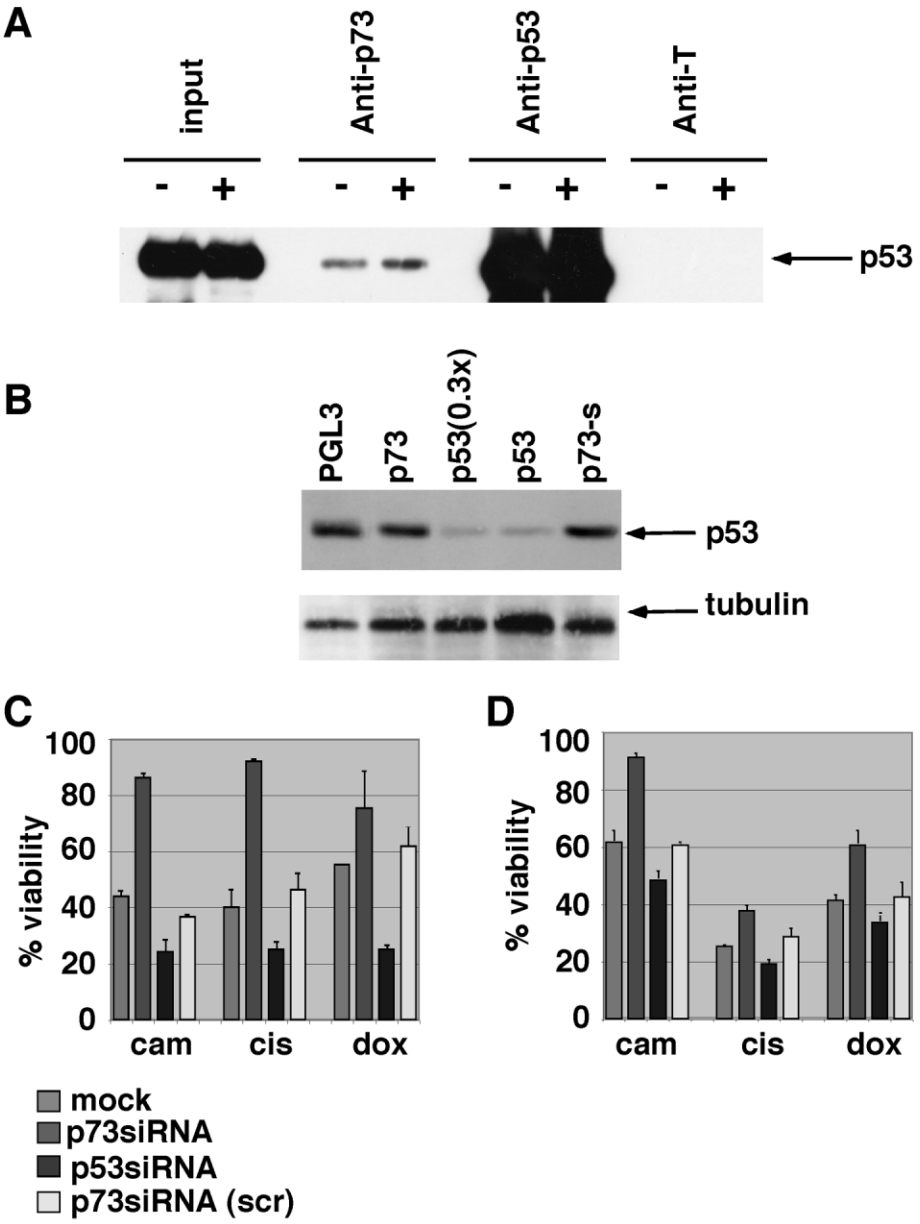

Figure 6. Downregulation of mutant p53 levels enhances chemosensitivity

A: Extracts of SW480 cells treated with camptothecin (1 μM) ("+") or DMSO ("−") were immunoprecipitated with anti-p73, anti-p53, or control (anti-T) antibodies. Bound p53 was detected by anti-p53 immunoblot analysis. Similar results were obtained using SW480 cells treated with etoposide (data not shown).

B: Anti-p53 immunoblot of SW480 cells transfected with siRNA directed against p53 (30 and 100 nM), luciferase (pGL3), or p73 (wild-type or scrambled [s]).

C and **D:** SW480 (**C**) and C33A (**D**) cells were transfected with the indicated siRNA oligonucleotides and subsequently treated with camptothecin (1 μM), cisplatinum (2 μg/ml), or doxorubicin (2 μM) for 72 hr prior to XTT analysis.

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