

Δ Np73, A Dominant-Negative Inhibitor of Wild-type p53 and TAp73, Is Up-regulated in Human Tumors

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Abstract

p73 has significant homology to p53. However, tumor-associated up-regulation of p73 and genetic data from human tumors and p73-deficient mice exclude a classical Knudson-type tumor suppressor role. We report that the human TP73 gene generates an NH₂ terminally truncated isoform. Δ Np73 derives from an alternative promoter in intron 3 and lacks the transactivation domain of full-length TAp73. Δ Np73 is frequently overexpressed in a variety of human cancers, but not in normal tissues. Δ Np73 acts as a potent transdominant inhibitor of wild-type p53 and transactivation-competent TAp73. Δ Np73 efficiently counteracts transactivation function, apoptosis, and growth suppression mediated by wild-type p53 and TAp73, and confers drug resistance to wild-type p53 harboring tumor cells. Conversely, down-regulation of endogenous Δ Np73 levels by antisense methods alleviates its suppressive action and enhances p53- and TAp73-mediated apoptosis. Δ Np73 is complexed with wild-type p53, as demonstrated by coimmunoprecipitation from cultured cells and primary tumors. Thus, Δ Np73 mediates a novel inactivation mechanism of p53 and TAp73 via a dominant-negative family network. Deregulated expression of Δ Np73 can bestow oncogenic activity upon the TP73 gene by functionally inactivating the suppressor action of p53 and TAp73. This trait might be selected for in human cancers.

Key words: p73 • Δ Np73 • Ex2Del p73 • apoptosis • deregulation in tumor

Introduction

The p53 family member p73 has significant homology to the p53 tumor suppressor. Human full-length p73 (TAp73) shares 63% amino acid identity with the DNA-binding region of TP53 including conservation of all DNA contact residues, as well as 38 and 29% identity with the tetramerization domain and transactivation domain, respectively (1). TAp73 also shows functional homology to p53. Ectopically overexpressed TAp73 α and TAp73 β (two COOH-terminal splice variants) largely mimic p53 activities, including the induction of apoptosis, cell cycle arrest, and the transactivation of an overlapping set of target genes (2, 3). Moreover, endogenous TAp73 is able to integrate death stimuli from three different pathways: cellular and viral oncogenes, some forms of DNA damage, and T cell receptor hyperactivation. We and others have shown that deregulation of the oncogenes E2F1, cMyc, and E1A induces apoptosis and growth suppression in tumor cells in a p53-

independent manner by transcriptionally inducing and activating endogenous TAp73 proteins (4–7). Moreover, during E2F1-mediated apoptosis in primary mouse embryo fibroblasts (MEFs),* a supra-additive cooperation exists between wild-type p53 and TAp73 (5). Although wild-type MEFs show 77% apoptosis after forced E2F1 expression, p53^{-/-} MEFs (containing TAp73) and p73^{-/-} MEFs (containing p53) alone show reduced killing ability of only 12 and 15%, respectively. The excessively weakened killing ability of p73^{-/-} MEFs, despite the presence of wild-type p53, is consistent with an important synergistic signal emanating from TAp73 that cooperates with p53 to induce oncogene-triggered death. Because oncogene deregulation of E2F1 is one of the most common genetic alterations in human tumors, this finding provides a possible physiologic cause for TAp73 overexpression in tumors. Furthermore,

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*Abbreviations used in this paper: EMSA, mobility shift assay; GAPDH, glyceraldehyde3-phosphate dehydrogenase; GFP, green fluorescent protein; MEF, mouse embryo fibroblast; TUNEL, Tdt-mediated dUTP-X nick-end labeling; UTR, untranslated region.

TAp73 is activated to mediate apoptosis by a restricted spectrum of DNA damage. Endogenous TAp73 is activated in response to cisplatin, and γ -IR in a pathway that depends on the nonreceptor tyrosine kinase c-abl (8–10). Moreover, doxorubicin stabilizes TAp73 protein by acetylation (11). Conversely, cells deficient in c-abl do not up-regulate their p73 and are resistant to killing by cisplatin. On the other hand, TAp73 is not activated by UV, actinomycin D, and mitomycin C, all of which activate p53 (1, 8). Lastly, peripheral T cells undergo apoptosis after hyperstimulation of their T cell receptors. This cell fate is mediated via the E2F1–TAp73 pathway (6). Consistent with this notion, E2F1 null mice exhibit a marked disruption of lymphatic homeostasis with increased T cells and splenomegaly (12, 13). Thus, TAp73 might function independently but synergistically with p53 in a tumor surveillance pathway in vivo.

However, despite this strong functional homology, data from human tumors and p73-deficient mice argue against a classical Knudson-type tumor suppressor role for the TP73 gene. TP73-deficient mice lack a spontaneous tumor phenotype (14) and inactivating mutations in human tumors are extremely rare (>900 tumors analyzed to date; for review see reference 15). Moreover, although all normal human tissues studied express very low levels of p73, multiple primary tumor types and tumor cell lines overexpress wild-type p73, including cancers of the breast, lung, esophagus, stomach, colon, bladder, ovary, liver, bile ducts, ependymal lining, myelogenous leukemia, and neuroblastoma (15). It is important to point out that to date, most studies identifying p73 overexpression in primary human tumors have examined total levels of p73, with only a few exceptions that specifically measured TAp73 (16, 17). Importantly, in the mouse, an N terminally truncated Δ Np73 protein has recently been found, generated from an alternative promoter in intron 3 and lacking a transactivation domain (18). Δ Np73 is the predominant form in the developing mouse brain and is the only form of p73 in the neonatal brain and sympathetic ganglia (14, 18). Mouse Δ Np73 plays an essential antiapoptotic role during developmental p53-driven neuronal death in vivo by acting as a dominant-negative inhibitor of p53 (18). Functional studies have shown that Δ Np73 is required to counteract p53-mediated neuronal death during normal “sculpting” of the developing mouse neuronal system (18). In primary neuronal cultures, withdrawal of the obligate survival factor nerve growth factor leads to p53 induction with p53-dependent cell death but a concomitant decrease of Δ Np73. Importantly, sympathetic neurons are rescued from cell death after nerve growth factor withdrawal or recombinant adenoviral p53 infection when Δ Np73 levels are maintained by viral delivery (18). Given the existence of this powerful transdominant p53 inhibitor in the mouse, the possibility arose that this isoform might in part be responsible for the overexpression seen in human tumors and, in fact, be the crucial component. Therefore, we sought the human counterpart of Δ Np73, determined whether human tumors express it and determined its potential role in cancer.

Materials and Methods

Tumor Samples and Cell Lines. Primary tumors and normal tissues were collected at University Hospital State University of New York, Stony Brook in compliance with and approved by the Institutional Review Board. Freshly harvested tumors (minimum 60% tumor cells) and normal tissues were immediately snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. The human breast cancer cell line MDA 231, the p53 null lines H1299 and SaOs2, and the wild-type p53 lines U2OS, RKO, and HeLa cells were maintained in DMEM/10% fetal calf serum.

Semiquantitative RT-PCR Assay. Total RNA was extracted from tissues as previously described (17). 1 μg total RNA, 10 pmoles each of a radiolabeled isoform-specific upstream primer, and a common TP73 exon 4 reverse primer were used in a 10- μl reaction (Titan Kit; Roche) for 25 cycles to ensure linearity of the assay (unpublished data) and subjected to phosphorimage analysis. Primer sequences were 5'-TGC TGT ACG TCG GTG ACC-3' (sense Δ Np73), 5'-CGA CGG CTG CAG AGC GAG-3' (sense TAp73), and 5'-TCG AAG GTG CAG CAG TGG TG-3' (antisense for both). Δ Np73 and TAp73 amplicon sizes were 175 and 365 bp, respectively. For Ex2Del p73, primers were 5'-GCG CCA GGC CAG CCG GGA CGG AC-3' (sense) and 5'-CGC GGC TGC TCA TCT GGT CCA TGG TGC-3' (antisense), which yielded a 320-bp band. Band intensities were normalized to their respective glyceraldehyde3-phosphate dehydrogenase (GAPDH) values. In matched samples, the up-regulation in tumors was calculated by dividing normalized Δ Np73^{Tumor} by Δ Np73^{Normal}, TAp73^{Tumor} by TAp73^{Normal}, or Ex2Del p73^{Tumor} by Ex2Del p73^{Normal}. To calculate the preferential up-regulation of Δ Np73 in tumors, the formula Δ Np73^{Tumor}/ Δ Np73^{Normal} divided by TAp73^{Tumor}/TAp73^{Normal} was used. The analogous formula was used for Ex2Del p73. Some samples were independently repeated and yielded highly reproducible results. Genomic sequencing of p53 exons 5–9 was performed with the Amplimer Panel (CLONTECH Laboratories, Inc.). Immunocytochemical staining for p53 was done on fixed tissue sections from the same tumor mass.

Luciferase Assays. A pcDNA3-based expression plasmid for Δ Np73 α was generated with an NH₂-terminal Flag tag. pcDNA3-Fp53 expressing human Flag-tagged wild-type p53, pcDNA3-p73 α and pcDNA3-p73 β expressing hemagglutinin-tagged human TAp73 α and TAp73 β (provided by G. Melino, University of Rome, Rome, Italy), murine stem cell virus expressing a tetramerization domain mutant of Δ Np73 α called Δ Np73L322P (corresponding to L371P in TAp73 α), and the p53/p73-specific reporter PG13-Luc were previously described (4). PG13 is a reporter plasmid containing 13 tandem repeats of the p53 consensus DNA binding site. H1299 and SaOs2 cells were transfected by Eugene (Roche). Luciferase activity was normalized for renilla luciferase activity (Promega).

Western Blot and Coimmunoprecipitation Analysis. HeLa and H1299 cells were transfected with expression plasmids for wild-type p53 (0.5 μg) or TAp73 β (0.5 μg) with either 1.5 μg Δ Np73 α or empty vector (see Fig. 3 C). The indicated molar ratios were used and green fluorescent protein (GFP) was cotransfected in all cases (see Fig. 3 B). Total cell lysates were prepared 24 h later and subjected to immunoblot analysis. Gel loading was normalized for equal vimentin or GFP levels (20 μg per lane). Antibodies to p73 were monoclonal ER15 (recognizes amino acids 380–495 of human p73 α isoforms and detects TA and Δ N forms; Oncogene Research Products), polyclonal anti-p73 (raised against the COOH-terminal; Chemicon), and the polyclonal

anti- Δ Np73 (raised in rabbit against the exon 3' peptide LY-VGDPARHLATA and immunopurified, and does not cross react with p53 or any TAp73 isoform). Antibodies against p53 (DO-1 and PAb 421), HDM2 (IF2), p21Waf1, and 14-3-3 σ were from Oncogene Research Products and Flag antibody (M2) was from Sigma-Aldrich. For the normalization of protein loading, blots were reprobbed with α -vimentin (BioGenex) or GFP (CLONTECH Laboratories, Inc., see Fig. 3 B). For coimmunoprecipitations (see Fig. 5, A–C), SaOs2 and U2OS cells were transfected with 2.4 μ g of the indicated plasmids in single transfections, or 0.6 μ g p53 plasmid plus 1.8 μ g Δ Np73 α in cotransfections. 24 h later, 600 μ g lysates were subjected to immunoprecipitation with 1 μ g of the indicated antibodies and analyzed by Western blot as previously described (19).

For the immunoprecipitation of Δ Np73 from tumors and normal tissues (see Fig. 2 D), frozen tissue was mechanically pulverized under liquid N₂, resuspended in 1.5 ml TENN buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, pH 8.0) containing a protease inhibitor cocktail (Roche), and homogenized using an electric homogenizer followed by sonication for 2 min. The lysates were centrifuged twice at 14,000 rpm for 15 min. 2 mg total protein in 500 μ l TENN was precleared four times using 70 μ l each time of a 1:1 mixture of protein A and G slurry (GIBCO BRL). The last supernatant was incubated with 1 μ g ER15 antibody for 30 min at 4°C, followed by the addition of 40 μ l slurry of Prot G beads. The mixture was then rotated overnight at 4°C. For controls, lysates were immunoprecipitated with monoclonal GC15 specific for p73 β (Oncogene Research Products) or Flag antibody (M2). Pelleted beads were washed four times in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% NaDeoxycholate, pH 7.4), boiled in sample buffer for 3 min, and loaded onto an SDS-PAGE gel. Membranes were immunoblotted with polyclonal anti- Δ Np73 (1:20) and bands were verified by reblotting with ER15 (1:200). For coimmunoprecipitation of a Δ Np73–p53 complex from tissues (see Fig. 5 D), 2 mg total protein in 500 μ l TENN was precleared as described above and incubated with a 1:1 mixture (40 μ l slurry) of agarose beads covalently coupled to the monoclonal p53 antibodies 1801 or DO-1 (Santa Cruz Biotechnology, Inc.). For controls, 40 μ l Prot G agarose slurry was used. Membranes were immunoblotted with polyclonal anti- Δ Np73 and bands were verified by reblotting with ER15.

Apoptosis Assay. HeLa and SaOs2 cells were seeded into 8-well chamber slides and cotransfected with 300 ng of the indicated pcDNA3-based expression plasmids per well using Fugene (see Fig. 4 A). Control wells (vector alone) received 600 ng. After 16 or 24 h, cells were stained with Annexin V or Tdt-mediated dUTP-X nick-end labeling (TUNEL), respectively, according to the manufacturer's instructions (Roche). Expression was determined by immunofluorescence in duplicate wells. Transfection efficiency was reproducibly \sim 30% of cells, similar among all constructs and evenly distributed throughout the wells. Annexin V or TUNEL positive cells (494 fields at 40 \times) and plasmid-expressing cells (15 random fields, >500 cells) were counted and the percentage of apoptosis in transfected cells was determined after correction for background with vector alone.

RKO cells were seeded into 8-well slides and transfected with either vector, the irrelevant control plasmid cRel, or with Δ Np73 α using Lipofectamine Plus (GIBCO BRL; see Fig. 4 D). LcRel is a transcriptionally and apoptotically inactive Flag-tagged, truncated version of the transcription factor cRel, fused to a mitochondrial targeting sequence (19) and cloned into pcDNA3. 5 h after transfection, cells were either stressed with 5 μ M camp-

tothecin or left untreated overnight before fixation with paraformaldehyde. Wells were processed for TUNEL staining followed by immunofluorescence staining with polyclonal anti- Δ Np73 (1:20) and donkey anti-rabbit IgG (H+L)-rhodamine conjugate (Jackson ImmunoResearch Laboratories). Apoptosis was quantitated as the percentage of Δ Np73 α -expressing cells and compared with vector-transfected cells.

Colony Suppression Assay. SaOs2 cells in 60-mm plates were transfected by Fugene with 1.5 μ g each of the indicated expression plasmids. Vector-only plates received 3 μ g plasmid. 24 h later, cells were transferred to 100-mm dishes and placed under G418 selection (500 μ g/ml) for 21 d, fixed, stained with crystal violet (20), and photographed. All foci were counted.

Mobility Shift Assays (EMSA). H1299 and U2OS cells were transfected with plasmids encoding wild-type p53, Δ Np73 α , or a combination of the two. 24 h later, nuclear extracts were prepared by Dounce homogenization as previously described (21). As probes, we used double stranded oligonucleotides of the p53 consensus DNA binding sequences: 5'-GGGCATGTCCGGGCATGTCC-3' (called p53CON) or 5'-CCTGCCTGGACTTGCC-TGGCCTGCCTGGACTTGCCCTGG-3'. EMSA was performed with 1 ng 32 P-labeled probe and 10 μ g nuclear extract in binding buffer (40 mM NaCl, 10 mM morpholino propane sulfonic acid, pH 7.0, 0.1% NP-40, 1 mM EDTA, 2.5% glycerol, 0.5 μ g poly[dl-dC]) for 30 min at room temperature. Samples were loaded onto a native 6% polyacrylamide gel and electrophoresed at 4°C and 200 V for 4 h. Where indicated, 0.5 μ g p53-specific monoclonal antibody PAb 421 was included to produce a supershift. Specific competition experiments included unlabeled p53CON in 50-fold molar excess of radiolabeled probe. Nonspecific competition consisted of 50-fold molar excess of the scrambled p53CON 5'-GGGAATTTCCGGGAATTTCC-3' or the mutant sequence CCTTAATGGACTTTAATGGCCTTAATGGACTTTAATGG (mutated nucleotides are underlined).

Antisense Suppression of Δ Np73. Wild-type p53-harboring RKO cells were seeded into 8-well slides and transfected with 360 ng wild-type p53 expression plasmid with 200 nM of either Δ Np73 antisense oligonucleotide directed against exon 3' with extension into the adjacent 5' untranslated region (UTR) (antisense, 5'-A*C*C*G*ACGTACAGC*A*T*T*G*G-3'; stars indicate the phosphorothioate-modified bases), or Δ Np73 sense oligonucleotide (sense, 5'-C*C*A*T*GCTGTACGT*C*G*G*T-3'; both HPLC-purified, Operon Inc.) using Lipofectamine Plus or Oligofectamine (GIBCO BRL) according to the manufacturer's instructions. Vector alone was also used. 20 h after transfection, cells were subjected to immunoblotting or TUNEL staining. Apoptosis was quantitated using a Nikon E800 microscope equipped with a Bio-Rad confocal laser scanning system. For each sample, the total fluorescence of five random fields per duplicate well was acquired with a 20 \times lens using identical acquisition and photomultiplier settings. Data were processed to calculate the integral optical density using the Laser Pix software package (Bio-Rad Laboratories). In other experiments, RKO cells were transfected with 200 nM each of the antisense and sense oligonucleotides listed above. After 8 h, cells were DNA damaged by adding 1 μ M camptothecin for an additional 16 h before TUNEL staining. Apoptosis was determined as described above.

Results

The Human TP73 Gene Can Produce Δ Np73. Mouse Δ Np73 differs from TAp73 by a novel exon 3', which re-

places the first 3 exons and is spliced in frame to exon 4 of the TP73 gene (18). To clone the human homologue of mouse Δ Np73, we performed a GenBank search using mouse exon 3'. By sequence alignment of a human genomic P1 artificial chromosome clone containing TP73 (sequence data are available from GenBank/EMBL/DDBJ under accession no. AL 136528), we identified a region with 77% identity to the 5' UTR of mouse Δ Np73 mRNA (sequence data are available from GenBank/EMBL/DDBJ under accession no. Y 19235). This allowed us to predict the human exon 3' and design isoform-specific primers for human Δ Np73. Full-length Δ Np73 α cDNA, spanning exons 3'-14 including 103 bases of 3' UTR, was cloned by RT-PCR from total RNA of human placenta and MDA 231 breast cancer cells, and sequence was confirmed (Fig. 1 A). To confirm that Δ Np73 derives from a separate promoter upstream of exon 3', we performed 5' rapid amplification of cDNA ends and sequenced the 5' UTR upstream of exon 3' (Fig. 1 C). Human exon 3' encodes 13 unique amino acids with almost complete identity to mouse exon 3' (12 out of 13 residues are identical; Fig. 1 A). The human Δ N promoter contains the predicted TATA box 25 nucleotides upstream of the transcriptional start site, which is located 7.6 kb downstream of exon 3.

Expression of Δ Np73 and Ex2Del p73 in Human Tumors. Unique cDNA primers were designed for amplification of Δ Np73 from tissues by semiquantitative RT-PCR (Fig. 1 B). Then, we determined if expression levels of Δ Np73 were tumor specifically up-regulated using a spectrum of human tumor pairs that were matched with the patients' normal tissues of origin (Table I and Fig. 2 A). They included 35 cancers (cancers of the ovary, endometrium, cervix, vulva, vagina, breast, kidney, and colon) and 2 large benign ovarian tumors (serous cystadenoma and serous cystadenofibroma). Indeed, in 27 of the 37 pairs (73%), Δ Np73 was specifically up-regulated between 2- and 150-fold in the patients' tumors compared with their matched normal tissues (Table I, second column). In a subset of our matched pairs, we also determined the expression of the previously described Ex2Del p73 (23). Ex2Del p73 is another isoform of p73 that lacks most of the transactivation domain, but in contrast to Δ Np73 it is generated from the same promoter as TAp73 by splicing out exon 2. Ex2Del p73 was shown to be up-regulated in some ovarian and vulval cancers and breast cancer cell lines and is a transdominant inhibitor of p53 (22-24). As already seen with Δ Np73, Ex2Del p73 was also specifically up-regulated 2.2-20-fold in 11 of 22 analyzed tumors (50%) compared with their matched normal tissues either alone or concomitant with Δ Np73 (Table I, third column, and Fig. 2 A). Taken together, 30 of 37 tumor pairs (81%) exhibited tumor-specific up-regulation of Δ Np73 and/or Ex2Del p73. On the other hand, because we previously showed that breast cancers can overexpress TAp73 (17), we next used isoform-specific RT-PCR to simultaneously measure Δ Np73 and TAp73. TAp73 was up-regulated in 18 of 37 tumors (49%; Table I, fourth column) compared

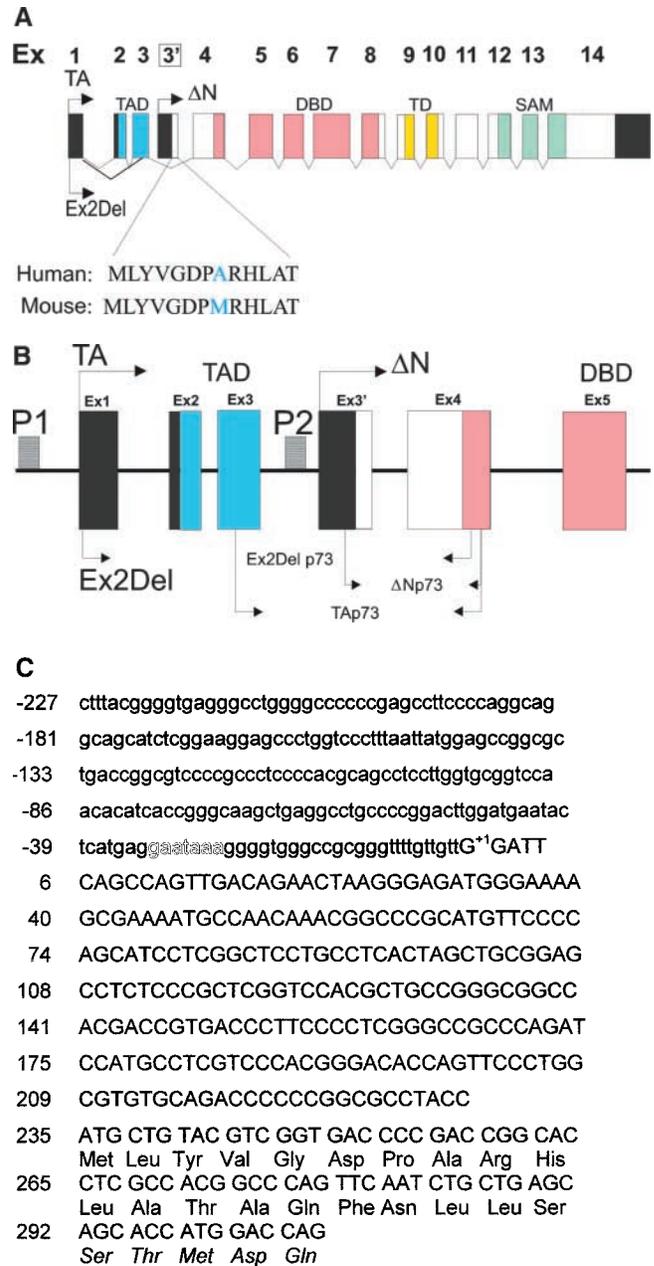


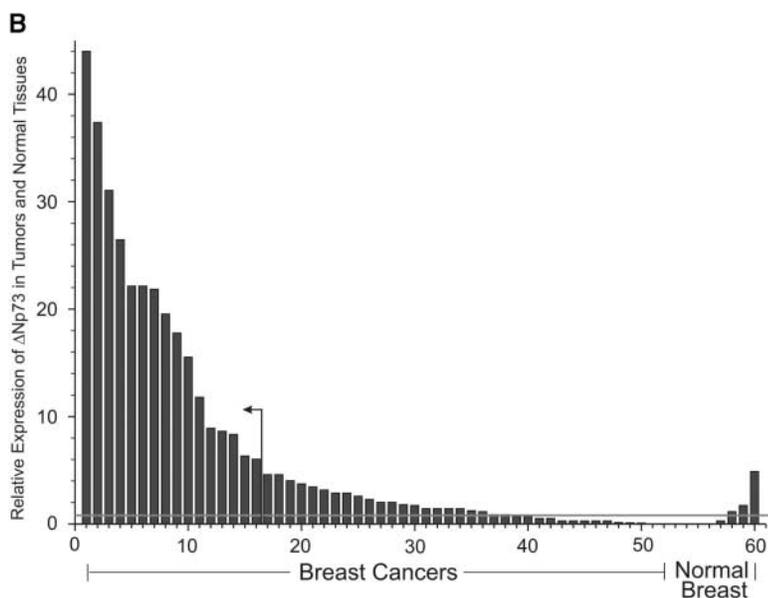
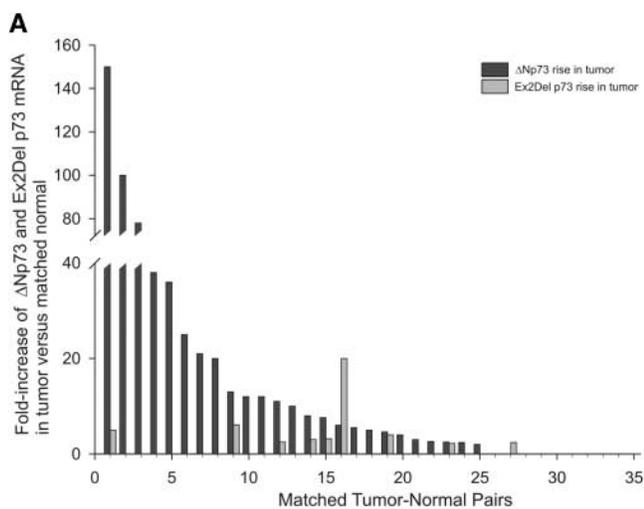
Figure 1. Gene architecture of human TP73. (A) In contrast to TP53, which harbors a single promoter generating a single protein composed of the transactivation domain (TAD), DNA-binding domain (DBD), and tetramerization domain (TD), the TP73 gene is complex and contains two promoters and an additional sterile α motif domain (SAM). The P1 promoter in the 5' UTR region produces transactivation-competent full-length proteins containing the TA domain (TAp73). The P2 promoter in intron 3 produces TA-deficient protein(s) (Δ Np73) with dominant-negative function toward TAp73 and wild-type p53. Δ Np73 starts with exon 3', which encodes 13 unique amino acids that are highly conserved between human and mouse. Another NH₂ terminally truncated p73, Ex2Del, also lacks the TA domain but is created by splicing out exon 2 from the P1 transcript (reference 1). The COOH-terminal of TAp73 undergoes additional exon splicing, which generates β - ϕ isoforms. (B) Positions of the P1 and P2 promoters. Positions of the RT-PCR primers are indicated: TAp73 (bottom), Δ Np73 (center), and Ex2Del p73 (top). (C) Sequence of the 5' UTR region of Δ Np73. The putative TATA box is indicated.

Table I. Expression of $\Delta Np73$, ExDel p73, and TAp73 in Matched Human Tumors

No.	Tumor	Increased $\Delta Np73$ expression in tumor vs. normal	Increased Ex2Del expression in tumor vs. normal	Increased TAp73 expression in tumor vs. normal	Preferential up-regulation of $\Delta Np73$ or Ex2Del vs. TAp73 in tumors	p53 status	
1	Endometrial Ca	150	5	6.5	23	—	wt, sequence confirmed
2	Endometrial Ca	100	—	7	14.2	—	mut
3	Cervical Ca ^a	78	nd	7	11	nd	wt
4	Vaginal Ca	38	—	—	2,794	—	mut
5	Ovarian Ca	36	nd	3	12	nd	wt, sequence confirmed
6	Cervical Ca	25	nd	155	—	nd	mut Ex 8, R273H CGT→CAT
7	Ovarian Ca	21	nd	4	5	nd	wt, sequence confirmed
8	Ser cystadenofibr	20	—	—	21	—	wt
9	Ovarian Ca	13	6.1	10.5	1.2	—	mut
10	Ovarian Ca	12	—	—	26.6	—	wt
11	Breast Ca	12	nd	—	11	nd	wt, sequence confirmed
12	Endometrial Ca	11	2.5	10	1.1	—	wt
13	Breast Ca ^a	10	nd	—	16	nd	wt, sequence confirmed
14	Endometrial Ca	8	3	32	—	—	wt
15	Vulvar Ca	7.6	3.2	5.3	1.4	—	mut
16	Endometrial CaSar	6	20	—	6	—	mut
17	Ovarian Ca	5.5	—	—	yes*	—	wt
18	Ovarian Ca	5	—	6.6	—	—	mut
19	Ovarian Ca	4.6	4	42	—	—	mut
20	Ovarian Ca	4	nd	10	—	nd	mut Ex 5, C176Y CGC→TGC mut Ex 6, R213STOP
21	Ovarian Ca ^a	yes*	nd	—	yes*	nd	CGA→TGA
22	Ovarian Ca	yes*	nd	yes*	yes*	nd	wt
23	Ser cystadenoma	3	nd	—	8	nd	wt
24	Ovarian Ca	2.6	—	13	—	—	mut
25	Vulvar Ca	2.5	2.2	3.7	—	—	wt
26	Bord Muc Ov Tu	2.4	—	—	2.3	—	wt
27	Ovarian Ca	2	—	3.3	—	—	mut
28	Breast Ca ^a	—	nd	29	—	nd	wt
29	Endometrial Ca	—	2.4	—	—	2	wt
30	Endometrial Ca	—	—	—	—	—	wt
31	Ovarian Ca	—	—	—	—	—	wt
32	Ovarian Ca	—	yes*	—	—	yes*	wt
33	Ovarian Ca ^a	—	nd	—	—	nd	wt
34	Breast Ca	—	nd	—	—	nd	wt
35	Ovarian Ca	—	nd	—	—	nd	mut
36	Renal Ca	—	nd	—	—	nd	wt
37	Colon Ca	—	yes*	—	—	yes*	wt

Numbers reflect fold increase. yes*, cannot be calculated as fold increase because level in normal tissue is undetectable; Ca, carcinoma; ICC, immunocytochemistry for p53 with monoclonal antibody DO-1 (negative suggests wild-type status, positive suggests mutant status); nd, not determined; Ser cystadenofibr, serous cystadenofibroma (a large benign ovarian tumor); Ser cystadenoma, serous cystadenoma (a large benign ovarian tumor); Bord Muc Ov Tu, borderline mucinous ovarian tumor (a large ovarian tumor of low or uncertain malignant potential).

^aAverage of two independent measurements. Values differed by <10%.



were standardized using the corresponding GAPDH value of each sample. The relative expression of Δ Np73 in breast cancers and normal breast tissues is shown. The average normal tissue expression (gray line) is indicated. The arrow marks an arbitrary cut-off, delineating tumors with fivefold or higher Δ Np73 overexpression. 16 of 52 breast cancers (31%) overexpress Δ Np73 levels that were between 6- and 44-fold higher than that of average normal breast tissue. (C) Characterization of the polyclonal anti- Δ Np73. Anti- Δ Np73 does not recognize TAp73 α , TAp73 β , or p53. H1299 cells were transfected with empty vector or the indicated expression plasmids and lysates (50 μ g protein for lanes 1–5 and 10 μ g for lanes 6–9) were immunoblotted with either a cocktail of ER15, GC15, and DO-1 (lanes 6–9) or anti- Δ Np73 (lanes 1–5). (D) Tumor-specific up-regulation of Δ Np73 α protein. Cases 9, 14, and 26 (top from left to right) and cases 10, 31, and 1 (bottom) from Table I are shown. Immunoprecipitations of equal amounts of total protein (2 mg each) from matched pairs of homogenized tumor/normal tissues with 1 μ g anti-p73 antibody ER15 followed by immunoblotting with polyclonal anti- Δ Np73. Hc, heavy chain. The last lane (top) is a positive control of H1299 lysate transfected with a Δ Np73 α expression vector.

with its respective normal tissues of origin. However, although tumors with deregulated Δ Np73 and/or Ex2Del p73 also tended to show up-regulation of TAp73, the magnitude of the Δ Np73 and/or Ex2Del p73 up-regulation was much higher than that of TAp73 in the majority of cases. Among the 31 tumor pairs with up-regulation of any one or all of these three p73 transcripts, 22 tumors (71%) exhibited preferential up-regulation of Δ Np73 (19 tumors; Table I, left side of fifth column) or Ex2Del p73 (3

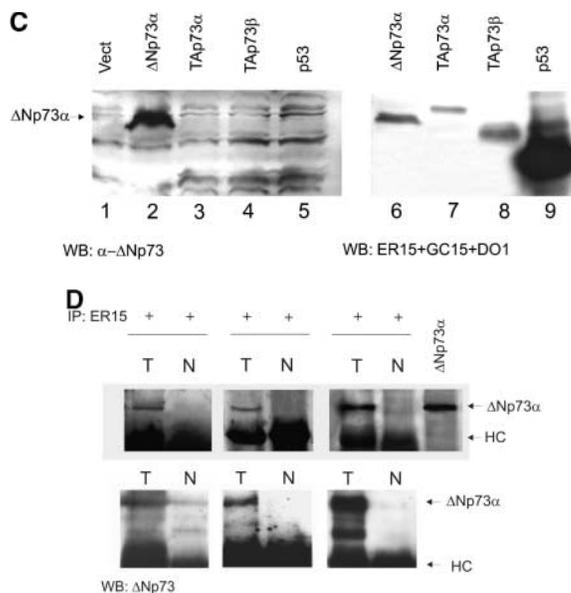


Figure 2. Δ Np73 is frequently overexpressed in a variety of primary human cancers. (A) Tumor-specific up-regulation of Δ Np73 (solid bars) transcripts in 35 tumor pairs, compared with their respective normal tissues of origin. In addition, Ex2Del p73 transcripts (gray bars) were measured in a subset of pairs. Ex2Del p73 is another previously described isoform of p73 that lacks most of the transactivation domain. In contrast to Δ Np73, it is generated from the same promoter as TAp73 by splicing out exon 2. It is also a transdominant inhibitor of p53 (references 22–24). Isoform-specific semiquantitative RT-PCR assay from total RNA extracted from human tissues (see Table I). Two tumor pairs (nos. 21 and 22) had readily detectable Δ Np73 levels in tumor tissues but failed to give detectable Δ Np73 levels in their corresponding normal tissues. Therefore, fold up-regulation could not be quantitated in those 2 cases reducing the total number plotted from 37 to 35 cases. Expression levels, standardized for their corresponding GAPDH values, were used to calculate fold induction as described in Materials and Methods. (B) Up-regulation of Δ Np73 transcripts in a series of unmatched 52 human breast cancers compared with 8 unrelated normal breast tissues. Δ Np73-specific semiquantitative RT-PCR assay from total RNA extracted from tissues. Expression levels

tumors; Table I, right side of fifth column). These included 13 ovarian cancers and 2 large benign ovarian tumors, 5 endometrial cancers, 2 breast cancers, 2 cervical cancers, and 2 vulvar cancers. Moreover, 14 of these 22 tumors (67%) exhibited exclusive up-regulation of Δ Np73 and/or Ex2Del p73. (“preferential” up-regulation means that the up-regulation of Δ Np73 and/or Ex2Del p73 was more marked than the up-regulation of TAp73). In one case, the tumor showed preferential Δ Np73 up-regulation of

>2,700-fold compared with TAp73 expression (tumor no. 4). Only nine tumors (29%) exhibited an inverse ratio with a disproportional rise of TAp73 compared with their rise in Δ Np73. Thus, taken together, in our series of 31 matched tumors with some form of tumor-specific deregulation of the TP73 gene, 22 tumors (71%) exhibit either exclusive or preferential up-regulation of dominant-negative p73 isoforms.

In our paired tumor series, we had an overall p53 mutational rate of 38%, as determined either by direct sequencing of the PCR-amplified DNA binding domain or by immunocytochemically shown nuclear overexpression (Table I). This prevalence is in agreement with the reported rates of p53 mutations in these tumor types (\sim 40%; reference 25). We reasoned that if Δ Np73 and Ex2Del p73 were indeed p53- and TAp73-directed inhibitors and would act as oncogenes, one would expect to see their expression preferentially in wild-type p53 tumors. Of the 22 tumors with preferential up-regulation of Δ Np73 and/or Ex2Del p73, 21 tumors were available for p53 mutational analysis. Of these 21 tumors, 15 tumors harbored wild-type p53 (71%). In contrast, among the nine cases with preferential up-regulation of TAp73, six tumors harbored mutant p53 (66%). We then analyzed this set of 30 tumors for a correlation between overexpression of dominant-negative forms of p73 and concomitant wild-type p53 status. When all tumors were included, a statistical trend but no significance was found by Student's *t* test. When the unusual tumor number 4 is excluded, which is characterized by a singularly high level of Δ Np73 up-regulation of almost 3,000-fold compared with TAp73 up-regulation plus a mutant p53 status, statistical significance was found ($P = 0.014$). Taken together, a correlation between tumor-specific up-regulation of Δ Np73 or Ex2Del p73 and wild-type p53 status of the tumor cannot be made with this limited set of tumors, although a trend is present. More tumor samples will need to be analyzed in the future to support the hypothesis that the expression of dominant-negative p73 isoforms alleviates the selection pressure for p53 mutations in tumors.

To further investigate whether tumors up-regulate Δ Np73, we determined Δ Np73 transcript levels in a series of 52 unmatched breast cancers and compared them to 8 available normal breast tissues from unrelated individuals (Fig. 2 B). 16 of 52 breast cancers (31%) overexpressed Δ Np73 levels that were between 6- and 44-fold higher than the average of the 8 normal breast tissues (Fig. 2 B, gray line). An additional 10 tumors showed Δ Np73 up-regulation between two- and sixfold above the normal average. In contrast, four normal breast tissues showed non-detectable levels of Δ Np73, two cases expressed at average level, and only two tissues were elevated two- to fourfold. Next, we used isoform-specific semiquantitative RT-PCR to simultaneously measure Δ Np73 and TAp73 because we previously showed that breast cancers can also overexpress TAp73 (17). Among the 16 cancers with a 6–44-fold increase of Δ Np73, 12 cancers again showed preferential up-regulation of Δ Np73 over TAp73 (unpublished data). Although the data is not complete enough to

make strong conclusions, as we had already seen in the gynecological cancers on Table I, our results on breast cancer again suggests that Δ Np73 might selectively be up-regulated during tumorigenesis.

To confirm that tumor-specific up-regulation of Δ Np73 transcripts translate to the up-regulation of proteins, we generated a Δ Np73-specific polyclonal antibody raised against the unique exon 3'. This antibody recognizes Δ Np73 but does not cross react with TAp73 α , TAp73 β , or p53 (Fig. 2 C). Using this reagent, we determined Δ Np73 protein expression on 10 matched pairs of homogenized tumor/normal tissues from Table I. Tissues were subjected to immunoprecipitations of equal amounts of total protein (2 mg each) with the anti-p73 specific antibody ER15 followed by immunoblotting with polyclonal anti- Δ Np73. Some examples are shown in Fig. 2 D, which represent cases 1, 9, 10, 14, 26, and 31. Tumor-specific up-regulation of Δ Np73 protein was found in all 10 cases as demonstrated by tumors yielding detectable Δ Np73 α protein, whereas their respective matched normal tissue showed a complete absence of Δ Np73 α protein in nine cases and only a minute amount in case number 10. Moreover, when tumor lysates in these cases (2 mg each) were immunoprecipitated with nonspecific Flag antibody, Δ Np73 protein could not be detected (unpublished data). Also, the immunoprecipitation of cases 9, 14, and 26 with β -specific anti-p73 antibody GC15 did not yield Δ Np73 protein, indicating that in contrast to Δ Np73 α , Δ Np73 β is not up-regulated to detectable levels in these cases. As expected, no strict correlation between the levels of tumor-associated protein and the ranking in Table I is present because the RT-PCR measurements indicate the relative fold increase of mRNA levels of tumor versus normal rather than absolute values. Interestingly, cases 26 and 31 did not exhibit increased Δ Np73 expression of their tumors at the transcript level, yet clearly do so on the protein level. This notion warrants additional investigation because it might suggest that the prevalence of tumor-associated Δ Np73 up-regulation is somewhat higher than transcript measurements would predict.

Δ Np73 Is an Efficient Dominant-Negative Inhibitor of the Transcription Function of Wild-type p53 and TAp73. To test the hypothesis that human Δ Np73 is a dominant-negative inhibitor of human wild-type p53 and TAp73, we first performed reporter assays with expression plasmids for wild-type p53, TAp73 α , TAp73 β , and a p53/TAp73-responsive luciferase reporter in the presence or absence of Δ Np73 α using p53 null H1299 and SaOs2 cells (Fig. 3, A–C, and unpublished data). Increasing ratios of Δ Np73 α expression plasmid were added to a constant amount of p53 or TAp73 α and TAp73 β plasmids. Fig. 3 B shows that the p53 expression levels in this assay were independent of the amount of Δ Np73 α added, and that p53 and TAp73 expression levels were not interfering with the amount of Δ Np73 α expressed. Surprisingly, the steady state levels of TAp73 β actually increased in proportion to the amount of Δ Np73 α added, despite the fact that the same amount of TAp73 β plasmid was used in all reactions. Of

note, the addition of Δ Np73 α exhibited complete suppression of the transcriptional activity of wild-type p53, TAp73 α , and TAp73 β in a dose-dependent manner (Fig. 3 A and unpublished data). Furthermore, Δ Np73 α also efficiently suppresses endogenous target gene products of wild-type p53 and TAp73 (Fig. 3 C and unpublished data). In HeLa and H1299 cells, the transfection of wild-type p53 or TAp73 β induces endogenous HDM2, 14-3-3 σ , and p21Waf1 compared with basal levels seen with empty vector. However, the concomitant expression of Δ Np73 α strongly suppresses each of these response gene products (compare lanes 2 and 3 and lanes 4 and 5). Expression of Δ Np73 α alone did not suppress these target genes (compare lanes 6 and 7).

Δ Np73 Is an Efficient Dominant-Negative Inhibitor of the Apoptosis and Suppressor Function of Wild-type p53 and TAp73. Moreover, Δ Np73 α is a strong inhibitor of apoptosis induced by wild-type p53 and TAp73 (Fig. 4 A). HeLa and SaOs2 cells undergo wild-type p53- and TAp73-dependent cell death as assessed by Annexin V staining and

TUNEL assay. This apoptotic activity is completely abolished by the coexpression of Δ Np73 α (Fig. 4 A and unpublished data). The inhibitory action of Δ Np73 α is dependent on the presence of transcription-competent wild-type p53 and TAp73 because Δ Np73 α alone cannot affect apoptosis (Fig. 4 A, left column). Furthermore, in agreement with the antiapoptotic effect, Δ Np73 α is an inhibitor of colony suppression mediated by wild-type p53 and TAp73 (Fig. 4 B and Table II). The reintroduction of wild-type p53 and TAp73 suppresses the growth of SaOs2 cells (2, 26) and this suppression is thought to be largely due to apoptosis (27). In keeping with these results, the transfection of wild-type p53 strongly suppresses macroscopic colony formation of SaOs2 cells compared with many visible colonies with vector backbone alone (4 foci for wild-type p53 vs. 1,778 for vector control; Fig. 4 B and Table II). As expected, the expression of Δ Np73 α alone has no growth-promoting effect compared with vector controls (1,383 vs. 1,778 foci), but was actually slightly

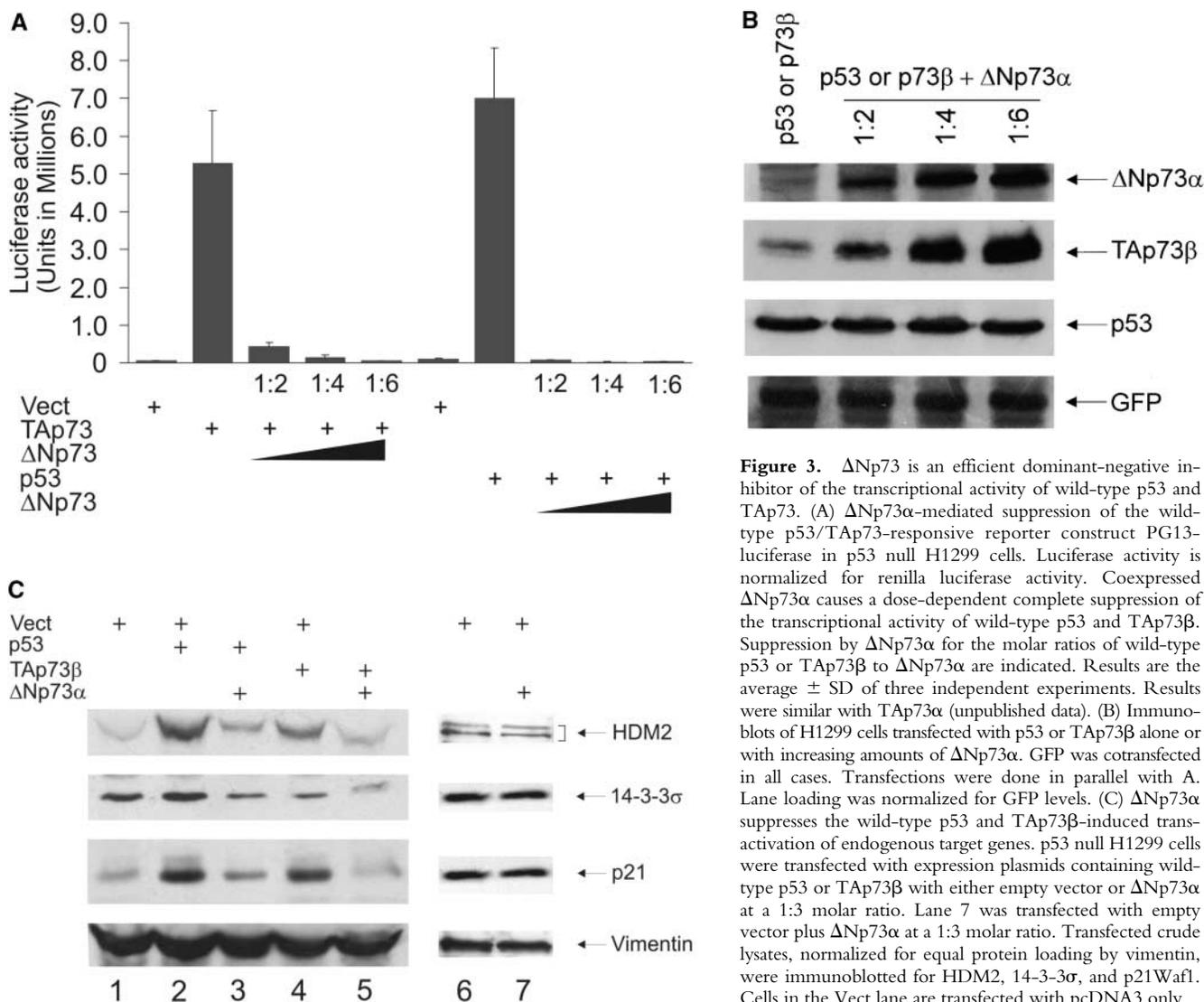


Figure 3. Δ Np73 is an efficient dominant-negative inhibitor of the transcriptional activity of wild-type p53 and TAp73. (A) Δ Np73 α -mediated suppression of the wild-type p53/TAp73-responsive reporter construct PG13-luciferase in p53 null H1299 cells. Luciferase activity is normalized for renilla luciferase activity. Coexpressed Δ Np73 α causes a dose-dependent complete suppression of the transcriptional activity of wild-type p53 and TAp73 β . Suppression by Δ Np73 α for the molar ratios of wild-type p53 or TAp73 β to Δ Np73 α are indicated. Results are the average \pm SD of three independent experiments. Results were similar with TAp73 α (unpublished data). (B) Immunoblots of H1299 cells transfected with p53 or TAp73 β alone or with increasing amounts of Δ Np73 α . GFP was cotransfected in all cases. Transfections were done in parallel with A. Lane loading was normalized for GFP levels. (C) Δ Np73 α suppresses the wild-type p53 and TAp73 β -induced transcriptional activation of endogenous target genes. p53 null H1299 cells were transfected with expression plasmids containing wild-type p53 or TAp73 β with either empty vector or Δ Np73 α at a 1:3 molar ratio. Lane 7 was transfected with empty vector plus Δ Np73 α at a 1:3 molar ratio. Transfected crude lysates, normalized for equal protein loading by vimentin, were immunoblotted for HDM2, 14-3-3 σ , and p21Waf1. Cells in the Vect lane are transfected with pcDNA3 only.

growth suppressive in these p53 null cells for reasons that are unclear. In contrast, the coexpression of Δ Np73 α with wild-type p53 at a 1:1 molar ratio counteracts this effect,

leading to a 12.5-fold increase in the number of colonies from 4 to 51 foci. Likewise, TAp73 α , although not quite as potent as wild-type p53, suppresses colony formation (82

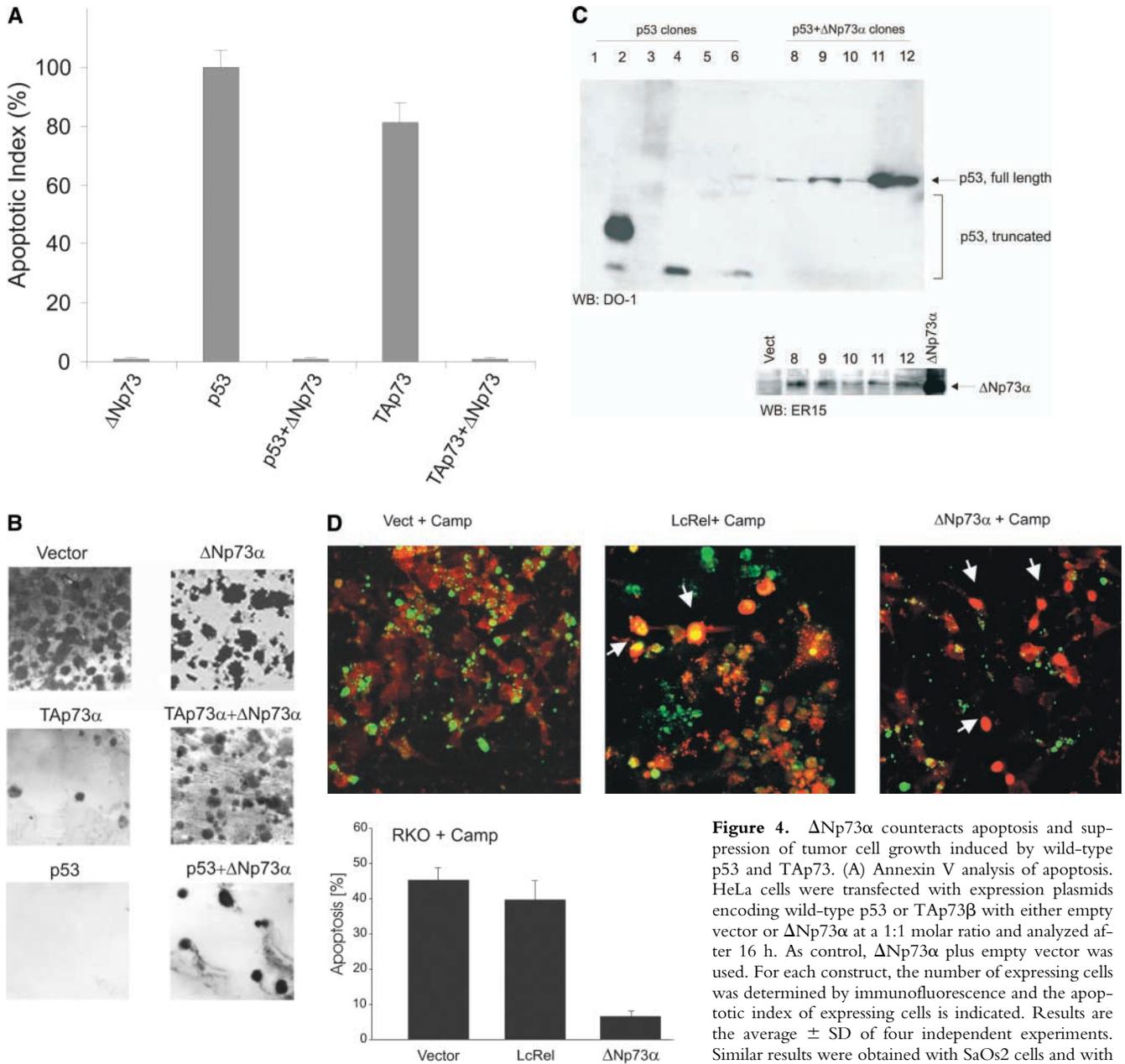


Figure 4. Δ Np73 α counteracts apoptosis and suppression of tumor cell growth induced by wild-type p53 and TAp73. (A) Annexin V analysis of apoptosis. HeLa cells were transfected with expression plasmids encoding wild-type p53 or TAp73 β with either empty vector or Δ Np73 α at a 1:1 molar ratio and analyzed after 16 h. As control, Δ Np73 α plus empty vector was used. For each construct, the number of expressing cells was determined by immunofluorescence and the apoptotic index of expressing cells is indicated. Results are the average \pm SD of four independent experiments. Similar results were obtained with SaOs2 cells and with TUNEL analysis. (B) SaOs2 colony suppression induced by wild-type p53 and TAp73 α is inhibited by Δ Np73 α . Number of colonies are shown in Table II. (C) p53 expression analysis of random SaOs2 clones derived from surviving colonies of a parallel experiment to the one shown in B. Immunoblots with anti-p53 antibody DO-1 using 30 μ g total cell lysate per lane. Clones from cells originally transfected with expression plasmids for wild-type p53 alone (left) or wild-type p53 plus Δ Np73 α (right). Except for clone 6, all other wild-type p53-transfected clones have lost full-length p53 protein expression. Clones 1, 3, and 5 show no detectable p53 protein at all, whereas clones 2, 4, and 6 express truncated p53 polypeptides (left). In contrast, all six colonies derived from cotransfection with wild-type p53 and Δ Np73 α show detectable levels of full-length p53 protein with sequence-confirmed wild-type status of the DNA binding domain in three of three tested clones (right, five clones are shown). Immunoblot to show Δ Np73 α expression in SaOs2 clones 8–12 compared with a SaOs2 clone transfected with empty vector (bottom). (D) Δ Np73 α confers drug resistance to wild-type p53/TAp73 harboring tumor cells. RKO cells were transfected with empty vector (left), the irrelevant expression plasmid LcRel (center), or with Δ Np73 α expression plasmid (right). 5 h after transfection, cells were treated with 5 μ M camptothecin overnight or left untreated (not depicted). After fixation with paraformaldehyde, each well underwent both TUNEL staining in green and immunofluorescence with Δ Np73 α -specific polyclonal antibody (left and right) or Flag antibody (center) in red to assess apoptosis and expression. In contrast to vector-only or LcRel-transfected cells, Δ Np73 α -expressing cells (right white arrows) are virtually protected from camptothecin-induced apoptosis. The p73 antibody produces a slight background staining in empty vector-transfected RKO cells, which allows the counting of all cells in the well (left). The percentage of apoptosis of Δ Np73 α -expressing and control-transfected cells after 24 h of treatment with 5 μ M camptothecin is shown.

foci), but the coexpression of Δ Np73 α with TAp73 α again antagonizes this effect and increases the number of macroscopic colonies by 8.1-fold to 669 foci. Taken together, Δ Np73 is an efficient dominant-negative inhibitor of wild-type p53 and TAp73, although the strong repressive effect of Δ Np73 α on p53 and TAp73 seen in transient assays (Fig. 4 A) is more modest in long-term assays (Fig. 4 B). Entirely consistent with this finding were data from a subsequent p53 expression analysis of SaOs2 cell clones that were established from surviving colonies of a duplicate experiment. A complete loss of full-length p53 protein expression was found in five out of six such randomly picked clones that were derived from plates transfected with wild-type p53 alone (Fig. 4 C, left). Although clones 1, 3, and 5 showed no detectable p53 protein at all, clones 2, 4, and 6 expressed only truncated and presumably nonfunctional p53 polypeptides, likely due to chromosomal rearrangement. The functional significance of the very small amounts of full-length p53 protein detectable in clone 6 is unclear, in light of the presence of coexpressed truncated p53. On the whole, this data is in agreement with the fact that wild-type p53 expression is incompatible with the outgrowth of clones in this assay, and rare colonies escape because they have lost wild-type p53 expression (28). In contrast, all six randomly picked colonies derived from plates cotransfected with wild-type p53 and Δ Np73 α exhibited only full-length p53 protein (Fig. 4 C, five clones are shown). Upon sequencing the DNA binding domain of three of these clones, all three revealed wild-type p53 genotypes. All clones expressed elevated levels of Δ Np73 α protein compared with endogenous levels (Fig. 4 C). Taken together, this data indicates that the coexpression of Δ Np73 α neutralizes the growth-suppressive effect of wild-type p53, thereby removing the selection pressure for the deletion or rearrangement of wild-type p53. Δ Np73 α is able to effectively counteract p53- and TAp73-induced colony suppression in transformed human cells.

Δ Np73 α Confers Drug Resistance to Wild-type p53-harboring Tumor Cells. Because we have shown that Δ Np73 is an efficient inhibitor of exogenous wild-type p53-mediated apoptosis and growth suppression, we next tested whether Δ Np73 α also inhibits endogenous wild-type p53-mediated

apoptosis after DNA damage. RKO cells, a human colon cancer line harboring wild-type p53 and TAp73, were transfected with empty vector, an irrelevant control plasmid (LcRel), or with Δ Np73 α expression plasmid and treated with either 5 μ M camptothecin or left untreated (Fig. 4 D). Camptothecin is a topoisomerase inhibitor generating single and double strand DNA breaks, which induce an increase of p53 and TAp73 protein levels (29). As expected, control cells treated with camptothecin and transfected with either empty vector or LcRel underwent marked apoptosis with 44 and 39% of cells dying after 24 h. In contrast, Δ Np73 α -expressing camptothecin-treated cells were strongly protected from drug-induced apoptosis, causing only 6% of cells to die. Thus, Δ Np73 α protects human cancer cells from p53- and TAp73-induced cell death mediated by a chemotherapeutic agent.

Δ Np73 Inhibits Wild-type p53 and TAp73 Function by Heterocomplex Formation. One explanation for the observed dominant-negative effect is a direct physical interaction between Δ Np73 and either wild-type p53 or TAp73 proteins, analogous to the dominant-negative mode of action of mutant p53 proteins toward wild-type p53. To test this hypothesis directly, lysates prepared from p53 null SaOs2 cells cotransfected with wild-type p53 and Δ Np73 α were immunoprecipitated with monoclonal antibody ER15, which recognizes Δ Np73 α . Immunoblot analysis with an antibody specific for p53 (CM1) revealed a complex of the two proteins (Fig. 5 A, lane 3). Of note, TAp73 isoforms are unable to form a protein complex with wild-type p53 (Fig. 5 A, lane 7, B, lane 3, and C, lane 1; references 18 and 30–32), excluding the possibility that the observed p53 band was coimmunoprecipitated via the endogenous TAp73 protein of SaOs2 cells. As control, no such complex was seen in SaOs2 cells transfected with either empty vector, TAp73 α , or Δ Np73 α alone (Fig. 5 A, lanes 1, 2, and 4), nor was a complex seen in SaOs2 cells transfected with p53 alone or with p53 plus TAp73 α (Fig. 5 A, lanes 6 and 7), indicating the specificity of the p53– Δ Np73 α complex. Moreover, a similar complex was seen in wild-type p53-expressing human U2OS cells after transfection with Δ Np73 α alone. Fig. 5 B shows a specific complex between endogenous wild-type p53 and ectopic Δ Np73 α that was immunoprecipitated by ER15 from U2OS cells (lane 1). No such complex was seen when an irrelevant monoclonal antibody against GFP was used (lane 2), or when TAp73 α or empty vector were expressed (lanes 3 and 4). The same specific complex can again be immunoprecipitated from U2OS cells using a monoclonal antibody specific for p53 (421) and immunoblotted with the polyclonal antibody specific for Δ Np73 that does not cross react with any TAp73 proteins (Fig. 5 C, lane 2). Again, no such complex is found with preimmune mouse IgG (Fig. 5 C, lane 3) or when U2Os cells were transfected with TAp73 α (lane 1) or vector (unpublished data). The lysate lane in Fig. 5 A represents 5% of the immunoprecipitation input and the lysate lane in Fig. 5 C represents 2–10% of the immunoprecipitation input, depending on the experiment. Together, this data indicates that a small

Table II. Inhibition of Colony Suppression by Δ Np73

Plasmid	No. of foci	SD ^a
vect	1,778	213
Δ Np73	1,383	194
wtp53	4	8
wtp53 plus Δ Np73	51	14
TAp73 α	82	16
TAp73 α plus Δ Np73	669	187

wtp53, wild-type p53.

^aData are derived from three independent experiments.

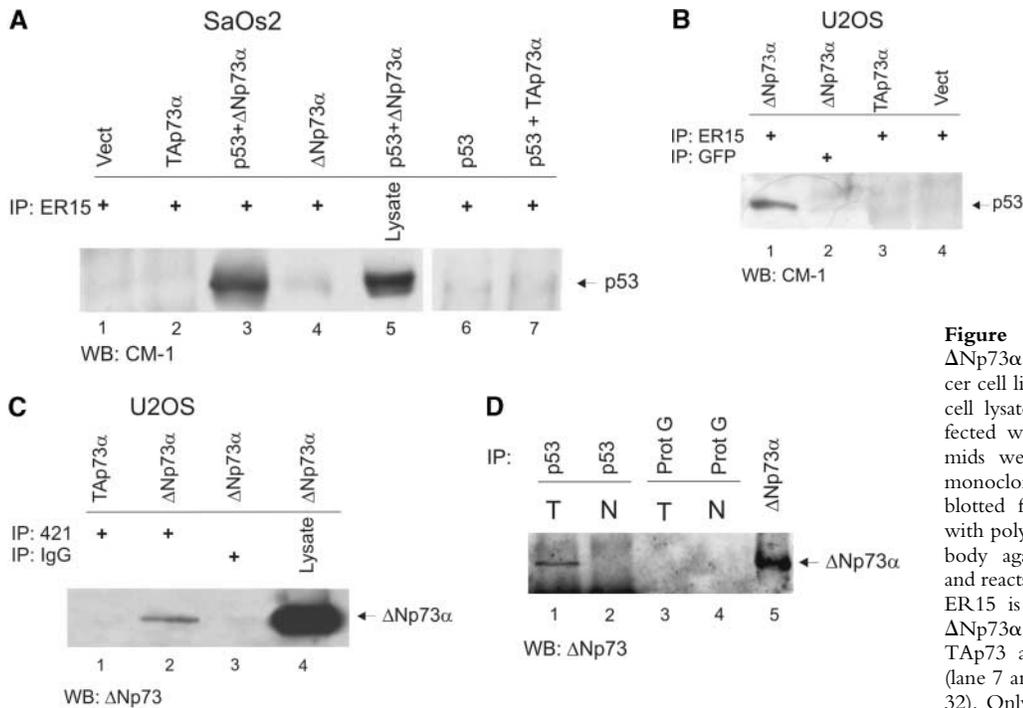


Figure 5. Physical interaction between Δ Np73 α and wild-type p53 proteins in cancer cell lines and human tumors. (A) Crude cell lysates of p53 null SaOs2 cells transfected with the indicated expression plasmids were immunoprecipitated with the monoclonal antibody ER15 and immunoblotted for coprecipitating wild-type p53 with polyclonal CM-1. ER15 is a p73 antibody against a COOH-terminal epitope and reacts with both Δ Np73 α and TAp73 α . ER15 is used here to immunoprecipitate Δ Np73 α because complexes between TAp73 and wild-type p53 do not occur (lane 7 and B and C; references 18 and 30–32). Only lysate was loaded in lane 5. The shadow band in lanes 4, 6, and 7 is derived

from the heavy chain of added ER15. (B) Crude cell lysates of wild-type p53 harboring U2OS cells transfected with the indicated plasmids were immunoprecipitated with ER15 or an irrelevant monoclonal antibody against GFP and immunoblotted for coprecipitating endogenous p53 with polyclonal CM-1. (C) Crude cell lysates of U2OS cells transfected with the indicated plasmids were immunoprecipitated with a monoclonal antibody against p53 (421) or irrelevant mouse IgG and immunoblotted for coprecipitating Δ Np73 α with polyclonal anti- Δ Np73. This antibody is raised and immunopurified against exon 3' and does not cross react with TAp73 isoforms or p53. Only lysate was loaded in the indicated lane. (D) Detection of tumor-specific protein complexes between Δ Np73 α and wild-type p53 in an ovarian carcinoma. Immunoprecipitations of equal amounts of total protein (2 mg each) from a matched pair of homogenized tumor/normal tissues using a mixture of anti-p53 antibodies DO-1 and 1801 covalently coupled to agarose beads, followed by immunoblotting with polyclonal anti- Δ Np73. The Δ Np73 α -p53 protein complex is detectable in the tumor but not in the respective normal tissue. Equal amounts of the same tumor and normal tissue lysates immunoprecipitated with protein G-coupled beads are used as an additional negative control. The last lane is a positive control of H1299 lysate transfected with a Δ Np73 α expression vector.

but highly reproducible portion of Δ Np73 forms a stable, specific complex with wild-type p53 in cells. To confirm the existence of this complex *in vivo* in tumor tissues, we coimmunoprecipitated the Δ Np73-p53 complex from an ovarian carcinoma and an endometrial carcinoma with wild-type p53 status (see example in Fig. 5 D, lane 1). Of note, this mixed complex was only seen in the patients' tumors but not in the corresponding normal ovarian tissues (Fig. 5 D, lane 2). Moreover, no specific complex was seen when the immunoprecipitating anti-p53 antibody beads were substituted by protein G beads (lanes 3 and 4).

We also examined the second possibility, namely direct competition for specific DNA binding between Δ Np73 α and wild-type p53 at the promoter level. Gel shift assays were performed with two different p53-specific DNA binding sequences as probes, using nuclear extracts of H1299 or U2OS cells transfected with expression plasmids for p53 alone or in combination with Δ Np73 α . Although nuclear extract transfected with empty vector showed no specific DNA complex (Fig. 6 A, lane 1), p53 formed a specific complex (lane 2) that could be supershifted with the p53-specific antibody PAb 421 (lane 3) and competed off with 50-fold excess of unlabeled specific probe (lane 4). Likewise, p53 only binds to the specific probe (Fig. 6 A,

lane 9), but not to a scrambled version of the specific probe (lane 11). The specific p53/DNA band (Fig. 6 A, lane 9) is supershifted by antibody 421 (lane 10), but 421 cannot produce a "false" supershifted band when p53 fails to bind to the scrambled probe (lane 12). Extracts from cells transfected with Δ Np73 α alone failed to bind to both probes despite high levels of Δ Np73 α expression on immunoblots (Fig. 6 A, lane 8, and unpublished data). However, nuclear extracts from cells cotransfected with p53 and Δ Np73 α at a 1:4 ratio showed a slight decrease in p53-DNA complex formation (Fig. 6 A, compare lanes 2 and 5), which became more pronounced at a 1:20 ratio of p53 to Δ Np73 α (compare lanes 6 and 7). Moreover, in a p53 reporter assay using PG13, a tetramerization-incompetent but DNA binding-competent mutant of Δ Np73 α , called Δ Np73 L322P (corresponding to L371P in TAp73 α ; reference 4), showed a significant but incomplete reversal of the dominant-negative inhibition of p53 transactivation by Δ Np73 α (Fig. 6 B). These results support the notion that competition at the promoter site is another mode of inhibition. Taken together, the formation of inactive heterooligomers between p53 and Δ Np73 appear to play a role in the dominant-negative inhibition of p53 by Δ Np73. Such complexes occur naturally in primary tumors and in cultured cells. On the

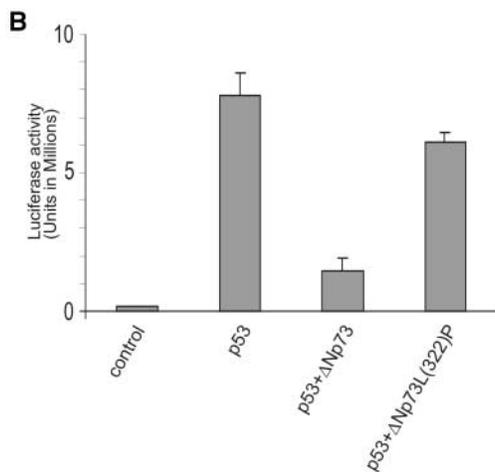
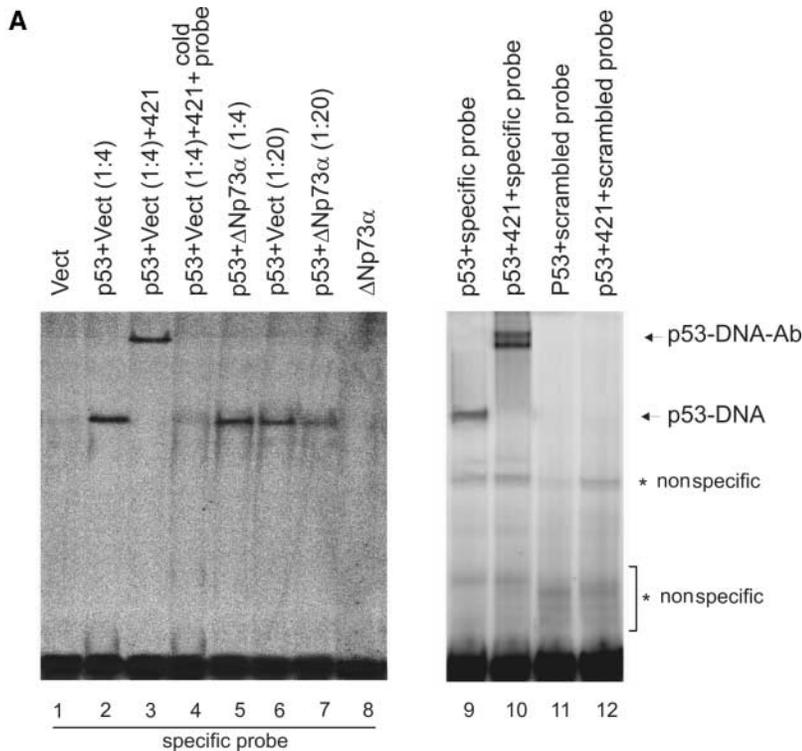


Figure 6. Mechanism of the dominant-negative effect of Δ Np73. (A) EMSA using nuclear extracts of H1299 cells transfected with expression plasmids for p53 or Δ Np73 α alone or in combination. Competition of sequence-specific binding of p53 by Δ Np73 α is shown (compare lanes 6 and 7). Δ Np73 α alone does not bind to the p53 cognate site, p53CON (lane 8). Anti-p53 antibody (421) was added as indicated. (B) p53 reporter assay using the p53-responsive reporter sequence PG13. A tetramerization-incompetent but DNA binding-competent mutant of Δ Np73 α , Δ Np73 L(322)P (corresponding to L371P in TAp73 α ; reference 4), shows a significant but incomplete reversal of the dominant-negative inhibition of p53 transactivation by Δ Np73 α .

other hand, competition between Δ Np73 and p53 at the level of the promoter, where the presence of Δ Np73 interferes with the ability of p53 to bind effectively to its cognate binding site, might be a second mechanism that is physiologically relevant. Using nuclear extracts of Δ Np73-transfected cells, however, we failed to obtain clear evidence that Δ Np73 α alone can directly bind to p53 cognate sites. This issue justifies additional studies.

Suppression of Δ Np73 Enhances Apoptosis Mediated by p53 and TAp73. Our studies demonstrate a dominant-negative action of Δ Np73 toward the apoptosis, focus suppression, and transactivation functions of p53 and TAp73 within the context of forced Δ Np73 expression. To test whether Δ Np73 would have the same inhibitory effect in cells when present at endogenous levels, we used antisense

oligonucleotides to suppress endogenous Δ Np73 expression in wild-type p53-harboring RKO cells that undergo apoptosis after DNA damage. RKO cells treated with camptothecin alone underwent a certain level of apoptosis (Fig. 7 A, left bar). In contrast, the preincubation of RKO cells with antisense oligonucleotides directed against the unique exon 3' of Δ Np73 showed a significant enhancement of p53-mediated apoptosis after camptothecin stress (Fig. 7 A, center bar), whereas camptothecin-stressed RKO cells pretreated with the sense version of the same oligonucleotide did not (Fig. 7 A, right bar). To further confirm that the gain in apoptotic ability seen after down-regulation of endogenous Δ Np73 was in fact due to specific derepression of p53-mediated apoptosis, we modified the above experiment by directly transfecting p53. RKO cells were

transfected with wild-type p53 expression plasmid before incubation with antisense or sense oligonucleotides. As already seen in Fig. 7 A, p53-expressing cells treated with antisense oligonucleotides showed significantly enhanced apoptosis compared with the same cells treated with sense oligonucleotides (Fig. 7 B). To confirm that our antisense strategy works, we determined Δ Np73 protein levels. As shown in Fig. 7 C, transfection of the antisense oligonucleotide clearly down-regulated endogenous Δ Np73 protein levels compared with the sense control oligonucleotide. Taken together, these studies clearly indicate that endogenous Δ Np73 exerts a significant transdominant inhibition of p53 function. Down-regulation of endogenous Δ Np73 levels alleviates its suppressive action on p53-mediated apoptosis after DNA damage.

Discussion

p53 controls a powerful stress response by integrating signals from many types of DNA damage or inappropriate oncogenic stimulation. Activation of p53 elicits a cellular response of apoptosis, cell cycle arrest, or senescence, thereby preventing tumor formation. Therefore, direct or indirect loss of p53 function is a preeminent defect in most human cancers whether via intragenic mutation of p53 itself (33), lack of p53 nuclear retention (34), loss of its upstream activator p14ARF (35), or inhibition by its antagonist HDM2 (36, 37). Here we show that the human TP73 gene, a homologue of p53, produces an NH₂ terminally truncated isoform driven by an alternative internal promoter in intron 3. Human Δ Np73 starts with a unique exon 3' that is highly conserved from mouse exon 3' and is in frame with exon 4. Δ Np73 lacks the transactivation domain and is therefore predicted to function as a transdominant inhibitor of p53. In physical and functional assays, we demonstrate dominant-negative interactions between human Δ Np73 and wild-type p53 or transcription-competent TAp73. Δ Np73 is a potent inhibitor of wild-type p53 and TAp73 with respect to their transcriptional activation, apoptotic ability, and growth suppressor function. Of note, Δ Np73-mediated interference with the biological p53 response occurs at endogenous Δ Np73 levels, because antisense-directed down-regulation of endogenous Δ Np73 expression leads to a derepression of the p53 response.

Importantly, in this study we provide the first clinical evidence that Δ Np73 is frequently up-regulated in a variety of primary cancers including cancers of the breast and the female genital tract. We show that tumor-specific up-regulation of Δ Np73 occurs at the mRNA and protein level in primary tumors. In a rigorous comparison of tumor/normal tissue pairs from 37 patients, 73% show Δ Np73 up-regulation specifically in their tumor tissues but not in their respective normal tissues of origin. In addition, a subset of those tumors also up-regulate the previously described Ex2Del p73, another transdominant inhibitor of p53, but one that is generated from the P1 promoter of TP73 via alternative splicing. Taken together, 81% of tumor pairs ex-

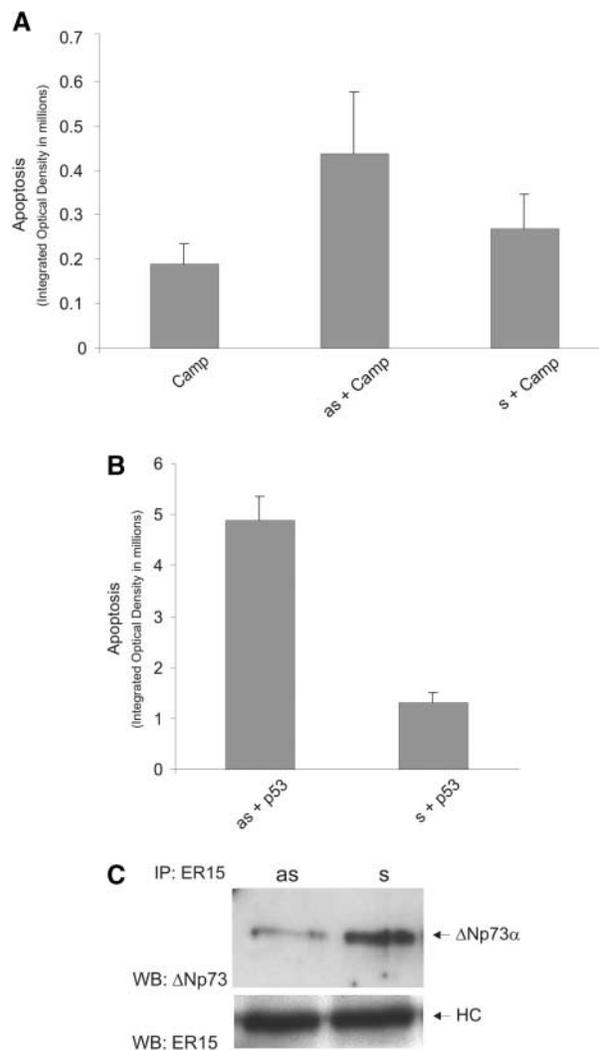


Figure 7. Down-regulation of endogenous Δ Np73 levels alleviates its suppressive action and thereby enhances apoptosis mediated by p53 and TAp73. (A) Wild-type p53 harboring RKO cells were transfected with antisense (as) or sense (s) oligonucleotides (200 nM each) directed against exon 3' of Δ Np73. After 8 h, cells were subjected to DNA damage by 1 mM camptothecin for an additional 16 h before TUNEL staining. Apoptosis was determined by measuring green fluorescence as described in Materials and Methods. Camp, cells treated with camptothecin alone. (B) RKO cells were transfected with 360 ng wild-type p53 expression plasmid with 200 nM of either Δ Np73 antisense (as) or sense (s) oligonucleotides. 20 h after transfection, TUNEL staining was performed. Apoptosis was quantitated as described above. (C) Cells were transfected with 200 nM each of antisense (as) or sense (s) oligonucleotides. 36 h later, equal amounts of lysates (2 mg total protein) were immunoprecipitated with ER15 and blotted with the polyclonal anti- Δ Np73.

hibited tumor-specific up-regulation of Δ Np73 and/or Ex2Del p73. Of note, among the tumors with up-regulation of any one or all three TP73 transcripts (Δ Np73, Ex2Del p73, and TAp73), 71% exhibited preferential up-regulation of Δ Np73 or Ex2Del p73. Moreover, two thirds of the latter group of tumors showed exclusive up-regulation of Δ Np73 and/or Ex2Del p73 without any concomitant rise in TAp73. Furthermore, among tumors with preferential up-regulation of Δ Np73 and Ex2Del p73, 71%

harbored wild-type p53. In contrast, among the (small) group of tumors with preferential TAp73 up-regulation, 66% harbored mutant p53. In summary, given the trend between tumor-specific up-regulation of Δ Np73 and/or Ex2Del p73 on the one hand and wild-type p53 status on the other, it is tempting to speculate that the expression of dominant-negative p73 isoforms alleviates the selection pressure for p53 mutations in tumors by functionally inactivating the suppressor action of p53. More tumor samples need to be analyzed to clarify this question. If confirmed, however, this mode of p53 inactivation would be functionally equivalent to the amplification of the HDM2 gene in some human sarcomas and leukemias (36, 37). While this work was in progress, Grob et al. (38) reported that p53 and TAp73 can directly induce Δ Np73 via a p53-responsive element in the Δ Np73 promoter in cultured cells. Moreover, Nakagawa et al. (39) confirmed that TAp73 directly transactivates Δ Np73, but such an activity for p53 could not be found by this group. Nevertheless, these results further fortify the analogy to the p53-HDM2 autoregulatory feedback loop. It will be interesting to determine whether p53 and/or TAp73 itself drive the overexpression of Δ Np73 in primary tumors as well.

We provide evidence that physical interaction between Δ Np73 α and wild-type p53 is one of the possible functional mechanisms of this inhibition. We showed that this mixed complex occurs naturally in cultured human tumor cells and tumor tissues. While this work was under review, Nakagawara et al. (39) also reported that Δ Np73 α and Δ Np73 β form stable mixed complexes with endogenous and ectopic wild-type p53, as well as with TAp73 α . Similarly, Pozniak et al. (18) reported a direct physical interaction of mouse Δ Np73 α and Δ Np73 β proteins with mouse wild-type p53, both of which inhibited the apoptosis-promoting activity of p53. Thus, heterocomplexes appear to interfere with the specific DNA binding activity of wild-type p53. In contrast to Δ Np73, TAp73 isoforms are unable to form a protein complex with wild-type p53 (Fig. 5, B and C; references 18 and 30–32). Therefore, the existence of mixed complexes suggests that the unique exon 3' at the NH₂ terminus of Δ Np73, in conjunction with the missing transactivation domain, induces a conformational change in Δ Np73 that allows the complex to form. In contrast, another recently described isoform named Δ TAp73 α , which is encoded by p73 (exons 4–14) but missing the unique exon 3', does not appear to form a complex with wild-type p53 (40). Heterooligomeric complexes mirror the ability of many missense p53 mutants in heterozygous tumors to abrogate the function of the remaining wild-type p53 allele (41, 42). Of note, a stoichiometry as low as 1:3 of mutant to wild-type p53 molecules already abrogates wild-type p53 function, suggesting that a similar stoichiometry might be effective for Δ Np73 as well. An additional mechanism of p53 inhibition might be direct promoter competition, with Δ Np73 displacing p53 from the DNA binding site (43). Such a phenomena has been seen in gel shift assays with high concentrations of in vitro translated p73 (exons 4–14) protein (Δ TAp73 α ; reference

40). In nuclear extracts of H1299 and U2OS cells cotransfected with p53 plus Δ Np73, which more closely mimics the physiological situation than in vitro translated proteins, we also observed a decrease in p53–DNA complex formation in the presence of Δ Np73 α , supporting the notion of promoter competition. Under these experimental conditions, however, we failed to obtain clear evidence that Δ Np73 α alone can directly bind to p53 cognate sites. Although both inhibitory mechanisms are likely, this point needs additional study.

Responsiveness to oncogenes and selected forms of DNA damage might suggest a putative tumor suppressor role of the TP73 gene analogous to TP53. However, tumor-associated overexpression of (total) p73 and in some cases TAp73 rather than loss of expression, is the most commonly observed tumor-specific abnormality of the TP73 gene. This fact, in conjunction with a conspicuous lack of p73 mutation in human tumors and a lack of a cancer phenotype in p73-deficient mice, provides clear evidence that the TP73 gene is not a classic Knudson-type tumor suppressor in vivo. Instead, our finding that a significant percentage of human tumors specifically select for dominant-negative p73 isoforms strongly argues for their oncogenic role during tumorigenesis in vivo. Preferential up-regulation of Δ Np73 and Ex2Del p73 in tumors might bestow oncogenic activity upon the TP73 gene that specifically interferes with the tumor suppressor functions of wild-type p53 and TAp73. In fact, this scenario suggests that the TP73 gene embodies the “two genes in one” idea with products that play opposing roles within the family circuitry. Their impact on tumor formation in vivo might therefore depend on the balance between tumor-promoting and -suppressing family members. The existence of this inhibitory family network might explain the paucity of p73 mutations in human tumors. In the developing mouse brain, Δ Np73 is the predominant form of TP73 and a powerful inhibitor of p53 (18). In vivo and in vitro studies showed that Δ Np73 plays an essential antiapoptotic role required to counteract p53-mediated neuronal death during the “sculpting” of the developing brain. This explains why p73^{-/-} mice, missing all forms of p73 including protective Δ Np73, underwent accelerated neuronal death in sympathetic ganglia (18). Thus, in keeping with a common theme in cancer, a developmental inhibitory network that is essential for normal physiology resurfaces in cancer, but in a corrupted and derailed fashion. In support of the idea that Δ Np73 can act as an oncogene in vivo, overexpression of Δ N isoforms of p63, a p73 homologue, is found in human cancers including lung cancer, squamous cell carcinoma of the head and neck (44), bladder cancer (45), and nasopharyngeal carcinoma (46). Importantly, a specific Δ Np63 isoform is oncogenic in nude mice and in the Rat 1a focus formation assay (44).

Evidence from some human cancers and mouse models indicates that the mutational status of p53 is an important clinical variable for prognosis as well as for guiding the aggressiveness of anticancer therapy. However, currently, p53 mutational status is not widely accepted as a clinical indica-

tor because many studies show that its prognostic power for survival and its predictive value for therapeutic response is poor (for review see reference 47). From a practical standpoint, the discovery of a $\Delta Np73$ -based p53-p73 interference network suggests that the p53 status of a tumor should no longer be considered in isolation. The existence of this inhibitory network might account for the inconsistencies of clinical studies that sought to use p53 status as a predictor of outcome. It mandates that we do a careful analysis of the functional consequences of this network in vivo. The establishment and clarification of an inhibitory p53-p73 network would have a major clinical impact ranging from fine tuning the prognostic power of p53 mutation status to rational p53-p73-targeted drug design.

In conclusion, we report here the cloning of human $\Delta Np73$ and show that $\Delta Np73$ mediates a novel inactivation mechanism of wild-type p53 and TAp73 via dominant-negative interference. Deregulated expression of $\Delta Np73$ can bestow oncogenic activity upon the TP73 gene by functionally inactivating the suppressor action of p53 and TAp73. This trait might be frequently selected for in a variety of human cancers.

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