ΔNp63α Up-Regulates the *Hsp70* Gene in Human Cancer

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Abstract

HSP70, a stress response protein, is known to be a determinant of cell death and cell transformation. We show that different isoforms of *p63* have different transcriptional activities on *hsp70* genes. ΔNp63α, an abundantly expressed isoform of *p63*, activates (*in vitro* and *in vivo*), whereas TAp63γ down-regulates the expression of *hsp70*. We further show that the transactivation domain at the NH_2 terminus of p63 represses, whereas the COOH terminus activates *hsp70* transcription. In addition, ΔNp63α regulates transcription of the *hsp70* gene through its interaction with the CCAAT binding factor and NF-Y transcription factors which are known to form a complex with the CCAAT box located in the *hsp70* promoter. Moreover, ΔNp63α expression correlates with HSP70 expression in all head and neck cancer cell lines. Finally, we show colocalization of ΔNp63α and HSP70 in the epithelium and coexpression of both proteins in 41 primary head and neck cancers. Our study provides strong evidence for the physiologic association between ΔNp63α and hsp70 in human cancer, thus further supporting the oncogenic potential of ΔNp63α. (Cancer Res 2005; 65(3): 758-66)

Introduction

The *p53* gene family consists of three members, *p53*, *p63*, and *p73*. Both *p63* and *p73* exhibit high amino acid identity with *p53* including their transactivation (25%), DNA binding (65%), and tetramerization domains (35%). Unlike *p53*, *p63* and *p73* each generate two major protein isoforms, transactivation (TA) and ΔN, through two different promoters and three alternative splicing sites at the 3′ end, denoted by α, β, and γ (Fig. 1*A*; refs. 1–3). Despite their structural similarity, it is thought that *p63* and *p73* play quite different biological roles compared with *p53*. In line with this reasoning, *p53* plays a major role in tumorigenesis, whereas *p63* and *p73* are involved mainly in normal development. These differences notwithstanding, recent research has brought to light several instances of functional overlap amongst the *p53* family members. Both p63 and p73 can bind to *p53* DNA binding sites *in vitro* and transcriptionally regulate common downstream target genes (2–4). In general, the TA isoforms of p63 and p73 act more like p53, whereas the ΔN isoforms display a dominant-negative function with other TA isoforms and p53, perhaps modulating the function of p53. This observation raised the possibility that *p63* and/or *p73* might be involved in the same *p53*-regulated pathways and the hypothesis that cell integrity might ultimately depend on the balance between all *p53* gene family members.

p53 can be activated in response to malignancy-associated stress signals that in turn provoke various responses including cell cycle arrest, differentiation, senescence, DNA repair, and apoptosis (5, 6). Many of the downstream target genes of *p53* are involved in the apoptosis signaling pathway, including *p21*, *BAX*, *PUMA*, and *hsp70* (7–12). HSP70 is one of the most abundant heat shock proteins (HSP) and accounts for as much as 1% to 2% of total cellular protein. HSP70 and other chaperones are also known to be determinants of cell death and cell transformation. The overexpression of HSP70 is associated with metastasis (13), whereas the repression of HSP70 results in the inhibition of tumor cell proliferation and the induction of apoptosis (14). HSP70 chaperone activity may also influence tumorigenesis by regulating the activity of proteins that are involved in the cell cycle machinery. The antiapoptotic function of HSP70 has prompted research into the relationship between *hsp70* and the proapoptotic function of *p53*. HSP70 family members participate in the cytoplasmic sequestration of wild-type (WT) p53 in cancer cells (neuroblastoma, breast cancer, colon cancer, and retinoblastoma) as well as in embryonic stem cells. In addition, p53 represses transcription from the human *hsp70* promoter via a direct protein-protein interaction with a specific CCAAT binding factor (CBF; ref. 15). The adenovirus E1A and c-MYC oncogenes also bind to CBF but (as opposed to p53) induce the expression of *hsp70* (16, 17).

We recently analyzed the downstream target genes of two *p63* isoforms and found that HSP70 was significantly up-regulated by ΔNp63α but not by TAp63α (4). We now show that the transactivation of *hsp70* by ΔNp63α is structure-related and provide evidence of the physiologic association between ΔNp63α and hsp70 in human cancer.

Materials and Methods

Cell Culture. Human osteosarcoma cell line Saos2 cells and head and neck cancer cell lines including O11, O12, O13, O28, and O29 were cultured in RPMI medium with 10% bovine calf serum and 1% penicillin-streptomycin. Cells were cultured at 37°C with 5% CO_2.

Establishment of Inducible Cell Line and Reverse Transcription-PCR. ΔNp63α and WTp53 Flp-in inducible Saos2 cell lines were generated according to the manufacturer's instructions, (Invitrogen, Carlsbad, CA). Briefly, pFRT/Laczeo Flp-in target site vector was transfected into Saos2 cells and the clones were screened by βgalactosidase assay. Then ΔNp63α or WTp53 and POG 44 were cotransfected into Flp-in host cell lines and positive clones were selected with hygromycin. Total RNA was extracted using Trizol reagent (Invitrogen) at different time periods adding

Note: Supplementary data for this article are available at *Cancer Research* Online (http://cancerres.aacrjournals.org/).

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1 μg/mL tetracycline to induce the expression of ΔNp63α or WTp53. Reverse transcription-PCR analysis was carried out as described previously (4).

Plasmids. The expression constructs for *p63* including TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ were cloned into the pRC/cytomegalovirus vector. The deletion constructs ΔNp63α and MWp63α were generated by PCR and subcloned into the pRC/cytomegalovirus vector. WT ΔNp63α was cloned into pAdtr vector and mutant ΔNp63α constructs: mutants 518, 534, and 541 were made using the Quickchange kit (Stratagene, La Jolla, CA) using the WT ΔNp63α construct as template. The sequences of these primers used for generating the constructs are available on request. WT *p53* and mutant *p53* ($Arg_{273}His$) were cloned into the pCDNA3.1 vector. The HSP-CBF full-length cDNA was cloned into the pMT2 eukaryotic expression vector.

***Hsp70* Gene Reporter Constructs.** The 1.4 kb basic Hsp70B promoter region in the p2500-CAT (Stressgen, Victoria, BC, Canada) vector was digested with *Bgl*II and *Hin*dIII and cloned into the pGL-3 basic luciferase vector (Promega, Madison, WI). Series deletion constructs generated by PCR were subcloned into the pGL-3 basic vector with *Bgl*II and *Hin*dIII restriction sites. The promoter constructs Hsp70-2 (1.2 kb) and Hsp70A (0.7 kb), as well as their series deletion constructs, were generated by PCR from genomic DNA and cloned into the pGL-3 basic vector. All constructs were confirmed by sequencing.

Luciferase Reporter Assay. Plasmid DNA for transient transfection was isolated using the plasmid maxi kit (Qiagen, Valencia, CA). Saos2 cells were plated at a density of 1×10^5 cells/well in six-well plates and grown overnight prior to transfection. All transfections were carried out using Fugene-6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Each transfection experiment was done in duplicate and repeated at least thrice. For cotransfection experiments, cells received 0.5 μg of *hsp70* gene promoter construct, 0.1 μg of pRL-TK *Renilla* luciferase vector (Promega), and 1 μg of the indicated expression plasmids and carrier DNA (empty vector). Firefly luciferase and *Renilla* luciferase assays were done using the Dual-Luciferase Reporter Assay System (Promega). Forty-eight hours after transfection, cells were washed with 1× PBS and harvested with 500 μL of passive lysis buffer (Promega). Cell lysates were cleared by centrifugation, and 10 μL was added to 50 μL of firefly luciferase substrate, and light units were measured in a luminometer. *Renilla* luciferase activities were measured in the same tube after addition of 50 μL of Stop and Glo reagent.

Immunofluorescence Analysis. Cos 7 cells were transiently transfected with different *p63* expression constructs. After 36 hours, cells were fixed and hybridized with *p63* 4A4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:400 dilution. Cells were washed with 1× PBS and incubated with goat anti-mouse secondary antibody conjugated with Texas red (Molecular Probes, Eugene, OR) at 1:800 and then incubated with 1 μg/mL Hoechst 33342. Images were obtained using a Zeiss confocal microscope.

Immunoprecipitation and Immunoblotting. NETN buffer [140 mmol NaCl, 20 mmol $NaPO_4$ (pH 7.4), 5 mmol EDTA, 1% NP40] was used for cell lysis. For a typical immunoprecipitation reaction, 1 to 2 mg of whole-cell extract in about 500 μL was incubated with 2 μg of antibody and 30 μL of antimouse or antirabbit IgG agarose gel (Sigma, St. Louis, MO) at 4°C overnight. Beads were washed thrice with 1× PBS. Protein bound to the beads was eluted by boiling in SDS gel sample buffer, separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Schleicher and Schuell, Riviera Beach, FL). Immunoblotting was done using the ECL kit according to the manufacturer's instructions (Amersham, Piscataway, NJ). The primary antibodies were routinely used at a concentration of 1 μg/mL, and the horseradish peroxidase-conjugated secondary antibodies (anti-mouse or antirabbit IgG; Amersham) at a 1:1,000 dilution. The primary antibodies were HSP70, HSP40 (Lab Vision, Freemont, CA), p63 4A4 (Santa Cruz Biotechnology), β-actin (Sigma), NF-YA, NF-YB (Rockland Immunochemicals, Gilbertsville, PA), NF-YC (Santa Cruz Biotechnology), HSP-CBF.

Electrophoretic Mobility Shift Assays. Double-strand oligonucleotides generated from single-strand oligonucleotides were used as electrophoretic mobility shift assay probes. The sequence of the oligonucleotides corresponded to the CCAAT binding box on the HSP70 gene promoter. The sense strand oligonucleotide is, ctc atc gag ctc ggt gat tgg ctc aga agg gaa aa; the antisense strand oligonucleotide is, ttt tcc ctt ctg agc caa tca ccg agc tcg atg ag. The gel shift assay and the competition experiments were carried out according to standard protocol (18). For supershift experiments, 2 μL of antibodies against NF-YA, NF-YB (Rockland Immunochemicals), NF-YC (Santa Cruz Biotechnology), and HSP-CBF were added to each binding reaction.

Immunohistochemistry Assay. Five-micron sections were stained using the EnVision+ System (Dako Cytomation, Carpinteria, USA). Briefly, sections were deparaffinized in xylene and rehydrated by graded ethanol before performing antigen retrieval using heat-induced epitope retrieval with 10 mmol citrate buffer. Endogenous peroxidase was inhibited by incubating the slides in 3% H_2O_2 for 10 minutes. Sections were then incubated with a rabbit polyclonal antibody against p40 (which recognizes all ΔNp63 isoforms but not TA p63 isoforms; Oncogene Research Products, Boston, USA) at a 1:400 dilution for 45 minutes or HSP70 (Santa Cruz Biotechnology) at a 1:25 dilution at 4°C overnight. After washing, the peroxidase labeled polymer was applied for 30 minutes. Visualization of the antibody-antigen reaction was done by incubation with diaminobenzidine.

The extent of staining was scored using a four-tiered system. A complete absence of staining was scored as 0. Staining that was weak and limited to < 20% of the tumor cells was scored as 1. Strong staining in 20% to 50% of tumor cells was scored as 2. Strong staining in over 50% of the tumor cells was scored as 3. Only nuclear staining was regarded as positive staining.

Results

HSP Family Members are Up-regulated by ΔNp63α. Based on cDNA microarray analysis, we previously found consistent up-regulation of HSP genes after transduction of ΔNp63α in an adenovirus system. There were five HSP members found to be up-regulated by ΔNp63α (but not by TAp63 isoforms), including three *hsp70* and two hsp40 genes (Supplementary data A). The most prominent of these were the *hsp70-2* and *hsp70B* genes demonstrating a 28-fold change. To explore the effects of more physiologic controlled *p63* expression, we established TAp63α and ΔNp63α Flp-in inducible systems based in Saos2 cells (*p53* and *p63* null) and used reverse transcription-PCR and Western blot to confirm the induced expression of *hsp70* and *hsp40* family members (Fig. 1*B* and *C*). The change in *hsp70* and *hsp40* gene expression in the inducible system parallels the different expression levels of ΔNp63α but not TAp63α, even though the fold change is not as dramatic as the microarray results. It is known that p53 can transcriptionally down-regulate HSP70 (15) and combined with our findings, it is likely that *hsp* genes are common targets for p53 family members.

To investigate whether the up-regulation of HSP70 is transcriptionally regulated by *p63*, we did a Luciferase reporter assay. ΔNp63α activated the *hsp70B* promoter more than 10-fold, whereas TAp63α transfection resulted in only 2-fold to 3-fold activation (Fig. 1*D*). As a control, we did the luciferase assay with both WT and mutant *p53* ($Arg_{273}His$). As expected, WT p53 down-regulated the *hsp70B* promoter more than 5-fold, whereas mutant p53 activated the *hsp70B* promoter approximately 2-fold (Fig. 1*E*), a much weaker effect than ΔNp63α on the *hsp70B* promoter. Transactivation of *hsp70* by mutant p53 was also confirmed by Western blotting (Supplementary data B). To further confirm the effect of ΔNp63α on *hsp70B*, we did a kinetic study. The relative luciferase activity of *hsp70B* was found to increase proportionally to increasing amounts of ΔNp63α and mutant p53 (Fig. 1*F*). In addition, we also observed a synergistic effect of ΔNp63α and mutant p53 on *hsp70* expression in Saos2 cells (Supplementary data B). These data support the notion that activation of *hsp70B* by ΔNp63α is through transcriptional regulation in a dose-dependent manner.

***p63* Isoforms Display Differential Transcriptional Effects on the HSP70 Promoter.** *p63* produces six main isoforms from two

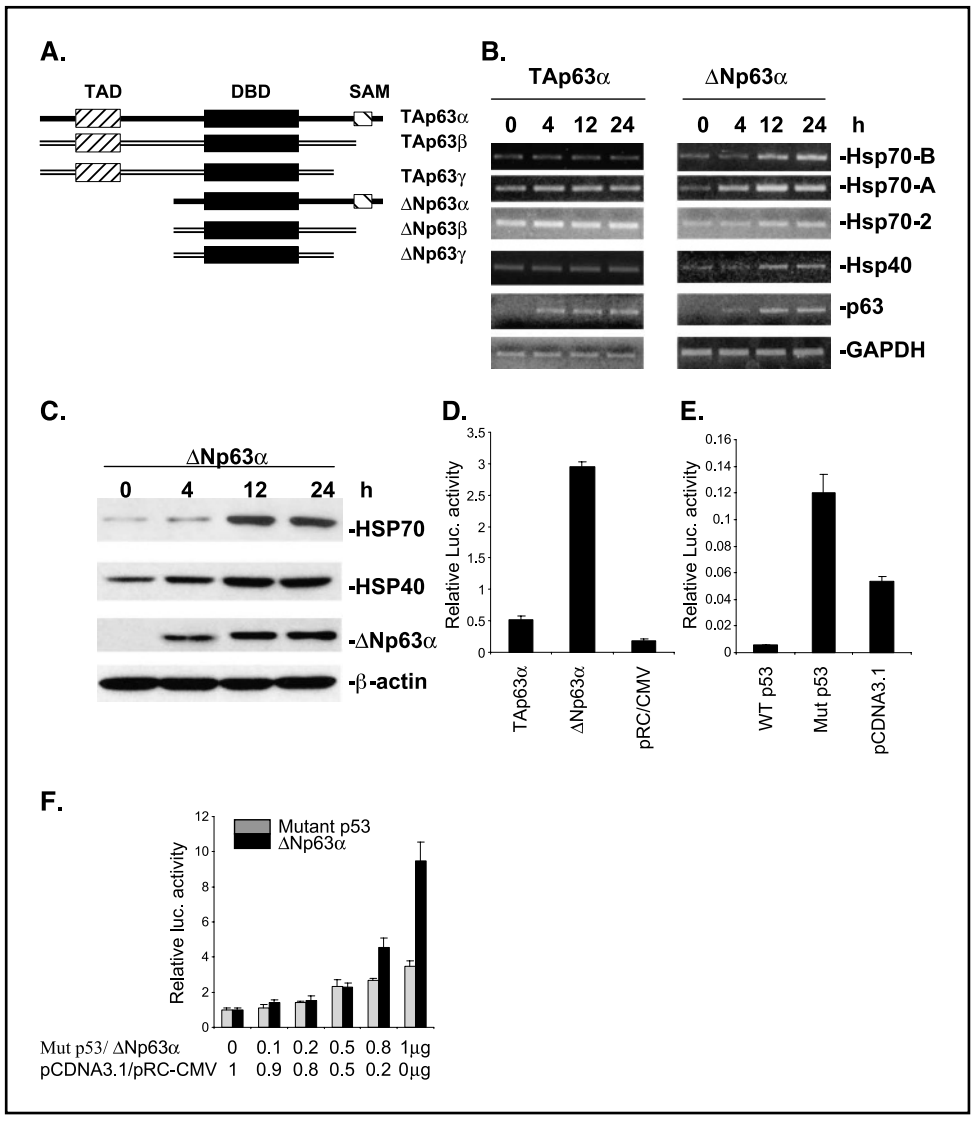

Figure 1. ΔNp63α up-regulates the expression of the *hsp70* gene. *A,* the structure of six isoforms of *p63*. The position of the transactivation domain (*TAD*), DNA binding domain (*DBD*), and sterile α motif (*SAM*) domains are shown. *B,* reverse transcription-PCR analysis of expression of *hsp70* and *hsp40* gene family members in TAp63α-inducible and ΔNp63α-inducible Saos2 cells. Data were taken at different time points after inducing cells with 1 μg/mL tetracycline. Glyceraldehyde-3-phosphate dehydrogenase was used as a normalization control. *C,* Western blot analysis showing HSP70 and HSP40 expression levels increasing in parallel with ΔNp63α induction at different time points. β-Actin was used as a protein loading control. *D,* luciferase reporter assay showing ΔNp63α and TAp63α having different effects on the *hsp70* promoter. *E,* luciferase reporter assay showing that WT p53 can repress, whereas mutant p53 (Arg_{273}His) can activate the *hsp70* gene. *F,* luciferase assay show that both ΔNp63α and mutant *p53* can up-regulate the *hsp70* promoter in a dose-dependent manner. *Samples 1-6,* variable amounts of ΔNp63α and mutant *p53* expression constructs cotransfected into Saos2 cells with *hsp70* promoter plasmid. Variable amounts of empty vector pRC/cytomegalovirus or pcDNA3.1 were added to adjust the final quantity to 1 μg. The basal activity of the reporters was set to 1. *Bars,* SD.

different promoters and differential COOH-terminal splicing (Supplementary data B). In order to explore this structural relationship further, we studied the transactivation potential of all six isoforms of p63 on Hsp70 activation. As shown in Fig. 2*A*, ΔNp63α displayed the highest transactivation effect on the *hsp70B* promoter; TAp63α, a low effect; and TAp63β, ΔNp63β, and ΔNp63γ, no transactivation effect (almost equal to empty vector). Moreover, TAp63γ actually suppressed the *hsp70B* promoter. In order to confirm the suppressive effect of TAp63γ, we did a kinetic analysis with WT p53. The luciferase analysis showed that TAp63γ like WT p53 suppressed the *hsp70* gene (Supplementary data C). We thus showed that (*a*), the transcriptional regulation of *p63* on the *hsp70* gene was structurally dependent, (*b*) the NH_2-terminal of p63 contained a suppression domain because the activation effect of TAp63α on *hsp70* was significantly lower than that of ΔNp63α, and (*c*) the COOH-terminal of p63 contained an activation domain because the activation effect of all α isoforms was stronger than the β and γ isoforms.

The NH_2 Terminus of TAp63 Contains a Repression Domain and the COOH Terminus of p63α Contains a Transactivation Domain. As seen in Fig. 2*A*, the transcriptional activity of ΔNp63α was higher than TAp63α (approximately 5-fold) and the activity of ΔNp63γ was higher than TAp63γ. This observation suggested that the TA domain might specifically repress transactivation of *hsp70* or affect overall protein stability leading to decreased p63 levels. To address this issue, we tested the protein stability of all isoforms of p63 by transfecting 1 μg of each construct into Saos2 cells. As shown in Fig. 2*B*, TAp63β, and TAp63γ were indeed unstable. However, TAp63α and ΔNp63α showed similar stable protein expression, indicating a specific contribution of the TA domain to transcriptional down-regulation of *hsp70* by *p63* isoforms.

To further test this hypothesis, we generated an artificial isoform of the *p63* gene, termed MWp63α. The start codon of the MWp63α construct is amino acid 68 of TAp63α (Supplementary data D). We then compared the transcriptional activity of TAp63α, ΔNp63α, and MWp63α on the *hsp70B* gene promoter and measured their protein stability by Western blotting. TAp63α and ΔNp63α constructs generated the same level of expression, whereas MWp63 showed a more than 10-fold increase in protein level (Fig. 2*C*), clearly indicating that MWp63α is more stable. On the other hand, the luciferase assay with equivalent amounts of MWp63α and ΔNp63α (1 μg) showed similar level of activity on the *hsp70B* gene promoter (approximately 10-fold), compared with

a 2-fold activation for TAp63α (Fig. 2*D*). We then decreased the MWp63α plasmid to 0.5 μg and found a similar protein expression level to 1 μg of ΔNp63α. The luciferase assay results showed that 0.5 μg MWp63α had only half the activity on the *hsp70* gene promoter compared with 1 μg of MWp63α or ΔNp63α (data not shown). This data clearly indicates that protein stability is not the significant element in regulation of *hsp70* by p63. Moreover, immunostaining results showed consistent nuclear localization of TAp63, ΔNp63α, and MWp63α indicating that the introduced structural alteration had no impact on subcellular localization (Supplementary data H). Taken together, our data suggest the existence of an NH_2-terminal repression domain in TAp63 isoforms.

To assess the transactivation potential of the *p63* COOH-terminal region, we generated a series of deletion constructs, termed hereafter Del 574, Del 548, Del 493, Del 400, Del 346, from the COOH-terminal end of ΔNp63α (Supplementary data E). Equal expression of all constructs was confirmed by Western blotting, showing that these constructs are structurally correct and that there is no protein degradation involved (Fig. 2*E*). We then checked the transactivation activity of all these constructs and found that the *hsp70B* promoter was strongly activated by WT ΔNp63α but not by any of the deletion constructs (Fig. 2*F*). These results clearly indicate that the minimal activation domain of ΔNp63α on the *hsp70B* promoter rests within the last 12 amino acids of the gene. In addition, using luciferase analysis, we reveal that the sterile α motif domain, a protein-protein interaction domain located in the COOH-terminal of both p63 and p73 proteins but not in *p53*, is not involved in *hsp70* transcription regulation (Supplementary data F and G).

ΔNp63α Expression Correlates with HSP70 Transcription *In vitro* and *In vivo*. Because ΔNp63α and WT p53 display opposite transcriptional effects on the *hsp70* promoter, we hypothesized that the balance of ΔNp63α and *p53* proteins might regulate *hsp70* gene expression. We cotransfected ΔNp63α and WT p53 constructs to judge the effects of both proteins on the *hsp70* promoter. *hsp70* activation by ΔNp63α was decreased with the addition of incremental amounts of WT p53 (Fig. 3*A*). Repression by WT p53 on the *hsp70* gene promoter was diminished in parallel with increasing amounts of ΔNp63α in Saos-2 cells (Fig. 3*B*).

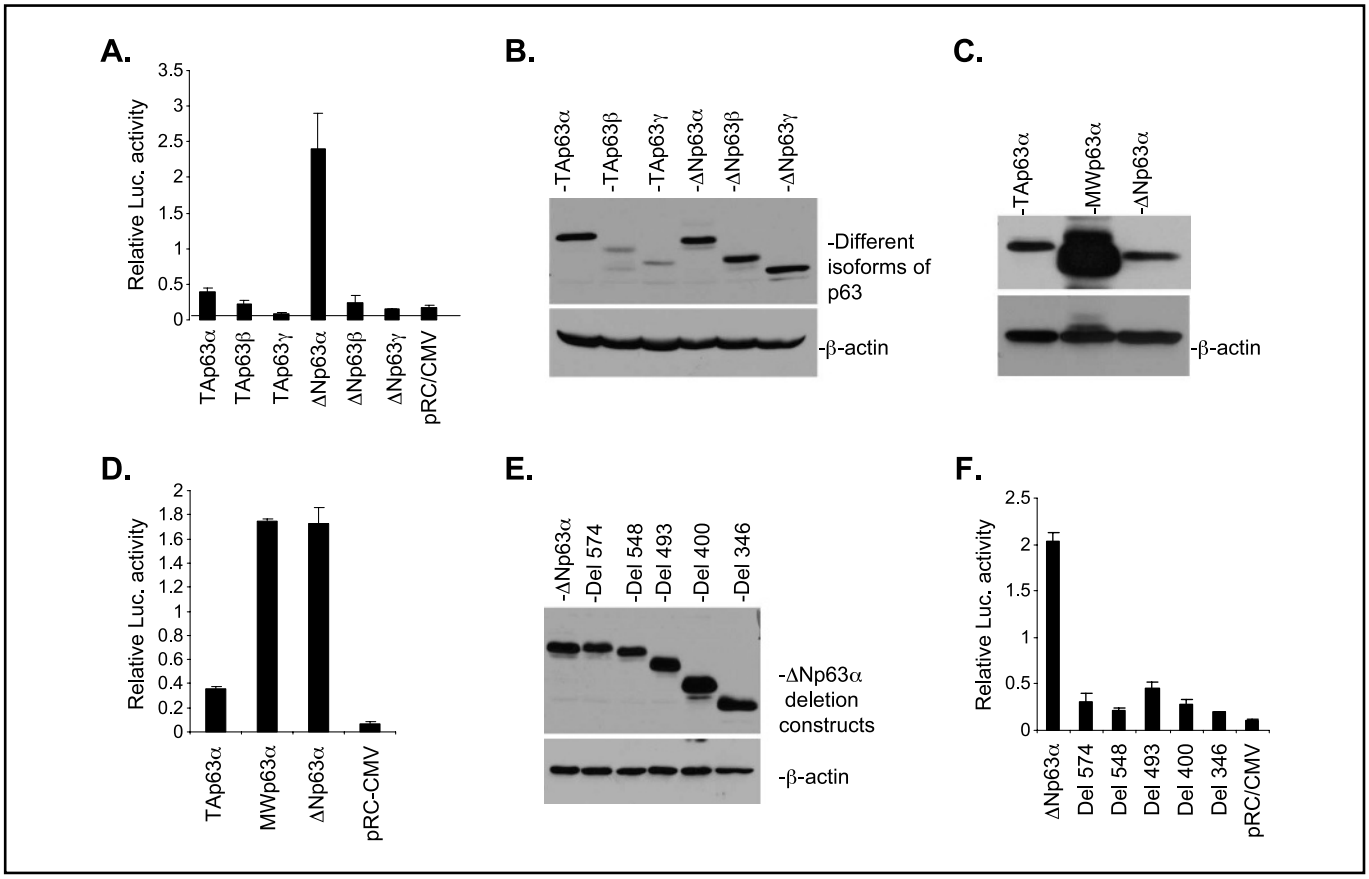

Figure 2. The regulation effect of p63 on *hsp70* gene is structure-related. *A,* six isoforms of *p63* gene differentially regulate the *hsp70* gene promoter. Saos2 cells were transfected with 1 μg each of the indicated plasmid DNA along with 0.5 μg *hsp70* gene promoter. Protein lysates were collected at 24 hours after transfection (all transitory transfection studies were done under the same conditions unless specifically indicated). *B,* Western blot analysis indicating protein stability of different isoforms of p63. β-Actin was used as a protein loading control. *C,* Western blot shows the size and amount of transfected proteins. Saos2 cells were transiently transfected with 1 μg of the indicated plasmids. β-Actin was used as a protein loading control. *D,* luciferase reporter assay showing differential reporter activity among TAp63α, MWp63α, and ΔNp63α. Saos2 cells were transfected with 1 μg each of the indicated plasmid DNA along with 0.5 μg *hsp70* gene promoter. *E,* Western blot showing protein stability of ΔNp63α and the five deletion derivates. One microgram of each indicated expression plasmid DNA was transfected into Saos2 cells. *F,* transactivation potential of ΔNp63α and the five COOH-terminal deletion constructs. Different amounts of expression plasmids for ΔNp63α and its five deletion derivates (up to 1 μg) were cotransfected with HSP70 reporter plasmids in Saos2 cells.

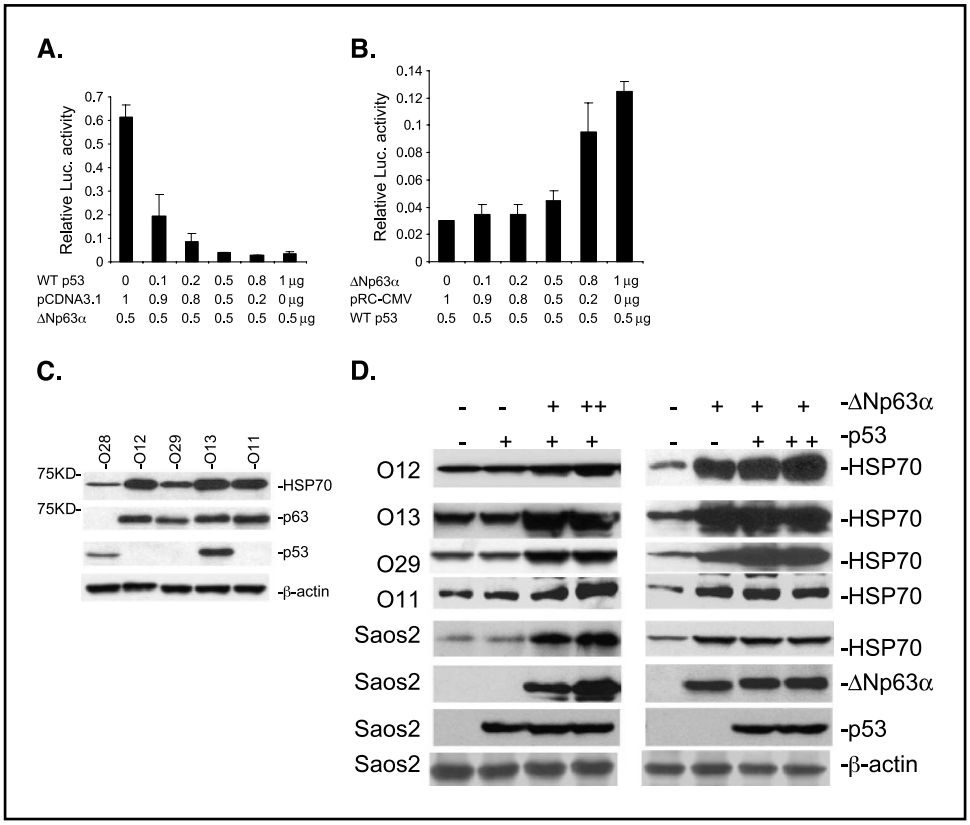

Figure 3. Regulation of ΔNp63α and p53 on the *hsp70* gene *in vitro* and *in vivo*. *A,* WT p53 can suppress the ΔNp63α activation of the *hsp70* gene. Different amounts of WT p53 along with empty vector pcDNA3.1 were cotransfected with ΔNp63α expression constructs into Saos2 cells. *B,* ΔNp63α can reverse the WT p53 suppression of the *hsp70* gene. Different amounts of ΔNp63α along with empty vector pRC/cytomegalovirus were cotransfected with WT p53 expression constructs into Saos2 cells. *C,* Western blot showing endogenous expression of HSP70, p63 and p53 in representative head and neck cancer cell lines. Protein lysates (50 μg) were used and immunoblotted with HSP70, p63 4A4, and p53 antibodies. β-Actin was used as a protein loading control. *D,* Western blot shows the effects of different dosages of p53 and ΔNp63α on the HSP70 expression level. Proteins (20 to 40 μg) were used and immunoblotted with the HSP70 antibody. *Top,* the variable dosages of p53 and ΔNp63α adenovirus are indicated, and control pAdtr adenovirus was used to equalize the amount of the total virus used in all the infections. *Left,* cell lines; *right,* antibodies.

These data indicate that in Saos2 cells (*p53* and *p63* null), ΔNp63α and WT p53 can mutually interfere with each other in their regulation of the *hsp70* gene promoter.

To further address these opposing regulatory functions of p53 and ΔNp63α *in vivo*, we first investigated whether HSP70 expression correlates with p53 and ΔNp63α in five head and neck cancer cell lines (O11, O12, O13, O28, and O29). The different cell lines vary greatly in their expression patterns for p53 and ΔNp63α. Most of the cells have abundantly expressed ΔNp63α and lack expression of *p53*. As a rule, WT p53 is unstable and mutant p53 is stable. Among our tested cells, O13 is ΔNp63α-positive and p53 mutant (positive); the others are ΔNp63α-positive and p53 WT (negative). However, O28 is ΔNp63α-negative and p53-positive (even though it is WT). HSP70 expression was closely correlated with ΔNp63α expression levels but not with the *p53* status in all cell lines tested (Fig. 3*C*). This observation indicates that ΔNp63α is one of the dominant regulators for HSP70 expression in cancer cells. Then variable amounts of adenovirus *p53* and Δ*Np63α* were infected into O11, O12, O13, O29, and Saos2 cells to see their effect on HSP70 expression. We observed up-regulation of HSP70 expression after ΔNp63α infection (Fig. 3*D*). Interestingly, p53 could not repress endogenous and induced HSP70 expression in these cells. To further confirm our observation, we did a detailed time course study on head and neck cancer cell lines and inducible Saos2 cells. The results consistently show that WTp53 has no effect on *hsp70* expression (Supplementary data I). Taken together, our data suggest that it is Δ*Np63α* expression and not *p53* expression which always correlates with *hsp70* gene expression in squamous cell carcinoma of the head and neck.

ΔNp63α Regulate *hsp70* through CBF, NF-Y and CCAAT Binding Box. We have shown that ΔNp63α could transcriptionally activate *hsp70* gene expression *in vitro* and *in vivo*. In order to localize the *cis*-response element of *hsp70* to ΔNp63α, we generated a series of deletion constructs on all *hsp70B*, *hsp70A*, and *hsp70-2* gene promoters. These constructs were then cotransfected with ΔNp63α expression constructs into Saos2 cells and luciferase analyses were done to detect activity. As shown in Fig. 4, the activity of the *hsp70B* gene promoter was proportional to the size of the deletion with the critical region located between −55 to −142 in the *hsp70B* promoter. The same critical regions were delineated on the *hsp70A* and *hsp70-2* promoters (data not shown). By searching the Motif database, we found that these regions contain a CCAAT binding box that was also shown to be essential for p53 repression of *hsp70* (15). These data suggest that ΔNp63α and p53 transcriptionally regulate the *hsp70* gene through the CCAAT binding box located in the *hsp70* promoter. The decrease in activity when additional regions on the *hsp70* promoter are deleted may be due to other factors such as HSF (19).

Several transcriptional factors including HSP-CBF, NF-YA, NF-YB, and NF-YC form a transcriptional complex that binds to a CCAAT box on several gene promoters (20, 21); p53 interaction with HSP-CBF was reported previously (15). We tested the interaction between ΔNp63α and HSP-CBF or NF-Y by cotransfection of the pRC/cytomegalovirus-Δ*Np63α* expression construct and the pMT2-HSP-CBF or NF-YA expression constructs in Saos2 cells. Immunoprecipitation was done with the p63 antibody and the control anti-FLAG antibody, followed by Western blotting with HSP-CBF or NF-YA antibodies. Specific bands were identified with the p63 but not by the FLAG antibody precipitation after Western

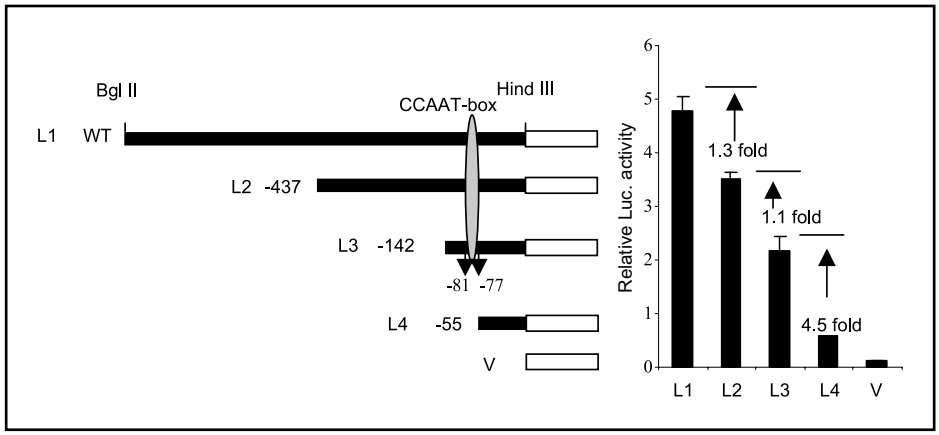

Figure 4. Regulation of ΔNp63α on the *hsp70* is through CCAAT box in *hsp70* promoter. Schematic diagram of a 1.4-kb fragment of the *hsp70B* gene promoter cloned into the pGL3 basic reporter construct. A series of deletion constructs is shown. The position of the most proximal nucleotide from the reporter region relative to ATG is shown for each construct. *Right,* luciferase activity of the *hsp70* gene reporter constructs in Saos2 cells. The differences in relative luciferase activity between different constructs are indicated. −77 to −81 is the location of CCAAT box.

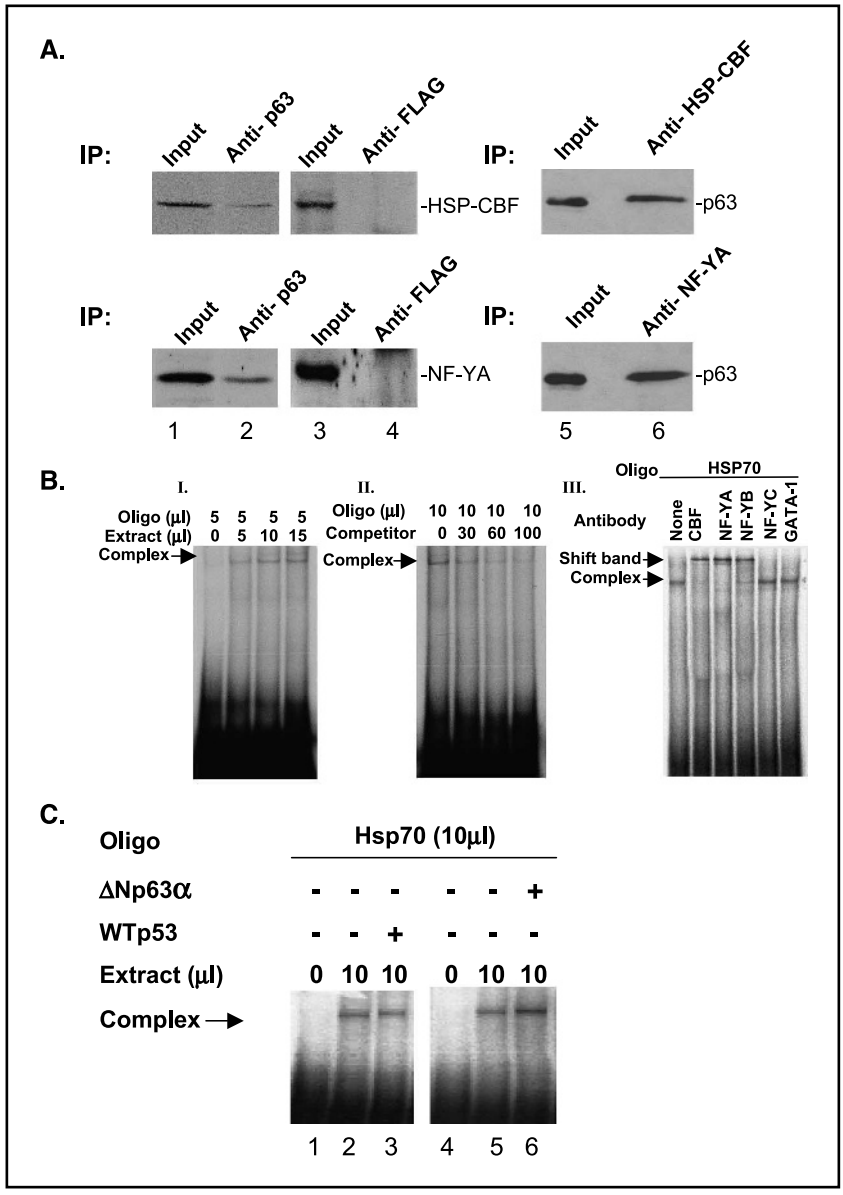

Figure 5. Physical interaction of ΔNp63α with CBF and NF-YA complex. *A,* Saos2 cell lysates were isolated following transient transfection of ΔNp63α with pMT2-CBF, or ΔNp63α with NF-YA (*lanes 1* and *3*) and were immunoprecipitated (*IP*) with p63 4A4 (*lane 2*) or a control Flag antibody (*lane 4*). Immunoblotting was done with either the CBF antibody (*top*) or the NF-YA antibody (*bottom*). Reciprocal IP-WB is presented on right panel. Cell lysates were immunoprecipitated with HSP-CBF and NF-YA antibodies and blotted with anti-*p63* antibody. *Lanes 1, 3,* and *5,* show input cell lysates. *B,* electrophoretic mobility shift assays were done using *hsp70* CCAAT box oligonucleotide probes and Saos2 cell protein extracts. The single protein complex band is indicated (*left*). Protein complex binds specifically to *hsp70* oligonucleotides. A competitive assay was done using increasing amounts of cold unlabeled oligonucleotide probes (*competitors*). Increasing amounts of cold to hot probes (*oligonucleotides*) were added as indicated (*middle*). CBF and NF-Y bind to the *hsp70* CCAAT box. Super-shift assays were done with one of the following antibodies (anti-CBF, -NF-YA, -NF-YB, -NF-YC, or -GATA-1) and the *hsp70* CCAAT box oligonucleotides probes together with Saos2 cell lysates. *Right,* the super-shifted complex containing the anti-CBF, anti-NF-YA, anti-NF-YB, and the gel shift complex. *C,* WT p53 decreases and ΔNp63α enhances the binding of CBF and NF-Y protein complex to the *hsp70* CCAAT oligonucleotides. *Lanes 1-6,* cell lysates from different Saos2 cells. *Lanes 1* and *4* do not contain protein extracts. *Lanes 2* and *5* contain normal Saos2 cell lysates. *Lane 3* contains the cell lysates from Saos2 infected with WT p53. *Lane 6* contains the cell lysates from Saos2 infected with ΔNp63α.

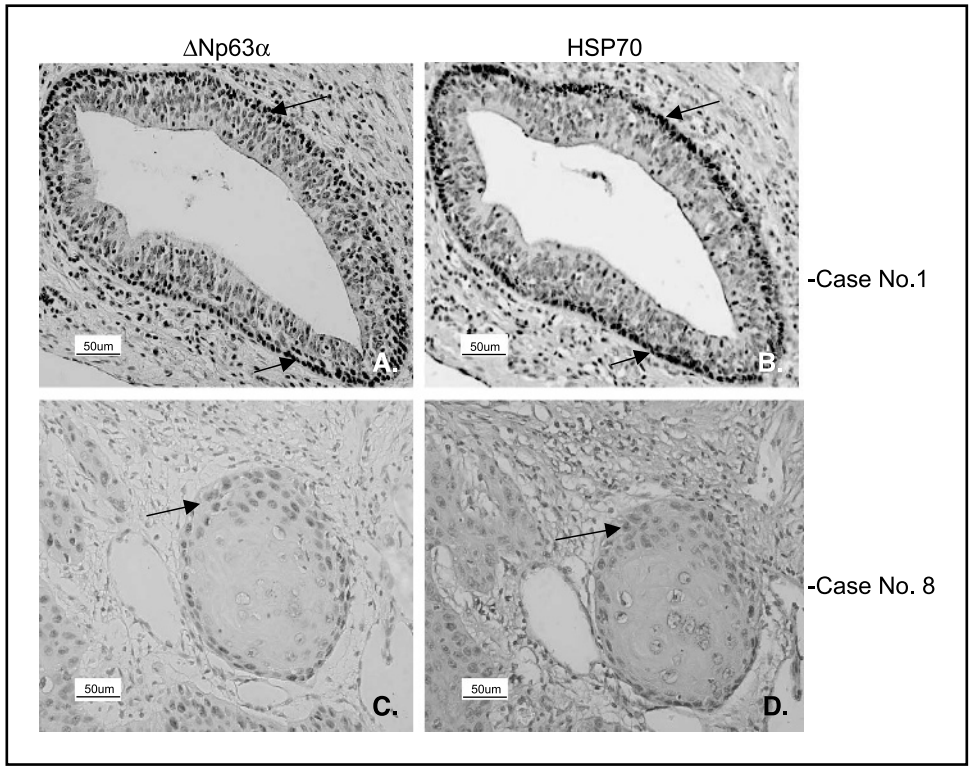

Figure 6. Coexpression of ΔNp63α and HSP70 in primary head and neck cancers. Representative cases of immunohistochemistry analysis. *A* and *C,* ΔNp63α staining; *B* and *D,* HSP70 staining. *A* and *B,* (*top*) case No. 1 with strong staining of both ΔNp63α and HSP70 in a glandular tumor. *Arrows,* tumor cells with strong positive signals. *C* and *D,* (*bottom*) case No. 8 with weak staining of both ΔNp63α and HSP70 in a highly differentiated squamous cell carcinoma. *Arrows,* tumor cells with weak staining signals. Bars, 50 μm.

blotting (Fig. 5*A*). This result was confirmed by immunoprecipitation with the HSP-CBF or NF-YA antibodies, followed by immunoblotting with the p63 antibody (Fig. 5*A*). These data clearly indicate a specific interaction between ΔNp63α and the HSP-CBF and NF-Y transcription factors and support the hypothesis that p53 and ΔNp63α regulate the *hsp70* gene through a common interaction with the HSP-CBF/NF-Y complex.

We next did gel shift analysis to verify the existence of the transcription complex in *hsp70* promoter. As was expected, the transcription factors do form a complex with the *hsp70* promoter CCAAT binding box in Saos2 cell lysates. This binding was specific because the intensity of the band decreased with increasing amounts of unlabeled oligonucleotide (Fig. 5*B*). These complexes were also super-shifted by anti-HSP-CBF, anti-NF-YA and anti-NF-YB antibodies but not by anti-NF-YC or negative control GATA-1 antibodies (Fig. 5*B*). Taken together, these results show that CBF and NF-Y form a complex that binds to the CCAAT box located in the *hsp70* promoter.

We then tested whether p53 and ΔNp63α expression could influence the binding of the CBF and NF-Y complex to the CCAAT box in the *hsp70* promoter. Gel shift results show that ΔNp63α can significantly increase the binding of the protein complex to the

Table 1. *p53* status and expression level of Np63 and HSP70 in head and neck tumors

Cases	ΔNp63α staining*	HSP70 staining*	*p53* status	Site	Histopathology
1	3	3	WT	Tonsil	SCC
2	3	3	WT	Buccal	SCC
3	3	3	WT	FOM	SCC
4	3	2/3	Mutant	Tongue	SCC
5	2	2	N/D	Tongue	SCC
6	2	2	Mutant	Mandible	SCC
7	2	2	WT	Alveolar ridge	SCC
8	1	1	WT	Buccal	SCC
9	1	1	Mutant	Larynx	SCC

Abbrevations: FOM, floor of mouth; SCC, squamous cell carcinoma.
*3 = strong and diffuse in over 50% of tumor cells; 2 = medium in 20% to 50% of tumor cells; 1 = weak, focal. 0 = negative; N/D = not done.

CCAAT box in the *hsp70* gene promoter, whereas the addition of increasing amounts of WT p53 decreases this binding (Fig. 5*C*). These results are in line with the *in vitro* luciferase reporter assay and show that ΔNp63α and *p53* have different effects on *hsp70* gene expression mediated through the CCAAT box in the *hsp70* gene promoter.

Colocalization and Coexpression of ΔNp63α and Hsp70 Protein in Head and Neck Cancers. To further confirm the physiologic interaction between ΔNp63α and hsp70, we next investigated the localization of both ΔNp63α and HSP70 in normal oral epithelium from human head and neck tissue. Both the HSP70 antibody and ΔNp63α antibody showed strong nuclear staining concentrated in the basal cells of the epithelium (data not shown). We then analyzed nine primary head and neck cancers to check for correlation of expression between these two proteins (Fig. 6). In all nine cases, ΔNp63α and HSP70 showed coordinate staining: strong in four cases, medium in three cases, and weak in two cases. In addition, the *p53* status in eight of these head and neck cancer samples was checked by sequence analysis of *p53*. Two cases with *p53* mutations were identified. When we compared the *p53* status and HSP70 expression levels in these eight cases, we found no correlation between *p53* status and HSP70 expression (Table 1). This observation is consistent with our findings in head and neck cancer cell lines (see above). As a follow-up to this study, 32 additional cases of head and neck primary tumors were analyzed for their ΔNp63α and HSP70 expression without *p53* sequencing data (Supplementary data J). The association between ΔNp63α and HSP70 expression in these 41 cases was calculated using the ^{2} test and a significant association was observed ($P < 0.0001$; Supplementary data K). Thus, our study provides strong evidence to support the physiologic association between ΔNp63α and HSP70 in human head and neck cancer.

Discussion

Numerous studies have investigated the transcriptional activity of the different isoforms of the *p63* gene in different systems. These data consistently show that TA isoforms of p63 and p73 transactivate *p53* downstream target genes by binding to *p53* recognition elements in the promoter of the target genes. ΔNp63 or ΔNp73 can act as dominant-negative molecules in blocking binding and thereby inhibiting their transactivation activity (2, 3). In contrast to these observations, our microarray analysis and the current study reveal an unanticipated function of the ΔNp63 protein in up-regulation of the *hsp70* gene. In addition, in comparing the WT p53 and mutant p53 activity on the *hsp70* promoter, we find that mutant p53 acts in the same way as ΔNp63α. These data clearly indicate that, in certain situations, proteins considered as dominant-negative in function also act as functional proteins *in vivo*.

The integration of our data and other previous studies leads to a comprehensive model in which *p53* family members influence transcription of target genes through two different mechanisms: (*a*) binding to *cis* elements directly, or (*b*) interacting with other transcription factors to influence the transcription of downstream targets. It is interesting to point out that the structural domains of the *p63* gene play totally opposite roles in these two different types of mechanisms. The NH_2-terminal TA domain of TAp63 has been widely accepted as a *trans*-activation element in up-regulating some *p53* downstream targets such as *p21*, *JAG2*, *PUMA*, etc. (2, 3, 22), although we show that it contains a repression domain for regulating HSP70. Although several reports suggest that the carboxyl terminal of p63α contains a repression domain for p53 target genes (23), we show that the carboxyl terminal of p63α contains an activation domain for the *hsp70* gene. Our findings and these other studies show that multiple transcription mechanisms might exist in parallel for *p53* and *p63*, thereby influencing different sets of target genes that control cell integrity and normal function.

Down-regulation of *hsp70* by WT p53 is well-documented (15). Interestingly, this report was done *in vitro* by luciferase analysis using the *hsp70* promoter with no *in vivo* data presented. The *in vitro* results of our study (luciferase and gel shift assay) are consistent with this report. In addition, we also show that endogenous expression of *hsp70* correlates with ΔNp63α but not with *p53* status in human head and neck cancer. Moreover, forced expression of WT p53 could not repress endogenous and induced *hsp70* expression levels *in vivo*. This difference between the *in vitro* and *in vivo* systems could be explained by the presence of many various transcription regulators *in vivo* leading to high expression levels of hsp70 in squamous cell carcinoma. These positive regulators might override the repression effort of WT p53 on *hsp70*. Further investigation is needed to uncover this mechanism that will facilitate and expand our understanding of *p53* function *in vivo*.

A lack of *p63* inactivation in human cancers has ruled out a typical tumor suppressor gene role for this *p53* homologue. Furthermore, there are several lines of evidence that strongly support its involvement in initiation and progression of cancer. *p63* gene is located at 3q27-29, a chromosomal region amplified in head and neck squamous cell carcinoma, cervical cancer and non-small cell lung cancer. The amplification of *p63* leads to a significant increase of the ΔN *p63* isoform expression level in different cancers. In addition, ΔNp63 mediates a decrease in the phosphorylation levels of β-catenin, which in turn induces its nuclear accumulation and activates the β-catenin signaling pathway. Thus, the ΔNp63 isoform acts as a positive regulator of the oncogenic β-catenin signaling pathway (24). The present study further adds to the idea that ΔNp63α could act as a functional oncogenic protein and actively up-regulates *hsp70* similarly to mutant p53. HSP70 proteins have been shown to play an important role in tumorigenesis. First, elevated expression levels of HSP70 members were reported in high-grade malignant tumors (13). In breast cancer, elevated expression levels of HSP70 were associated with short-term disease-free survival, metastasis, and poor prognosis. Consistent with these observations, HSP70 induction has been suggested to play a role as an antiapoptotic molecule. In addition, several oncogenes including adenovirus oncogene E1A and c-MYC, have been shown to activate the *hsp70* gene through its promoter (16, 17). The consistent coexpression of C-MYC and HSP70 was detected in varied kinds of tumors including melanoma and cervical carcinoma (25). Colocalization of ΔNp63α and HSP-70 proteins and up-regulation of HSP70 expression by ΔNp63α *in vitro* and *in vivo* further supports the potential oncogenic role of ΔNp63α in cell transformation.

The question remains as to what circumstances lead to changes in ΔNp63α activity and in turn HSP70 activity. When we treated head and neck cancer cell lines cells with damaging agents, ΔNp63α was phosphorylated and degraded, whereas p53 protein was stabilized, resulting in cell cycle arrest (data not shown). It is likely but unproven that strong proliferation signals are likely to induce ΔNp63α accumulation and in turn appropriate survival signals through *hsp70* and other targets. Further studies have

documented that overexpression of HSPs including *hsp90*, *hsp70*, and the small HSP, *hsp27*, is closely correlated with chemotherapeutic resistance (26). Inhibition of *hsp90*, using 17-allylamino, 17-demethoxygeldanamycin, increases the sensitivity of the cancer cells to chemotherapy and has been used in clinical trials (27). Therefore, our discovery of ΔNp63α activation of *hsp70* in cancer cells may provide a new broad therapeutic target for cancer treatment.

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