The known to form a complex with the CCAAT box located in binding factor and NF-Y transcription factors which are the hsp70 transcription. In addition, p63 represses, whereas the COOH terminus activates. Neck Cancer Research, 818 Ross Research Building, 720 Rutland Avenue, Baltimore, Maryland.

### 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 NH2 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. 1A; 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 adenosine 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% CO2.

**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 galactosidase 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...
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, TAp63δ, 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 pAd4R vector and mutant ΔNp63α constructs mutants 518, 534, and 541 were made using the Quick change 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 (Arg273His) 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 BglII and HindIII and cloned into the pG-3 basic luciferase vector (Promega, Madison, WI). Series deletion constructs generated by PCR were subcloned into the pG-3 basic vector with BglII and HindIII 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 pG-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 three times. 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 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. NET buffer [140 mmol NaCl, 20 mmol NaPO4 (pH 7.4), 5 mmol EDTA, 1% NP-40] 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 antiserum 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), p-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, etc atc gag ctc ggt gat tgc ctc aag gaa aac the antisense strand oligonucleotide is, ttg ctc tct cgg caa tca cgg cag tgg atg. 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, Carpenteria, 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% H2O2 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 (18 and C). The change in hsp70 and hsp40 gene expression in the inducible system parallels the different expression levels of ΔNp63α but not ΔTAp63α, 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. 1D). As a control, we did the luciferase assay with both WT and mutant p53 (Arg273His). As expected, WT p53 down-regulated the hsp70B promoter more than 5-fold, whereas mutant p53 activated the hsp70B promoter approximately 2-fold (Fig. 1F), 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 (1F). 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
of all of p63 contained an activation domain because the activation effect was significantly lower than that of TAp63 and the COOH Terminus of p63 was higher than TAp63 Domain.

As seen in Fig. 2A, 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 NH₂-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 NH₂ Terminus of TAp63 Contains a Repression Domain and the COOH Terminus of p63α Contains a Transactivation Domain. As seen in Fig. 2A, the transactivation 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. 2B, TAp63β, and TAp63α 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 MWp63α showed a more than 10-fold increase in protein level (Fig. 2C), 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

**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 (Arg273His) 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 pcDNA3.1 were added to adjust the final quantity to 1 μg. The basal activity of the reporters was set to 1.

Bars, SD.
a 2-fold activation for TAp63x (Fig. 2D). We then decreased the MWp63x plasmid to 0.5 μg and found a similar protein expression level to 1 μg of ΔNp63x. The luciferase assay results showed that 0.5 μg MWp63x had only half the activity on the hsp70 gene promoter compared with 1 μg of MWp63x or ΔNp63x (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, ΔNp63x, and MWp63x indicating that the introduced structural alteration had no impact on subcellular localization (Supplementary data H). Taken together, our data suggest the existence of an NH2-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 ΔNp63x (Supplementary data E). 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. 2E). We then checked the transactivation activity of all these constructs and found that the hsp70B promoter was strongly activated by WT ΔNp63x but not by any of the deletion constructs (Fig. 2F). These results clearly indicate that the minimal activation domain of ΔNp63x 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).

ΔNp63x Expression Correlates with HSP70 Transcription In vitro and In vivo. Because ΔNp63x and WT p53 display opposite transcriptional effects on the hsp70 promoter, we hypothesized that the balance of ΔNp63x and p53 proteins might regulate hsp70 gene expression. We cotransfected ΔNp63x and WT p53 constructs to judge the effects of both proteins on the hsp70 promoter. hsp70 activation by ΔNp63x was decreased with the addition of incremental amounts of WT p53 (Fig. 3A). Repression by WT p53 on the hsp70 gene promoter was diminished in parallel with increasing amounts of ΔNp63x in Saos-2 cells (Fig. 3B).

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**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 TAp63x, MWp63x, and ΔNp63x. 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 ΔNp63x and the five deletion derivates. One microgram of each indicated expression plasmid DNA was transfected into Saos2 cells. F, transactivation potential of ΔNp63x and the five COOH-terminal deletion constructs. Different amounts of expression plasmids for ΔNp63x and its five deletion derivates (up to 1 μg) were cotransfected with HSP70 reporter plasmids in Saos2 cells.
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-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. 3C). This observation indicates that ΔNp63α is one of the dominant regulators for HSP70 expression in cancer cells. Then variable amounts of adenovirus p53 and ΔNp63α were infected into O11, O12, O13, O28, and Saos2 cells to see their effect on HSP70 expression. We observed up-regulation of HSP70 expression after ΔNp63α infection (Fig. 3D). 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 1). Taken together, our data suggest that it is ΔNp63α 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 135 to 172 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-FC 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-ΔNp63α 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 ∆Np63α 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 ∆Np63α with CBF and NF-YA complex. A, Saos2 cell lysates were isolated following transient transfection of ∆Np63α with pMT2-CBF, or ∆Np63α 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 labeled 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 ∆Np63α 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 ∆Np63α.
blotting (Fig. 5A). This result was confirmed by immunoprecipitation with the HSP-CBF or NF-YA antibodies, followed by immunoblotting with the p63 antibody (Fig. 5A). 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. 5B). 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. 5B). 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

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Abbreviations: FOM, floor of mouth; SCC, squamous cell carcinoma.

*p3 = 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. 5C). These results are in line with the in vitro luciferase reporter assay and show that △Np63α and p53 have different effects on hsp70 gene expression mediated through the CCAAT box in the hsp70 gene promoter.

**Colocalization and Coexpression of △Np63α and Hsp70 Protein in Head and Neck Cancers.** To further confirm the physiologic interaction between △Np63α and hsp70, we next investigated the localization of both △Np63α and HSP70 in normal oral epithelium from human head and neck tissue. Both the HSP70 antibody and △Np63α 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, △Np63α 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 △Np63α and HSP70 expression without p53 sequencing data (Supplementary data J). The association between △Np63α and HSP70 expression in these 41 cases was calculated using the χ2 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 △Np63α 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. △Np63α or △Np73 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 △Np63α 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 △Np63α. 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 NH2-terminal TA domain of Tp63 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 △Np63α 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 △N p63 isoform expression level in different cancers. In addition, △Np63 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 △Np63 isoform acts as a positive regulator of the oncogenic β-catenin signaling pathway (24). The present study further adds to the idea that △Np63α 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 adenosine 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 △Np63α and HSP70 proteins and up-regulation of HSP70 expression by △Np63α in vitro and in vivo further supports the potential oncogenic role of △Np63α in cell transformation.

The question remains as to what circumstances lead to changes in △Np63α activity and in turn HSP70 activity. When we treated head and neck cancer cell lines cells with damaging agents, △Np63α 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 △Np63α 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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Hsp70 Up-Regulates the $\Delta$Np63$\alpha$ Gene in Human Cancer

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