p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis

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Summary

We demonstrate that ΔNp63α is an essential survival factor in head and neck squamous cell carcinoma (HNSCC) through its ability to suppress p73-dependent apoptosis. Inhibition of endogenous p63 expression by RNAi induces apoptosis selectively in HNSCC cells that overexpress ΔNp63α. Knockdown of p63 induces the proapoptotic bcl-2 family members Puma and Noxa, and both their induction and subsequent cell death are p53 independent but require transactivating isoforms of p73. Inhibition of p73-dependent transcription by ΔNp63α involves both direct promoter binding and physical interaction with p73. In HNSCC cells lacking endogenous ΔNp63α expression, bcl-2 is instead upregulated and can suppress p73-mediated death. Together, these data define a pathway whereby ΔNp63α promotes survival in squamous epithelial malignancy by repressing a p73-dependent proapoptotic transcriptional program.

Introduction

Apoptosis represents a fundamental roadblock to tumorigenesis. During tumor formation, genome instability, oncogenic stress, hypoxia, and other stresses trigger the apoptotic response (Lowe et al., 2004; Vogelstein and Kinzler, 2004). Inactivation of pathways mediating apoptosis is therefore essential to nascent tumor cells. One common mechanism for disabling apoptosis involves inactivation of *p53*, the prototypical tumor suppressor that is mutated in more than 50% of human cancers (Vogelstein et al., 2000; Vousden, 2000). Indeed, recent data from model organisms has demonstrated directly that loss of p53-mediated apoptosis, rather than other p53-dependent functions, is the critical target of selection during tumor formation (Schmitt et al., 2002).

Like p53, the p53 family member p73 is known to be an important mediator of apoptosis in response to DNA damage, chemotherapy, and other stimuli (Gong et al., 1999; Irwin et al., 2003; Urist et al., 2004; Yuan et al., 1999). Nevertheless, mutation of *p73* is not observed at a significant frequency in human cancers (Melino et al., 2003; Moll and Slade, 2004). Instead, recent evidence suggests that other mechanisms may contribute to functional inactivation of p73 in human tumors. These include promoter methylation, overexpression of inhibitory isoforms of p73 itself, and interaction with a subset of mutant p53 proteins (Bergamaschi et al., 2003; Chim et al., 2002; Gaiddon et al., 2001; Irwin et al., 2003; Zaika et al., 2002). These findings support the notion that inhibition of p73 function may be critical to the pathogenesis of some human tumors.

Head and neck squamous cell carcinomas (HNSCC) represent a group of treatment-refractory malignancies derived from cells within the basal epithelia of the aerodigestive mucosa (Forastiere et al., 2001). A common molecular abnormality observed in these tumors is overexpression of the p53 family member p63. Numerous studies have documented increased p63 expression in up to 80% of primary HNSCC tumors, and its overexpression is also commonly observed in other squamous epithelial malignancies, including lung and esophagus (Hu et al., 2002; Massion et al., 2003; Sniezek et al., 2004; Weber et al., 2002). *p63* maps to chromosome 3q27-28, and human squamous cell carcinomas (SCCs) frequently exhibit genomic amplification at 3q (Bjorkqvist et al., 1998). The relevance of these observations is supported by data showing that increased *p63* mRNA levels correlate with increased *p63* gene copy number in SCCs of the lung and head and neck (Hibi et al., 2000; Tonon et al., 2005). In some cases, overexpression of p63 is likely to involve mechanisms independent of genomic amplification (Redon et al., 2001). In either case, it is apparent that

SIGNIFICANCE

Genomic amplification and/or overexpression of the *p53* family member *p63* is commonly observed in HNSCC, yet the precise contribution of p63 in tumor cells remains uncertain. While some studies have posited that ΔNp63α might inhibit p53 function within cancer cells, this model is challenged by the lack of consistent correlation between *p53* mutation and p63 expression in these tumors. The p73 protein is thought to exert a proapoptotic function, yet unlike *p53*, *p73* is not targeted for mutation in HNSCC. Here, we demonstrate that endogenous ΔNp63α promotes survival of HNSCC cells by repressing p73-dependent apoptosis. These findings provide an explanation for p63 overexpression in HNSCC, and they represent direct evidence for inhibitory interactions between endogenous p63 and p73.

overexpression of p63 is one of the most common molecular abnormalities identified in HNSCC. Nevertheless, the precise contribution of p63 overexpression to HNSCC remains undefined.

The essential function of p63 in the epithelium is evidenced by the phenotype of *p63* null mice, which exhibit profound developmental failure of the epidermis and oral epithelium, as well as abnormalities of limb, prostate, and mammary development (Mills et al., 1999; Yang et al., 1999). Like p53 and p73, p63 is a sequence-specific DNA binding factor that regulates transcription of critical downstream target genes. All three p53 family members possess a highly homologous DNA binding domain, through which they regulate a shared subset of transcriptional targets (Harms et al., 2004). Expression from two distinct *p63* promoters produces protein isoforms that either contain or lack the N-terminal transactivation domain (TAp63 and ΔNp63, respectively). Differential mRNA splicing also gives rise to multiple C-terminal variants (Yang et al., 1998). In both normal epithelia and in HNSCC cells, the predominant p63 isoform expressed is ΔNp63α (Parsa et al., 1999; Yang et al., 1998). While few bona fide transcriptional target genes of p63 have been identified, ΔNp63α is known to function as a transcriptional repressor of endogenous cell cycle inhibitors including $p21^{CIP1}$, implying a contribution by p63 to cellular proliferation (Westfall et al., 2003). Other studies have proposed roles for p63 in cell survival, cellular differentiation, and morphogenesis (King et al., 2003; Mills et al., 1999; Yang et al., 1999).

In addition to its role in normal epithelia, ΔNp63α has been hypothesized to contribute to tumorigenesis based on its ability to inhibit p53-dependent transactivation in vitro following ectopic expression of these proteins (King et al., 2003; Yang et al., 1998). Such observations supported a model whereby overexpression of p63 might inactivate p53, therefore abrogating the requirement for its loss during tumorigenesis. Whether these findings reflect an endogenous function of p63 remains uncertain, since no consistent correlation has been proven between p53 mutation and p63 overexpression in SCCs (Choi et al., 2002; Hibi et al., 2000; Sniezek et al., 2004; Weber et al., 2002). Similarly, although ectopic ΔNp63α expression can block p73-dependent reporter transactivation, and p73 and p63 associate in cotransfection assays (Chan et al., 2004), it remains to be determined whether endogenous p63 exhibits either a physical or functional interaction with p73 in tumor cells. Indeed, it has been proposed that p63 promotes oncogenesis in HNSCC cells by a distinct mechanism involving enhancement of β-catenin-dependent transcription (Patturajan et al., 2002). Thus, the contribution of any potential interaction between p63 and other p53 family members in SCC remains to be defined.

We wished to focus on the role of endogenous p63 in SCC. We find that HNSCC-derived cell lines, like the majority of primary tumors, exhibit overexpression of both ΔNp63α mRNA and protein relative to normal primary epidermal cells, and we demonstrate that this p63 isoform promotes survival of HNSCC cells by virtue of its ability to suppress a p73-dependent pro-apoptotic transcriptional program.

Results

Expression of p63 isoforms in HNSCC cells

Numerous studies have used immunohistochemical analysis to demonstrate overexpression of p63 protein in SCCs of the head and neck, lung, and esophagus. Few studies have examined the expression of multiple p63 isoforms in a quantitative manner. We have used human HNSCC-derived cell lines as a model to explore the function and biochemical mechanisms of p63. These squamous carcinoma lines are derived from primary tumors, are growth factor independent, and have been characterized for the most common genetic abnormalities present in HNSCC (Richtsmeier and Carey, 1987; Scher et al., 1993). We chose two representative cell lines that differ in their p53 status for our analysis. JHU-029 expresses wild-type p53 as demonstrated by functional and mutational analysis (Figure 5A, below, and data not shown). In contrast, JHU-011 expresses only truncated mutant p53 (Hoque et al., 2003), resulting from a splice donor mutation following exon 6 (Figure S1 in the Supplemental Data available with this article online). We first compared the level of p63 expression in these cell lines to that observed in primary human epidermal keratinocytes (HK). As a control, we examined p63 expression in the human osteosarcoma-derived line Saos-2, which is reported not to express p63 (Hibi et al., 2000). By Western analysis, p63 is expressed in JHU-029 and JHU-011 at three to five times the level observed in normal HK (Figure 1A). The size of the predominant p63 band detected corresponds to the ΔNp63α isoform, consistent with prior studies showing ΔNp63α to be the major isoform expressed in both normal and malignant epithelial tissues (Parsa et al., 1999; Sniezek et al., 2004). To extend and quantitate these results, we performed isoform-specific real-time quantitative RT-PCR (QRT-PCR) to detect isoforms containing or lacking the N-terminal transactivation domain (TAp63 and ΔNp63, respectively). As anticipated, ΔNp63 mRNA is overexpressed in HNSCC cells to the same degree as its protein product, ranging in abundance from 3.5 to 8 times that observed in primary HK (Figure 1B). TAp63 mRNA is rare in all cells tested, and in all cases ΔNp63 mRNA is

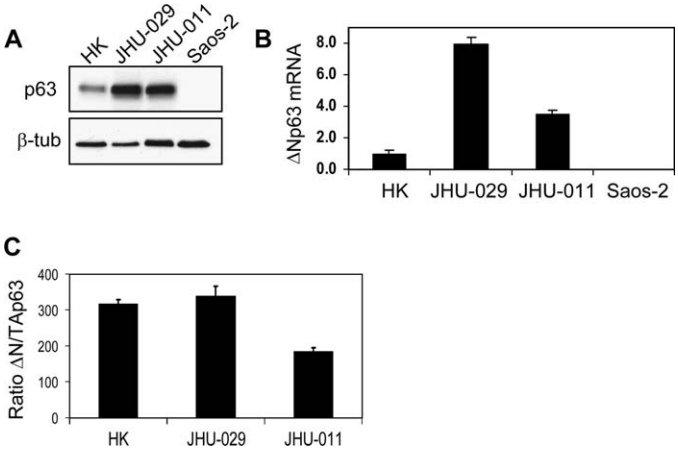

Figure 1. p63 is overexpressed in HNSCC cells

A: Immunoblot for p63 protein in cultured human keratinocytes (HK), two HNSCC-derived cells lines (JHU-029, JHU-011), and the p63-negative line Saos-2. The predominant band detected corresponds to ΔNp63α. β tubulin (β-tub) serves as a loading control.

B: Levels of ΔNp63 mRNA correlate with those of ΔNp63α protein (**A**). Real-time QRT-PCR was used to assay ΔNp63 mRNA levels, which are shown normalized to GAPDH expression. The ΔNp63 mRNA level in HK is arbitrarily designated as 1.0.

C: ΔNp63 is highly expressed relative to TAp63. The ratio of ΔN/TAp63 mRNA was determined by real-time QRT-PCR.

Error bars for **B** and **C** represent the standard deviation of three independent experiments.

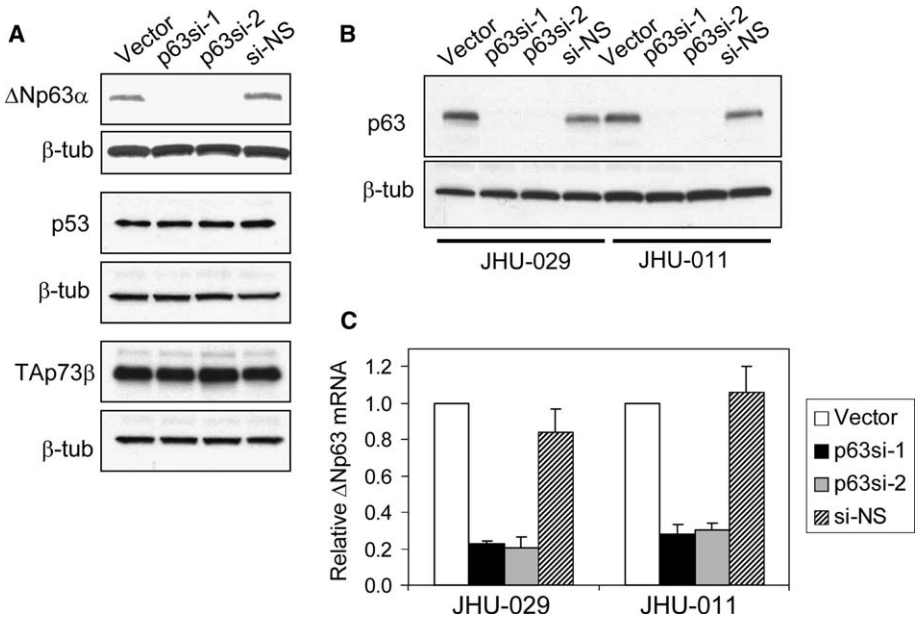

Figure 2. Inhibition of p63 by RNAi

A: p63-directed RNAi does not inhibit p53 or p73 expression. Immunoblot of lysates from Saos-2 cells following cotransfection of the indicated p53 family member with plasmids expressing either of two independent p63-directed shRNA constructs (p63si-1,2), a nonspecific shRNA (si-NS), or the control vector.

B: Inhibition of endogenous p63 expression in HNSCC cells by lentiviral RNAi. Immunoblot of lysates harvested 48 hr following infection with the indicated lentiviral RNAi construct or the control vector. Note that cells are propagated in the absence of drug selection.

C: Quantitation of endogenous p63 inhibition by lentiviral RNAi. Real-time QRT-PCR was used to examine ΔNp63 mRNA levels in samples treated as in **B**. Levels were normalized to GAPDH expression. The level in each vector-treated sample is designated as 1.0. Error bars represent the standard error of three independent experiments.

more than 100-fold more abundant than TAp63 mRNA (Figure 1C). This observation is consistent with our inability to detect TAp63 protein isoforms by Western analysis in either primary HK or HNSCC cells (data not shown). As expected, Saos-2 expresses no detectable p63 mRNA or protein (Figures 1A and 1B). Thus, ΔNp63α is the predominant p63 isoform expressed in HNSCC cells.

RNAi-mediated inhibition of p63

In order to determine the functional role of endogenous p63 in HNSCC cells, we used an RNAi (RNA-mediated interference) approach. We designed multiple constructs expressing p63-targeted small hairpin RNA (shRNA) species from the U6 RNA Pol III promoter (Sui et al., 2002). We first tested the ability of these shRNA species to inhibit expression of ΔNp63α in a cotransfection assay. Two independent shRNA species efficiently inhibited ΔNp63α expression, while the control U6 promoter vector and a nonspecific shRNA species had no effect (Figure 2A). Importantly, none of these shRNA species inhibited expression of p53 or p73 (Figure 2A).

To efficiently inhibit endogenous p63, we created lentiviral vectors expressing these shRNA species. We were able to optimize lentiviral production and infection conditions in order to ensure essentially 100% infection of JHU-029, JHU-011, and Saos-2 cells, as assessed initially by using viruses coexpressing either a puromycin resistance gene or a GFP protein (data not shown). Importantly, this high infection efficiency allowed us to carry out subsequent experiments on lentiviral-infected cell populations in the absence of drug or other selection. Under these conditions, we observed approximately 80% knockdown of endogenous ΔNp63α protein and mRNA in both JHU-029 and JHU-011, as assessed by Western analysis (Figure 2B), and by real-time QRT-PCR (Figure 2C), respectively. Of note, the efficacy of the two p63-directed shRNA species for p63 knockdown is comparable (Figures 2B and 2C). Infection with a nonspecific shRNA species did not affect endogenous p63 protein or mRNA levels (Figures 2B and 2C, respectively).

Induction of apoptosis following p63 inhibition

No significant effects on cell viability were observed at early time points (24–36 hr) following infection with a p63-directed shRNA lentivirus in JHU-029 or JHU-011 cells. However, between 48 and 72 hr a significant fraction of both JHU-029 and JHU-011 cells underwent obvious death and ultimately detached (Figures 3A and 3D). Cell death was accompanied by cleavage of the poly(ADP-ribosylating) enzyme PARP-1, a specific hallmark of apoptotic cell death (Figure 3A). In contrast, infection of either cell line with the lentiviral vector or a nonspecific shRNA lentivirus did not alter p63 expression, induce PARP cleavage, or cause cell death (Figures 3A and 3D). As an additional control for specificity, we infected Saos-2 cells (which do not express p63) with a p63-directed lentiviral shRNA. Neither PARP cleavage nor cell death was observed under these conditions (Figures 3A and 3D).

To quantitate the fraction of apoptotic cells, we stained unfixed cells 96 hr following lentiviral infection with annexin V/propidium iodide (PI) and performed flow cytometry analysis. This assay detects both early apoptotic (annexin V-positive/PI-negative) and late apoptotic (annexin V-positive/PI-positive) cells (Martin et al., 1995). Figure 3B shows representative annexin V/PI profiles, and the data are summarized for all cell lines in Figure 3C. Approximately one-third of JHU-029 and JHU-011 cells underwent apoptosis within 96 hr of infection with the p63-specific shRNA lentivirus (Figures 3B and 3C). Similar results were obtained using either of the two p63-directed shRNA species, supporting the specificity of this effect (Figures 3B and 3C). As above, no increase in cell death was observed following infection of either cell line with the control lentiviral vector or nonspecific lentiviral shRNA. As expected, no death was observed in Saos-2 following infection with any of these vectors (Figure 3C). Thus, specific inhibition of p63 triggers apoptotic cell death in tumor cells in which it is expressed.

As noted above, the most abundant p63 protein present within HNSCC cells is ΔNp63α. Thus, we reasoned that the p63-mediated survival effect that we observe should be attributed primarily to expression of this p63 isoform. To test this

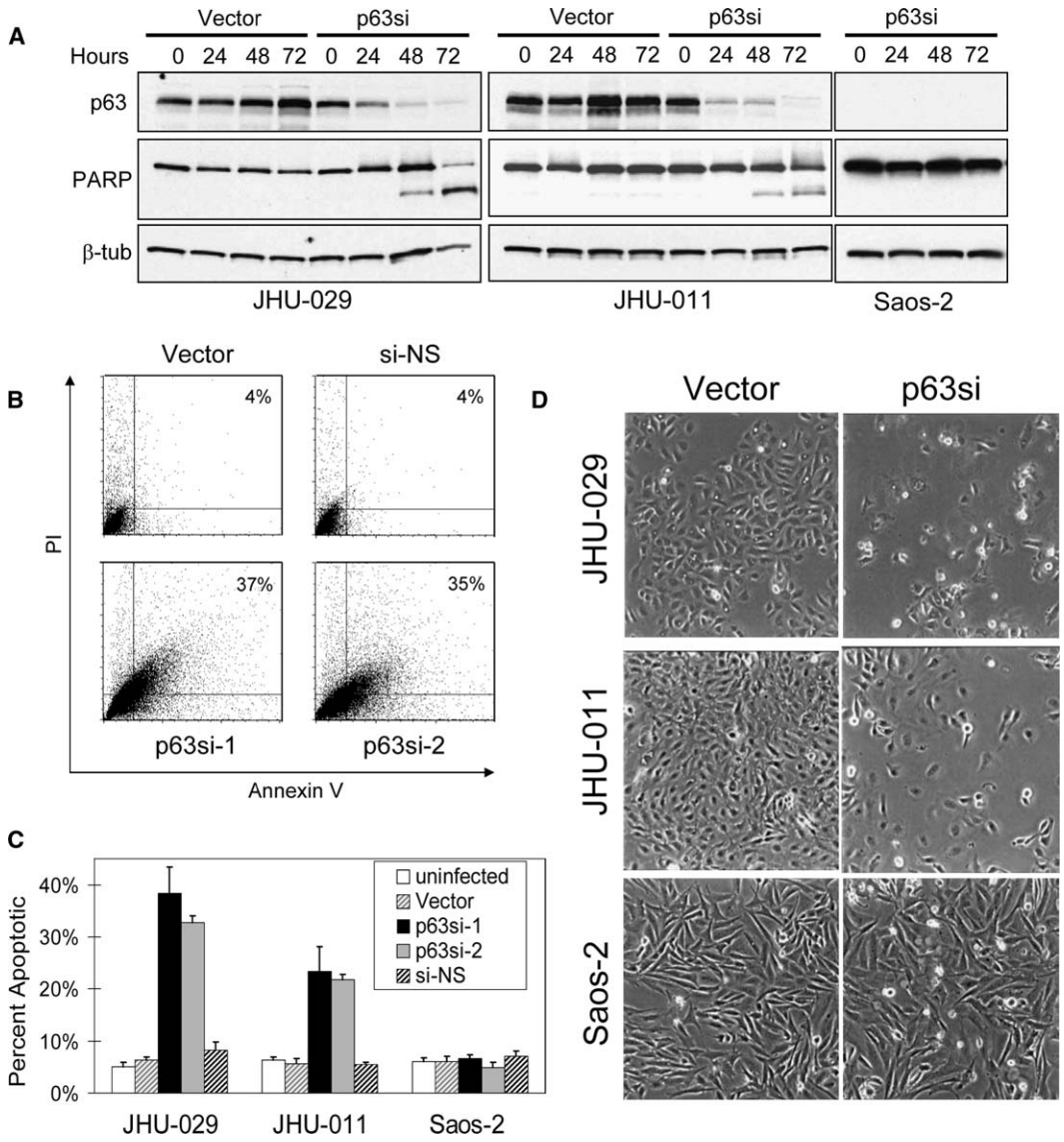

Figure 3. Inhibition of endogenous p63 by RNAi induces apoptosis in HNSCC cells

A: Knockdown of p63 induces PARP cleavage in HNSCC cells. Immunoblots of lysates harvested at the indicated times following infection with a p63 shRNA-expressing lentivirus (p63si) or the control vector. Infection with the p63si lentivirus does not induce PARP cleavage in Saos-2, which does not express p63.

B: Induction of apoptosis in JHU-029 cells following infection with either p63-directed shRNA (p63si-1,2), but not with a nonspecific shRNA (si-NS) or the vector control. Unfixed cells were stained with annexin V and propidium iodide (PI) 96 hr following infection with the indicated lentivirus, then analyzed by flow cytometry. Numbers refer to the percent annexin V- and/or PI-positive cells (UL + UR + LR quadrants) in this representative experiment.

C: Apoptosis in HNSCC cells is specific to p63 RNAi. Quantitation of apoptotic cells (annexin V- and/or PI-positive) treated and analyzed as in **B**. The mean values of three independent experiments for each cell line are shown. Error bars represent one standard deviation.

D: Loss of HNSCC cells following infection with the p63si lentivirus compared with the control vector. Representative fields were photographed 72 hr following lentiviral infection. Saos-2 cells are unaffected by the p63si lentivirus. As above, cells are propagated in the absence of drug selection.

possibility directly, we examined the effect of p63 knockdown in JHU-029 cells expressing a shRNA-resistant ΔNp63α. The amino acid sequence of the human and murine ΔNp63α proteins is highly conserved, but murine ΔNp63α is insensitive to our human p63-directed shRNA by virtue of a nucleotide sequence difference in the targeted region. We therefore used retroviral transduction to establish pools of cells expressing murine ΔNp63α or the control retroviral vector, followed by lentiviral shRNA expression. We found that constitutive ΔNp63α expression substantially blocked PARP cleavage and cell death following knockdown of endogenous p63 (Figures S2A and S2B). Together, these data demonstrate that ΔNp63α functions to promote the survival of HNSCC cells.

Apoptosis following p63 inhibition involves induction of Puma and Noxa

In normal epithelia, ΔNp63α is known to function as a transcriptional repressor of cell cycle regulatory genes that are positively regulated by p53 (Westfall et al., 2003). In addition, ΔNp63α has been implicated based on cotransfection studies as a repressor of p73-dependent transcription (Chan et al., 2004; Yang et al., 1998). Therefore, we hypothesized that in tumor cells p63 might

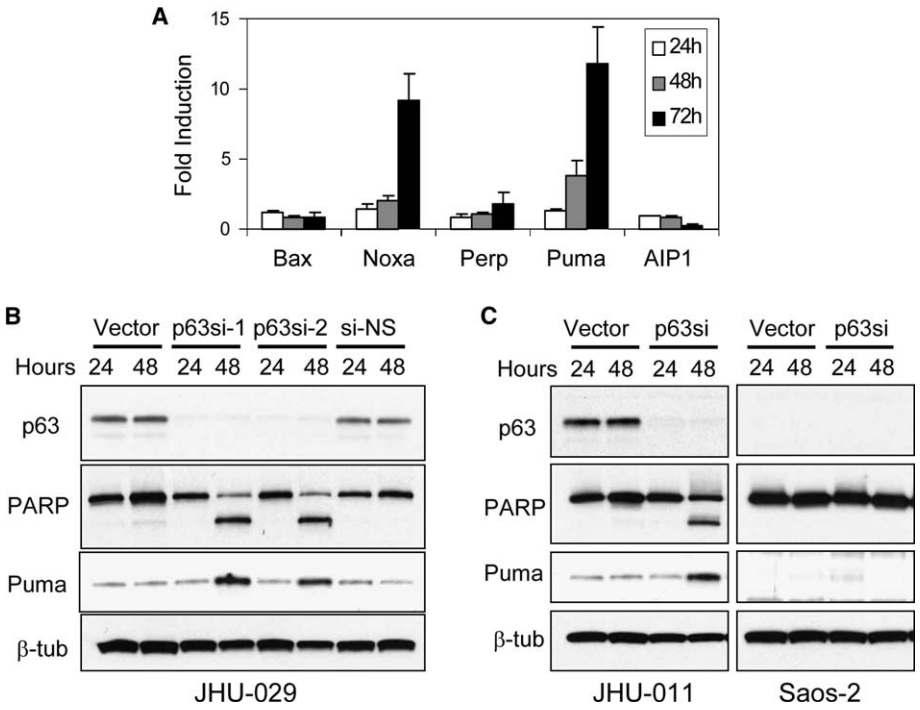

Figure 4. Inhibition of endogenous p63 in HNSCC cells induces Puma and Noxa

A: Induction of *Puma* and *Noxa* mRNA following p63 RNAi. Real-time QRT-PCR for candidate proapoptotic genes was performed at the indicated times following infection with a p63 shRNA-expressing lentivirus. Values shown are relative to infection with the control lentiviral vector and normalized to GAPDH expression. Error bars represent the standard error of three independent experiments.

B: Induction of Puma protein correlates with PARP cleavage and is specific to p63 inhibition. Immunoblot of lysates from JHU-029 cells infected with lentivirus expressing either of two independent p63-directed shRNA constructs (p63si-1,2), a nonspecific shRNA (si-NS), or the control vector, harvested at the indicated times.

C: Induction of Puma protein following p63 inhibition correlates with PARP cleavage in JHU-011. Immunoblot of lysates from cells treated as in **B**. Saos-2 cells exhibit neither PARP cleavage nor Puma induction following lentiviral infection.

promote survival through either direct or indirect repression of proapoptotic genes regulated by p53 or p73. We initially assayed expression of the proapoptotic genes *Bax*, *Noxa*, *Perp*, *Puma*, and *AIP1* in JHU-029 by QRT-PCR following p63 RNAi. Consistently, both *Puma* and *Noxa* but not other proapoptotic genes were induced by p63-directed RNAi (Figure 4A). Induction of Puma mRNA and protein were detectable in both JHU-029 and JHU-011 cells within 48 hr, which is the earliest time at which we observe PARP cleavage (Figures 4B and 4C). Noxa induction occurs somewhat later, as the mRNA and protein were both detectable within 72 hr (Figures 4A and 5B, respectively). No induction of Puma or Noxa was observed following infection with the lentiviral vector or the nonspecific shRNA. In addition, neither gene was induced in Saos-2 following infection with either the control or the p63 shRNA lentivirus (Figures 4B and 4C and data not shown). Thus, specific inhibition of p63 in HNSCC cells leads to induction of proapoptotic effector genes, PARP cleavage, and cell death. These findings imply that p63 expression is essential for survival of HNSCC cells due to its suppression of a proapoptotic transcriptional program.

Cell death and Puma/Noxa induction following p63 inhibition require p73 but not p53 function

Both p53 and p73 have been identified as regulators of *Puma* and *Noxa* transcription (Melino et al., 2004; Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). Therefore, we wished to determine whether p63-mediated repression of these genes and cell death involved a functional interaction with endogenous p53 or p73. Both JHU-029 and JHU-011 exhibit induction of Puma, Noxa, and cell death following p63 knockdown. Since JHU-011 is effectively p53 null (lacking expression of either wild-type or stabilized mutant p53), it appeared that p63 represses the apoptotic program through a mechanism other than inhibition of p53 function. To address this issue in an isogenic setting, we blocked p53 function in JHU-029 cells and then tested the effect of p63 knockdown. JHU-029 cells were infected with a retrovirus encoding a C-terminal truncated p53 fragment (p53DD) that is well characterized as a potent dominant inhibitor of p53 function (Bowman et al., 1996). Importantly, this protein, like wild-type p53, does not physically interact with endogenous p63 or p73 in HNSCC cells (data not shown). To demonstrate p53 inactivation by p53DD in JHU-029, we first examined expression of the p53-regulated gene $p21^{CIP1}$ following DNA damage by doxorubicin treatment, which is known to elicit p53-dependent transcription (Lowe et al., 1993). We found that $p21^{CIP1}$ was induced in control JHU-029 cells following doxorubicin treatment, but not in JHU-029 cells expressing p53DD (Figure 5A). Next, we infected these cells with p63-directed or control lentiviral shRNA. We observed no effect of p53DD expression on the p63-dependent induction of Puma or Noxa (Figure 5B). In addition, p53DD expression had no effect on cell death following p63 knockdown, as assessed by annexin V/PI staining (Figure 5E). Together, these data suggest that p53 does not contribute to the apoptotic program induced following loss of p63.

In some cellular contexts, p73 promotes apoptosis by activating a subset of p53-regulated proapoptotic genes (Melino et al., 2004). Therefore, we asked whether $\Delta Np63\alpha$ might promote survival through inhibition of p73 function. We first tested the ability of p73 to regulate the expression of Puma and Noxa in JHU-029 cells. We found that retroviral expression of p73 induced both Puma and Noxa relative to control vector-infected cells, and that both genes were further induced following treatment of cells with lentiviral p63-directed shRNA (Figure 5C). Thus, overexpression of p73 in HNSCC cells opposes the specific repressive effect of p63, and simultaneous p73 overexpression and p63 knockdown leads to the highest induction of proapoptotic genes.

We next examined expression of endogenous p73 in JHU-029. Using isoform-specific QRT-PCR, we detected expression

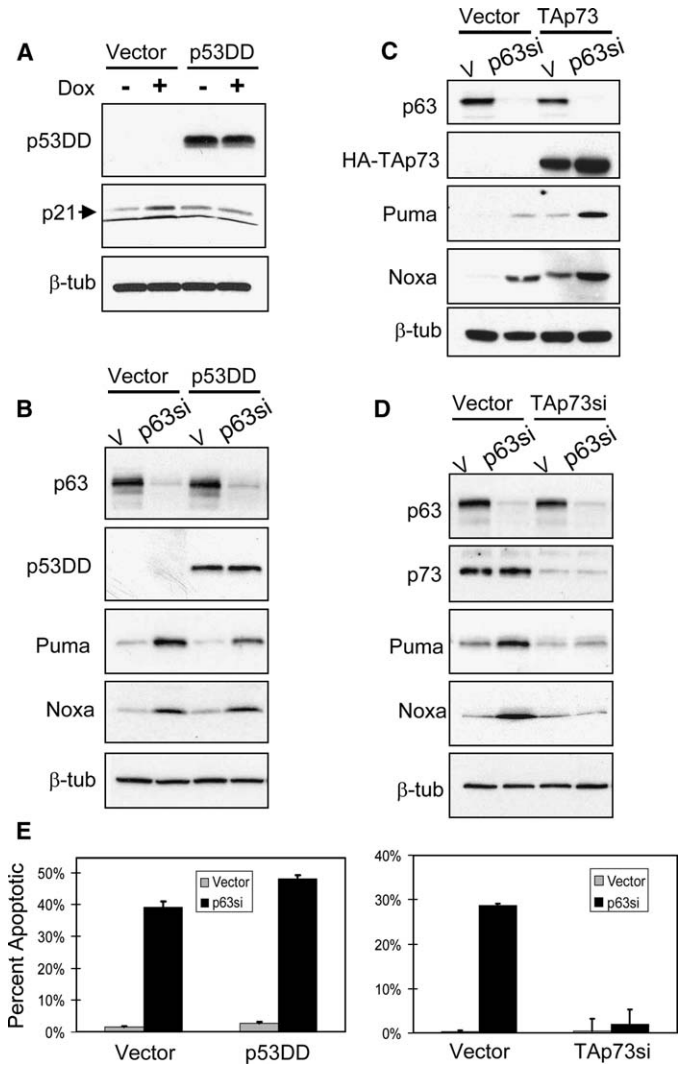

Figure 5. Puma and Noxa induction and cell death following p63 inhibition are p53 independent but require transactivating p73 isoforms

A: Inhibition of p53 function by the dominant-negative p53DD. Immunoblot of lysates from JHU-029 expressing either p53DD or the control retroviral vector, 6 hr following treatment with doxorubicin (Dox; 0.75 μM). Induction of $p21^{CIP1}$ is inhibited by p53DD.

B: Puma and Noxa induction following p63 RNAi are not inhibited by p53DD expression. JHU-029 cells expressing the control vector or p53DD were harvested 72 hr following treatment with a p63 shRNA-expressing lentivirus (p63si) or the control lentiviral vector.

C: TAp73 induces Puma and Noxa in a p63-dependent manner in HNSCC cells. JHU-029 cells expressing retroviral TAp73 or the control vector underwent lentiviral infection as in **B** and were harvested at 72 hr for immunoblot.

D: Puma and Noxa induction following p63 RNAi require endogenous TAp73. Stable pools of JHU-029 expressing a TAp73-directed shRNA (TAp73si) or the control vector were infected with a p63 shRNA-expressing lentivirus or control vector and were harvested at 72 hr for immunoblot. Note that endogenous p73 levels are unchanged following p63 RNAi.

E: Cell death following p63 RNAi is p53 independent but requires endogenous TAp73. Quantitation of annexin V- and/or PI-positive JHU-029 cells treated as in **B** (left graph) and **D** (right graph), harvested 96 hr following p63 shRNA lentiviral (p63si) or control vector infection. Error bars show standard deviation for three independent experiments.

only of TAp73 isoforms and not ΔNp73 isoforms (data not shown). Western analysis demonstrates that the major detectable p73 species corresponds in its migration to TAp73β (Figure 5D). To test directly whether endogenous p73 is required for cell death following loss of p63, we assayed the effect of p63 knockdown in cells in which p73 expression was ablated by RNAi. We first generated lentiviral shRNA species directed against the p73 N-terminal transactivation domain and infected JHU-029 with this virus followed by brief drug selection. This selected cell pool exhibited at least 75% knockdown of p73 compared to control vector-infected cells (Figure 5D). Then we expressed the p63-directed shRNA in these cells. Remarkably, p73 knockdown substantially and consistently abrogated the effects of p63 inhibition. Thus, in the absence of p73 we observed little or no Puma induction, Noxa induction, or cell death as assessed by annexin V/PI staining (Figures 5D and 5E). Therefore, TAp73 is required for the apoptotic program elicited following loss of p63. Together, these data suggest that p63 suppresses a proapoptotic function of p73 in squamous carcinoma cells.

TAp73β is complexed to ΔNp63α in HNSCC cells

Several possible models could explain functional inhibition of p73 by p63. We first examined whether p63 knockdown increased expression of either the p73 mRNA or protein. No change in the *p73* mRNA was detected by QRT-PCR, and no change in the p73 protein was evident following p63 knockdown in either JHU-029 or JHU-011 (Figure 5D and data not shown). The p63 and p73 proteins contain a highly homologous (>60% identical) oligomerization domain, and they are reported to interact physically when coexpressed (Chan et al., 2004; Davison et al., 1999). Therefore, we tested the ability of ectopically expressed ΔNp63α and TAp73β to interact by coimmunoprecipitation using antibodies directed against either protein. We transfected ΔNp63α, TAp73β, or both proteins into U2OS osteosarcoma cells, which express little if any endogenous p63 and p73. These two proteins bound one another quantitatively when coexpressed in U2OS, even under stringent detergent conditions (Figure 6A). These experiments also demonstrate that the p63 and p73 antibodies used for these studies do not cross-react (Figure 6A).

Next, we tested whether the endogenous ΔNp63α and TAp73β proteins could be coimmunoprecipitated in JHU-029 cells. Endogenous TAp73β protein was readily detectable in lysates following p63 IP, and endogenous ΔNp63α was coimmunoprecipitated using p73-specific antisera (Figure 6B). Thus, endogenous ΔNp63α and TAp73β physically interact in HNSCC cells. Similar results were obtained using either whole-cell lysates (Figure 6B) or nuclear lysates (data not shown), in keeping with the predominantly nuclear localization of both proteins.

We observed that the amount of coimmunoprecipitated ΔNp63α following IP for p73 represented only a small fraction of endogenous ΔNp63α, whereas a much larger fraction of endogenous TAp73β was brought down by p63 IP. (Compare the ratios of input to immunoprecipitated proteins in Figure 6B.) These findings are consistent with the large molar excess of ΔNp63α within the cell relative to TAp73β. Furthermore, they suggested that a high fraction of cellular TAp73β is bound to ΔNp63α. To address this issue directly, we immunodepleted lysates from JHU-029 cells for ΔNp63α, then examined the fraction of ΔNp63α versus TAp73β remaining in these lysates. We achieved approximately 90% immunodepletion of endogenous ΔNp63α (Figure 6C). Remarkably, the same lysate showed approximately 90% depletion for TAp73β as compared to the control (mock immunodepleted) lysate. These data suggest

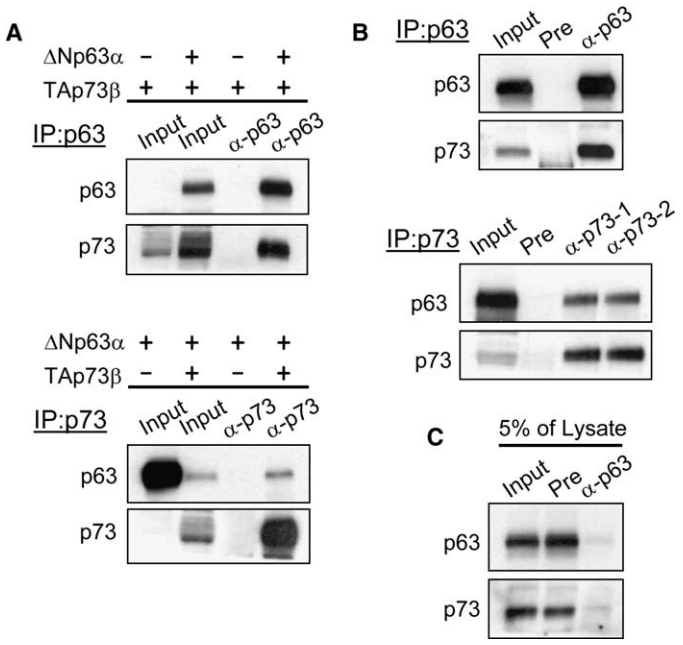

Figure 6. P73 binds p63 in HNSCC cells

A: p63 and p73 coimmunoprecipitate (co-IP) when coexpressed. Immunoblots of U2OS cells transfected with plasmids encoding ΔNp63α or TAp73β as shown, followed by IP using antibodies against the indicated proteins. Ten percent of each lysate prior to IP (Input) is also shown. Note the absence of antibody crossreactivity.

B: Endogenous p63 and p73 co-IP in HNSCC cells. Lysates from JHU-029 were immunoprecipitated using preimmune sera (Pre) or antibodies against the indicated proteins.

C: The vast majority of endogenous p73 in bound to p63. Lysates from JHU-029 were immunodepleted with preimmune sera (Pre) or α-p63 antisera, followed by immunoblot.

that the vast majority of TAp73β within the cell is complexed with ΔNp63α.

ΔNp63α inhibits p73-dependent transcription through direct promoter binding

Together, our findings argue that TAp73β promotes apoptosis following knockdown of ΔNp63α through activation of effector genes including *Puma*. This model implies that TAp73β is a direct transcriptional regulator of *Puma* and that p73-dependent activation of *Puma* is inhibited in the presence of ΔNp63α. To test these two predictions, we examined p73-mediated regulation of the *Puma* promoter. The human *Puma* promoter contains two putative p53 family binding sites within a 500 bp region upstream of the major transcription start site; however, only one of these sites is conserved in mouse (Yu et al., 2001). We used the wild-type *Puma* promoter reporter (Yu et al., 2001), and in addition created a mutant reporter in which the critical consensus residues within the evolutionarily conserved p53 family binding site were changed (Figure 7A). As expected, transfection of p53 significantly induced expression of the wild-type reporter, while minimal induction of the mutant promoter was observed (Figure 7B). Of note, expression of TAp73β, the major p73 protein expressed in JHU-029 cells, induced the reporter even more strongly than p53. Induction by TAp73β requires the p53 family binding motif, as minimal induction of the mutant reporter was observed (Figure 7B). These findings suggest that TAp73β, like p53, is a potent and direct regulator of *Puma* transcription. We then tested the ability of ΔNp63α coexpression to inhibit p73-mediated *Puma* promoter regulation. Coexpression of ΔNp63α with TAp73β blocked p73-mediated transcription in a dose-dependent manner (Figure 7C). Taken together, these data imply that high-level ΔNp63α in squamous carcinoma cells binds p73 to inhibit its transactivation of proapoptotic genes and subsequent cell death.

Two possible models could explain inhibition of p73-dependent transcription resulting from binding of ΔNp63α. ΔNp63α could inhibit p73-dependent transactivation by an "off-promoter" mechanism involving exclusively sequestration of p73 protein. Alternatively, ΔNp63α-containing complexes might be localized to the *Puma* promoter, suggesting that ΔNp63α also functions as a direct repressor of *Puma* transcription. To distinguish between these possibilities, we performed chromatin immunoprecipitation (ChIP) for p63 in JHU-029 cells. We observed strong enrichment of *Puma* promoter sequences relative to a control genomic locus following ChIP for p63 (Figure 7D). In contrast, we do not detect *Puma* promoter sequences following IP using preimmune sera. The findings argue that ΔNp63α functions to repress *Puma* transcription through both its physical interaction with p73 and its direct binding to the *Puma* promoter.

Under normal growth conditions, we detect little if any enrichment for *Puma* promoter sequences following ChIP using p73-specific antibodies in JHU-029 cells, suggesting that most complexes localized to this promoter are ΔNp63α homo-tetramers. Our model predicts, however, that p73 should be recruited to the *Puma* promoter following knockdown of p63. To test this possibility, we performed ChIP using JHU-029 cells shown in Figure 5C that express p73 and that induce *Puma* in a p63-dependent manner. We find limited p73 on the *Puma* promoter in the presence of p63, but we observe a significant increase in p73 localization following p63 knockdown using either of two p63-directed shRNA species (Figure 7E). These findings are consistent with the large increase in Puma expression that we observe following p63 knockdown in these cells (Figure 5C). All together, these data strongly support our model that in HNSCC cells p63 suppresses p73, which is recruited to the *Puma* promoter in the absence of p63 to activate an apoptotic program.

Bcl-2 expression abrogates the requirement for ΔNp63α overexpression in HNSCC cells

While the majority of HNSCC tumors and cell lines express p63 at high levels, a minority exhibit little or no p63 expression (Hu et al., 2002; Sniezek et al., 2004; Weber et al., 2002). Since our data demonstrate that HNSCC cells undergo apoptosis in the absence of a p63-mediated survival signal, it seemed that cells with little or no p63 must somehow circumvent the requirement for p63 expression. To investigate this possibility, we first assayed p63 expression in a well-characterized panel of HNSCC tumor-derived cell lines (Richtsmeier and Carey, 1987; Scher et al., 1993). Of seven HNSCC cell lines initially tested, five showed high-level ΔNp63α protein expression (at levels comparable to those in JHU-029 and JHU-011), while two showed little or no ΔNp63α expression (Figure 8A). We then tested p73 expression in all these cell lines, reasoning that the absence of p73 might explain cell survival in the absence of p63 expression. Surprisingly, all these cell lines express TAp73β at nearly the same level as in JHU-029 (data not shown). Since we had

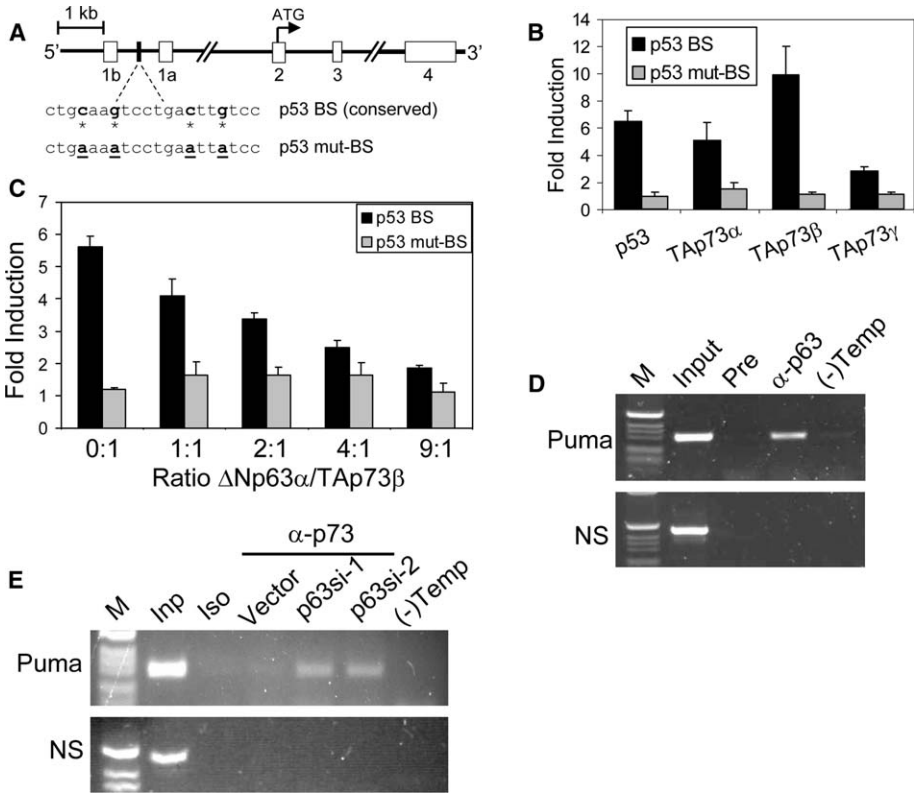

Figure 7. ΔNp63α blocks *Puma* transcriptional regulation by TAp73β

A: Schematic representation of the human *Puma* promoter. The conserved mammalian p53 consensus binding site (p53 BS; black box) is located between alternate exons 1a and 1b (white boxes). Critical residues for p53 family binding (asterisks) were changed (underlined) to create a mutant site (p53 mut-BS).

B: Potent transactivation of the *Puma* reporter by TAp73β requires the p53 binding site. A 200 bp fragment containing the *Puma* p53 BS was used to demonstrate luciferase reporter activation following cotransfection with the indicated expression plasmids in Saos-2 cells. Values shown are relative to the control vector and are normalized for transfection efficiency. Error bars show the standard error for three independent experiments.

C: ΔNp63α blocks *Puma* transactivation by TAp73β. *Puma* luciferase reporter activation following cotransfection with ΔNp63α and TAp73β at the indicated molar ratios is shown, normalized as in **B**. The amount of transfected TAp73β is the same in each case.

D: p63 is localized to the *Puma* promoter in HNSCC cells. PCR of the *Puma* p53 BS region (*Puma*) following ChIP of crosslinked JHU-029 lysates, using preimmune sera (Pre) or α-p63 sera. PCR for a nonspecific genomic locus (NS) is shown as an additional specificity control.

E: P73 is recruited to the *Puma* promoter following knockdown of p63. JHU-029 cells expressing TAp73 were infected with the lentiviral vector or with p63 shRNA-expressing lentivirus (p63si), then were subjected to ChIP 48 hr later using anti-p73 antibody (α-p73), followed by PCR as in **D**. ChIP using isotype-matched antibody (Iso), and PCR for a nonspecific genomic locus (NS) serve as negative controls, while PCR of input total genomic DNA (Inp) controls for PCR efficiency.

identified proapoptotic bcl-2 family proteins as critical mediators of death following loss of p63, we hypothesized that overexpression of bcl-2 itself might provide a compensatory survival signal. In agreement with this possibility, we found that both of the cell lines lacking ΔNp63α expression exhibited high levels of bcl-2, while those expressing ΔNp63α showed low or no bcl-2 expression (Figure 8A). To extend these results, we obtained a second panel of tumor-derived HNSCC cell lines (Heo et al., 1989). As in our initial panel, a striking inverse correlation between ΔNp63α and bcl-2 levels was observed. Cells that showed high levels of ΔNp63α did not express bcl-2, while those with little or no ΔNp63α expression had robust bcl-2 levels (Figure 8B). These findings imply that upregulation of endogenous bcl-2 may be a mechanism to subvert the requirement for p63 expression in HNSCC cells.

To determine directly whether bcl-2 expression conveyed survival on HNSCC cells lacking ΔNp63α, we tested the effect of constitutive bcl-2 expression in JHU-029 cells. We first generated stable pools of JHU-029 expressing bcl-2 via retroviral infection followed by brief drug selection. We then examined the effect of p63 knockdown in these cells compared to control retroviral vector-expressing cells. We observed a dramatic rescue of cell death following p63 knockdown in cells expressing bcl-2 versus the control vector, as evidenced by diminished PARP cleavage (Figure 8C) and annexin V/PI staining (Figure 8D). Consistent with our model, the p73-dependent transcriptional program is still intact following bcl-2 expression, as both Puma and Noxa proteins were still induced following p63 loss in the presence of bcl-2 (Figure 8C). Together, these data are consistent with a role for ΔNp63α in mediating a survival effect that contributes to the pathogenesis of HNSCC, and that can be superseded by high-level bcl-2 expression in the minority of HNSCC cases that lack p63 expression.

Discussion

We find that ΔNp63α is significantly overexpressed in human HNSCC cells relative to normal epidermal cells, consistent with the large number of pathologic studies demonstrating high p63 expression in HNSCC. While p63 is essential for normal epithelial development in both mice and humans, precisely how p63 might contribute to the pathogenesis of HNSCC remains uncertain. Here, we show that endogenous ΔNp63α is required for the survival of tumor cells by virtue of its ability to suppress p73-dependent apoptosis. Ablating p63 expression by RNAi triggers induction of Puma, Noxa, and apoptotic cell death. While these events are p53 independent, they all require transactivating isoforms of p73. ΔNp63α could conceivably inhibit the activity of p73 by a variety of mechanisms, given the amino acid sequence homology within both the DNA binding and

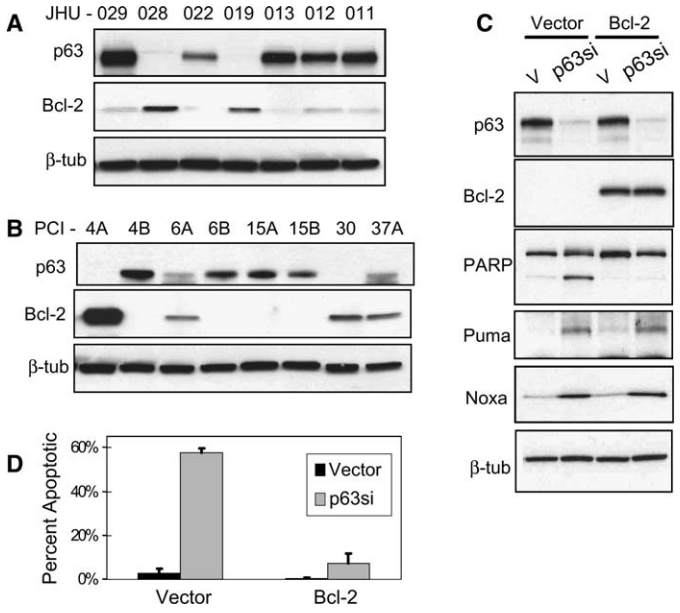

Figure 8. Bcl-2 upregulation abrogates the requirement for p63 expression in HNSCC

A: Bcl-2 expression correlates inversely with p63 expression in HNSCC cells. Immunoblots were performed using lysates from the indicated HNSCC tumor-derived cell lines.

B: A second panel of HNSCC tumor-derived cell lines supports the pattern observed in **A**.

C: Bcl-2 expression blocks PARP cleavage but not Puma and Noxa induction following p63 inhibition. JHU-029 cells expressing retroviral bcl-2 or the control vector were harvested 72 hr following treatment with a p63 shRNA-expressing lentivirus (p63si) or the control lentiviral vector.

D: Bcl-2 expression blocks apoptosis following p63 inhibition. Quantitation of annexin V- and/or PI-positive JHU-029 cells treated as in **C**, harvested 96 hr following p63 shRNA lentiviral (p63si) or control vector infection. Error bars show standard deviation for three independent experiments.

oligomerization domains of these two p53 family members. We find that virtually all endogenous p73 is complexed to endogenous ΔNp63α within HNSCC cells. In addition, p63 itself binds the Puma promoter, and while we detect little if any p73 on the Puma promoter in the presence of p63, p73 relocalizes to this promoter following p63 knockdown. Relocalization of p73, *Puma* induction, and PARP cleavage all occur within 48 hr of lentiviral infection. This time interval presumably reflects the time required for ΔNp63α degradation, for release of p73, and finally for assembly of an active p73-containing transcriptional complex at the *Puma* promoter. Together, our findings support the view that endogenous ΔNp63α suppresses the proapoptotic activity of p73 both through its direct association with p73, and through direct repression of p73-dependent transcription.

Early studies involving ectopic overexpression of ΔNp63α suggested that it might serve to inhibit apoptosis mediated by both p53 and p73 (Yang et al., 1998). Therefore, it was initially speculated that increased ΔNp63α expression might obviate the need for p53 mutation in HNSCC. However, reports have varied as to whether p53 mutation and p63 overexpression are inversely correlated in HNSCC (Choi et al., 2002; Hibi et al., 2000; Sniezek et al., 2004; Weber et al., 2002). Our finding that ΔNp63α mediates a survival effect that is independent of p53 may provide an explanation for the lack of a consistent correlation. Our data might also explain in part the finding that mutations in either p73 or *PUMA* are very uncommon in HNSCC cells (Hoque et al., 2003; Weber et al., 2002). We find that p73 is inactivated by ΔNp63α overexpression in the majority of such cancers, and its downstream effectors are neutralized by overexpression of bcl-2 in a significant minority.

Our findings also shed light on previous studies demonstrating that p73 is a critical mediator of cell death following chemotherapy in HNSCC (Bergamaschi et al., 2003; Irwin et al., 2003). These studies demonstrated that p73 is modestly induced following DNA-damaging agents. Of note, we and others have observed that ΔNp63α itself is dramatically downregulated following DNA damage (Liefer et al., 2000). Thus, p73-mediated cell death following DNA damage may represent the cumulative effect of increased p73 levels and decreased p63-mediated inhibition. As a pilot experiment to test this possibility, we examined the effect of p73 loss on cell survival following treatment with the chemotherapeutic agent cisplatin, which rapidly downregulates p63 expression (Fomenkov et al., 2004). For these experiments, we used JHU-029 cells in which TAp73 expression was ablated by RNAi (Figure 5D). We found that loss of TAp73 indeed attenuated cellular sensitivity to cisplatin (Figure S3). These findings support a role for p73 in DNA damage-induced cell death following downregulation of p63. This pathway may be restricted to particular cell types, however, since genetic deletion of p73 does not affect sensitivity to ionizing radiation in murine T cells (Senoo et al., 2004). Posttranslational modification of p73 following DNA damage is also thought to play a role in its activation and promoter selectivity (Strano et al., 2005). This fact may explain differences in proapoptotic genes induced by p73 following DNA damage versus those we find induced following inhibition of p63 by RNAi (Bergamaschi et al., 2003; Strano et al., 2005). Consistent with our findings, a prior study found that overexpression of p73 in the absence of DNA damage leads to induction of endogenous Puma mRNA and protein (Melino et al., 2004).

Although our studies show that p73 is necessary for the apoptotic program activated following loss of p63, our findings do not rule out additional mechanisms by which p63 may promote tumor cell survival. Indeed, one recent study identified the insulin-like growth factor binding protein 3 (IGFBP-3) as a potential transcriptional target of p63 in HNSCC (Barbieri et al., 2005). This study did not directly address a potential contribution of IGFBP-3 to HNSCC survival; however, p63 is likely to regulate a number of factors that contribute to the pathogenesis of these tumors. Nevertheless, we find that bcl-2 expression rescues cells from death following loss of p63 and is inversely correlated with p63 in HNSCC cells. These observations are consistent with a primary role for p63 as a suppressor of the mitochondrial apoptotic pathway downstream of p73.

A minority of HNSCC tumors exhibit low or absent p63 expression, and our data begin to address their particular biology. Rather than evade apoptosis via loss of p73, these tumors may instead upregulate bcl-2, which we show to be a potent suppressor of the p63-dependent apoptotic program. A recently reported mouse tumor model may be of use in furthering our understanding of such p63-low tumors. This model involves mice engineered for p63 heterozygosity, 10% of which develop squamous carcinomas characterized by loss of the remaining p63 allele (Flores et al., 2005). Like human tumors, these tumors do not commonly exhibit loss of p73, even in a p73 heterozygous background. It will therefore be of interest to determine whether

upregulation of bcl-2 is a cooperating event observed during tumorigenesis in this model.

Finally, our data may in part explain previously observed prognostic correlations involving p63 and bcl-2. Head and neck cancers are commonly treated with a combination of DNA-damaging agents, including radiation and/or chemotherapy. For cancers that express ΔNp63α, its downregulation and the subsequent activation of p73 may be important mediators of a favorable response to treatment (Massion et al., 2003). In contrast, upregulation of bcl-2 and the corresponding loss of ΔNp63α may signify tumors that are resistant to the proapoptotic effect of these treatment modalities. In fact, several studies support the use of bcl-2 expression as a marker of poor response to standard radiation and chemotherapy in HNSCC (Nix et al., 2005; Ogawa et al., 2003). Thus, tumors that have circumvented the requirement for p63-mediated survival may exhibit resistance to common cancer treatments, making them good candidates for alternative therapeutic approaches.

Experimental procedures

Cell lines

The HNSCC cell lines designated JHU were the generous gift of David Sidransky, MD, Johns Hopkins University, Baltimore, MD (Richtsmeier and Carey, 1987; Scher et al., 1993), and those designated PCI were the generous gift of Robert Ferris, MD, Ph.D., University of Pittsburgh Cancer Institute, Pittsburgh, PA (Heo et al., 1989).

Lentiviral and retroviral production and infection

The shRNA lentiviral constructs were created by transferring the U6 promoter-shRNA cassette into a lentiviral backbone. High-titer amphotrophic retroviral and lentiviral stocks were generated as described (Ellisen et al., 2001; Shin et al., 2004; Rubinson et al., 2003). The p63si-1 lentiviral vector used in some experiments was the generous gift of Dr. William Hahn. The targeted sequences for p63 were 5′-GGGTGAGCGTGTTATTGATGCT-3′ and 5′-GAGTGGAATGACTTCAACTTT-3′. The targeted sequence for TAp73 was 5′-GGATTCCAGCATGGACGTCTT-3′. Further details are available in the Supplemental Data.

QRT-PCR analysis

First-strand cDNA was synthesized from total RNA using random hexamer primers and the SuperScript II system for RT-PCR (Invitrogen). Gene expression levels were measured by real-time QRT-PCR using the iQ SYBR Green Supermix reagent (Bio-Rad) and an Opticon real-time PCR detector system (MJ Research). Data analysis was performed using Opticon Monitor Analysis Software V1.08 (MJ Research). The expression of each gene was normalized to GAPDH as a reference, and relative levels were calculated from a 4-point standard curve. All experiments were performed in triplicate. Further details and primer sequences are available in the Supplemental Data.

Luciferase promoter reporter and apoptosis assays

Saos-2 cells were seeded in 24-well plates and were transfected with the indicated expression construct, reporter plasmid, and SV40-renilla control vector, and lysates were analyzed at 48 hr using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's recommendations. Further details are available in the Supplemental Data. Apoptotic cell death was determined using the BD ApoAlert annexin V-FITC Apoptosis Kit (BD Biosciences) according to the manufacturer's instructions, and cells were analyzed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Biosciences).

Immunoprecipitation and ChIP

Transfected U2OS cells or untransfected JHU-029 cells were lysed on ice in lysis buffer (0.75% NP-40, 1 mM DTT, and protease inhibitors in PBS). Precleared lysates (1.0 mg) were incubated with either 2.0 μg/sample of anti-p63 polyclonal antibody (H-129, Santa Cruz) or 1.0 μg/sample of anti-p73 monoclonal antibody (Ab-1 and Ab-2, CalBiochem) for 2 hr at 4ºC, and immunocomplexes were precipitated using protein A or protein G Sepharose (both from Amersham Biosciences), then washed four times with lysis buffer prior to analysis by SDS-PAGE. ChIP assays were performed essentially as described (Meluh and Broach, 1999), with modifications as detailed in the Supplemental Data. "Input" templates were purified from 10% of the original lysates in parallel with the eluted IP products. PCR was carried out using 10% of the IP product with primers spanning the conserved p53 family binding motif within the *Puma* promoter or targeting the nonspecific (*loricrin*) gene.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, three supplemental figures, and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/1/45/DC1/.

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References

Barbieri, C.E., Perez, C.A., Johnson, K.N., Ely, K.A., Billheimer, D., and Pietenpol, J.A. (2005). IGFBP-3 is a direct target of transcriptional regulation by ΔNp63α in squamous epithelium. Cancer Res. *65*, 2314–2320.

Bergamaschi, D., Gasco, M., Hiller, L., Sullivan, A., Syed, N., Trigiante, G., Yulug, I., Merlano, M., Numico, G., Comino, A., et al. (2003). p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell *3*, 387–402.

Bjorkqvist, A.M., Husgafvel-Pursiainen, K., Anttila, S., Karjalainen, A., Tammilehto, L., Mattson, K., Vainio, H., and Knuutila, S. (1998). DNA gains in 3q occur frequently in squamous cell carcinoma of the lung, but not in adenocarcinoma. Genes Chromosomes Cancer *22*, 79–82.

Bowman, T., Symonds, H., Gu, L., Yin, C., Oren, M., and Van Dyke, T. (1996). Tissue-specific inactivation of p53 tumor suppression in the mouse. Genes Dev. *10*, 826–835.

Chan, W.M., Siu, W.Y., Lau, A., and Poon, R.Y. (2004). How many mutant p53 molecules are needed to inactivate a tetramer? Mol. Cell. Biol. *24*, 3536–3551.

Chim, C.S., Liang, R., and Kwong, Y.L. (2002). Hypermethylation of gene promoters in hematological neoplasia. Hematol. Oncol. *20*, 167–176.

Choi, H.R., Batsakis, J.G., Zhan, F., Sturgis, E., Luna, M.A., and El-Naggar, A.K. (2002). Differential expression of p53 gene family members p63 and p73 in head and neck squamous tumorigenesis. Hum. Pathol. *33*, 158–164.

Davison, T.S., Vagner, C., Kaghad, M., Ayed, A., Caput, D., and Arrowsmith, C.H. (1999). p73 and p63 are homotetramers capable of weak heterotypic interactions with each other but not with p53. J. Biol. Chem. *274*, 18709–18714.

Ellisen, L.W., Carlesso, N., Cheng, T., Scadden, D.T., and Haber, D.A. (2001). The Wilms tumor suppressor WT1 directs stage-specific quiescence and differentiation of human hematopoietic progenitor cells. EMBO J. *20*, 1897–1909.

Flores, E.R., Sengupta, S., Miller, J.B., Newman, J.J., Bronson, R., Crowley, D., Yang, A., McKeon, F., and Jacks, T. (2005). Tumor predisposition in mice

mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell *7*, 363–373.

Fomenkov, A., Zangen, R., Huang, Y.P., Osada, M., Guo, Z., Fomenkov, T., Trink, B., Sidransky, D., and Ratovitski, E.A. (2004). RACK1 and stratifin target ΔNp63α for a proteasome degradation in head and neck squamous cell carcinoma cells upon DNA damage. Cell Cycle *3*, 1285–1295.

Forastiere, A., Koch, W., Trotti, A., and Sidransky, D. (2001). Head and neck cancer. N. Engl. J. Med. *345*, 1890–1900.

Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol. Cell. Biol. *21*, 1874–1887.

Gong, J.G., Costanzo, A., Yang, H.Q., Melino, G., Kaelin, W.G., Levrero, M., and Wang, J.Y.J. (1999). The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature *399*, 806–809.

Harms, K., Nozell, S., and Chen, X. (2004). The common and distinct target genes of the p53 family transcription factors. Cell. Mol. Life Sci. *61*, 822–842.

Heo, D.S., Snyderman, C., Gollin, S.M., Pan, S., Walker, E., Deka, R., Barnes, E.L., Johnson, J.T., Herberman, R.B., and Whiteside, T.L. (1989). Biology, cytogenetics, and sensitivity to immunological effector cells of new head and neck squamous cell carcinoma lines. Cancer Res. *49*, 5167–5175.

Hibi, K., Trink, B., Patturajan, M., Westra, W.H., Caballero, O.L., Hill, D.E., Ratovitski, E.A., Jen, J., and Sidransky, D. (2000). AIS is an oncogene amplified in squamous cell carcinoma. Proc. Natl. Acad. Sci. USA *97*, 5462–5467.

Hoque, M.O., Begum, S., Sommer, M., Lee, T., Trink, B., Ratovitski, E., and Sidransky, D. (2003). PUMA in head and neck cancer. Cancer Lett. *199*, 75–81.

Hu, H., Xia, S.H., Li, A.D., Xu, X., Cai, Y., Han, Y.L., Wei, F., Chen, B.S., Huang, X.P., Han, Y.S., et al. (2002). Elevated expression of p63 protein in human esophageal squamous cell carcinomas. Int. J. Cancer *102*, 580–583.

Irwin, M.S., Kondo, K., Marin, M.C., Cheng, L.S., Hahn, W.C., and Kaelin, W.G., Jr. (2003). Chemosensitivity linked to p73 function. Cancer Cell *3*, 403–410.

King, K.E., Ponnamperuma, R.M., Yamashita, T., Tokino, T., Lee, L.A., Young, M.F., and Weinberg, W.C. (2003). ΔNp63α functions as both a positive and a negative transcriptional regulator and blocks in vitro differentiation of murine keratinocytes. Oncogene *22*, 3635–3644.

Liefer, K.M., Koster, M.I., Wang, X.J., Yang, A., McKeon, F., and Roop, D.R. (2000). Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. Cancer Res. *60*, 4016–4020.

Lowe, S.W., Ruley, H.E., Jacks, T., and Houseman, D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957–967.

Lowe, S.W., Cepero, E., and Evan, G. (2004). Intrinsic tumour suppression. Nature *432*, 307–315.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. *182*, 1545–1556.

Massion, P.P., Taflan, P.M., Jamshedur Rahman, S.M., Yildiz, P., Shyr, Y., Edgerton, M.E., Westfall, M.D., Roberts, J.R., Pietenpol, J.A., Carbone, D.P., and Gonzalez, A.L. (2003). Significance of p63 amplification and overexpression in lung cancer development and prognosis. Cancer Res. *63*, 7113–7121.

Melino, G., Lu, X., Gasco, M., Crook, T., and Knight, R.A. (2003). Functional regulation of p73 and p63: development and cancer. Trends Biochem. Sci. *28*, 663–670.

Melino, G., Bernassola, F., Ranalli, M., Yee, K., Zong, W.X., Corazzari, M., Knight, R.A., Green, D.R., Thompson, C., and Vousden, K.H. (2004). p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation. J. Biol. Chem. *279*, 8076–8083.

Meluh, P.B., and Broach, J.R. (1999). Immunological analysis of yeast chromatin. Methods Enzymol. *304*, 414–430.

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature *398*, 708–713.

Moll, U.M., and Slade, N. (2004). p63 and p73: roles in development and tumor formation. Mol. Cancer Res. *2*, 371–386.

Nakano, K., and Vousden, K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol. Cell *7*, 683–694.

Nix, P., Cawkwell, L., Patmore, H., Greenman, J., and Stafford, N. (2005). Bcl-2 expression predicts radiotherapy failure in laryngeal cancer. Br. J. Cancer *92*, 2185–2189.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Nature *288*, 1053–1058.

Ogawa, T., Shiga, K., Tateda, M., Saijo, S., Suzuki, T., Sasano, H., Miyagi, T., and Kobayashi, T. (2003). Protein expression of p53 and Bcl-2 has a strong correlation with radiation resistance of laryngeal squamous cell carcinoma but does not predict the radiation failure before treatment. Oncol. Rep. *10*, 1461–1466.

Parsa, R., Yang, A., McKeon, F., and Green, H. (1999). Association of p63 with proliferative potential in normal and neoplastic keratinocytes. J. Invest. Dermatol. *113*, 1099–1104.

Patturajan, M., Nomoto, S., Sommer, M., Fomenkov, A., Hibi, K., Zangen, R., Poliak, N., Califano, J., Trink, B., Ratovitski, E., and Sidransky, D. (2002). ΔNp63 induces β-catenin nuclear accumulation and signaling. Cancer Cell *1*, 369–379.

Redon, R., Muller, D., Caulee, K., Wanherdrick, K., Abecassis, J., and du Manoir, S. (2001). A simple specific pattern of chromosomal aberrations at early stages of head and neck squamous cell carcinomas: PIK3CA but not p63 gene as a likely target of 3q26-qter gains. Cancer Res. *61*, 4122–4129.

Richtsmeier, W.J., and Carey, T.E. (1987). Rationalized nomenclature for head and neck carcinomas. Arch. Otolaryngol. Head Neck Surg. *113*, 1339–1340.

Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Rooney, D.L., Ihrig, M.M., McManus, M.T., Gertler, F.B., et al. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat. Genet. *33*, 401–406.

Scher, R.L., Koch, W.M., and Richtsmeier, W.J. (1993). Induction of the intercellular adhesion molecule (ICAM-1) on squamous cell carcinoma by interferon gamma. Arch. Otolaryngol. Head Neck Surg. *119*, 432–438.

Schmitt, C.A., Fridman, J.S., Yang, M., Baranov, E., Hoffman, R.M., and Lowe, S.W. (2002). Dissecting p53 tumor suppressor functions in vivo. Cancer Cell *1*, 281–298.

Senoo, M., Manis, J.P., Alt, F.W., and McKeon, F. (2004). p63 and p73 are not required for the development and p53-dependent apoptosis of T cells. Cancer Cell *6*, 85–89.

Shin, J.J., Katayama, T., Michaud, W.A., and Rocco, J.W. (2004). Short hairpin RNA system to inhibit human p16 in squamous cell carcinoma. Arch. Otolaryngol. Head Neck Surg. *130*, 68–73.

Sniezek, J.C., Matheny, K.E., Westfall, M.D., and Pietenpol, J.A. (2004). Dominant negative p63 isoform expression in head and neck squamous cell carcinoma. Laryngoscope *114*, 2063–2072.

Strano, S., Monti, O., Pediconi, N., Baccarini, A., Fontemaggi, G., Lapi, E., Mantovani, F., Damalas, A., Citro, G., Sacchi, A., et al. (2005). The transcriptional coactivator Yes-associated protein drives p73 gene-target specificity in response to DNA Damage. Mol. Cell *18*, 447–459.

Sui, G., Soohoo, C., Affar, E.B., Gay, F., Shi, Y., Forrester, W.C., and Shi, Y. (2002). A DNA-based RNAi technology to suppress gene expression in mammlian cells. Proc. Natl. Acad. Sci. USA *99*, 5515–5520.

Tonon, G., Wong, K.K., Maulik, G., Brennan, C., Feng, B., Zhang, Y., Khatry, D.B., Protopopov, A., You, M.J., Aguirre, A.J., et al. (2005). High-resolution genomic profiles of human lung cancer. Proc. Natl. Acad. Sci. USA *102*, 9625–9630.

Urist, M., Tanaka, T., Poyurovsky, M.V., and Prives, C. (2004). p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. Genes Dev. *18*, 3041–3054.

Vogelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. Nat. Med. *10*, 789–799.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.

Vousden, K.H. (2000). p53: Death star. Cell *103*, 691–694.

Weber, A., Bellmann, U., Bootz, F., Wittekind, C., and Tannapfel, A. (2002). Expression of p53 and its homologues in primary and recurrent squamous cell carcinomas of the head and neck. Int. J. Cancer *99*, 22–28.

Westfall, M.D., Mays, D.J., Sniezek, J.C., and Pietenpol, J.A. (2003). The ΔNp63α phosphoprotein binds the p21 and 14-3-3σ promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutants. Mol. Cell. Biol. *23*, 2264–2276.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dotsch, V., Andrews, N.C., Caput, D., and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death inducing, and dominant negative activities. Mol. Cell *2*, 305–316.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature *398*, 714–718.

Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W., and Volgelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. Mol. Cell *7*, 673–682.

Yuan, Z.M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y.Y., Lu, H., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1999). p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. Nature *399*, 814–817.

Zaika, A.I., Slade, N., Erster, S.H., Sansome, C., Joseph, T.W., Pearl, M., Chalas, E., and Moll, U.M. (2002). ΔNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. J. Exp. Med. *196*, 765–780.