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ΔNp63 induces β-catenin nuclear accumulation and signaling

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Summary

The P53 homolog *p63* encodes multiple proteins with transactivating, apoptosis-inducing, and oncogenic activities. We showed that *p63* is amplified and that ΔNp63 isotypes are overexpressed in squamous cell carcinoma (SCC) and enhance oncogenic growth in vitro and in vivo. Moreover, p53 associated with ΔNp63α and mediated its degradation. Here, we report that ΔNp63 associates with the B56α regulatory subunit of protein phosphatase 2A (PP2A) and glycogen synthase kinase 3β (GSK3β), leading to a dramatic inhibition of PP2A-mediated GSK3β reactivation. The inhibitory effect of ΔNp63 on GSK3β mediates a decrease in phosphorylation levels of β-catenin, which induces intranuclear accumulation of β-catenin and activates β-catenin-dependent transcription. Our results suggest that ΔNp63 isotypes act as positive regulators of the β-catenin signaling pathway, providing a basis for their oncogenic properties.

Introduction

Wnt signal transduction pathways represent a very complex signaling network, which actively contributes to the control of cell proliferation during development and tumorigenesis (Barker and Clevers, 2000; Polakis, 2000; Taipale and Beachy, 2001). One target of the Wnt signaling cascade is β-catenin, which plays an important role in many human cancers. The association of Wnt ligands with their cell surface receptors triggers the activation of numerous downstream signaling events, which abolish the phosphorylation and ubiquitin-mediated degradation of β-catenin, leading to the intranuclear accumulation of β-catenin. β-catenin associates with the T cell factor (Tcf)/lymphocyte enhancer binding factor (Lef) complex and mediates the regulation of Tcf/Lef-responsive genes that result in cell proliferation and dedifferentiation (He et al., 1998; Barker and Clevers, 2000).

In the absence of Wnt, the association of β-catenin with glycogen synthase kinase 3β (GSK3β), Axin, and the adenomatous polyposis coli (APC) protein leads to GSK3β-mediated phosphorylation and targeting of β-catenin into a proteasome degradation pathway (Frame and Cohen, 2001; Janssens and Goris, 2001). Protein phosphatase 2A (PP2A) is a serine-threonine protein phosphatase associated with APC, Axin, and GSK3β and also contributes to β-catenin destruction (Kikuchi et al., 2000; Janssens and Goris, 2001). PP2A consists of a catalytic (C) subunit, a structural (A) subunit, and a variable regulatory (B) subunit (Janssens and Goris, 2001). B56 regulatory subunits (α, β, δ, ϵ, and γ) were shown to interact with APC and direct PP2A to dephosphorylate GSK3β, leading to the activation of GSK3β and subsequent degradation of β-catenin (McCright et al., 1996; McCright and Virshup, 1998; Seeling et al., 1999; Li et al., 2001a). The overexpression of B56α led to activation of GSK3β by PP2A-mediated dephosphorylation, which dramatically reduced the level of β-catenin and inhibited Lef-mediated gene transactivation (Seeling et al., 1999; Li et al., 2001a).

The β-catenin nuclear accumulation is a critical event in many cancers. Retroviral insertion of Wnt-expressing constructs in the mammary gland dramatically induces accumulation of β-catenin and promotes tumor formation in mice (reviewed in Li et al., 2001b). Transfection of murine cells with the Wnt constructs led to a tumorigenic phenotype and β-catenin nuclear accumulation (Wong et al., 1994; Bafico et al., 1998). In humans, mutations in the *APC* gene that produce a truncated polypeptide unable to promote degradation of β-catenin are abundant in sporadic colon cancers (Morin et al., 1997). Point mutations in β-catenin that alter N-terminal putative GSK3β phosphorylation

SIGNIFICANCE

Unlike p53, its homolog p63 is rarely mutated in cancers. P63 encodes multiple proteins with transactivating, apoptosis-inducing, and oncogenic activities. In this report, we show that ΔNp63 dramatically induces intranuclear accumulation of β-catenin and activates β-catenin-responsive transcription. The mechanism underlying this novel function of ΔNp63 involves the physical association of ΔNp63 with B56α, which inhibits phosphorylation of β-catenin by GSK3β and its degradation. In squamous cell carcinomas (SCC), mutations of the APC complex are virtually absent; however, SCC cells commonly harbor *p53* mutations accompanied by *p63* gene amplification and/or overexpression of ΔNp63 isotypes. Thus, our observations support the oncogenic function of ΔNp63 and elucidate molecular events leading to neoplastic progression in SCC.

sites have been found in colon, prostate, and skin cancers and in medulloblastomas and hepatocellular carcinomas (Rubinfeld et al., 1997; Sparks et al., 1998; Zurawel et al., 1998; Voeller et al., 1998; de La Coste et al., 1998; Chan et al., 1999). Mutations in the N-terminal region of β-catenin abrogate the phosphorylation of β-catenin and, subsequently, its degradation. Axin mutations have also been found in hepatocellular carcinomas and other tumor types that lack APC or β-catenin mutations (Satoh et al., 2000; Dahmen et al., 2001).

Positive (oncogenic) and negative (tumor suppressive) regulators of Wnt signaling have opposite effects on cell proliferation and tumorigenesis (Barker and Clevers, 2000; Polakis, 2000; Taipale and Beachy, 2001). In contrast to adenocarcinomas, genetic changes in the components of Wnt signaling have not been identified in human squamous cell carcinomas (SCC) from the lung, head and neck, skin, and cervix. Recently, we reported increased expression of ΔNp63 isotypes and amplification of *p63* in these types of human cancers, which suggests that ΔNp63 isotypes play the role of putative oncoproteins (Hibi et al., 2000). Moreover, the overexpression of ΔNp63 isotypes in transfected cells mediated an increase in tumor growth rates in vitro and in vivo (Hibi et al., 2000). Overexpression of ΔNp63 isotypes was also described in many other types of epithelial tumors (Parsa et al., 1999; Crook et al., 2000; Nylander et al., 2000; Park et al., 2000).

To further understand the oncogenic properties of ΔNp63 isotypes, we identified additional p63 protein binding partners, known to function as key signaling intermediates that regulate β-catenin degradation. As described below, our results support the notion that ΔNp63 isotypes activate β-catenin signaling and elucidate a molecular mechanism that underlies the oncogenic function of ΔNp63 proteins in human squamous epithelial tumors.

Results

To better understand the role of *p63* in cell proliferation and tumorigenesis, we applied a yeast two-hybrid screen to identify possible p63-interacting proteins. In addition to p53 (Ratovitski et al., 2001), we identified B56α, the regulatory subunit of PP2A, as a p63-interacting candidate.

To examine the regions required for the association of B56α with p63, we generated truncated variants of B56α and the p63 core domain (p40). Truncated fragments of p40 or B56α were fused to the Gal4 binding domain or the Gal4-activation domain, respectively. Using the two-hybrid assay, we found that the protein domain of PP2A-B56α (residues 76–150) is necessary to mediate the interaction with p63. Likewise, the N-terminal domain of p40 (residues 1–20) mediates the association with B56α (Figure 1A, Table 1).

To further confirm these observations, we analyzed the interaction of B56α and p40 ectopically expressed in Saos-2 cells (Figure 1B) using coprecipitation assays. We also transfected Saos-2 cells with plasmids harboring ΔNp63α, TAp63α, and GSKβ to evaluate the association between these proteins. Complexes between p63 isotypes and B56α were precipitated from total lysates with an antibody recognizing the DNA binding domain of p63 proteins (4A4), followed by immunoblotting with an antibody to B56α (Figure 1B). A significant amount of B56α could be coprecipitated from extracts containing p40, but not in extracts lacking p40, confirming the yeast two-hybrid screen data (Figure 1B, Table 1). In addition, GSK3β also forms complexes with p40 when the latter is expressed together with B56α (Figure 1B). Ectopically expressed ΔNp63α was also shown to bind B56α, while TAp63α failed to associate with B56α in these conditions (Figure 1C, central panel, lanes 3 and 4). Used as a negative control, nonrelevant HA-tagged PTN (pleiotrophin) did not associate with any p63 isotypes (Figure 1C, lower panel, lanes 3 and 4). The HNSCC cell line 013 expressed varied amounts of ΔNp63α, B56α, GSK3β, PP2A(C), APC, and Axin (Figure 1D, lane 1). By coprecipitation, we showed a physical association between endogenous ΔNp63α and B56α (Figure 1D, lane 2). Moreover, we demonstrated that GSK3β is also part of the ΔNp63α/B56α complex (Figure 1D, lane 2). However, APC, Axin, and PP2A(C) were not detected in the ΔNp63α precipitates (Figure 1D, lane 2).

We wondered whether β-catenin could be part of the ΔNp63 complex. We had evidence that the carboxy terminus of p63α (including the sterile α motif) binds β-catenin itself in yeast (data not shown). Coprecipitation studies using HNSCC 013 cells confirmed that ΔNp63α binds to β-catenin in the nucleus (Figure 2). In addition, β-catenin and ΔNp63 were found to colocalize within intranuclear formations of 013 cells, further strengthening the notion that ΔNp63α is an important component of β-catenin regulation.

We next examined whether the overexpression of ΔNp63α affects the protein level of β-catenin. Our initial experiments failed to show any effect of p63 isotypes on the RNA level of β-catenin (data not shown). We transfected HEK-293 cells with expression constructs for TAp63α, ΔNp63α, and deletion mutants ΔNp63α-Δ20 and ΔNp63α-Δ41, lacking the first 20 or 41 residues, respectively. The latter constructs were generated based on results of the two-hybrid screens, which demonstrated that residues 1–20 are necessary to mediate binding of ΔNp63 to B56α (Table 1). As negative and positive controls, we used HEK-293 cells grown in the presence of either the conditioned medium (CM) from uninfected mammary epithelial cells or CM from C57MG-MV-Wnt-1 mammary epithelial cells that secrete the physiologically active Wnt-1, respectively (Bradley and Brown, 1995). As shown, the extracellular Wnt ligand mediates an increase in β-catenin protein levels (Figure 3A, lanes 1 and 2). We also observed that ectopic expression of ΔNp63α (Figure 3A, lane 6) led to dramatic accumulation of β-catenin. However, ΔNp63α-Δ41, ΔNp63α-Δ20, and TAp63α failed to increase the protein levels of β-catenin (Figure 3A, lanes 3, 4, and 5, respectively). Thus, these data strongly suggest that residues 1–20 are critical for the interaction between ΔNp63 and B56α and for the ΔNp63-induced β-catenin accumulation (Figure 3A).

We further examined whether ΔNp63α overexpression affects the subcellular localization of β-catenin. We used HEK-293 cells transfected with an empty pCEP4 vector (−ΔNp63α) or with the pCEP4-ΔNp63α expression construct (+ΔNp63α) as a source of nuclear and cytoplasmic fractions. The purity of nuclei and the cytoplasm was confirmed by immunoblotting with antibodies to specific markers as indicated (Figure 3B). As shown, overexpression of ΔNp63α dramatically increased the intranuclear protein level of β-catenin. Interestingly, the protein levels of both B56α and GSK3β also rise in the nuclei (Figure 3B). Conversely, we observed that APC, Axin, and PP2A(C) are predominantly located in the cytoplasm in the presence or absence of ΔNp63α expression (data not shown).

The nuclear accumulation of ΔNp63 associated with B56α,

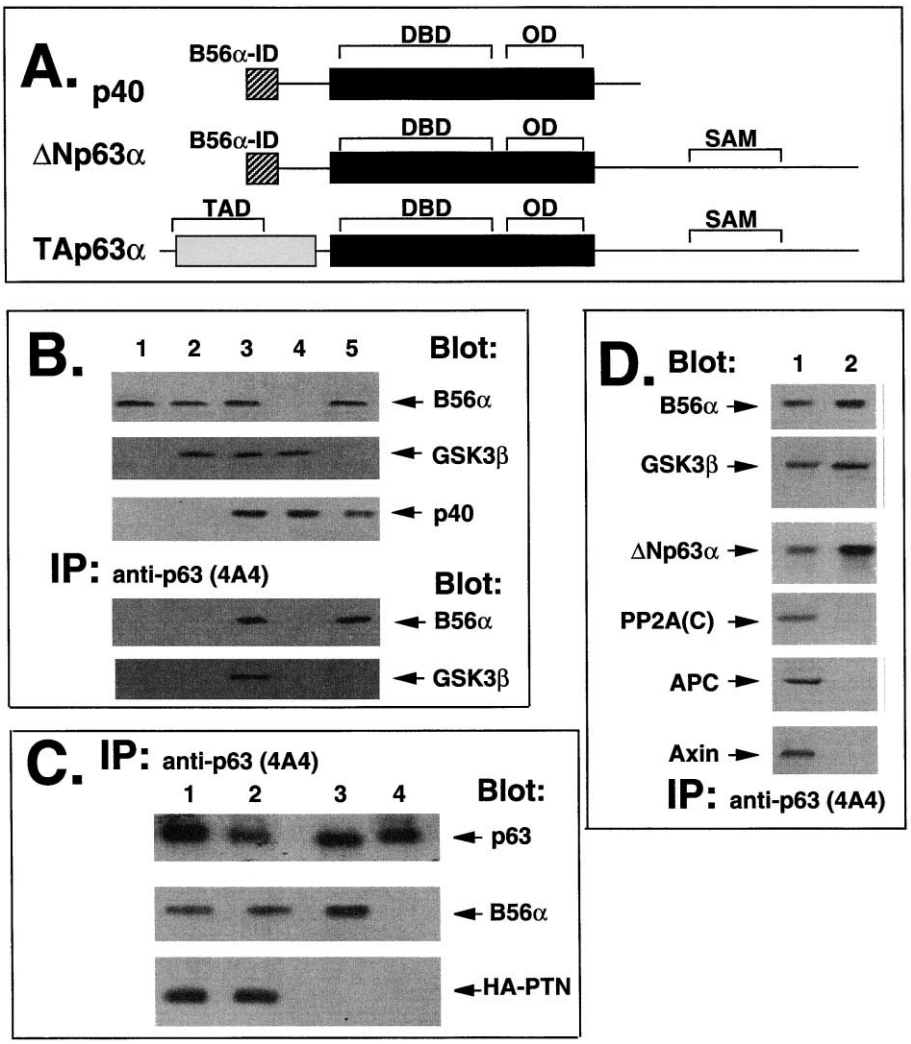


Figure 1. ΔNp63 isotypes physically associate with B56α

A: A schematic representation of p63 isotypes (p40 and ΔNp63α versus TAp63α); the N-terminal domain of ΔNp63 isotypes is thought to mediate binding to B56α. **B:** Interaction of ΔNp63 isotypes with the B56α subunit of PP2A. Saos-2 cells were transfected (~50% efficiency) with various plasmids (2 μg each): pCEP4-HA-B56α, pCEP4-FLAG-GSK3β, pCEP4-p40, pCEP4-TAp63α, or pCEP4-HA-PTN. Total lysates expressing ectopic B56α (lanes 1–3 and 5), GSK3β (lanes 2–4), and p40 (lanes 3–5) were used. Protein levels were detected by immunoblotting (~30 μg of cell lysate) with antibodies to B56α (upper panel), GSK3β (central panel), or ΔNp63 (lower panel). P63 complexes were immunoprecipitated (IP, ~100 μg cell lysate) using antibody to p63 (4A4), followed by immunobloting with antibodies to B56α (upper panel) or GSK3β (lower panel). **C:** Total lysates expressing TAp63α (lanes 1 and 4) or ΔNp63α (lanes 2 and 3) were analyzed by immunoblotting (lanes 1 and 2, ~40 μg cell lysate) with antibodies to p63 (4A4, upper panel), B56α (central panel), or HA (lower panel) or were immunoprecipitated (IP, ~120 μg) with antibody 4A4 (lanes 3 and 4), followed by immunoblotting with indicated antibodies. **D:** Endogenous B56α/GSK3β/ΔNp63α complexes in HNSCC 013 cells. Total lysates from HNSCC 013 cells were assayed for expression of B56α, GSK3β, ΔNp63α, PP2A(C), APC, and Axin (lane 1) by immunoblotting (~50 μg). ΔNp63α complexes were immunoprecipitated (IP, ~150 μg cell lysate) with antibody to ΔNp63 and were immunoblotted with antibodies to B56α, GSK3β, ΔNp63, PP2A(C), APC, and Axin (lane 2). Endogenous ΔNp63α forms complexes with B56α and GSK3β, but does not associate with APC, Axin, or PP2A(C). Moreover, TAp63α does not interact with B56α.

GSK3β, and β-catenin suggests its positive role in β-catenin signaling and oncogenic growth. This observation led us to explore a correlation between the intranuclear levels of ΔNp63 (p40) and β-catenin in mouse tumor xenografts. The xenografts were initially obtained by injection of Rat1α cells transfected with pCEP-p40 expression construct versus an empty pCEP4 vector (Hibi et al., 2000). We previously reported that transfection of Rat1α cells with pCEP-p40 led to a dramatic increase in tumorigenic foci formation and, in turn, to a greater growth of tumors in athymic nude mice (Hibi et al., 2000). We found that these tumor xenografts exhibiting increased growth (Hibi et al., 2000) due to p40 overexpression also demonstrated abundant intranuclear accumulation of β-catenin (Figure 4A). The intranuclear levels of β-catenin also correlated with those of ΔNp63α in isogenic HNSCC cell lines 012 and 013 (Figure 4B). 013 cells harbor mutated p53, leading to an increase of intranuclear ΔNp63α and β-catenin levels (Figure 4B), and higher rates of cell proliferation than isogenic 012 cells (see below). Thus, the increased nuclear accumulation of β-catenin correlates with the oncogenic potential of ΔNp63.

To further evaluate the effect of ΔNp63α on the nuclear accumulation of β-catenin, we infected HNSCC 013 cells (expressing endogenous ΔNp63α) with an empty Ad5 or Ad-ΔNp63α-AS adenovirus. Our data clearly show that overexpression of ΔNp63α-AS decreases levels of both ΔNp63α and β-catenin (Figure 5A, top and central panels, respectively). Taking into consideration that a decrease in ΔNp63α might cause apoptotic changes, we examined the protein levels of β-actin and found no change (Figure 5A, lower panels). Furthermore, transfection of these cells with pCEP4-ΔNp63α-AS (versus cells transfected with an empty vector) modulated the proliferation rate of both HNSCC 012 (wild-type p53, curve 3 versus curve 1) and 013 (mutated p53, curve 4 versus curve 2) cells, supporting a critical role for ΔNp63α in the regulation of cell proliferation (Figure 5B).

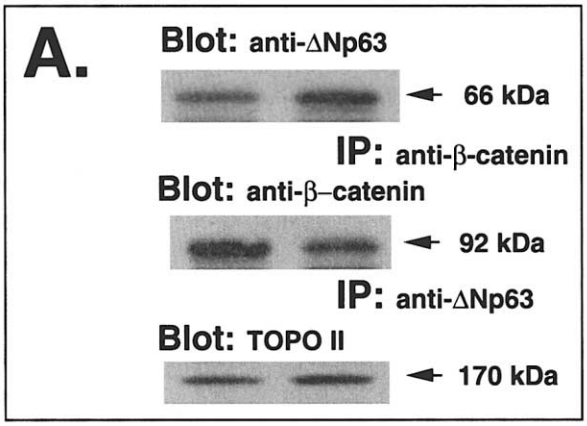
To determine whether the increase in the β-catenin intranuclear accumulation induced by ΔNp63 leads to an increase in the β-catenin-mediated activation of transcription, we transfected HEK-293 cells (which contain intact β-catenin signaling machinery) with expression plasmids (indicated in the legend of Figure 5C) and analyzed their effect on the Lef-1: luciferase reporter activity. As shown, expression of B56α reduced luciferase activity by ~50%, while expression of ΔNp63α activated luciferase activity by 3- to 4-fold (Figure 5C, samples 2 and 3

Table 1. P63 core domain (p40) interacts with B56α in a yeast genetic assay

No	Bait	Prey	Trp-	Leu-	His-	β-galactosidase (nmole/min/mg)
1.	pGal4-BD-p53^{WT}	pGal4-AD-SV-40	+	+	+	61.8 ± 5.9
2.	pGal4-BD-lamin C	pGal4-AD-SV-40	+	+	--	2.1 ± 0.5
3.	pGal4-BD-p40 (1–356)	pGal4-AD	+	+	--	2.9 ± 0.9
4.	pGal4-BD	pGal4-AD-B56α (1–408)	+	+	--	3.6 ± 0.7
5.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (1–408)	+	+	+	39.1 ± 4.1
6.	pGal4-BD-p40 (1–74)	pGal4-AD-B56α (1–408)	+	+	+	38.8 ± 3.4
7.	pGal4-BD-p40 (75–266)	pGal4-AD-B56α (1–408)	+	+	--	6.7 ± 2.5
8.	pGal4-BD-p40 (267–356)	pGal4-AD-B56α (1–408)	+	+	--	3.7 ± 0.9
9.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (1–75)	+	+	--	6.5 ± 2.4
10.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (1–150)	+	+	+	38.5 ± 3.6
11.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (1–225)	+	+	+	38.8 ± 3.7
12.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (1–300)	+	+	+	38.6 ± 3.4
13.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (1–408)	+	+	+	38.9 ± 4.1
14.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (76–408)	+	+	+	38.5 ± 3.6
15.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (150–408)	+	+	--	5.8 ± 1.7
16.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (225–408)	+	+	--	5.6 ± 1.4
17.	pGal4-BD-p40 (1–356)	pGal4-AD-B56α (300–408)	+	+	--	5.9 ± 1.1
18.	pGal4-BD-p40 (21–356)	pGal4-AD-B56α (1–408)	+	+	--	5.4 ± 1.6
19.	pGal4-BD-p40 (42–356)	pGal4-AD-B56α (1–408)	+	+	--	5.1 ± 1.3

Amino acid residues are in parentheses.

versus sample 1, respectively). In addition, an antisense ΔNp63α construct (ΔNp63α-AS) reduced luciferase activity mediated by the endogenous β-catenin/Lef-1 complex, while TAp63α had no effect on this activity (Figure 5C, samples 4 and 5 versus sample 1, respectively). We then transfected HEK-293 cells with pCEP4-myc-tagged FL-β-catenin and various combinations of other plasmids (Figure 5C). We observed that expression of B56α reduced the luciferase activity (by ~30%–40%) induced by ectopic β-catenin, whereas ΔNp63α expression dramatically increased (by ~8-fold) this activity (Figure 5C, samples 7 and 8 versus sample 6, respectively). However, ΔNp63α-AS (sample 9) counteracts the inducible effect of ΔNp63α, while TAp63α



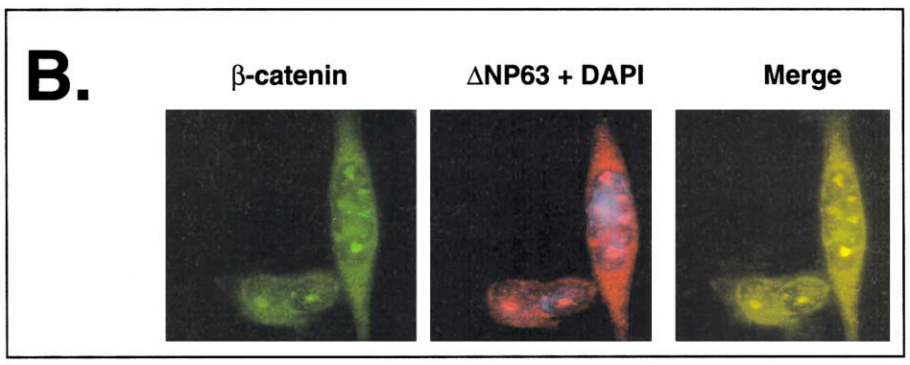
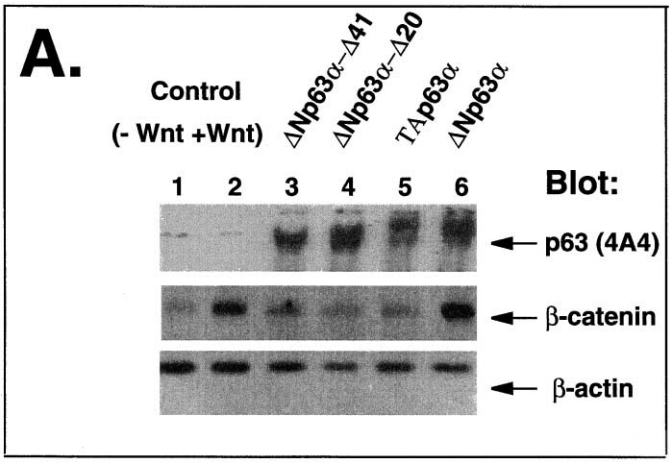


Figure 2. Association of ΔNp63α and β-catenin

A: The physical interaction of endogenous ΔNp63α and β-catenin. Nuclear lysates (~30 μg) from HNSCC 013 cells were analyzed for protein levels of ΔNp63α and β-catenin by immunoblotting. Levels of DNA topoisomerase IIα (TOPO II) served as a loading and fractionation control. For immunoprecipation (IP) with the indicated antibodies, 150 μg nuclear lysates was used. **B:** Nuclear colocalization of ΔNp63α and β-catenin. HNSCC 013 cells were stained with antibody to β-catenin (green) or antibody to ΔNp63α (red) and were counterstained with DAPI for nuclear DNA (blue). Merging of the two stainings shows colocalization of the two proteins (ΔNp63α and β-catenin) in the nucleus (yellow).



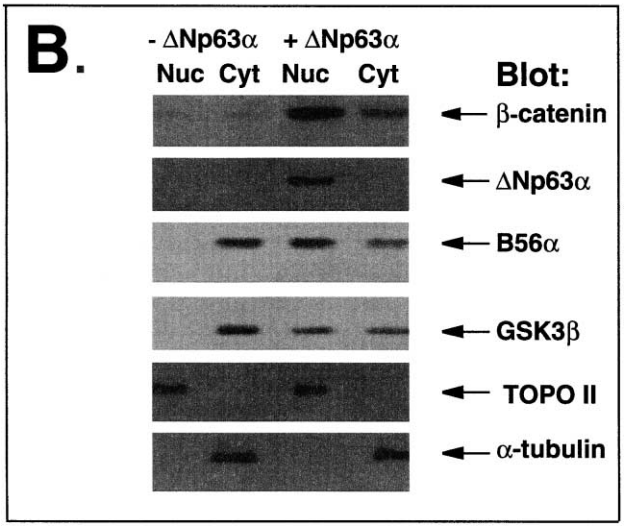
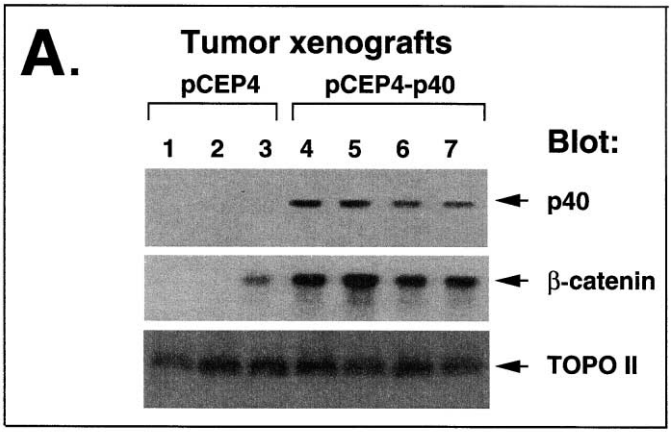


Figure 3. ΔNp63α inhibits degradation of β-catenin and induces its intranuclear accumulation

A: HEK-293 cells were grown in the presence of conditioned medium (CM) with Wnt-1 or control CM (lanes 1 and 2) or were transfected (~70% efficiency) with 2 μg TAp63α, ΔNp63α (lanes 5 and 6), or ΔNp63α-Δ41 or ΔNp63α-Δ20 expression constructs (lanes 3 and 4). Total lysates (~40 μg) were resolved by 10% SDS-PAGE and were immunoblotted with antibodies to p63 (4A4, upper panel) or β-catenin (central panel). Loading levels were examined by immunoblotting with antibody to β-actin (lower panel). **B:** Nuclear and cytoplasmic fractions were isolated from HEK-293 cells transfected (~60% efficiency) with 2 μg empty pCEP4 vector (−ΔNp63α) or pCEP4-ΔNp63α (+ΔNp63α). Proteins obtained from nuclear (~30 μg) and cytoplasmic (~50 μg) fractions were resolved by SDS-PAGE and were immunoblotted with antibodies to β-catenin, ΔNp63, B56α, or GSK3β. As loading and fractionation controls, we used antibodies to DNA topoisomerase IIα (nuclei) or α-tubulin (cytoplasm). ΔNp63α increases the protein level of β-catenin, while the expression of TAp63α, ΔNp63α-Δ20, or ΔNp63α-Δ41 had no effect on β-catenin levels. ΔNp63α dramatically induces the intranuclear abundance of β-catenin, while B56α and GSK3β also undergo nuclear accumulation.

(sample 10) and deletion mutants of ΔNp63α (ΔNp63α-Δ20, ΔNp63α-Δ41, and ΔNp63α-Δ74) failed to activate the luciferase activity (Figure 5C, samples 11–13). Thus, these results strongly support the notion that overexpression of ΔNp63α causes increased activation of transcription at β-catenin-dependent promoters.



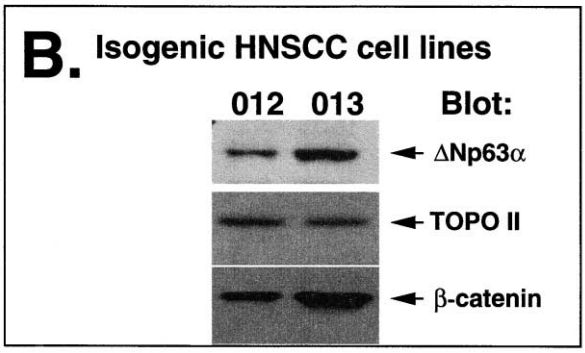


Figure 4. Endogenous ΔNp63α increases the intranuclear levels of β-catenin in tumor xenografts and HNSCC cell lines

A: Tumor xenografts were obtained from nude mice injected with Rat1α cells transfected with the control vector pCEP4 (lanes 1–3) or with pCEP4-p40 (lanes 4–7). Nuclear proteins (~40 μg) were analyzed by immunoblotting with antibodies to ΔNp63 (upper panel) or β-catenin (central panel). **B:** Nuclear proteins from isogenic HNSCC cell lines 012 and 013 were analyzed by immunoblotting with antibodies to ΔNp63 (upper panel) or β-catenin (central panel). Loading levels were examined by immunoblotting with antibody to DNA topoisomerase IIα. ΔNp63 (p40) overexpression correlates with an increase in the intranuclear levels of β-catenin in tumor xenografts and HNSCC cell lines.

To examine the molecular mechanism of ΔNp63-induced β-catenin accumulation, we evaluated the effect of ΔNp63α on the phosphorylation of β-catenin by GSK3β. HEK-293 cells proven to maintain a putative APC destruction complex (Seeling et al., 1999), and tested for the presence of the components of the APC complex and β-TrCP (data not shown), were transfected with plasmids: (i) pCEP4-FLAG-GSK3β and pCEP4-myc-FL-β-catenin, (ii) pCEP4-GSK3β, pCEP4-HA-B56α, and pCEP4-myc-FL-β-catenin, (iii) pCEP4-FLAG-GSK3β, pCEP4-ΔNp63α, and pCEP4-myc-FL-β-catenin, (iv) pCEP4-FLAG-GSK3β, pCEP4-HA-B56α, pCEP4-ΔNp63α, and pCEP4-myc-FL-β-catenin. As a control, we used HEK-293 cells transfected with an empty pCEP4 vector. In addition to endogenous GSK3β, ectopic expression of GSK3β greatly induced phosphorylation of β-catenin (by ~3-fold, Figure 6A, lane 2 versus lane 1), while the coexpression of B56α increased the phosphorylation level of β-catenin by ~60% (Figure 6A, lane 3 versus lane 2). However, when ΔNp63α was coexpressed with B56α, it led to a

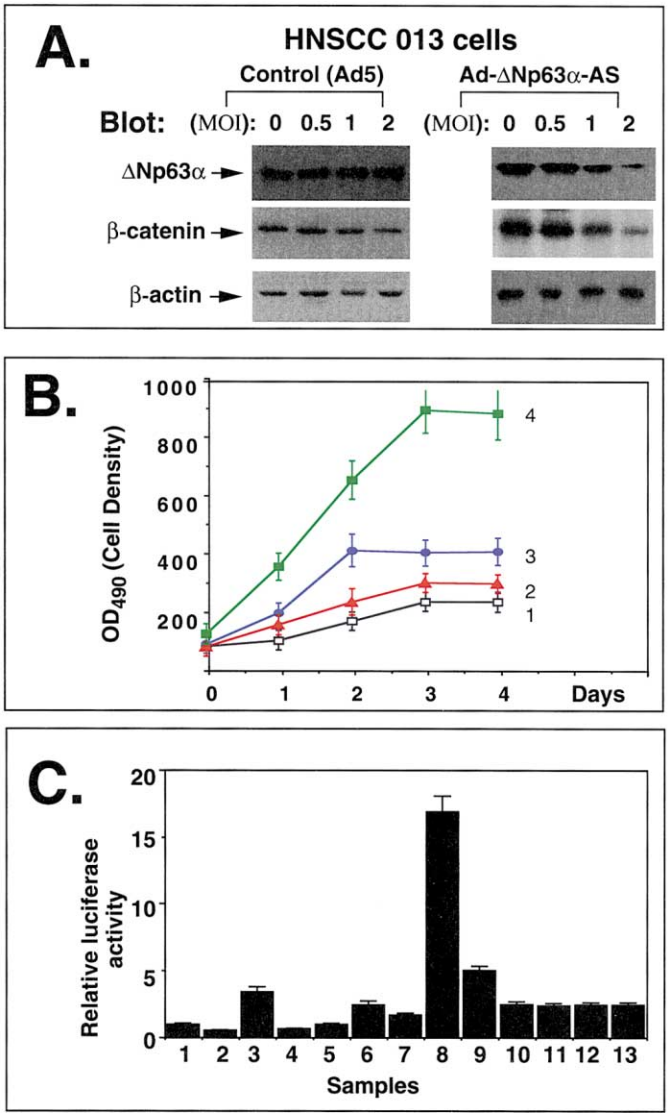


Figure 5. ΔNp63 induces β-catenin-mediated transcription and cell proliferation

A: The inhibitory effect of anti-sense ΔNp63α on the protein levels of β-catenin. HNSCC 013 cells (with endogenous ΔNp63α) were infected with variable amounts (MOI) of control Ad5 or Ad-ΔNp63α-AS as indicated (~95% efficiency). Total lysates (~30 μg) were analyzed by immunoblotting with antibodies to ΔNp63 (top panels) or β-catenin (central panels). Protein levels of β-actin served as loading controls (bottom panels). **B:** The expression of ΔNp63α-AS cell proliferation. HNSCC 012 (curves 1 and 3) or 013 (curves 2 and 4) cells were transfected (~80% efficiency) with 5 μg empty pCEP4 vector (curves 3 and 4) or pCEP4-ΔNp63α-AS expression construct (curves 1 and 2). Cell growth was assayed with the MTS/PMS nonradioactive kit (Promega), and cell density was followed for the times indicated. **C:** ΔNp63α dramatically increases the β-catenin-mediated induction of Lef-1: luciferase reporter activity. HEK-293 cells were transfected with the Lef-1: luciferase reporter (or mutant Lef-1: luciferase reporter) plasmid. All transfections included a CMV:β-galactosidase construct to control for transfection efficiency. Lef-1-dependent activity was defined as the ratio of activity from the wild-type Lef-1: luciferase reporter plasmid divided by luciferase activity from the mutant Lef-1: luciferase reporter plasmid. This value was normalized to Lef-1: luciferase activity obtained from control cells transfected with an empty pCEP4 vector (Sample 1). Luciferase activity was determined with luciferase reagent (Promega), and β-galactosidase was assayed using the GALACTON reagent (Tropix). Cells were cotransfected with various plasmids (Samples: 2, pCEP4-HA-B56α; 3, pCEP4-ΔNp63α; 4, pCEP4-ΔNp63α-AS; 5, TAp63α; 6, pCEP4-myc-tagged FL-β-catenin; 7, pCEP4-HA-B56α; 8, pCEP4-ΔNp63α; 9, pCEP4-ΔNp63α-AS; 10, pCEP4-TAp63α; 11, pCEP4-ΔNp63α-Δ20; 12, pCEP4-ΔNp63α-Δ41; 13, pCEP4-ΔNp63α-Δ74). All reporter assays were performed in triplicate (mean + SD is shown).

dramatic inhibition of GSK3β-mediated phosphorylation of β-catenin by ~8-fold (Figure 6A, lane 5 versus lane 2) and, therefore, to an accumulation of total β-catenin (Figure 6A). Interestingly, the addition of ΔNp63α alone to GSK3β and β-catenin had a slight inhibitory effect on the phosphorylation level of β-catenin (~15%, Figure 6A, lane 4 versus lane 2), suggesting that ectopic ΔNp63α may titrate down some endogenous PP2A regulatory proteins present in HEK-293 cells. Conversely, even ΔNp63α in the presence of B56α failed to induce accumulation of ΔN90-β-catenin, which lacks the residues normally phosphorylated by GSK3β (Figure 6A).

To further evaluate whether ΔNp63α affects GSK3β kinase activity, we transfected HEK-293 cells with individual plasmids: (i) an empty pCEP4 vector, (ii) GSK3β, (iii) B56α, (iv) ΔNp63α, and (v) β-catenin. Total lysates were mixed in various combinations and were preincubated for 30 min at 4°C, then complexes were immunoprecipitated with antibody to GSK3β, and the bead-bound proteins were incubated with [γ^{32}P]ATP and a prephosphorylated synthetic peptide (YRRAVPPSPSLSRHSSPH QSEDEE), as a substrate for GSK3β, as described (Ikeda et al., 1998). We observed (Figure 6B) that the addition of B56α (sample 3 versus sample 2) activated GSK3β kinase activity (by ~50%), while ΔNp63α alone (sample 4 versus sample 2) had little inhibitory effect on GSK3β activity (~10%). However, the addition of both B56α and ΔNp63α dramatically reduced GSK3β activity by ~40% compared to samples containing ectopic GSK3β alone (sample 5 versus sample 2) and reduced activity 4-fold less than a mix of GSK3β and B56α (sample 5 versus sample 3).

Immunoblotting analysis (Figure 6C) demonstrated that phosphorylation levels of the GSK3β protein itself changed accordingly in the presence of various components: levels decreased in the presence of B56α (by ~50%), slightly increased in the presence of ΔNp63α alone (10%), and greatly increased in the presence of both B56α and ΔNp63α (~3-fold), while ΔNp63α-AS successfully counteracted the effect of ΔNp63α (Figure 6C).

Since the B56α regulatory subunit was reported to activate PP2A, mediating GSK3β dephosphorylation and increasing its activity (Seeling et al., 1999), we examined whether the activation of β-catenin by ΔNp63α is due to the inhibitory effect of ΔNp63α on GSK3β or to its effect on PP2A, which could directly dephosphorylate β-catenin. ΔNp63α overexpression greatly decreased the phosphorylation levels of β-catenin in the presence of NaCl (~5-fold, Figure 7A, lane 2 versus lane 1) and without OA (okadaic acid) (~5-fold, Figure 7A, lane 4 versus lane 3), whereas inhibition of GSK3β with LiCl almost entirely inhibits β-catenin phosphorylation regardless of ΔNp63α levels (Figure 7A, lane 6 versus lane 5). Inhibition of PP2A with OA negligibly affects the phosphorylation levels of β-catenin similarly in both conditions (Figure 7A, lane 8 versus lane 7). The phosphorylation levels of β-catenin inversely correlate with β-catenin protein levels (Figure 7A). Regarding phospho-GSK3β, we observed that ΔNp63α increases the phosphorylation levels of GSK3β in the presence of NaCl or LiCl and in the absence of OA (~4- to 6-fold, Figure 7A, lanes 2, 4, and 6 versus lanes 1, 3, and 5, respectively), while inhibition of PP2A (+OA) led to a slight but equal increase of phospho-GSK3β under both conditions (~25%, Figure 7A, lane 8 versus lane 7). To directly test the effect of ΔNp63α on PP2A activity, we performed a phosphatase assay on HEK-293 cell lysates without or with ΔNp63α. Our

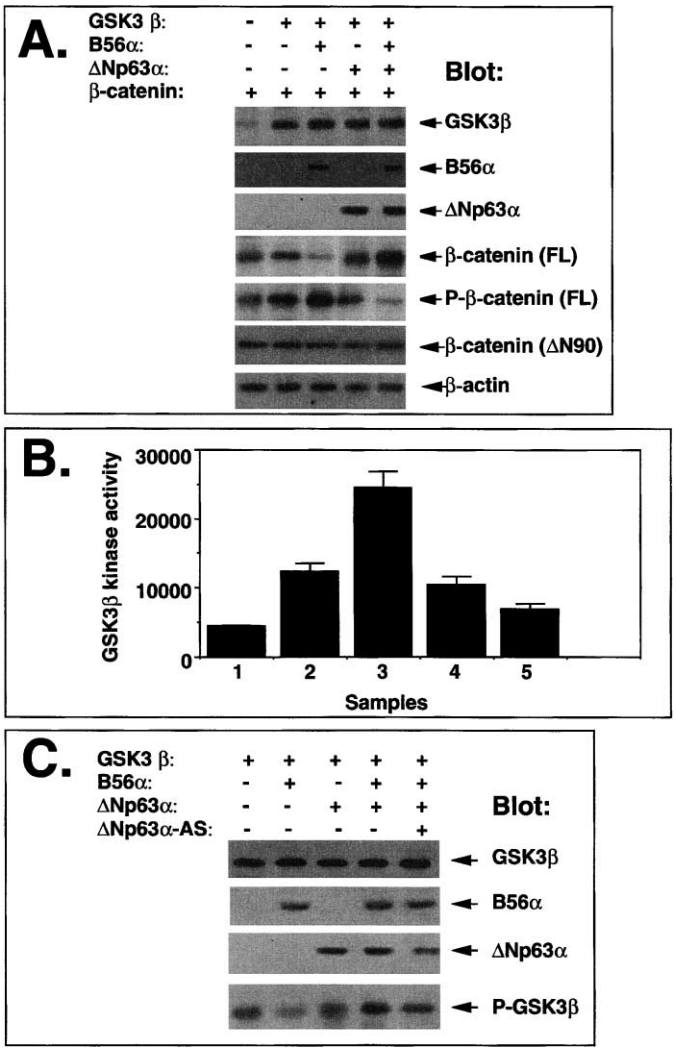


Figure 6. ΔNp63α inhibits GSKβ-mediated phosphorylation of β-catenin

A: HEK-293 cells were transfected (~75% efficiency) with plasmids (2 μg of each): (i) β-catenin (FL), (ii) GSK3β and β-catenin, (iii) GSK3β, B56α, and β-catenin, (iv) GSK3β, ΔNp63α, and β-catenin, and (v) GSK3β, B56α, ΔNp63α, and β-catenin. Total lysates (~50 μg) were resolved by 7.5% SDS-PAGE, followed by immunoblotting with the indicated antibodies. In some experiments, cells were transfected (instead of β-catenin [FL]) with the pCEP-ΔN90-β-catenin construct, which encodes a truncated β-catenin lacking the N-terminal region. Protein levels of β-actin served as loading controls. **B:** ΔNp63α inhibits GSKβ kinase activity in vitro. Samples: HEK-293 cells were transfected (~75% efficiency) with individual plasmids (2 μg of each): (i) empty pCEP4, (ii) GSK3β, (iii) B56α, (iv) ΔNp63α, and (v) β-catenin. Total lysates were prepared and mixed for 30 min at 4°C in various combinations as follows: (1) empty pCEP4; (2) pCEP4 plus GSK3β; (3) pCEP4 plus GSK3β and B56α; (4) pCEP4 plus GSK3β and ΔNp63α; and (5) pCEP4 plus GSK3β, B56α, and ΔNp63α. GSK3β immunoprecipitates (~200 μg total lysates) were assayed for kinase activity in triplicate. **C:** ΔNp63α inhibits the PP2A-mediated dephosphorylation of GSK3β. Saos-2 cells were transfected (~70% efficiency) with the following plasmids (1 μg each): (1) GSK3β and β-catenin, (2) B56α, GSK3β, and β-catenin, (3) ΔNp63α, GSK3β, and β-catenin, (4) GSK3β, β-catenin, B56α, and ΔNp63α, or (5) GSK3β, β-catenin, B56α, 1 μg ΔNp63α, and 5 μg ΔNp63α-AS. Immunoblotting of total lysates with the phospho-GSK3β (Ser9) antibody shows that the B56α regulatory subunit of PP2A mediates dephosphorylation of GSK3β, while ΔNp63α (in the presence of B56α) inhibits this process.

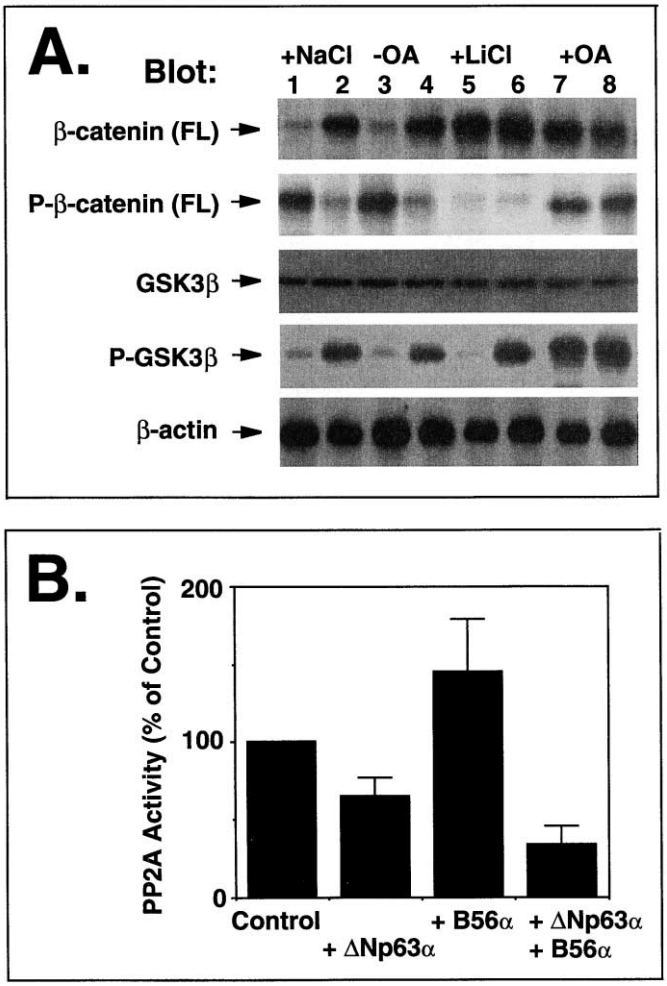


Figure 7. ΔNp63α inhibits phosphorylation of β-catenin through modulation of GSK3β reactivation

A: HEK-293 cells were transfected (2 μg each, ~70% efficiency) with an empty pCEP4 vector (lanes 1, 3, 5, and 7) or pCEP4-ΔNp63α (lanes 2, 4, 6, and 8) and were treated overnight with 30 mM NaCl or 30 mM LiCl (an inhibitor of GSK3β) or with 20 nM okadaic acid (OA, an inhibitor of PP2A). Total lysates (~50 μg) were resolved by 7.5% SDS-PAGE and were immunoblotted with the antibodies indicated. ΔNp63α increased GSK3β phosphorylation levels and conversely decreased β-catenin phosphorylation levels. However, inhibitor of PP2A negligibly affects these levels. **B:** ΔNp63α inhibits PP2A activity in vitro. Immunoprecipitates of PP2A (~200 μg total lysate) from HEK-293 cells transfected with an empty pCEP4 vector or pCEP4-ΔNp63α were analyzed for phosphatase activity as described. ΔNp63α inhibits β-catenin phosphorylation via PP2A-dependent inhibition of GSK3β rather than by activating PP2A to directly dephosphorylate β-catenin.

results clearly show an inhibitory effect of ΔNp63α on PP2A activity in vitro (Figure 7B).

Discussion

Recent reports show that ΔNp63α is the most abundantly expressed isotype in tumor cells, and it has been implicated in cell proliferation and oncogenic growth (Hibi et al., 2000; Parsa et al., 1999; Crook et al., 2000; Nylander et al., 2000; Park et al., 2000). The studies of p63 null mice further substantiate a critical role for p63 in regulating cell proliferation and in mediat-

ing epidermal stem cell renewal during early development (Yang et al., 1999; Mills et al., 1999). These findings raise the intriguing possibility that p63 functions as a regulator of cell growth in normal tissues and in cancer. Our studies now strongly suggest that ΔNp63 isotypes function as positive regulators of β-catenin signaling.

We have presented data demonstrating a physical interaction between ΔNp63 and B56α, GSK3β, and β-catenin. This association was initially shown for p40 (p63 core domain) and B56α in yeast by coimmunoprecipitation of the ectopically expressed p40 and B56α proteins and endogenous ΔNp63α, B56α, GSK3β, and β-catenin.

The B56α regulatory subunit associates with the N terminus of APC and, therefore, recruits the PP2A heteromeric enzyme to the APC complex consisting of APC, Axin, GSK3β, β-catenin, and other accessory elements (Seeling et al., 1999; Kikuchi, 2000; van Es et al., 2001). Moreover, B56α was shown to negatively regulate β-catenin signaling by tethering PP2A to GSK3β and thereby inducing the ability of GSK3β to phosphorylate β-catenin and target β-catenin into a proteasome degradation pathway (Seeling et al., 1999; Li et al. 2001a). The association of ΔNp63 with B56α prompted us to investigate the molecular mechanisms underlying the effect of p63 on β-catenin degradation mediated by B56α. We observed that overexpression of the ΔNp63 isotypes (p40 or ΔNp63α) dramatically increases the intranuclear accumulation of β-catenin. Moreover, ΔNp63α coprecipitated with B56α and GSK3β in the nuclei, while APC and Axin retained their cytoplasmic location. In addition, the ectopic expression of ΔNp63α led to inhibition of the GSK3β-mediated phosphorylation of β-catenin, apparently by directly affecting the ability of B56α to reactivate GSK3β activity and, therefore, blocking the degradation of β-catenin. Furthermore, the inhibitory effect of ΔNp63α on GSK3β counteracts and modulates the activation of GSK3β by B56α/PP2A, which can be seen in the dramatic intranuclear accumulation and decreased phosphorylation levels of β-catenin and increased phosphorylation (and reduced activity) of GSK3β mediated by ΔNp63α. Moreover, the association of ΔNp63α with B56α and β-catenin targets B56α, β-catenin, and GSK3β into the nucleus, while leaving APC and Axin in the cytoplasm. Thus, ΔNp63 isotypes appear to act as positive regulators of the β-catenin signaling pathway, perhaps by contributing to disassembly and inactivation of the APC degradation complex.

Interestingly, TAp63α is not able to affect the protein level of β-catenin, despite sharing the same carboxyl terminus as ΔNp63α. By comparative alignment of p40 and TAp63 isotypes, we observed that some portions of the B56α-interacting domain present in p40 are different compared to the TA isotypes (residues 1–6 and 8–12). Also, additional sequences (26 residues between 14 and 15) are present in TAp63 isotypes that are likely to affect the 3D configuration of this critical domain. In contrast to ΔNp63α, TA isotypes contain the N-terminal TA domain, which affects its stability and targets TAp63α into a proteasome degradation pathway (Osada et al., 2001). Many transcription factors are known to be unstable proteins that are destroyed by ubiquitin (Ub)-mediated proteolysis through the 26S proteasome (Thomas and Tyers, 2000; Salghetti et al., 2000, 2001). Recently, it was shown that two distinct regions mediating transcriptional activation (TA domain) or Ub-dependent degradation ("degron") of several transcriptional factors (i.e., p53; TA domain, residues 13–52; degron, residues 1–40) demonstrate structural overlap (Thomas and Tyers, 2000). Therefore, the TA domain is likely to play a dual role in transcriptional activation and subsequently its own destruction (Salghetti et al., 2001).

These and other features may account for the high abundance of the ΔNp63α isotype in human cancer cells (Parsa et al., 1999; Crook et al., 2000; Nylander et al., 2000; Park et al., 2000).

The intriguing association between p63 and B56α further emphasizes the role of these two proteins in both embryogenesis and the regulation of cell proliferation in adult tissues. Both embryogenesis and tumorigenesis rely on cell communication via the Wnt signaling pathway, resulting in the presence or absence of the intranuclear β-catenin (Barker and Clevers, 2000; Polakis, 2000; Taipale and Beachy, 2001). In the absence of the Wnt ligand, a multiprotein complex containing APC, GSK3β, and Axin normally facilitates the addition of phosphate groups to β-catenin by GSK3β, which, in turn, results in the ubiquitination of β-catenin and its proteasome-dependent degradation (Ikeda et al., 1998, 2000). Activation of the Wnt pathway results in disassembly of the APC-Axin complex and leads to inhibition of β-catenin degradation by decreasing the ability of GSK3β to phosphorylate β-catenin (Barker and Clevers, 2000; Polakis, 2000). This reduces β-catenin susceptibility to degradation, leading to its intranuclear accumulation. We have shown an inhibitory effect of ΔNp63α on GSK3β kinase activity. Moreover, ΔNp63α decreases the phosphorylation level and increases the nuclear abundance of β-catenin. In adenocarcinomas (e.g., colon cancers), the oncogenic mutations in genes encoding various components of the Wnt pathway (APC and Axin) and mutations of phosphorylation sites in β-catenin are common and increase the stability of β-catenin (Morin et al., 1997; Sparks et al., 1998; de La Coste et al., 1998). In SCC (e.g., lung cancer), these mutations are virtually absent; however, SCC cells often harbor *p63* gene amplification and/or overexpression of ΔNp63 isotypes (Hibi et al., 2000). P40-overexpressing xenografts with increased growth rates in vitro and in vivo (Hibi et al., 2000) were shown here to harbor higher β-catenin levels in nuclei compared to control xenografts. Thus, the increase in ΔNp63α, which induces intranuclear accumulation of β-catenin, represents an alternative mechanism of β-catenin signaling activation that induces positive regulation of β-catenin-responsive genes in SCC.

Interestingly, the aberrant intranuclear accumulation of β-catenin in tumors is often associated with mutational inactivation of the p53 tumor suppressor. The overexpression of p53, by either transfection or DNA damage, was recently shown to downregulate nuclear levels of β-catenin in human and mouse cells accompanied by inhibition of its transactivation potential (Sadot et al., 2001). This effect was not obtained with transcriptionally inactive mutant p53. The inhibitory effect of p53 on β-catenin is apparently mediated by the ubiquitin-proteasome system and requires an active GSK3β. Mutations in β-catenin, which compromise its phosphorylation and therefore the degradation by proteasomes or the inhibition of GSK3β activity, all rendered β-catenin resistant to downregulation by p53. These findings outline a negative-feedback loop involving β-catenin and p53, in which excess β-catenin induces the accumulation of p53, while high p53 levels downregulate β-catenin. In contrast to p53, ΔNp63 isotypes mediate intranuclear accumulation of β-catenin and, therefore, oppose the function of p53 as a tumor suppressor and support the function of ΔNp63 as an oncopro-

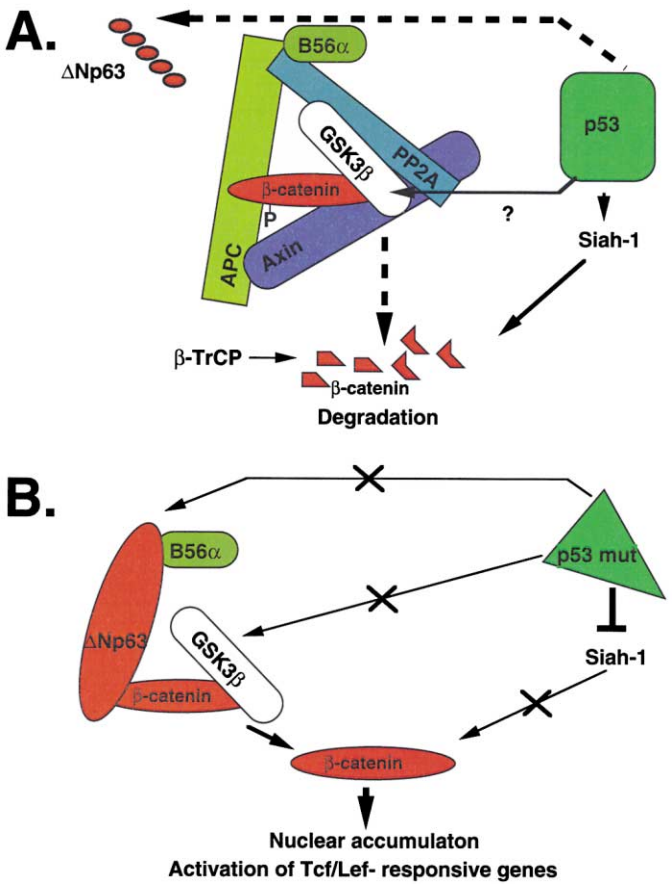


Figure 8. Opposing effects of p53 and ΔNp63 on β-catenin in normal and cancer cells

A: In normal cells, p53 induction leads to direct degradation of ΔNp63 (Ratovitski et al., 2001) and proposed activation of GSK3β (Sadot et al., 2001), resulting in β-catenin degradation. **B:** In SCC cancer cells, p53 mutations commonly lead to the accumulation of ΔNp63. ΔNp63 binds to B56α and alters its conformation, resulting in dramatic inhibition of GSK3β activity by modulating the PP2A-mediated reactivation of GSK3β and probably dismantling the APC complex. Thus, nonphosphorylated β-catenin is aberrantly accumulated in the nucleus, which leads to activation of β-catenin/Tcf/Lef-responsive genes, controlling tumor cell growth and contributing to the neoplastic phenotype. The organization of the APC complex is based on structural studies (Kikuchi, 2000; Spink et al., 2000; van Es et al., 2001).

tein. The inhibition of p53-mediated degradation of β-catenin may further unleash the oncogenic activity of β-catenin, thereby contributing to tumor progression. This effect may occur through inactivation of the *p53* gene (e.g., by mutation), leading to an increase in ΔNp63-mediated intranuclear accumulation of β-catenin (Figure 8).

We thus propose a schematic model in which induction of p53 (Figure 8A) leads to p53-mediated degradation of ΔNp63 isotypes (Ratovitski et al., 2001) and β-catenin (Sadot et al., 2001), resulting in abrogation of β-catenin signaling. For cancers, mutated p53 and/or overexpression of ΔNp63 lead to inactivation of GSK3β, resulting in a probable disassembly of the APC complex and aberrant intranuclear accumulation of β-catenin (Figure 8B). However, complementary GSK3β-independent mechanisms may also play a role in the regulation of β-catenin signaling. Recent observations showed that the p53-inducible gene *Siah-1* actively functions in the destruction of β-catenin and, therefore, in the downregulation of β-catenin signaling (Liu et al., 2001; Matsuzawa and Reed, 2001). Our initial experiments failed to demonstrate that ΔNp63α affects the expression of *Siah-1* (data not shown).

Other mechanisms may also underlie the oncogenic function of ΔNp63 isotypes and may contribute to tumorigenesis. For example, ΔNp63 proteins may function as dominant-negative regulators of p53-mediated transactivation of specific genes implicated in cell cycle control and apoptosis, as demonstrated by in vitro luciferase reporter assays (Yang et al., 1998). However, many primary SCC harbor p53 mutations accompanied by overexpression of ΔNp63 (Hibi et al., 2000; Yamaguchi et al., 2000). The common clonal expansion of cells that harbor both p53 inactivation and p63 amplification suggests that ΔNp63's oncogenic properties, as described here, extend beyond a dominant-negative effect on p53 function. The respective contribution of each mechanism underlying p63 oncogenic function in tumorigenesis remains to be fully explored.

Experimental procedures

Two-hybrid yeast expression screens

We screened the Hybri-Zap mouse embryonic cDNA library (B6: C57BL/6, 14.5, Stratagene) with the pGal4-BD-p40. Positive transformants were selected on a medium lacking tryptophan, leucine, and histidine and were identified by a β-galactosidase filter lift assay and by the quantitative β-galactosidase liquid assay using o-nitrophenyl-β-D-galactopyranoside as a substrate (Ratovitski et al., 2001).

Cells, antibodies, and plasmids

We used osteosarcoma cells (Saos-2), head and neck squamous cell carcinoma cells (HNSCC, line 012 and 013), and human embryonic kidney (HEK)-293 cells. Saos-2 and HEK-293 cells were obtained from American Tissue Culture Collection and were maintained accordingly. HNSCC 012 and 013 cells were defined at the Johns Hopkins Medical Institutions, grown, and harvested (at 60%–80% confluency) as described (Ratovitski et al., 2001). We used rabbit polyclonal antibodies to ΔNp63 (Oncogene Research), to phospho-(Ser33/37/Thr41)-β-catenin and phospho-(Ser9)-GSK3β (both from Cell Signaling Technology/New England Biolabs), and to APC (C-20); goat polyclonal antibodies to Axin (R-20), to PP2A-B56α (C-18), to DNA topoisomerase IIα (designated as TOPO II, L-17), and to β-TrCP/E3RS (C-18) (all from Santa Cruz Biotechnology); monoclonal antibodies to p63 (4A4) and α-tubulin (TU-02) (from Santa Cruz Biotechnology); monoclonal antibodies to β-catenin and GSK3β (Transduction Laboratories) and hemagglutinin (HA, 12CA5, Roche Molecular Biochemicals); and rabbit polyclonal antibody to catalytic subunit (C) of PP2A (Upstate Biotechnology). We used mammalian expression cassettes; pCEP4-HA-tagged B56α, pCEP4-FLAG-tagged GSK3β, pCEP4-myc-tagged FL-β-catenin, and pCEP4-myc-tagged ΔN90-β-catenin (kindly provided by David M. Virshup), pCEP4-TAp63α, pCEP4-ΔNp63α, pCEP4-p40, in which the expression of desirable proteins was driven by the cytomegalovirus (CMV) promoter. The full-length human Siah-1 cDNA was obtained from John C. Reed (through Science Reagents). For activation of Wnt signaling, we used CM from C57MG-MV-Wnt-1 mammary epithelial cells secreting Wnt-1 protein. This cell line infected with retroviral vector pMV-Wnt-1was kindly provided by Anthony M.C. Brown. To obtain CM from C57MG cells infected with MV-Wnt-1 or empty MV-7, cells were plated at a density of 10^5 per 10-cm^2 dish and were grown to confluence, at which time CM was harvested, centrifuged for 10 min at 2,000 × g, and frozen at –80°C. Target HEK-293 cells were plated at 10^5 cells per 6-cm^2 dishes and were grown to confluence. The culture medium was then replaced with 2 ml CM to be tested. After 24 hr, the cells were collected, and protein lysates were analyzed by immunoblotting or immunoprecipitation as described (Ratovitski et al., 2001). Blots were developed using the enhanced chemiluminescence method using Hyperfilm-ECL (Amersham). The films were scanned using an UltroScan XL laser densitometer (Pharmacia LKB Biotechnology).

Transfections, luciferase reporter, and proliferation assays

Cells were transiently transfected for 24 hr with plasmids expressing desirable proteins using Fugene-6 (Boehringer Mannheim) according to the manufacturer's recommendations.

Transfection efficiency was analyzed by the introduction of pCMV-β-galactosidase into cells and subsequent measuring of β-galactosidase activity. In some experiments, we used recombinant adenoviruses (Ad-ΔNp63α, Ad-ΔNp63α-AS, or an empty adenovirus, Ad5) to infect target cells for 18 hr with MOI as indicated (Ratovitski et al., 2001). Cells were resuspended in lysis buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.5% Brij-50, 1 mM PMSF, 0.5 mM NaF, 0.1 mM Na_3VO_4, 2× complete protease inhibitor cocktail), sonicated for 5 times for 10-s time intervals, and clarified for 30 min at 15,000 × g. Supernanatants were resolved by 10% (for APC in 5%) SDS-PAGE and were then analyzed by immunoblotting (~30–60 μg/lane) or immunoprecipitation (~150–200 μg/lane).

For the luciferase reporter assay, HEK-293 cells grown in 24-well plates were transfected with 0.01 μg LEF-1: luciferase reporter plasmid (wild-type or mutant) and 0.15 μg β-galactosidase plasmid. Various combinations of plasmids expressing desirable proteins were also introduced into the cells with the total amount not exceeding 1 μg. After 24 hr, transfected cells were lysed and clarified for 20 min at 15,000 × g, and luciferase activities were determined. Luminescence was measured using a TD-20e luminometer (Turner Design) from triplicate plates. The luciferase activities were normalized against the levels of β-galactosidase activity. Luciferase activity values obtained from the mutant Lef-1 reporter were subtracted from values obtained from the wild-type Lef-1 reporter and were latter plotted as relative fold values compared to control experiment designated as 1. The results represent data from three independent experiments, and error bars represent standard deviations.

Transfected HNSCC cells were assayed for cell growth using a CellTiter 96™ AQ_{ueous} nonradioactive colorimetric proliferative kit according to the manufacturer's recommendations (Promega). A total of 20 μl combined MTS:PMS (20:1) reagent was added to 96-well, cell-free plates (control) and plates with cells, and plates were incubated for 4 hr at 37°C. Plates were analyzed on plate reader Labsystems Multiscan MCC/340 at A_{490}–A_{690}. Data obtained from cell-free plates were subtracted from results obtained from plates containing cells, individually. All subtracted data were in quadruplicate.

Subcellular fractionation

Cells were resuspended in buffer A (10 mM KCl, 10 mM Tris [pH 7.9], 1 mM DTT, 250 mM sucrose, 60 μM PMSF with a proteinase inhibitor cocktail) at 4°C, incubated for 20 min on ice with swirling, and lysed by 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 3000 × g for 5 min, and the supernatant (cytoplasm) was clarified by centrifugation for 30 min at 15,000 × g. The first pellet (crude nuclei) was resuspended in 5 volumes of buffer A with 0.3% Nonidet P-40, and this suspension was mixed with an equal volume of 10 mM Tris (pH 8.0), containing 2.2 M sucrose, 5 mM magnesium acetate, and 0.1 mM EDTA. Purified nuclei were collected by centrifugation through the 2.2 M sucrose cushion. Nuclei were resuspended in buffer B (20 mM HEPES [pH 7.9], 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF with a proteinase inhibitor cocktail) plus 400 mM NaCl and were lysed by vigorous shaking on a Vortex mixer. For subsequent analysis, nuclear extracts were diluted with buffer B three times. Both nuclear and cytoplasmic fractions were analyzed for purity by immunoblotting with antibody to DNA topoisomerase IIα (nuclear marker) and antibody to α-tubulin (cytoplasmic marker).

Enzymatic assays

Following immunoprecipitation of the total lysates with antibody to GSK3β, the bead-bound proteins were washed in kinase assay buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiotreitol) and were incubated for 30 min at 30°C with 5 μCi [γ^{32}P]ATP (500–2000 cpm/pmol, Amersham-Pharmacia-Biotech) and a prephosphorylated synthetic peptide (YRRAVPPSPSL SRHSSPHQSEDEE) as a substrate for GSK3β, as described (Ikeda et al., 2000). Kinase reaction products were applied to Whatman paper, washed, and analyzed using a scintillation counter. All assays were performed in triplicate. Equivalent aliquots of each GSK3β immunoprecipitate used for kinase assay were analyzed by immunoblotting to ensure equal amounts of GSK3β protein. For the protein phosphatase assay, following immunoprecipitation of total lysates with antibody to PP2A(C), bead-bound proteins were washed with lysis buffer and then with assay buffer (50 mM Tris [pH 7.0], 0.1 mM $CaCl_2$), resuspended in assay buffer containing 2.5 mM $NiCl_2$ and 900 μg p-nitrophenyl phosphate/ml (Sigma), and incubated for 30 min at 30°C. The amount of p-nitrophenol produced was determined by measuring the absorbance at 405 nm.

Immunofluorescence imaging

Cells were than fixed in 3% paraformaldehyde for 2 min at 4°C, permealized with 0.5% Triton X-100 in PBS for 1 min, and blocked with blocking solution (PBS, 0.1% Tween 20, 5% normal goat serum) for 1 hr at room temperature. Cells were incubated with antibodies to ΔNp63α (1:500) or β-catenin (1:100) overnight at 4°C. Slides were washed with blocking solution and were incubated with FITC-conjugated anti-mouse (or anti-goat) immunoglobulins (1:1000) and rhodamine-conjugated anti-rabbit (or anti-mouse) immunoglobulins (1:1000) for 2 hr in the dark. DNA in nuclei was stained with 4, 6-diamino-2-phenylindole (DAPI, 1:10,000) for 5 min. Images were acquired using the DeltaVision system equipped with a Zeiss Axiovert 100 microscope and a Photometrics 300 cooled charge-coupled device camera.

Acknowledgments

The plasmids expressing HA-tagged B56α, FLAG-tagged GSK3β, myc-tagged-FL-β-catenin, myc-tagged-ΔN90-β-catenin, and Lef-1: luciferase reporter plasmids (wild-type and mutant) were a kind gift from David M. Virshup (Huntsman Cancer Institute). We are grateful to Bert Vogelstein (Johns Hopkins University School of Medicine) for many reagents and critical comments. We thank L. Lee (Denver Health Medical Center) for CUSP (ΔNp63α) cDNA and S. Ikawa (Institute of Development, Aging and Cancer, Tohoku University) for TAp63α p51B. The C57MG mammary epithelial cells (expressing Wnt-1) were kindly provided by Anthony M.C. Brown (Cornell University Medical College).

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