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# A Role For P63 In The Regulati On Of Cell Cycle Progression And Cell Death

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A DISSERTATION

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ABSTRACT

p63 is a member of the p53 family of transcription factors that is a critical regulator of epithelial development. Studies have shown that p63 does not appear to function as a classical tumor suppressor like p53. Instead, the expression pattern of p63 in cancers suggests that p63 retains the potential to function as either a tumor suppressor or an onco-protein. Here, we provide evidence describing the transcriptional activity of the p63 isoforms and provide mechanisms whereby p63 function is regulated in a context dependent manner. Our data shows that both the ΔNp63 and TAp63 variants retain the potential to regulate transcription and inhibit proliferation. We suggest that the intrinsic transactivation potential of p63 isoforms is established at the level of transcription. Promoter selection determines which N-terminal activation domain is encoded in the p63 protein. For example, we propose that transactivation by ΔNp63 is more dependent upon co-activator binding to the C-terminal proline-rich region than the TAp63 variant that possesses the strong, acidic activation domain. In addition, alternative splicing regulates expression of either the α, β, or γ C-termini. The α C-terminus has been previously described as inhibitory. Here we demonstrate that expression of the PPXY motif in the α and β, but not γ, termini affords additional transactivation potential to these isoforms. Finally, we show that p63 transactivation potential is dependent upon PXXP and PPXY motifs suggesting that signaling pathways can modulate p63 function through post-translational modifications or protein-protein interactions occurring at these proline-rich motifs.

## DEDICATION

This dissertation is dedicated to the memory of my father Leon Gavin Helton, who died at the age of 65 on October 20th 2006, after a long battle with prostate cancer. He will forever be remembered as a loving father and trusted friend, possessed of unequalled generosity to both his friends and family. His commitment to excellence in the field of anesthesia is evidenced by the lives he saved and the knowledge he imparted with fellow anesthesia professionals and students. He will be sorely missed by all that knew him.

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I would like to convey my genuine appreciation for the mentorship provided by Professor Xinbin Chen. Foremost, I would like to express my gratitude for his patience and commitment during my doctoral training. His enthusiasm for science has been an inspiration during my doctoral training, and the lessons that I have learned under his tutelage will serve to guide me throughout my scientific career.

I am grateful for the effort put forth by the members of my dissertation committee: Chenbei Chen, David F. Crawford, J. Michael Ruppert, and Rosa A. Serra. Their guidance and encouragement throughout my training were thankfully accepted. I am appreciative to all of the members of Professor Xinbin Chen's laboratory, both current and former: Amy Willis Amara, Alana Cozier, Anita Chen, Kelly McNaughton Harms, Eun Joo Jung, Gang Liu, Susan Nozell, Yingjuan Qian, Ariane Scoumanne, Limin Shu, Teresa Wakefield, Hui Xiao, Yang Xu, Wensheng Yan, Jin Zhang, and Jianhui Zhu. Your constant companionship and exemplar performance were a constant source of encouragement over my graduate career.

I am indebted to my family members who have been a constant source of support throughout my life. My parents, Susan Bream and Leon Gavin Helton, provided a loving home for their children.

The work that I have presented in this dissertation is the culmination of countless hours spent apart from my wife, Amanda Jill Helton. She is the love of my life. Any advancement that I might make in the field of scientific endeavors is only achieved at great personal sacrifice by me and my wife.

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# INTRODUCTION

p63 is a member of the p53 family of sequence-specific transcription factors. Identified in 1998 by multiple laboratories, early nomenclature for p63 includes p51, p40, p73-like (p73L), keratinocyte transcription factor (KET), chronic ulcerative stomatitis protein (CUSP) and amplified in squamous cell carcinoma (AIS) [Reviewed in 1].

## *Comparison of p53 Family Members*

In addition to p63, the p53 family of sequence-specific transcription factors includes p53 and p73. These family members share strong identity in their activation domains (AD), DNA-binding domains (DBD), and tetramerization domains (TD) (Fig. 1). Interestingly, phylogenetic analysis across higher eukaryotes reveals that p53 family members are derived from a common ancestor more closely resembling the sterile alpha motif (SAM) containing TAp63α isoform [2]. In addition, while the *TP53* locus

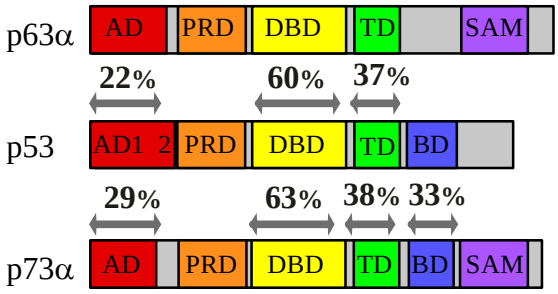


**Fig. 1. p53 family members share strong sequence similarity.**
Abbreviations: Activation Domain (AD), Proline-Rich Domain (PRD), DNA-Binding Domain (DBD), Tetramerization Domain (TD) Basic Domain (BD), and Sterile-Alpha Motif (SAM).

primarily expresses a single p53 protein with strong transactivation potential, both the *TP63* and *TP73* loci express multiple isoform with variable abilities to affect transcription [reviewed in 1]. Significantly, all p53 family members bind to the consensus p53-response element (p53 RE), RRRC(A/T)(T/A)GYYY, where R is any purine and Y is any pyrimidine [3], and regulate expression from p53-target gene promoters [2]. In addition, recent studies have identified target genes that are specifically bound by p63 and 73, but not p53. These target genes include: envoplakin and SMARCD3 [4], pigment epithelium-derived factor (*PEDF*) [5], the antioxidant glutathione reductase (*GPX2*) [6], sonic hedgehog (*SHH*) [7], *WNT4* [8], and the vitamin D receptor (*VDR*) [9]. Significantly, regulation of specific target genes provides a mechanism whereby p63 and 73 functions can functionally diverge from that of p53.

*Review of p53 function*

p53 functions as a sequence-specific transcription factor that functions as a potent tumor suppressor. Under normal cell growth conditions p53 levels are kept low by the E3 ubiquitin ligase, mouse double minute (MDM2), that ubiquitinates p53 and targets p53 for proteosomal degradation. DNA damage, oncogene activation, and other forms of cellular stress stabilize and activate p53, promoting nuclear accumulation and p53 transactivation of both cell survival and cell death pathways. Structurally, p53 is organized into several domains, each of which contributes to p53 transcriptional activity. p53 contains two amino-terminal activation domains, AD1 within residues 1 to 42 and AD2 within residues 43 to 92 including the proline-rich domain, which enable p53 to form direct associations with transcriptional co-activators. The DNA-binding domain

(DBD) allows for sequence-specific recognition of response elements in p53 target gene promoters. The tetramerization domain (TD) directs formation of the p53 tetramer required for DNA binding. Finally, the carboxy-terminal basic domain (BD) has a regulatory function by controlling p53 stability, transcriptional activity, and sequence independent DNA-binding [10].

p53 is a critical tumor suppressor as evidenced by the fact that greater than 50% of tumors are p53 null. During normal cell growth, p53 function is inhibited primarily by MDM2, an E3-ubiquitin ligase that promotes proteosomal degradation of p53 [reviewed in 11]. DNA damage, oncogene activation, or other forms of cellular stress stimulate signaling pathways that stabilize and activate p53 through a series of post-translational modifications and protein-protein interactions [reviewed in 12, 13].Once p53 accumulates in the nucleus and induces target genes that promote either cell survival, including those regulating cell cycle arrest (e.g., *P21*, *BTG2*, and *GADD45*) and DNA repair (e.g., *XPC*, *XPE*, *POLH*, and *MSH2*), or cell death (e.g., *BAX*, *PUMA*, *PIG3*, and *FDXR*) [reviewed by 14, 15]. Loss of p53 function either through mutation, gene silencing, or oncogene inactivation promotes genomic instability and increases the likelihood of tumor formation. For example, p53 knockout mice are phenotypically normal at birth, but display a high rate of gene amplifications and spontaneous tumor formation [reviewed in 16]. Similarly, Li-Fraumeni syndrome patients carrying germline mutations in the *TP53* locus are prone to develop chromosome abnormalities and have a higher incidence of tumor formation [17]. In conclusion, p53 appears to possess dual functions as both a pro-survival and pro-apoptotic protein. Having been termed “the defender of the genome,” p53 is well known to promote genomic integrity by initiating cell cycle arrest, promoting

DNA repair, inhibiting excess recombination, and triggering apoptosis. Still, additional studies are required to define the pathways involved in p53 regulation and to determine the functional interaction of p53 with its other family members, p63 and p73.

*Characterization of the TP63 locus*

The *TP63* locus is capable of expressing two major variants, called TA and ΔN, both of which have multiple isoforms as a result of alternative splicing at the carboxy-terminus. Transcribed from the upstream promoter, the TA variant contains an acidic, transcriptional activation domain (AD) similar to p53. In contrast, the ΔNp63 variant, transcribed from the cryptic promoter in intron 3, lacks the activation domain encoded by exons 2 and 3, but gains fourteen unique residues in its N-terminus. *TP63* also encodes for a PRD, DBD, and TD homologous to p53, however C-terminal sequences in p63, including a sterile alpha motif (SAM), differ significantly from that of p53 and may

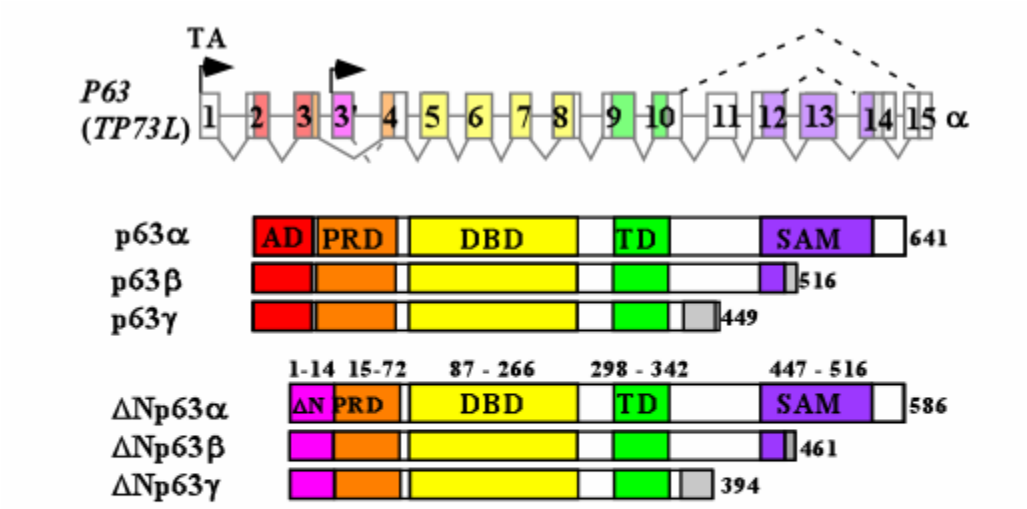


**Fig. 2. The *TP63* locus expresses multiple gene products.**
Abbreviations: Activation Domain (AD), Proline-Rich Domain (PRD), DNA-Binding Domain (DBD), Tetramerization Domain (TD), and Sterile-Alpha Motif (SAM).

contribute to unique p63 functions [18]. Alternative splicing of p63 transcripts results in expression of at least three C-termini, α, β, and γ. Thus, the *TP63* gene is capable of producing at least six transcripts: p63α, p63β, p63γ, ΔNp63α, ΔNp63β, and ΔNp63γ (Fig. 2) [reviewed in 1].

*Determination of p63 functio*n

p63 is a member of the p53 family of transcription factors. Identified in 1998 by multiple laboratories, early nomenclature for p63 includes p51, p40, p73-like (p73L), keratinocyte transcription factor (KET), chronic ulcerative stomatitis protein (CUSP) and amplified in squamous cell carcinoma (AIS) [Reviewed in 1]. Like p73, another p53 family member, p63 is competent to bind to p53 RE. Accordingly, p63 is able to regulate many p53 target genes. However, determining p63 function is difficult because *TP63* can express multiple gene products with different transactivation potentials. In addition, p63 expression is temporally and spatially regulated (19, 20), whereas p53 is ubiquitously expressed. Finally, very little is known about the mechanisms that regulate p63 function.

*Role of p63 in development*

A role for p63 in development has been well documented. *TP63* null mice display defects in ectodermal development and fail to maintain a population of epithelial stem cells [19, 20]. Furthermore, overexpression of TAp63α in the epidermis has been shown to induce epithelial stratification [21]. In contrast to *TP53*, mutations in the *TP63* gene are linked to developmental disorders and not spontaneous tumor formation. Mutations in p63 are associated with ectrodactyly, ectodermal dysplasia, and cleft

lip/palate syndrome (EEC3), limb-mammary syndrome (LMS), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT), ankyloblepharon-ectodermal dysplasia-cleft (AEC), split hand/split foot malformations (SHFM), and Rapp-Hodgkin's syndrome (RHS) [Reviewed in 22] (Fig.2). Recently, p63 has been shown to regulate expression of *WNT4*, *VDR*, and *SHH* providing potential mechanisms whereby p63 can regulate development [7, 8, 9].

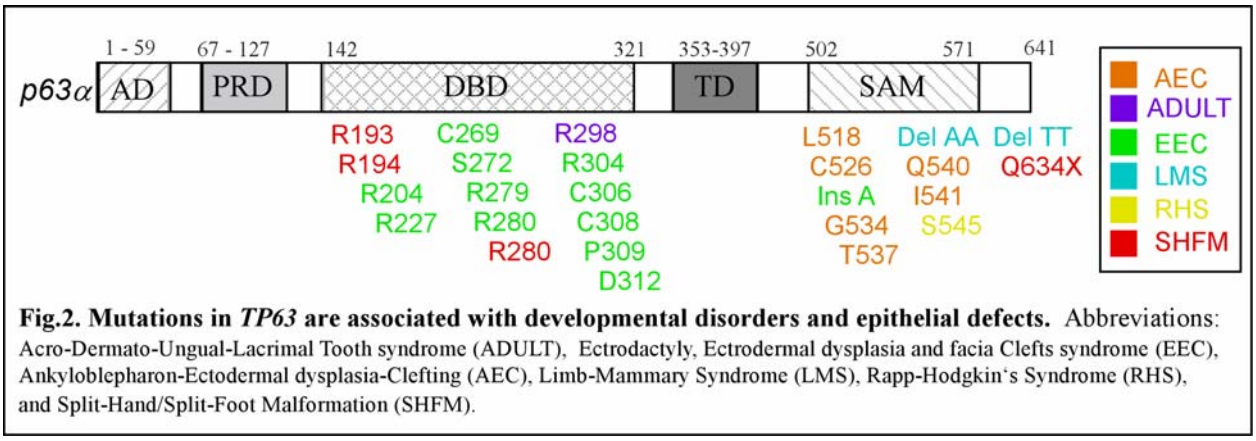


**Fig.2. Mutations in *TP63* are associated with developmental disorders and epithelial defects.** Abbreviations: Acro-Dermato-Ungual-Lacrimal Tooth syndrome (ADULT), Ectrodactyly, Ectrodermal dysplasia and facia Clefts syndrome (EEC), Ankyloblepharon-Ectodermal dysplasia-Clefting (AEC), Limb-Mammary Syndrome (LMS), Rapp-Hodgkin's Syndrome (RHS), and Split-Hand/Split-Foot Malformation (SHFM).

*Role of p63 in Cancer*

Initial studies failed to classify p63 as a classical tumor suppressor since p63 mutations could not be associated with increased tumorigenesis [23]. In fact, early studies suggested that ΔNp63α was an oncogene since overexpression of ΔNp63α in RatE1A cells promoted cell proliferation in vitro and increased tumor growth upon mouse xenograft transplantation [24]. In contrast conditional overexpression of ΔNp63α in mice did not alter cell proliferation, but did protect cells from apoptosis after UV-B treatment, demonstrating that ΔNp63α is not transforming when expressed in the epidermis [25]. Interestingly, subsequent studies from the same laboratory found that the

TA variant p63α induced epithelial stratification [21]. Furthermore, reactivation of p63α during chemically-induced skin carcinogenesis promoted Twist and N-cadherin expression consistent with cells undergoing epithelial to mesenchymal transitions and resulted in an increased incidence of spindle cell carcinomas and lung metastases [26]. Altogether these data suggest that p63 function is context dependent. Thus several factors including cell type, micro-environment, and specific isoforms expressed must be considered when drawing conclusions on p63 function.

# THE UNIQUE $NH_2$-TERMINALLY DELETED (ΔN) RESIDUES, THE P*XX*P MOTIF, AND THE PP*X*Y MOTIF ARE REQUIRED FOR THE TRANSCRIPTIONAL ACTIVITY OF THE ΔN VARIANT OF p63

by

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## Abstract

p63, a member of the p53 family of transcription factors, is known to be involved in epithelial development. However, its role in tumorigenesis is unclear. Contributing to this uncertainty, the *TP63* locus can express multiple gene products from two different promoters. Utilization of the upstream promoter results in expression of the TAp63 variant with an activation domain similar to p53. In contrast, the $NH_2$-terminally deleted (ΔN) p63 variant, transcribed from a cryptic promoter in intron 3, lacks such an activation domain. Thus, the TAp63 and ΔNp63 variants possess a wide ranging ability to up-regulate p53 target genes. Consequentially, the disparity in transactivation potential between p63 variants has given rise to the hypothesis that the ΔNp63 variant can serve as onco-protein by opposing the activity of the TAp63 variant and p53. However, recent studies have revealed a transcriptional activity for ΔNp63. This study was undertaken to address the transcriptional activity of the ΔNp63 variant. Here, we showed that all $NH_2$-terminally deleted p63 isoforms retain a potential in transactivation and growth suppression. Interestingly, ΔNp63β possesses a remarkable ability to suppress cell proliferation and transactivate target genes, which is consistently higher than that seen with ΔNp63α. In contrast, ΔNp63γ has a weak or undetectable activity dependent upon the cell lines used. We also demonstrate that an intact DNA-binding domain is required for ΔNp63 function. In addition, we found that the novel activation domain for the ΔNp63 variant is composed of the 14 unique ΔN residues along with the adjacent region, including a P*XX*P motif. Finally, we demonstrated that a PP*X*Y motif shared by ΔNp63α and ΔNp63β is required for optimal transactivation of target gene promoters, suggesting that the PP*X*Y motif is requisite for ΔNp63 function.

# Introduction

p53 functions as a tumor suppressor by transactivating target genes that mediate cell cycle arrest, apoptosis, and other p53-dependent activities. In response to DNA damage, oncogene activation, or other forms of cellular stress, p53 is stabilized by a complex series of post-translational modifications and protein-protein interactions that allow p53 to carry out its tumor suppression activity. Structurally, p53 is organized into several domains, each of which contributes to p53 transcriptional activity. p53 contains two amino-terminal activation domains, AD1 within residues 1–42 and AD2 within residues 43–92, including the proline-rich domain which enables p53 to form direct associations with transcriptional co-activators. The DNA-binding domain allows for sequence-specific recognition of response elements in p53 target gene promoters. The tetramerization domain directs formation of the p53 tetramer required for DNA binding. Finally, the carboxyl-terminal basic domain has a regulatory function by controlling p53 stability and transcriptional activity (reviewed in Ref. 1).

p63, a member of the p53 family of transcription factors, was identified in 1998 (2, 3). Like p53, p63 contains a proline-rich domain, a DNA-binding domain, and a tetramerization domain, all of which share strong sequence similarity with p53 (reviewed in Ref. 4). Accordingly, p63 is able to regulate many p53 target genes. However, due to two transcriptional start sites, the *TP63* locus is capable of expressing two major variants, called $TA^2$ and ΔN, both of which have multiple isoforms as a result of alternative splicing at the COOH terminus. Transcribed from the upstream promoter, the TA variant contains an acidic, transcriptional activation domain (AD) similar to p53 and is able to transactivate p53 target genes as well as inhibit cell proliferation and induce apoptosis. In contrast, the ΔNp63 variant, transcribed from the cryptic promoter in intron 3, lacks the

activation domain encoded by exons 2 and 3 but gains 14 unique residues in its $NH_2$ terminus. Alternative splicing of p63 transcripts results in expression of at least three COOH-terminal isoforms, α, β, and γ. Thus, the *TP63* gene is capable of producing at least six transcripts: p63α, p63β, p63γ, ΔNp63α, ΔNp63β, and ΔNp63γ (Fig. 1*A*).

The discovery of p53 family members, p73 in 1997 (5) and p63 in 1998 (2, 3), was first thought to introduce two new tumor suppressors in the fight against cancer. Although enthusiasm was initially tempered by findings that p63 and p73 do not function as classic tumor suppressors, recent evidence suggests that p63 might serve an important role in tumor suppression. Amplification and overexpression of p63 has been linked to increased survival rates in lung cancer patients (6), and loss of p63 expression has been linked to an increase in metastasis in bladder (7–9) and breast cancers (10). Likewise, fibroblasts derived from p63 knock-out mice were shown to be defective in the p53-mediated apoptotic response (11). In addition, a follow up study has demonstrated that mice heterozygous for p63 developed an increased tumor burden and metastasis rate, which was compounded in mice harboring heterozygous alleles of p53 and/or p73 (12).

Among all p63 isoforms, ΔNp63α is the most predominantly expressed. Our laboratory and others have demonstrated that ΔNp63α retains transcriptional activity and is competent to induce growth suppression (13). When expressed in H1299 cells, ΔNp63α is able to inhibit cell proliferation, induce cell death, and up-regulate GADD45 (growth arrest and DNA damage-45) and p21. Importantly, these activities are dependent on the presence of the $NH_2$ terminus, since the ΔΔNp63α construct lacking the first 26 amino acids was nonfunctional. These findings have forced the scientific community to reconsider the role of ΔNp63α in tumor formation and development. Evidence supporting

a transcriptional activity for ΔNp63α has been demonstrated by others. For example, ΔNp63α was shown to function both as a positive regulator of p53-responsive promoters and as a negative regulator of maturation-specific genes during $Ca^{2+}$-induced keratinocyte differentiation (14). Similarly, small interfering RNA knockdown of ΔNp63α in immortalized mammary epithelial cells revealed that ΔNp63α promotes transcription of PUMA and NOXA but negatively regulates the *P21* and *P53* promoters (15). Finally, ΔNp63α activates the *HSP70* promoter, as well as induces hsp70 protein expression (16).

Whereas a tumor suppressor function has recently been ascribed to p63, previous studies have suggested that p63 might function as an oncogene. For example, ΔNp63α was characterized as transcriptionally incompetent and shown to be a dominant negative to TAp63 and p53 in luciferase reporter assays (3, 17). In addition, loricrin promoter-driven expression of ΔNp63α in the epidermis of transgenic mice hindered apoptosis induced by UV-B but did not alter epithelial cell proliferation or stratification (18). Furthermore, chromosomal arm 3q, which contains the *TP63* locus, is amplified, and the ΔNp63α transcript is overexpressed in lung cancer and squamous cell carcinomas of the head and neck (19). Likewise, TAp63 isoforms are overexpressed in certain lymphomas (20).

Debate on the function of ΔNp63α will continue until regulation of p63-mediated target gene expression is elucidated. To gain a better understanding of the diverse activities found in the p63 isoforms, we mutated or deleted specific regions in p63. By comparing the transcriptional activity of wild-type and mutant p63 proteins in stable, inducible cell lines and in transient expression studies, we have characterized several functional domains that regulate ΔNp63 transactivation potential. Our results

demonstrated that ΔNp63α and ΔNp63β are capable of inducing target gene expression and inhibiting cell proliferation. We showed that these activities are dependent upon DNA binding, since DNA-binding domain mutants were inactive. In addition, we have defined the activation domain in ΔNp63, which includes the 14 unique ΔN residues and the adjacent proline-rich domain. Finally, our results demonstrate that a PP*X*Y motif, present in ΔNp63α and ΔNp63β, modulates ΔNp63 function.

## Experimental Procedures

### *Reagents*

HF-Taq polymerase and tetracycline-free fetal bovine serum were obtained from Clontech. The dual luciferase kit was obtained from Promega. The site-directed mutagenesis kit was obtained from Stratagene. Primary anti-Myc antibody was collected from the 9e10.2 hybridoma cell line. The p21(C-19) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit anti-β-actin antibody and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma. Unless otherwise indicated, all remaining reagents were purchased from Sigma.

### *Plasmids and Mutagenesis*

Various ΔNp63 constructs were generated by PCR amplification using Myc-tagged ΔNp63α or ΔNp63β cDNA as a template. To generate ΔNp63β(Δ2–14), a cDNA fragment encoding residues 15–211 was amplified using 5' end primer p63–58 (5'-GAA TTC CTC GAG CCA CAG TAC ACG AAC CTG GG-3') and 3' primer p63-2 (5'-TTG TAC AGG ACT GTT GTG AA TTC AG-3'). To generate ΔNp63β(Δ2–19), a cDNA

fragment encoding residues 20–211 was amplified using 5' end primer p63-67 (5'-ACT CGA GCT GGG GCT CCT GAA CAG C-3') and the 3' primer p63-2. To generate ΔNp63α(N6H), a cDNA fragment encoding residues 2–211 was amplified using 5' end primer p63-26 (5'-CCT CGA GTT GTA CCT GGA ACA CAA TGC CCA GAC TC-3') and the 3' primer p63-2. These cDNA fragments were digested with XhoI and BsrGI and used to replace the corresponding region in the wild-type construct.

To generate ΔNp63(Δ457-ter), a cDNA fragment encoding residues 199–456 was amplified using 5' end primer p63-59 (5'-CCA CAG GTT GGC ACT GAA TTC ACA ACA GTC C-3') and 3' primer p63-60 (5'-TGG ATC CTC AGA CAA TGC TGC AGT CTG TGG-3'). To generate ΔNp63(Δ431-ter), a cDNA fragment encoding residues 199–430 was amplified using the 5' end primer p63-59 and 3' primer p63-61 (5'-TGG ATC CTC AAG CTT GGG TAG GGC TGA GTC C-3'). To generate ΔNp63(Δ406-ter), a cDNA fragment encoding residues 199–405 was amplified using the 5' end primer p63-59 and 3' primer p63-62 (5'-TGG ATC CTC ACA TGC CCT CAG GCA TGG-3'). To generate ΔNp63(Δ381-ter), a cDNA fragment encoding residues 199–380 was amplified using the 5' end primer p63-59 and 3' primer p63-63 (5'-TGG ATC CTC AGA CAA TGC TGC AGT CTG TGG-3'). To generate ΔNp63(Δ356-ter), a cDNA fragment encoding residues 199–355 was amplified using 5' end primer p63-59 and 3' primer p63-355 (5'-ACG GAT CCT CAT TTC TGA AGT AGG TGC TG-3'). To generate ΔNp63β(Y449A), a cDNA fragment encoding residues 199–456 was amplified using the 5' end primer p63-59 and 3' primer p63-71 (5'-TGG ATC CTC AGA CTT GCC AAA TCC TGA CAA TGC TGC AGT CTG TGG GGG CGG GCG GTG GTG GGG T-3'). To generate ΔNp63β(Y449D), a cDNA fragment encoding residues 199–456 was amplified

using the 5' end primer p63-59 and 3' primer p63-72 (5'-TGG ATC CTC AGA CTT GCC AAA TCC TGA CAA TGC TGC AGT CTG TGG GGT CGG GCG GTG TGG GT-3'). To generate ΔNp63β(Δ445–449), a cDNA fragment encoding residues 199–456 was amplified using the 5' end primer p63-59 and 3' primer p63-73 (5'-TGG TCC CAG CTT CCA ATC TGA AAT CTG AGT TGT GGG TGC GTG GAG -3'). These cDNA fragments were digested with BamHI and BsrGI and used to replace the corresponding region in the wild-type construct.

To generate ΔNp63α(R149W), a cDNA fragment encoding residues 135–215 was amplified using 5' end primer p63-1 (5'-CCT TCT CAA AAA CTG GCA GTC CCG GGT GTG AAT GTG CCT ACC T-3') and the 3' primer p63-2. This cDNA fragment was digested with AccI and BsrGI and used to replace the corresponding region in the wild-type construct.

To generate ΔNp63α(L22Q,L23S), a cDNA fragment encoding residues 35–215 was amplified using 5' end primer p63-5QS (5'-TTC GAA GGA CCT GTC ACC GCC-3') and the 3' primer p63-2. An additional cDNA fragment encoding residues 1–34 with point mutations at residues 22 and 23 (L22Q,L23S) was amplified from wild-type ΔNp63α in pcDNA3 using 5' end primer T7 (5'-TAA ACG CTC CTA AGG -3') and 3' primer p63-3QS (5'-GGA GAG ATG GTT TGA TCT CTG TCC TGC GTT GAC GCC CAG TTC-3'). Following digestion with EcoRI and BamHI, a three-piece ligation was used to replace the corresponding region in the wild-type construct.

Site-directed mutagenesis was performed using the Stratagene QuikChange kit. To generate ΔNp63α(R243Q), the entire construct was amplified using 5' end primer (5'-GCA GTC TGG CCA CGG GCT TGA G-3') and 3' end primer (5'-CGT CAG ACC GGT

GCC CGA ACT C-3'). To generate ΔNp63α(R249W), the entire construct was amplified using 5' end primer (5'-CGG GCT TGA GCC GGA CTG GCT GCC A-3') and 3' end primer (5'-GGG AAG ACA ATC AGG CTC AAG ACC -3'). To generate ΔNp63α(P72A), the entire construct was amplified using 5' end primer (5'-CTC ATC CCT CCA TGC TCC ACA AGA TAC CG-3') and 3' end primer (5'-CGG TAA CTG GTT GAG CAA GGC GGG ATG AG-3'). To generate ΔNp63α(ΔP*XX*P), in which residues thus allowing for deletion of residues 69–72 are deleted, the entire construct was amplified using 5' end primer (5'-GCC TCT TCC TCC CCA CAC GAT ACC G-3') and 3' end primer (5'-CGG TAA CTG GTT GAG ATG AGA AGG C-3'). All mutations were confirmed by DNA sequencing.

The reporter constructs, pGL2-basic-p21 (full-length promoter), pGL2-Fos-GADD45 (intron 1 RE), and pGL2-basic-FDXR (ferredoxin reductase) (–56 to +11 promoter) were described in previous studies (21–23).

### *Cell Culture and Stable Transfections*

Stable cell lines were generated using the tetracycline-off system as previously described (24). Transfections were carried out using the calcium phosphate precipitation method (25).

### *Growth Curve Analysis*

The overall viability of each cell line was addressed by growth curve analysis, as previously described (26). Briefly, $5 \times 10^4$ H1299 and $1 \times 10^5$ MCF-7 cells were plated per 6-cm dish and either induced (without tetracycline) or un-induced (with tetracycline).

Adherent cells were counted at 24-h intervals over 5 days in culture using a Coulter cell counter. Medium was changed at day 3 for both control and experimental cell groups.

### *DNA Histogram Analysis*

The assay was carried out using a FACS-Caliber cell sorter (BD Biosciences) as previously described (24). Briefly, $3 \times 10^5$ cells were plated per 10-cm dish. H1299 and MCF-7 cells were induced or un-induced to express various ΔNp63 proteins for 3 days. Trypsinized, adherent cells and floating cells were combined, washed with phosphate-buffered saline, pH 7.4 (PBS), and fixed in 100% ethanol. When ready to stain, cells were washed with PBS and resuspended in staining buffer with 100 µg/ml RNase A and 50 µg/ml propidium iodine (Molecular Probes, Inc., Eugene, OR). RNA was digested, and cells were stained for 30 min at room temperature. The percentage of cells in each phase of the cell cycle ($G_1$, S, and $G_2$-M) was analyzed using Cell Quest software, with sub-$G_1$ accumulation being used as a marker for apoptosis.

### *Trypan Blue Dye Exclusion*

H1299 and MCF-7 cells were induced or un-induced to express various ΔNp63 proteins for 3 days. Trypsinized, adherent cells and floating cells were collected and stained with trypan blue dye for 5 min. Both live (unstained) and dead (stained) cells were counted two times using a hemocytometer. Results are expressed as a percentage of dead cells over total cells counted.

*Western Blot Analysis*

Cells were plated at a density of $2.5 \times 10^6$ cells/10-cm dish and incubated for 24 h. Following induction, plates were washed with cold PBS (pH 7.4) and collected in 500 µlof2x SDS sample buffer and heated for 7 min at 95 °C. SDS-PAGE and Western blots were carried out as previously described (27). Nitrocellulose membranes were blocked with 5% dry milk in PBS with 0.1% Tween 20 (PBST) for 30 min, incubated in primary antibody overnight at 4 °C or for 2 h at room temperature, and washed three times for 10 min in PBST, and then the membrane was incubated in horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and washed four times in PBST. Blots were developed using Pierce Pico-West reagents, followed by exposure to x-ray film. All antibody dilutions were carried out with 2% dry milk in PBST.

*Northern Blot Analysis*

Cells were plated at a density of $2.5 \times 10^6$ cells/10-cm dish and incubated for 24 h. Following induction, plates were washed with cold PBS (pH 7.4) and collected in 1.0 ml of Trizol reagent (Invitrogen). Total RNA was isolated following the manufacturer's instructions. Northern blots were prepared using 10 µg of total RNA. The *P21, DKK1* (*DICKKOPF-1*), *PIG3* (p53-induced gene-3), *GADD45, FDXR,* and *GAPDH* probes were prepared as previously described (27, 28). Blots were exposed to x-ray film and quantified by densitometry.

### *Luciferase Assays*

Transient transfections were carried out using the calcium phosphate precipitation method (25). Briefly, p53-null H1299 cells were plated at $5 \times 10^4$ cells/well in 12-well plates and allowed to recover overnight. Using the calcium phosphate method, cells were co-transfected with 100 ng of wild-type or mutant p63 construct in pcDNA3 and 100 ng of pGL2 reporter vector/well. As an internal control, 5 ng of pRL-CMV, a *Renilla* luciferase vector (Promega), was also co-transfected per well. 36 h post-transfection, cells were washed with cold PBS and lysed in 150 µl of passive lysis buffer. Luciferase activity was measured using the Promega dual luciferase kit and a Turner Designs luminometer. The fold increase in relative luciferase activity was the product of the luciferase activity induced by pcDNA3 constructs expressing wild-type or mutant p63 divided by that induced by a pcDNA3 empty control vector.

## Results

### *All ΔNp63 Isoforms Have the Potential to Inhibit Cell Proliferation*

To determine the role of ΔNp63 isoforms in cell cycle progression and cell death, stable cell lines were created using the tetracycline-off system. H1299, a p53-null lung cancer cell line, was utilized to determine the p53-independent function of ΔNp63 isoforms. Previously published data from our laboratory demonstrate that tetracycline does not alter H1299 cell proliferation (29). Multiple cell lines were generated, and two representative cell lines for each ΔNp63 isoform were selected for further study. As shown in Fig. 1*B*, these cell lines were able to inducibly express ΔNp63α, ΔNp63β, or ΔNp63γ. In addition, the protein level of each isoform was comparable (Fig. 1*B*). To

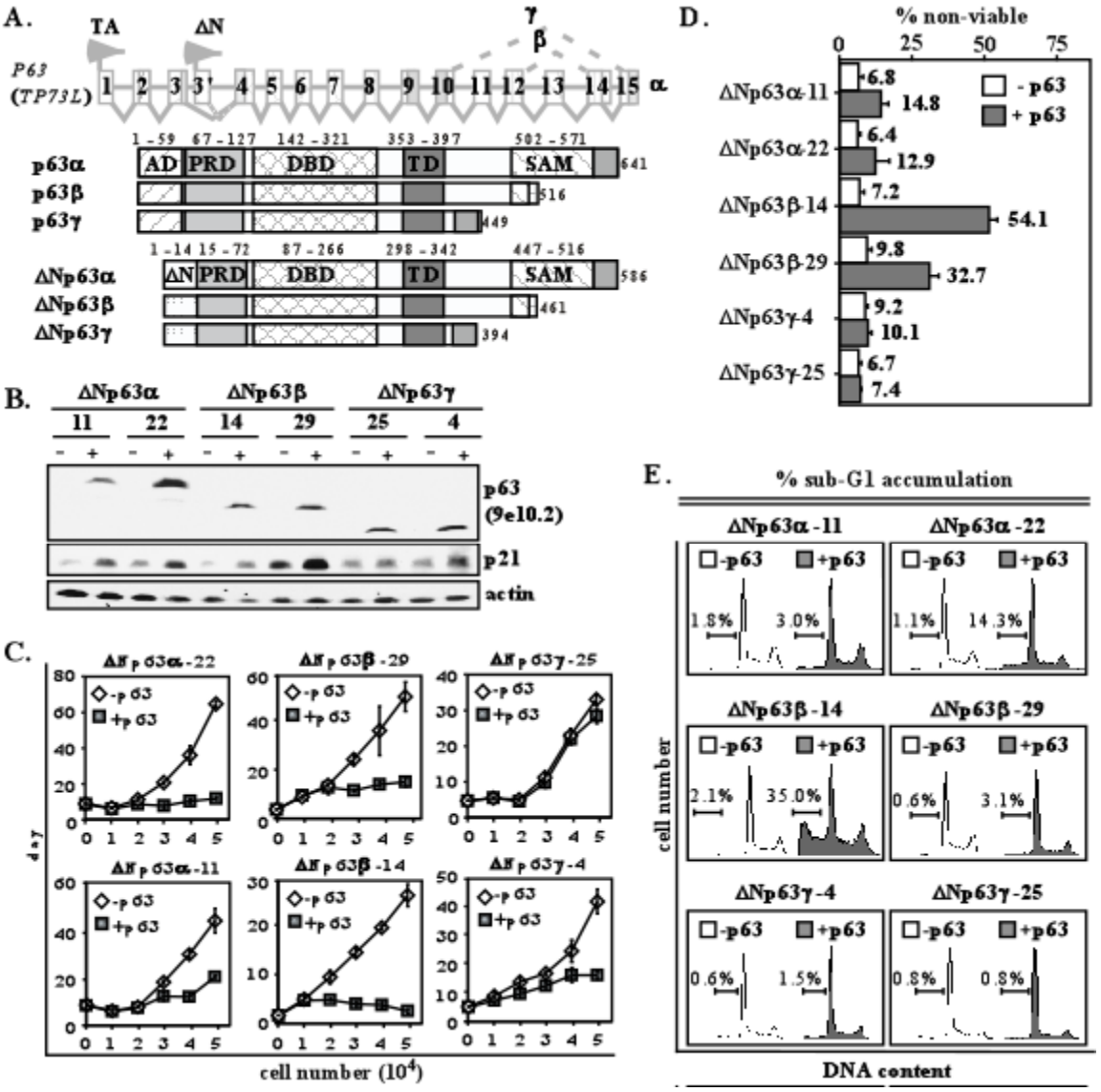


FIG. 1. ΔNp63 isoforms possess varied abilities to induce target gene expression and inhibit cell proliferation in H1299 cells. *A*, schematic representation of *TP63* gene and gene products. *PRD*, proline-rich domain; *DBD*, DNA-binding domain; *TD*, tetramerization domain; *SAM*, sterile-alpha motif. *B*, generation of stable H1299 cell lines inducibly expressing ΔNp63 isoforms. Two cell line clones for each isoform (ΔNp63α-11, ΔNp63α-22, ΔNp63β-14, ΔNp63β-29, ΔNp63γ-25, and ΔNp63γ-4) were utilized in this study. Western blots were performed on extracts collected 24 h post-induction from control cells (*–p63*) and induced cells (*+p63*). Myc-tagged p63 was detected with anti-Myc antibody (9e10.2). p21 was detected with anti-p21 antibody (C19). Actin was detected with an anti-actin polyclonal antibody. *C*, growth curve analysis was performed over a 5-day period as described under "Experimental Procedures" (*numerals* in the *graph titles* refer to clonal isolate number). *D*, cell viability was determined by counting live (unstained) and dead (stained) cells following staining with trypan blue dye. *E*, DNA content was quantified following propidium iodide staining of fixed cells that were cultured for 3 days in the presence (*–p63*) or absence (*+p63*) of tetracycline.

determine the transcriptional activity of each isoform, we monitored the level of $p21^{WAF1}$, a known p53 target gene. Similar to our previous finding (13), ΔNp63α was able to induce a modest increase in p21 expression. Whereas p21 expression was weakly induced by ΔNp63γ, ΔNp63β was highly active in inducing this p53 target gene. To determine the ability of ΔNp63 isoforms to inhibit cell proliferation, growth rate analysis was performed (Fig. 1*C*). Consistent with our previous report, ΔNp63α reduced the proliferation potential of H1299 cells by day 3 (13). Furthermore, we showed that ΔNp63β and ΔNp63γ were capable of inhibiting cell proliferation, with ΔNp63β displaying the greatest ability to induce growth suppression, followed by ΔNp63α and then ΔNp63γ (Fig. 1*C*).

To characterize the decreased proliferative potential of H1299 cells seen in our growth curve assay, trypan blue dye exclusion studies were performed as an indicator of cell viability (Fig. 1*D*). Following induction of ΔNp63β, we observed a significant increase in the number of trypan blue-positive, nonviable cells over that seen for un-induced controls. Likewise, we observed a moderate increase in nonviable cells upon induction of ΔNp63α. In contrast, there was no difference in the viability of control cells and those induced to express ΔNp63γ. In addition, DNA histogram analysis was performed to determine the cell cycle profile of these cells in the presence or absence of each ΔNp63 isoform (Fig. 1*E*). We found that sub-$G_1$ accumulation, associated with apoptotic cell death, was increased upon induction of ΔNp63α and ΔNp63β. We also found that ΔNp63α and ΔNp63β induced cell cycle arrest, primarily in $G_1$ phase. Upon induction of ΔNp63γ, cell cycle arrest in $G_1$ phase, but not significant apoptosis, was detected in both ΔNp63γ-expressing cell lines. Taken together, these results demonstrate that ΔNp63α and ΔNp63β can induce cell death.

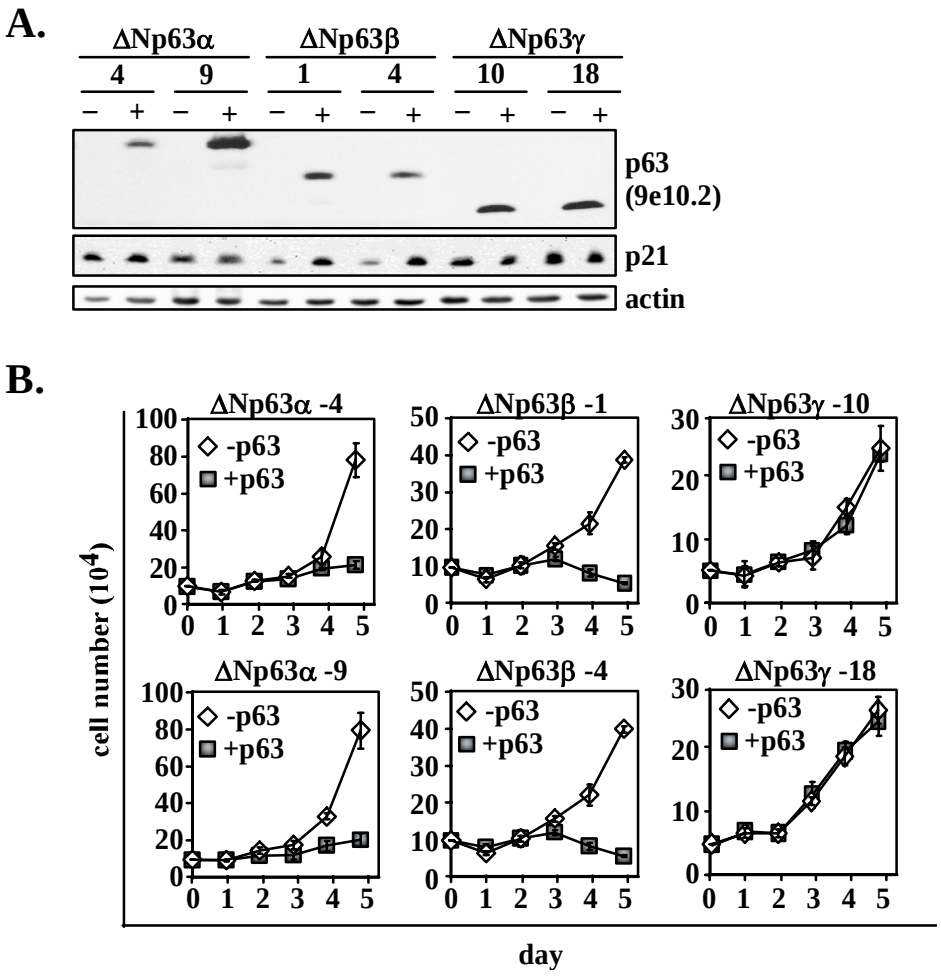


FIG. 2. The ΔNp63 isoforms are functional in MCF-7 cells. *A*, generation of stable MCF-7 cell lines inducibly expressing ΔNp63 isoforms. Two cell line clones for each isoform (ΔNp63α-4, ΔNp63α-9, ΔNp63β-1, ΔNp63β-4, ΔNp63γ-10, and ΔNp63γ-18) were utilized in this study (numerals refer to clonal isolate number). Western blot analysis was performed to determine levels of p63, p21, and actin in extracts collected 24 h post-induction from control cells (*–p63*) and induced cells (*+p63*). *B*, growth curve analysis was performed over a 5-day period as described under "Experimental Procedures" (*numerals* in *graph titles* refer to clonal isolate number).

To rule out the possibility that these ΔNp63 activities are cell type-specific, we used MCF-7 breast adenocarcinoma cells to produce additional stable cell lines. Unpublished results from our laboratory have demonstrated that tetracycline does not alter MCF-7 cell proliferation. Multiple cell lines inducibly expressing ΔNp63α, ΔNp63β, or ΔNp63γ were generated, and two representative clones were chosen for

further studies. Western blot analysis was performed to determine the level of protein expressed (Fig. 2*A*). We found that these ΔNp63 isoforms were expressed at a comparable level and that p21 was up-regulated by ΔNp63β but not by ΔNp63α and ΔNp63γ. Similar to our results observed in H1299 cells, growth curve analysis demonstrated that ΔNp63α and ΔNp63β inhibited cell proliferation, whereas ΔNp63γ was almost inert (Fig. 2*B*). It is noteworthy that p21 induction was low in ΔNp63α, suggesting that a target gene other than p21 was responsible for mediating ΔNp63α-dependent growth suppression.

To further demonstrate the transcriptional activity of p63 isoforms, Northern blot analysis was performed on MCF-7 cell lines induced to express TAp63α, ΔNp63α, TAp63γ, and ΔNp63γ for 1 and 7 days (Fig. 3). As expected, TAp63γ possessed the greatest potential to up-regulate *PIG3*, a pro-apoptotic gene, and *P21*, a cyclin-dependent kinase inhibitor, whereas TAp63α had reduced transactivation potential due to its inhibitory COOH terminus. In addition, ΔNp63α was able to induce expression of *GADD45* in MCF-7 cells similar to our previously published results seen in H1299 cells (13). Interestingly, ΔNp63α was able to induce *DKK1*, an inhibitor of Wnt signaling. These data effectively demonstrate the differential transcriptional activity of the p63 isoforms and highlight the need for additional studies involving isoform-specific gene activation.

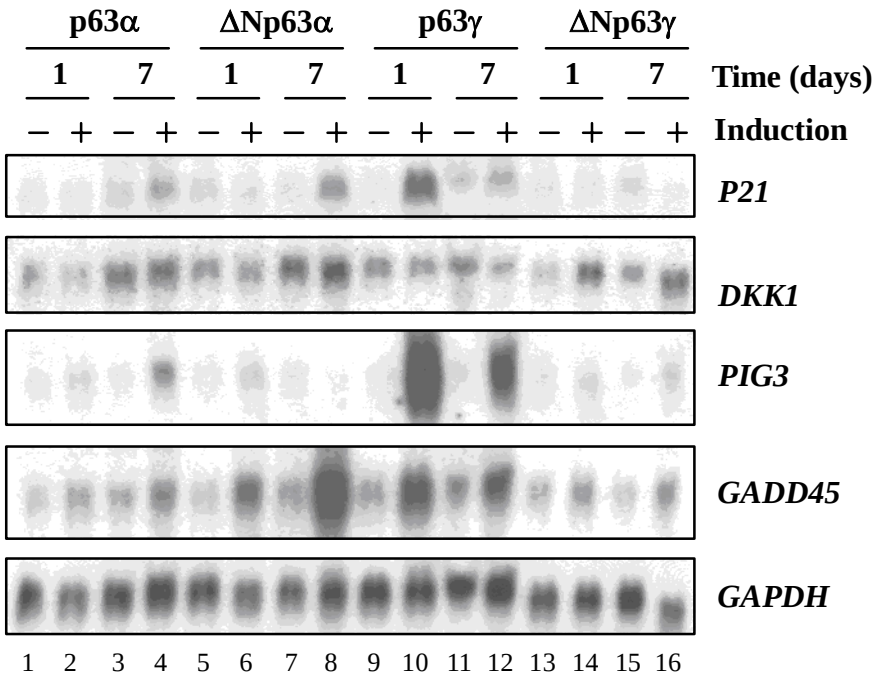


FIG. 3. Unique sequences in the $NH_2$ terminus and COOH terminus of p63 isoforms differentially regulate their ability to induce endogenous target genes. Northern blots were prepared using 10 μg of RNA purified from un-induced (*–p63*) and induced cells (*+p63*) cultured for 1 day or 7 days post-induction. Blots were analyzed with cDNA probes for *P21*, *DKK1*, *PIG3*, and *GADD45*, as indicated to the *right* of the *blot*. *GAPDH* was utilized as a loading control. *Lanes 1–16* are labeled at the *bottom*.

*DNA Binding Is Essential for p63 Function*

Most hotspot mutations found in *TP53* are located in the DNA-binding domain. These mutations, characterized as either contact site or conformational mutants, are often associated with loss of tumor suppressor function. Similar mutations, associated with EEC syndrome (reviewed in Ref. 30), have been identified in the DNA-binding domain of *TP63*. To test the effect of DNA-binding domain mutations on p63 function, we established MCF-7 cell lines inducibly expressing ΔNp63α(R149W), carrying a point mutation corresponding to arginine 204 in TAp63 and to arginine 175 in p53 (Fig. 4*A*). Multiple cell lines were generated, and two representative clones were used for further study. Western blot analysis was performed and revealed that the level of

ΔNp63α(R149W) was equal to or greater than wild-type ΔNp63α (Fig. 4*B*). Consistent with our earlier published reports (13), Northern blot analysis demonstrated that ΔNp63α was transcriptionally active in MCF-7 cells and induced expression of the *FDXR*, *GADD45*, and *P21* genes (Fig. 4*C*). However, contrary to wild-type ΔNp63α, the transcriptional activity of the R149W mutant was abrogated (Fig. 4*C*). Consequentially, ΔNp63α(R149W) were unable to suppress cell proliferation (Fig. 4*D*). These results suggest that an intact DNA-binding domain is required for ΔNp63α function.

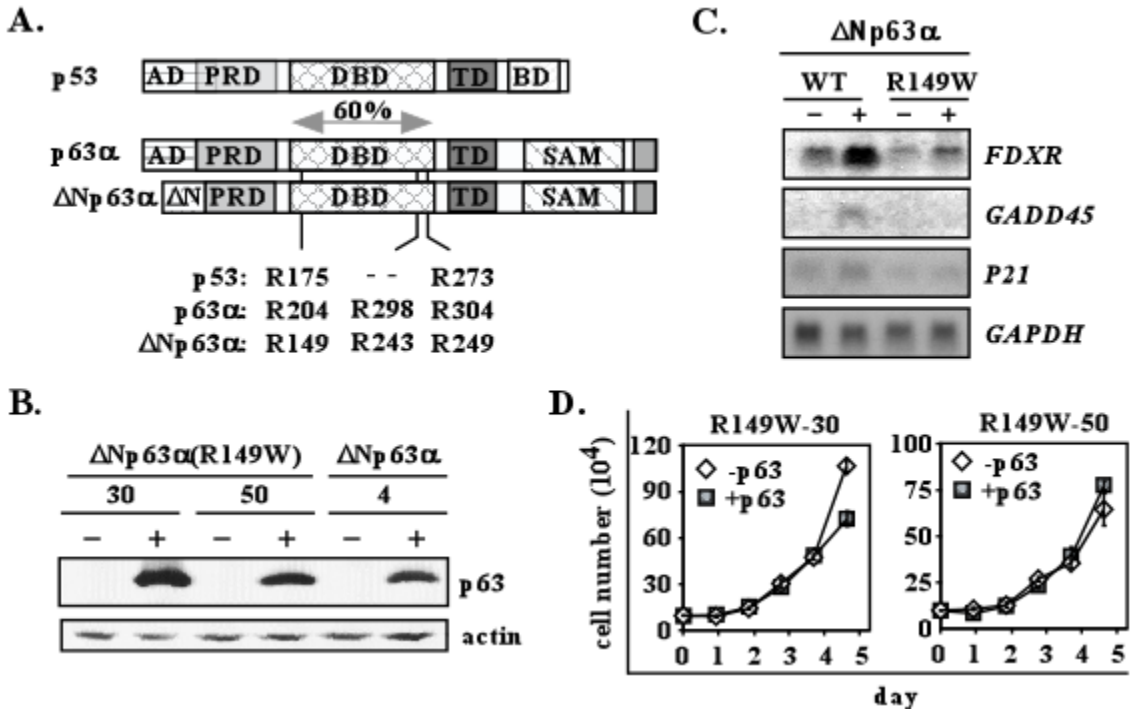


FIG. 4. The DNA-binding domain is required for ΔNp63α to inhibit cell proliferation in MCF-7 cells. *A*, the DNA-binding domains of p63 and p53 are 60% identical. Residues identified as hot spot mutations for p53 are conserved in p63. Non-conserved residues are denoted by a *dash*. *B*, generation of stable MCF-7 cell lines, clones 30 and 50, inducibly expressing ΔNp63α(R149W). Western blot analysis of Myc-tagged p63 and actin was performed on extracts collected 24 h post-induction from ΔNp63α(R149W) control cells (*–p63*) and induced cells (*+p63*). *C*, Northern blots were prepared using 10 μg of RNA purified from un-induced (*–p63*) and induced cells (*+p63*) cultured for 24 h post-induction. Blots were analyzed with cDNA probes for *FDXR*, *GADD45*, and *P21* genes as indicated to the *right* of the blot. *GAPDH* was utilized as a loading control. *D*, growth curve analysis was performed over a 5-day period as described under "Experimental Procedures" (*numerals* in *graph titles* refer to clonal isolate number).

*N6H Mutation Modestly Compromises ΔNp63α Function*

A patient diagnosed with ADULT syndrome was found to have a mutation in exon 3' of the *TP63* gene resulting in the substitution of an asparagine with a histidine at codon 6 in all ΔNp63 isoforms (31). To date, the N6H mutation is the only naturally occurring mutation discovered in the unique $NH_2$-terminal region of ΔNp63, but its effect on ΔNp63 function is unclear. To address this, multiple stable cell lines expressing ΔNp63α(N6H) were generated in MCF-7 cells. Western blot analysis demonstrated that the level of ΔNp63α(N6H) expressed was comparable with that of the wild-type (Fig. 5*A*). To examine the effect of this mutation on p63 function, growth curve analysis was performed. Following induction of ΔNp63α(N6H), cell proliferation was inhibited (Fig. 5*B*), but to a lesser extent compared with wild-type ΔNp63α in MCF-7 cells (Fig. 2*B*).

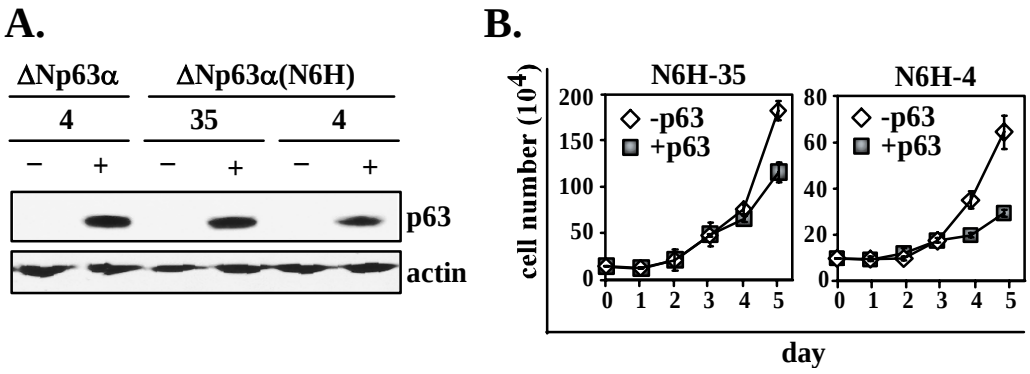


FIG. 5. The ΔNp63α(N6H) mutation associated with ADULT syndrome modestly compromises protein function. *A*, generation of stable MCF-7 cell lines, clones 4 and 35, inducibly expressing ΔNp63α(N6H). Western blot analysis of Myc-tagged p63 and actin was performed on extracts collected 24 h post-induction from control cells (*–p63*) and induced cells (*+p63*). *B*, growth curve analysis was performed over a 5-day period as described under "Experimental Procedures" (*numerals* in *graph titles* refer to clonal isolate number).

*The Proline-rich Domain Contributes to ΔNp63 Transcriptional Activity*

Src homology 3 domains have been shown to function as protein-protein interaction motifs in various signaling pathways (reviewed in Ref. 32). P*XX*P motifs,

identified in the proline-rich regions of p53 and p73, serve as ligand binding sites for Src homology 3 domain-containing proteins and regulate the transcriptional activity of these p53 family members (reviewed in Ref. 33). To explore the effect of the proline-rich domain on ΔNp63 function, we mutated the P*XX*P motif at residues 69–72 to P*XX*A. Since ΔNp63α exhibited a strong growth suppression in MCF-7 cells (Fig. 2*C*), cell lines expressing ΔNp63α(P72A) were generated, and two representative clones were selected for further study. As demonstrated in Fig. 6*A*, Western blot analysis revealed that the mutant ΔNp63α(P72A) protein was expressed at levels equal to or greater than that of the wild-type. Growth curve analysis was performed and showed that, in comparison with ΔNp63α, ΔNp63α(P72A) had a diminished ability to inhibit cell proliferation (Fig. 6*B*).

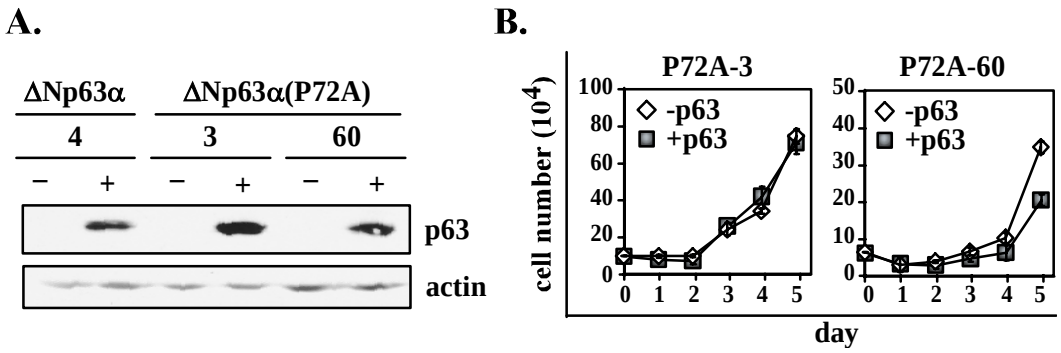


FIG. 6. Mutation of a P*XX*P motif in ΔNp63α adversely affects protein function. *A*, generation of stable MCF-7 cell lines, clones 3 and 60, inducibly expressing ΔNp63α(P72A). Western blot analysis of Myc-tagged p63 and actin was performed on extracts collected 24 h post-induction from control cells (*–p63*) and induced cells (*+p63*). *B*, growth curve analysis was performed over a 5-day period as described under "Experimental Procedures" (*numerals* in *graph title* refer to clonal isolate number).

*ΔNp63 Isoforms Differ in Their Ability to Activate the p53 Target Gene Promoters*

To better understand the contribution of specific amino acid sequences to p63 function, we characterized the ability of wild-type and mutant p63 constructs to activate p53-responsive promoters. As determined by Western blot analysis, ΔNp63β and ΔNp63γ

were expressed at equivalent levels, whereas the level of ΔNp63α was slightly higher following transient transfection in H1299 (Fig. 7*A*). To measure the transactivation potential of the wild-type ΔNp63 isoform, each ΔNp63-expressing vector was co-transfected with a luciferase reporter construct into the p53-null H1299 cells (Fig. 7*B*). We showed that ΔNp63α, ΔNp63β, and ΔNp63γ differentially regulated the *P21*, *GADD45*, and *FDXR* reporters (Fig. 7*B*). Similar to the results obtained in the stable cell line studies, luciferase reporter assays demonstrated that ΔNp63β was highly potent, whereas ΔNp63γ was limited, to transactivate *P21*, *GADD45*, and *FDXR* promoters. However, transiently expressed ΔNp63α was unable to transactivate these promoters.

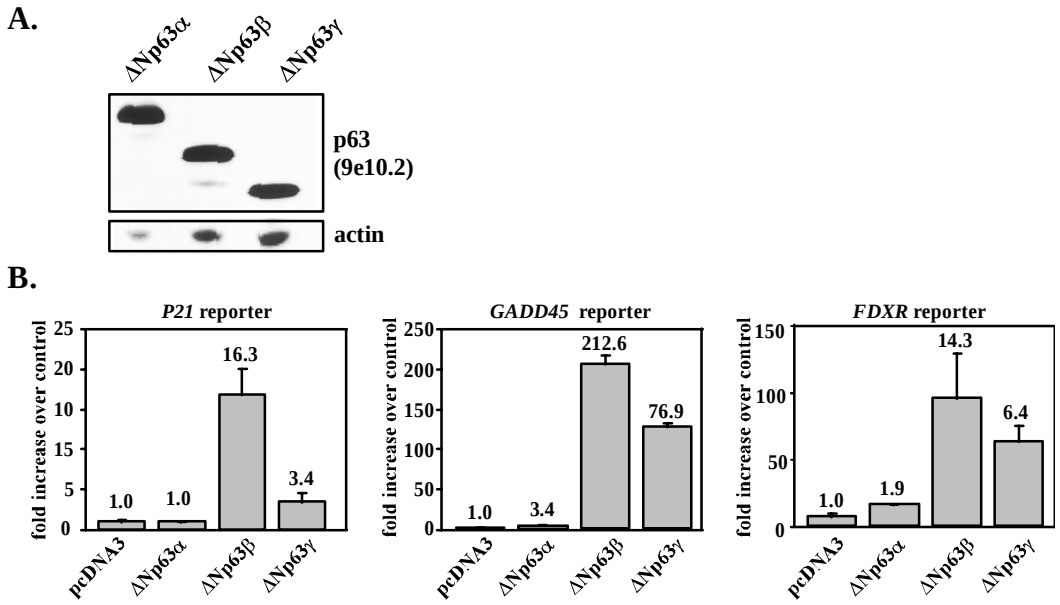


FIG. 7. ΔNp63 isoforms differ in their ability to activate p53 target gene promoters following transient transfection. *A*, the levels of ΔNp63 isoforms and actin were assayed by Western blot analysis of extracts collected 24 h following transient transfection utilizing anti-Myc antibody for Myc-tagged p63 and anti-actin antibody. *B*, transcriptional activity of ΔNp63α, ΔNp63β, and ΔNp63γ was determined by luciferase assay following co-transfection with pGL2-p21, pGL2-GADD45, or pGL2-FDXR. The -fold increase in relative luciferase activity was calculated using an empty pcDNA3 vector as a control.

*ΔNp63β DNA-binding Domain Mutants Exhibit Loss of Function*

Among ΔNp63 isoforms, ΔNp63β is the most potent in transactivation and in inducing cell cycle arrest and apoptosis. Thus, the remaining studies to analyze the domain and function of ΔNp63 were carried out with the ΔNp63β isoform. Since mutations in the DNA-binding domain of p63 have been identified in several developmental disorders, we utilized these mutants to demonstrate a requirement for DNA binding for p63 function (reviewed in Refs. 30 and 34). For example, codons corresponding to arginine 149 and arginine 249 were found to be mutated in EEC syndrome, whereas arginine 243 was found to be mutated in ADULT syndrome (Fig. 4*A*). These mutants were generated and used for luciferase assay. As revealed in Fig. 8*A*, both wild-type and mutant ΔNp63β constructs were expressed at comparable levels. To determine their transactivation potential, these DNA-binding domain mutants were analyzed for their ability to activate the *P21, GADD45*, and *FDXR* reporter constructs (Fig. 8*B*). Consistent with data obtained from ΔNp63β(R149W)-expressing cell lines (Fig. 4), ΔNp63β(R149W) showed a complete lack of transcriptional activity for all reporter constructs (Fig. 8*B*). Likewise, ΔNp63β(R243Q) was inactive. Surprisingly, we found that ΔNp63β(R249W) was able to activate the *P21, GADD45*, and *FDXR* reporters, albeit less than the wild-type protein (Fig. 8*B*). These data suggest that the DNA-binding domain is necessary for ΔNp63 activity and that some critical residues in the p53 DNA-binding domain are conserved in p63, whereas others are not.

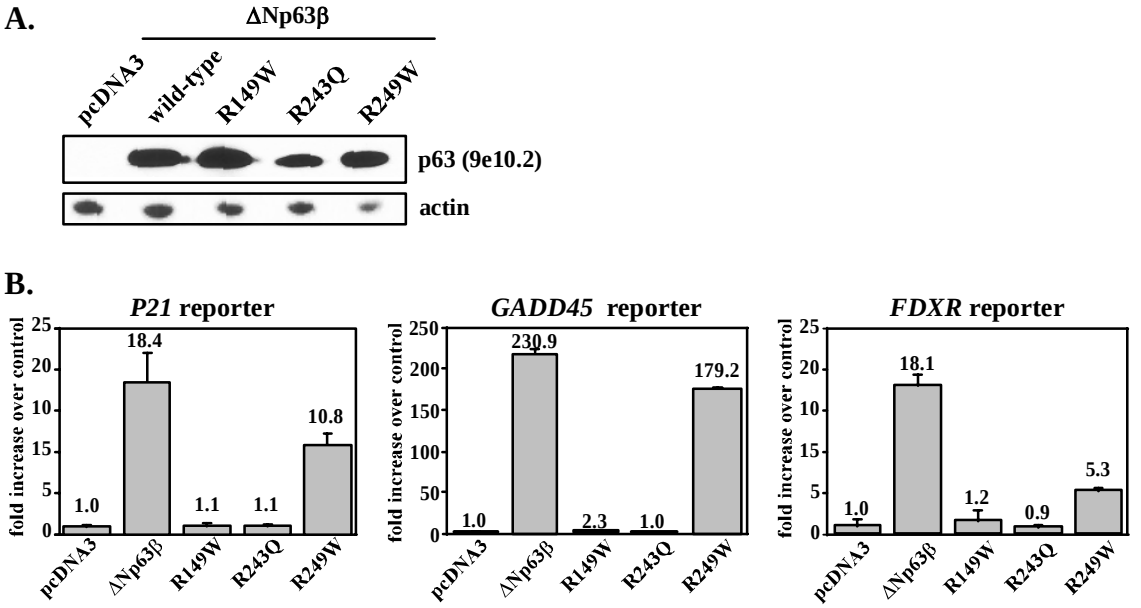


FIG. 8. DNA-binding domain mutants greatly reduce the transactivation potential of ΔNp63β. *A*, the levels of wild type, DNA-binding domain mutants, and actin were assayed by Western blot analysis of extracts collected 24 h following transient transfection utilizing anti-Myc antibody for Myc-tagged p63 and anti-actin antibody. *B*, transcriptional activity of DNA-binding domain mutants for ΔNp63β was determined by luciferase assay following co-transfection with pGL2-p21, pGL2-GADD45, or pGL2-FDXR.

*The 14 Unique ΔN Residues and Adjacent Proline-rich Domain Constitute the Activation Domain for ΔNp63*

Previously, we showed that a deletion construct lacking the first 26 residues in the $NH_2$ terminus of ΔNp63α, termed ΔΔNp63α, lost the ability to induce *P21*$^{WAF1}$ and *GADD45* (13). In order to extend this observation, additional $NH_2$-terminal mutants were constructed to uncover the residues required for transactivation by ΔNp63 (Fig. 9*A*). Following transfection into H1299 cells, Western blot analysis revealed that all $NH_2$-terminal constructs were expressed at similar levels (Fig. 9*B*). The *P21* reporter construct was used to measure the transactivation potential for these mutant constructs. As shown in Fig. 9*C*, we found that the luciferase activity induced by ΔNp63β(Δ2–14) was substantially reduced. In addition, subsequent deletion of additional $NH_2$-terminal residues, as in ΔNp63β(Δ2–19), led to a further reduction in its transactivation potential. Consistent with our previous finding that ΔΔNp63α is inert (13), deletion of the first 26 residues of ΔNp63β completely abolished its transcriptional activity.

To further analyze the ΔN activation domain, we generated point mutations at residues deemed likely to contribute to ΔNp63 function. First, we generated ΔNp63β carrying the N6H mutation associated with ADULT syndrome (31). Although the N6H mutation consistently decreased the ability of ΔNp63β to transactivate the *P21* reporter, the data failed to rise to the level of statistical significance (Fig. 9*C*). However, when taken together with the ability of the ΔNp63α(N6H) mutation to attenuate growth suppression (Fig. 4*B*), it is likely that the N6H mutation can alter p63 function, thus accounting for the ADULT syndrome phenotype (31). Mutation of hydrophobic residues in the $NH_2$-terminal activation domain (AD1) has been shown to reduce the transcriptional activity of p53 (35). Therefore, the presence of two analogous hydrophobic residues in the $NH_2$ terminus of ΔNp63 led us to construct the ΔNp63β(L22Q,L23S) mutant. As measured in a luciferase reporter gene assay, the (L22Q,L23S) mutation resulted in a small, but reproducible, decrease in promoter activation (Fig. 9*C*).

Several studies have demonstrated that the proline-rich domain in p53 and p73 contributes to their transcriptional activity (reviewed in Ref. 33). Likewise, we showed that the P*XX*P motif in ΔNp63α is necessary for inducing growth suppression (Fig. 6*B*). To determine the effect of the P*XX*P motif on the transcriptional activity of ΔNp63β, we constructed ΔNp63β(P72A), in which the P*XX*P motif was mutated to P*XX*A, and ΔNp63β(Δ69–72), in which the P*XX*P motif was deleted (Fig. 9*A*). Although ΔNp63β(P72A) and ΔNp63β(Δ69–72) were expressed at a higher level than wild-type ΔNp63β (Fig. 9*B*), we found that the ability of these mutants to activate the *P21* promoter was greatly reduced compared with wild-type ΔNp63β(Fig. 9*C*). To further analyze the

function of the P*XX*P motif, cell lines expressing the ΔNp63β(P72A) mutant were created in the H1299 background. Multiple clones were collected, and two clones were selected for further study. Western blot analysis revealed that the mutant was expressed at levels comparable with that of wild-type ΔNp63β (Fig. 9*D*). To determine the transcriptional activity of ΔNp63β(P72A), endogenous p21 protein levels were monitored. Consistent with the transient transfection assay, p21 was not up-regulated in cells induced to express ΔNp63β(P72A) (Fig. 9*D*, *p21 panel*). Coincidentally, a commonly used p63 monoclonal antibody (p63 Ab-1; Oncogene) only partially recognized the P72A mutant and did not recognize the Δ69–72 mutant. Taken together, these data suggest that the 14 unique residues in the $NH_2$ terminus and adjacent residues, including the P*XX*P motif, are required for transactivation by all Δ p63 isoforms. FIG. 9. The 14 unique ΔN residues and adjacent region form an activation domain for ΔNp63 isoforms. *A*, schematic diagram of $NH_2$-terminal deletion and point mutation constructs for ΔNp63β. *B*, levels of p63 and actin were assayed by Western blot analysis using anti-Myc antibody for Myc-tagged p63 and anti-actin antibody. *C*, transcriptional activity of ΔNp63β carrying mutations in $NH_2$-terminal residues was determined by luciferase assay following co-transfection with pGL2-p21. The -fold increase in relative luciferase activity was calculated using an empty pcDNA3 vector as a control. *D*, the levels of p63, p21, and actin expressed in wild-type ΔNp63β and ΔNp63β(P72A) were assayed by Western blot analysis.

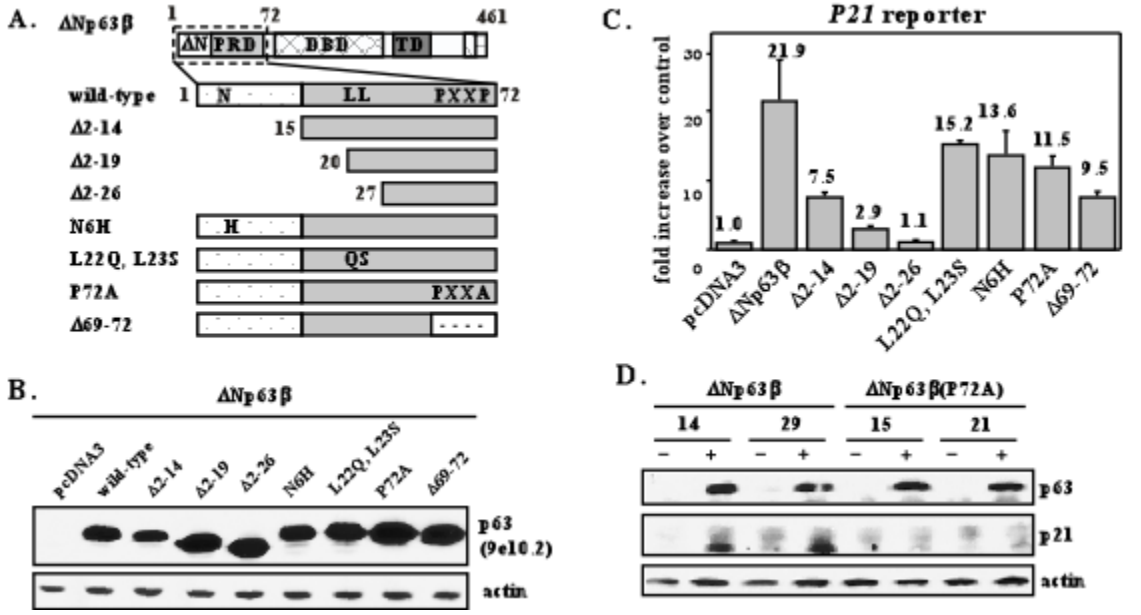


FIG. 9. The 14 unique ΔN residues and adjacent region form an activation domain for ΔNp63 isoforms. *A*, schematic diagram of $NH_2$-terminal deletion and point mutation constructs for ΔNp63β. *B*, levels of p63 and actin were assayed by Western blot analysis using anti-Myc antibody for Myc-tagged p63 and anti-actin antibody. *C*, transcriptional activity of ΔNp63β carrying mutations in $NH_2$-terminal residues was determined by luciferase assay following co-transfection with pGL2-p21. The -fold increase in relative luciferase activity was calculated using an empty pcDNA3 vector as a control. *D*, the levels of p63, p21, and actin expressed in wild-type ΔNp63β and ΔNp63β(P72A) were assayed by Western blot analysis.

*A Region Present in ΔNp63α and ΔNp63β, but Not ΔNp63γ, Contributes to Their Transcriptional Activity*

The data above clearly indicate that ΔNp63β is the most potent transcription factor among the ΔNp63 isoforms, suggesting that a positive regulatory domain exists in the COOH terminus of ΔNp63β. Therefore, five COOH-terminal truncation mutants were generated to map this domain in ΔNp63β (Fig. 10*A*). Western blot analysis showed that when transfected into H1299 cells, these constructs were expressed at equivalent levels (Fig. 10*B*). Of importance, the transcriptional activity of ΔNp63(Δ457-ter), which lacks the unique residues associated with the β-isoform, and ΔNp63(Δ356-ter), which lacks the

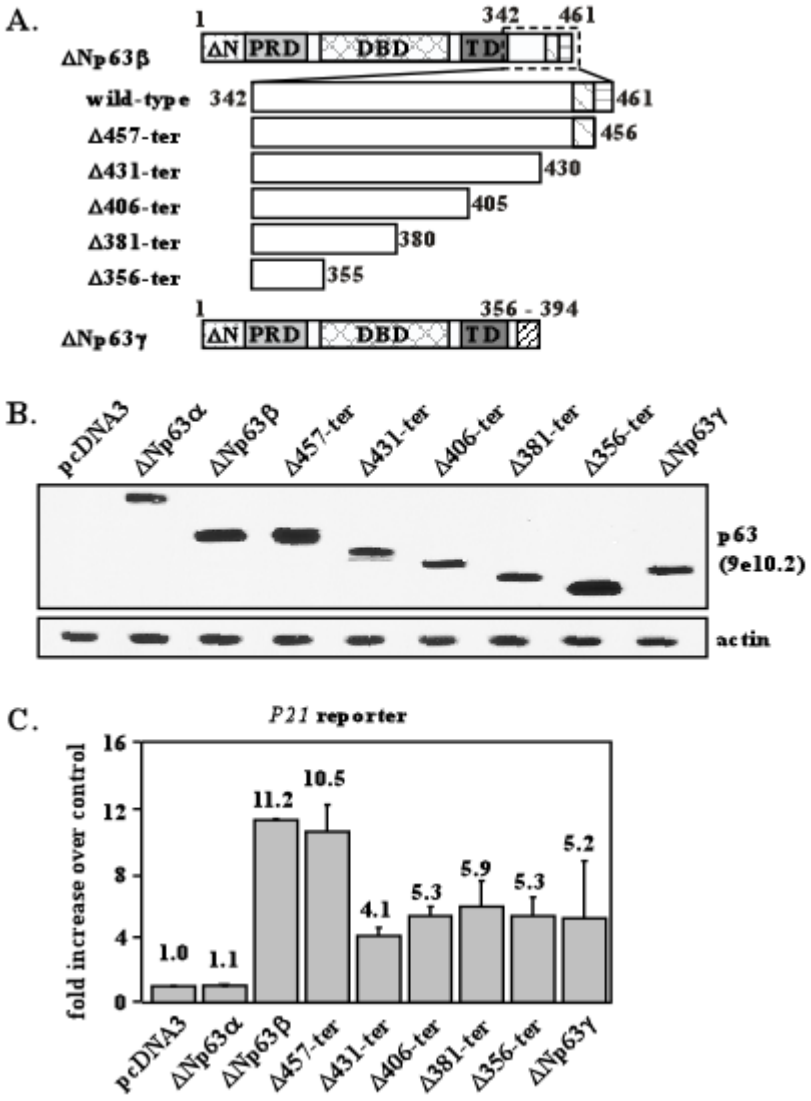


FIG. 10. Residues 431–455 in ΔNp63β are critical for its strong transcriptional activity. *A*, schematic diagram of COOH-terminal deletion constructs. *B*, levels of p63 and actin were assayed by Western blot analysis. *C*, transcriptional activity of ΔNp63 isoforms and COOH-terminal truncation mutants was determined by luciferase assay following co-transfection with pGL2-p21.

unique residues associated with the γ-isoform, was not altered compared with wild-type ΔNp63β and ΔNp63γ, respectively (Fig. 10*C*). Therefore, the five unique β and 39 unique γ residues present in their COOH termini do not appear to have any effect on ΔNp63

activity. However, the ability of ΔNp63(Δ431-ter), ΔNp63(Δ406-ter), and ΔNp63(Δ381-ter) to activate the *P21* promoter was diminished compared with that of ΔNp63β. In sum, these data suggest that residues 431–455 are responsible for the differential transcriptional activities exhibited by the three ΔNp63 isoforms.

*A COOH-terminal PPXY Motif Confers Increased Transactivation Potential to ΔNp63β*

PP*X*Y motifs serve as ligands for WW domain-containing proteins. Recently, studies showed that p73, another p53 family member, contains a PP*X*Y motif that can be regulated by WW domain-containing proteins (36–39). Thus, we searched for such a motif between residues 431 and 455 and found a PP*X*Y motif present in the α and β isoforms (Fig. 11*A*). To explore the role of the PP*X*Y motif in p63 function, three mutants, which disrupt the PP*X*Y motif, were generated (Fig. 11*B*). Western blot showed that both wild-type and mutant ΔNp63β constructs were expressed at equivalent levels (Fig. 11*C*). Interestingly, these mutants exhibited a reduced ability to activate the *P21* promoter (Fig. 11*D*). Nevertheless, the PP*X*Y mutants of ΔNp63β were still more potent than ΔNp63γ to activate the *P21* promoter (Fig. 11*D*). These data suggest that the PP*X*Y motif and additional amino acids between residues 431 and 455 are capable of enhancing the transcriptional activity of ΔNp63β.

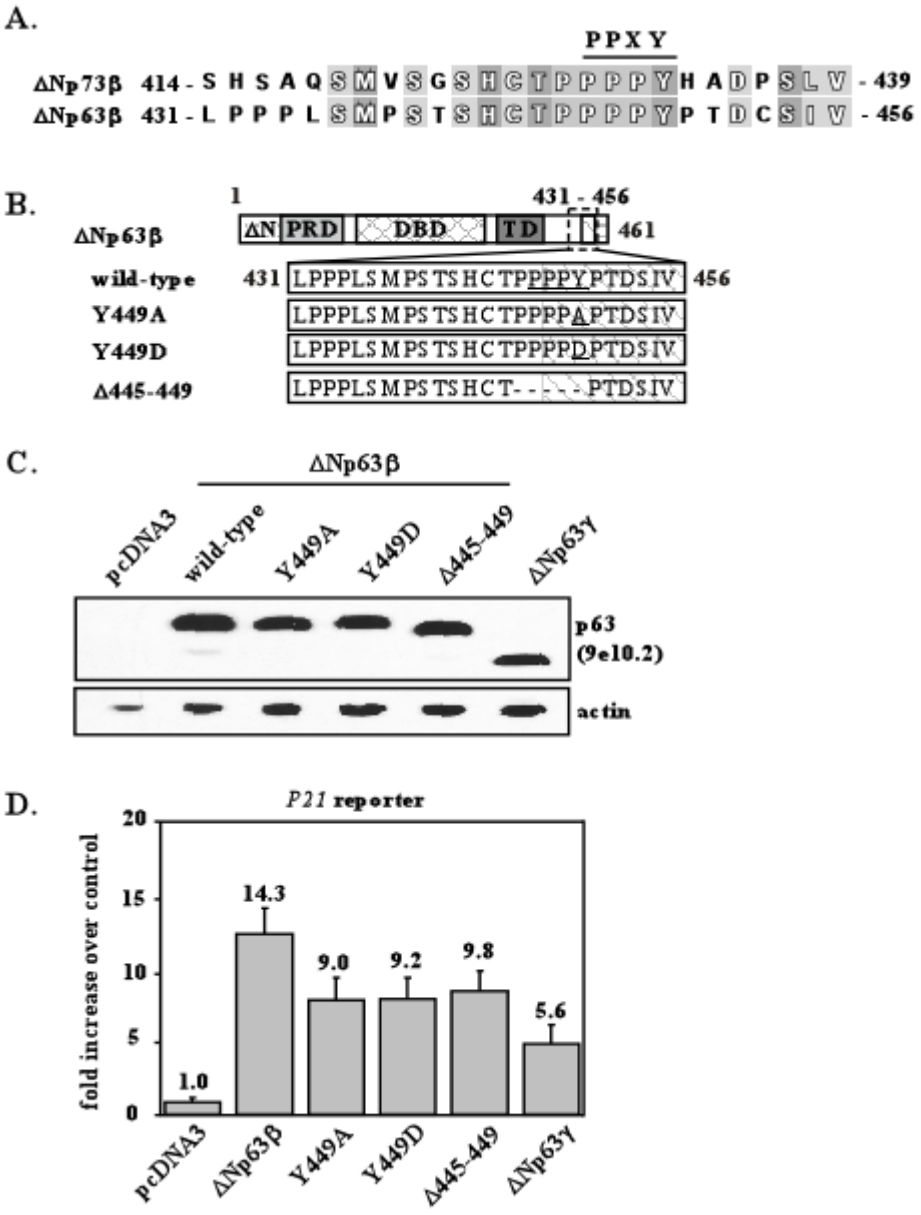


FIG. 11. Point mutation or deletion of the PP*X*Y motif attenuates the ability of ΔNp63β to transactivate the p21 promoter. *A*, alignment of p63 and p73 COOH-terminal residues including a conserved PP*X*Y motif. *B*, schematic presentation of the ΔNp63β COOH terminus proline-rich domain. The PP*X*Y motif is disrupted by point mutation in ΔNp63β(Y449A) and ΔNp63β(Y449D) or by deletion in ΔNp63β(Δ445–449). *Dashes* represent deleted residues. *C*, Western blot analysis of p63 and actin in H1299 cells transfected with wild-type ΔNp63β and PP*X*Y mutants. *D*, transcriptional activity of ΔNp63β and mutants carrying mutations in the PP*X*Y motif was determined by luciferase reporter assay following co-transfection with pGL2-p21.

Discussion

The role of ΔNp63 in cancer is highly debatable. Previous studies have suggested that ΔNp63α is dominant negative toward p53 and TAp63, since it is capable of inhibiting these proteins in reporter assays (3). However, we and others have demonstrated a role for ΔNp63α in the induction of gene expression (13–16). In addition, some studies have suggested that ΔNp63α is a potential onco-protein, because it is overexpressed in lung cancer and squamous cell carcinomas of the head and neck (19), whereas others have observed a correlation between loss of p63 expression and an increase in tumorigenesis and metastasis (9, 12). To clarify the ambiguous role of p63 in cancer, we further characterized the ΔNp63 isoforms. We found that all three ΔNp63 isoforms, although lacking an activation domain similar to p53 or TAp63, retain the ability to induce target genes and inhibit cell proliferation. Of significance, the ability of ΔNp63β to induce target gene expression is the strongest among ΔNp63 isoforms, followed by ΔNp63α and then ΔNp63γ.

Having demonstrated a transcriptional activity for the ΔN variants, we searched for the activation domain. Previous work in our laboratory demonstrated that ΔNp63α requires residues 1–26 to be transcriptionally active (13). Here, we found that the first 26 residues in ΔNp63β were also required for transactivation of *P21*. Interestingly, deletion of the 14 unique ΔN residues (Δ1–14) or of additional residues reaching into the proline-rich domain (Δ1–19) diminished, but did not abolish, the activity of ΔNp63α. Of interest, we observed that an intact P*XX*P motif is required for ΔNp63α to suppress cell proliferation and for ΔNp63β to transactivate the *P21* gene. Thus, the 14 residues unique

to the ΔN variant and the adjacent region, including the P*XX*P motif, comprise an activation domain for the ΔN variant.

Our data indicate that a COOH-terminal proline-rich domain within residues 431–455, including a PP*X*Y motif found at residues 446–449, is to some extent responsible for the differential biochemical and biological activities associated with the ΔNp63 isoforms. Supporting our hypothesis, deletion of the COOH-terminal proline-rich domain or mutation of the PP*X*Y motif revealed that this domain is required for optimal transactivation of the *P21* promoter. Additionally, ΔNp63α and ΔNp63β, which contain the COOH-terminal proline-rich domain, are effective in suppressing cell proliferation. In contrast, ΔNp63γ, which lacks the COOH-terminal proline-rich domain, possesses a much weaker potential in growth suppression. Of relevance to these latter results, fusion of *TP63* exons 11 or 12 to the DNA-binding domain of GAL4 revealed a weak transcriptional activity associated with the COOH-terminal proline-rich domain (40). These findings suggest that the COOH-terminal proline-rich domain augments the transcriptional activity of the ΔN variant. However, it is unlikely that this COOH-terminal region constitutes an independent activation domain, since deletion of the $NH_2$-terminal activation domain makes ΔNp63 transcriptionally inactive. Thus, alternative splicing confers an enhanced transactivation potential to ΔNp63α and ΔNp63β, which is not extended to ΔNp63γ.

The question remains as to how the COOH-terminal PP*X*Y motif modulates the transcriptional activity of ΔNp63. Instead of functioning as an activation domain, we hypothesize that the PP*X*Y motif within the COOH-terminal proline-rich domain is likely to alter p63 function by promoting interaction with WW domain-containing proteins.

Several studies have demonstrated that WW domain-containing proteins can alter the function of transcription factors. For example, Strano *et al.* (41) observed that yes-associated protein binds to a PP*X*Y motif in TAp73α and enhances p73-mediated Bax promoter activation. In addition, WW-containing ubiquitin ligases, including Itch and NEDL2, can interact with the PP*X*Y motif in p73 and regulate p73 protein stability (38, 39). Since mutation of the PP*X*Y motif in ΔNp63β reduced its ability to transactivate the *P21* reporter, it is likely that WW domain-containing proteins modulate ΔNp63β function in a manner similar to that seen with p73.

We have established that following inducible expression in stably transfected cells, ΔNp63α is able to induce growth suppression and transactivate target genes. In contrast, following transient transfection, we and others demonstrated that ΔNp63α is unable to transactivate known p53 target gene reporters (3, 17) but can transactivate the *HSP70* reporter, a non-p53 target gene (16). In addition, several recent studies have described a positive and negative role for ΔNp63α in the transcriptional regulation of endogenous target genes (14, 15). Given these observations, the transcriptional activity of ΔNp63α is likely to require complex regulation found at the level of chromatin DNA, which is not extended to the naked DNA utilized in most reporter assays. Furthermore, it is apparent that the effect of the α-COOH terminus on transcription is context-specific, since some genes are induced whereas others are repressed in the presence of the α-COOH terminus. However, it is still unclear how the α-COOH terminus modulates p63 function. Previous studies have demonstrated that the transcriptional activity of TAp63α is inhibited by its COOH terminus (40, 42). One hypothesis suggests that transactivation by TAp63α is inhibited through an interaction between the α-COOH terminus and the

TA variant activation domain (42). Importantly, our data indicate that ΔNp63α is less active than ΔNp63β to induce growth suppression, suggesting that the α-COOH terminus can also attenuate the activity of the ΔN variant (Figs. 1 and 2). Thus, the currently accepted model cannot be applied to ΔNp63α, since the ΔN variant does not encode the residues required for this inhibitory interaction. Therefore, at least for the ΔN variant, the inhibitory effect of the α-COOH terminus must function through an unknown mechanism. These observations point to a complex regulation of ΔNp63 transcriptional activity, which may account for the differential activities attributed to ΔNp63α (12–15, 19).

In conclusion, this study demonstrates a transcriptional activity for all three ΔNp63 isoforms and defines the activation domain for the ΔN variant to include the 14 unique ΔN residues and the adjacent region, including the proline-rich domain. We found that the DNA-binding domain is necessary for ΔNp63 activity and that some critical residues in the p53 DNA-binding domain are conserved in p63, whereas others are not. Interestingly, our results suggest that regulation of and by the PP*X*Y motif is likely to confer isoform-specific function to the ΔN variant. Furthermore, it is probable that regulation of proteins interacting with the P*XX*P and PP*X*Y motifs accounts for the paradoxical functions attributed to p63 in cancer.

Footnotes

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[2] The abbreviations used are: TA, transcriptional activation; ADULT, acro-dermato-ungual-lacrimal-tooth; EEC, ectrodactyly-ectodermal dysplasia-cleft lip/palate; ΔN, $NH_2$-terminally deleted; AD, activation domain; PBS, phosphate-buffered saline.

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# THE PROLINE-RICH DOMAIN OF p63 REGULATES THE TRANSCRIPTIONAL ACTIVITY OF THE TAp63 VARIANT

by

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In preparation for submission to *Oncogene*

Format adapted for dissertation

## Abstract

p63, a member of the p53 family of transcription factors, is integral to epithelial development. Recently, studies have demonstrated that p63 has the potential to function as a tumor suppressor or an onco-protein. To advance our understanding of p63 function and its potential to promote either cell survival or cell death, we generated stable cell lines that inducibly express p63α, p63β, or p63γ. We show that TAp63 isoforms, containing a strong acidic activation domain similar to activation domain 1 of p53, are competent to induce the p53 responsive genes, to inhibit cell proliferation, and to promote apoptosis. Interestingly, we discovered that both the activation domain (residues 1 to 59) and the proline-rich domain (residues 67 to 127) are required for the TAp63 variant to transactivate exogenous promoter constructs following transient transfection. Likewise, TAp63β(ΔPRD), deleted of residues 60 to 133, possessed a greatly attenuated ability to induce endogenous target gene expression and promote apoptosis, but retained the ability to inhibit cell proliferation when expressed in stable, inducible cell lines. In addition, p63β(ΔPRD) functioned as a dominant negative to wild-type p63β in a dose dependent manner. Furthermore, the loss of function seen with deletion of the proline-rich domain was not due to a DNA-binding defect, as p63β(ΔPRD) was found to strongly bind endogenous promoters using the chromatin immunoprecipitation. Finally, mutational analysis reveals that a PXXP motif at residues 124 to 127, contributes to the transcriptional activity of the TAp63 variant. Altogether, our findings suggest that the transcriptional activity of TAp63 isoforms can be regulated by modification of or protein-protein interactions at the p63 proline-rich domain.

## Introduction

Despite strong homology in their activation domains (AD), DNA-binding domains (DBD), and oligomerization domains (OD), studies have demonstrated that p53 family members play diverse biological roles. p53 is a critical tumor suppressor as evidenced by the fact that greater than 50% of tumors are p53 null. Loss of p53 function either through mutation, gene silencing, or oncogene inactivation promotes genomic instability and increases the likelihood of tumor formation. For example, $p53^{-/-}$ knockout mice are phenotypically normal at birth, but display a high rate of gene amplifications and spontaneous tumor formation [reviewed in 1]. Similarly, Li-Fraumeni syndrome patients carrying germline mutations in the *TP53* locus are prone to develop chromosome abnormalities and have a higher incidence of tumor formation [2].

p63 was identified as p53 homologue in 1998 and is thought to play a major role in epithelial development. Germline mutations in *TP63* are associated with skin, hair, tooth, and skeletal patterning defects [3]. In addition, $p63^{-/-}$ mice display defects in epithelial development, fail to maintain epithelial stem cells, and die shortly after birth due to dehydration [4, 5]. Unfortunately, studies linking p63 function to cancer have frequently described conflicting results. For example, p63 is often over expressed in head and neck squamous cell carcinomas and is thought to provide promote tumorigenesis by blocking the apoptotic function of other p53 family members. In contrast, loss of p63 expression in bladder and breast cancer may lead to increased metastatic potential. Further confusing matters, studies by Flores et al, demonstrated that mice heterozygous for p63 or p73 were more prone to tumor formation, while those generated in another laboratory failed to show any predisposition to cancer.

While the tumor suppressor role of p53 is indisputable, defining the role of p63 in cancer is problematic. For example, the *TP53* locus primarily expresses a single transcript with high transactivation potential, whereas the *TP63* locus expresses multiple isoforms with variable transcriptional activities [6].. The transactivation p63 (TAp63) variant is expressed from the upstream promoter and contains an activation domain (AD) similar to the strong, acidic activation domain 1 (AD1) of p53. Initiation of transcription from a cryptic promoter in intron 3 resulting in exclusion of the strong, acidic activation domain from the N-terminal deleted p63 (ΔNp63) variant. In addition, alternative splicing of both TAp63 and ΔNp63 transcripts yields the α, β, and γ isoforms encoding unique carboxy-termini. Altogether, six unique isoforms are expressed from the TP63 locus: p63α, p63β, p63γ, ΔNp63α, ΔNp63β, ΔNp63γ (Note: the TA prefix is often omitted when referring to the TAp63 variant). In addition, p53 is ubiquitously expressed in all tissues and throughout the lifespan of higher eukaryotes; whereas p63 expression is tightly regulated resulting in an expression pattern that is limited both temporally and spatially. Finally, the tumor suppressor function of p53 is dependent upon DNA damage signals, oncogene activation, or other forms of cellular stress that stabilize and activate p53 [reviewed in 7, 8]. In contrast, very little is known about the signaling mechanisms, including post-translation modifications, protein-protein interactions, and regulation of protein stability, that modulate p63 transactivation potential.

Initially, the transcriptional activity of the p63 variants was though to be determined at the level of transcription with the TAp63 variant functioning as a transcriptional activator and the ΔNp63 as a dominant negative inhibitor. However, work in our laboratory showed that the ΔNp63 variants retained transcriptional activity [9].

Furthermore, we defined the ΔNp63 activation domain and identified proline-rich motifs that contribute to ΔNp63 transcriptional activity [10]. These data suggest that there are post-translation mechanisms of regulating p63 activity that have yet to be described.

Given that p63 can bind to p53-responsive elements, uncovering mechanisms that potentate p63 transcriptional activity could provide a new tool in the fight against cancer. In this study, we show that all TAp63 isoforms possess the ability to transactivate p53-target genes, inhibit proliferation, and induce apoptosis. Surprisingly, we found that the transcriptional activity of the TA variant requires the presence of both the activation domain and the proline-rich domain. In addition, we demonstrate that deletion of the proline-rich domain greatly attenuates the transactivation and apoptotic potential of TAp63β without affecting its ability to bind DNA or inhibit cell proliferation. Finally, we identified a PXXP motif at residues 124 to 127 as a contributor to TAp63 transactivation function.

## Results

### *TAp63 isoforms are potent transcriptional activators*

To characterize the function of the TAp63 isoforms we used the tetracycline-off system to generate stable, inducible cells lines in p53-null H1299 cells derived from a lung adenocarcinoma. Two cell line clones for each isoform (p63α-11, p63α-14, p63β-17, p63β-30, p63γ-1, and p63γ-17) were selected for further study. Western blots performed on protein extracts revealed that each isoform was expressed and that all TAp63 variants were strong transcriptional activators of p21, a cyclin-dependent kinase inhibitor that is associated with both G1 and G2 arrest (Fig. 1a). To further analyze the transcriptional

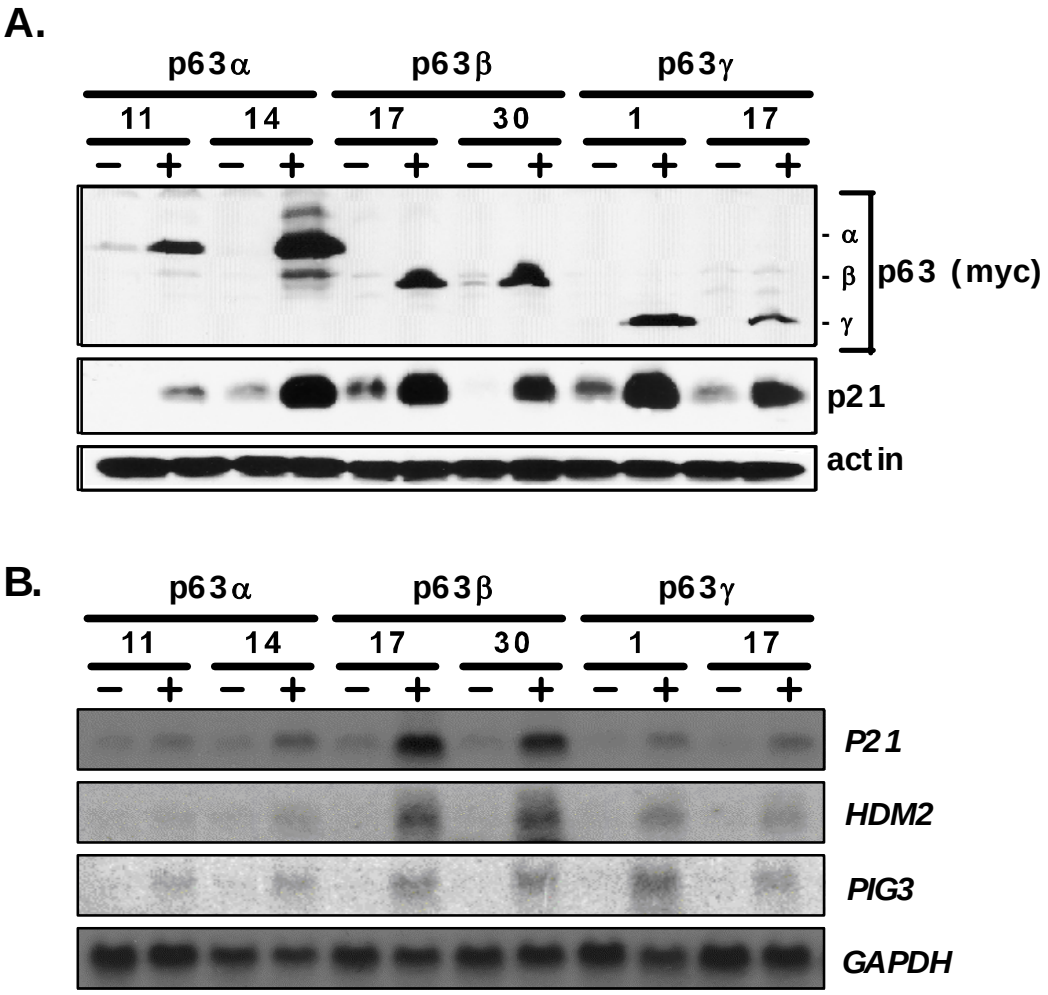


**Figure 1. TAp63 isoforms are potent transcriptional activators. (a) The tetracycline-off system was employed to generate stable, inducible cell lines expressing p63 isoforms. Two cell line clones for each isoform (p63α-11, p63α-14, p63β-17, p63β-30, p63γ-1, and p63γ-17) were selected for further study. Western blots performed on protein collected 24 h following induction from control cells (-p63) and induced cells (+p63) was analyzed for myc-tagged p63 expression using an anti-Myc antibody (Abcam, Cambridge, UK). All p63 isoforms strongly induced expression of p21, as detected with anti-p21 antibody (C19, Santa Cruz Biotechnology, CA). Protein loading was normalized to actin levels using an anti-actin polyclonal antibody (Sigma, St. Louis, MO). (b) RNA isolated from un-induced (-p63) and induced cells (+p63)incubated for 24 h post-induction was analyzed by Northern blot and hybridization with radio-labeled cDNA probes for P21, HDM2, and PIG3 genes as indicated to the right of the blot. GAPDH was utilized as a loading control.**

activity of these isoforms, we performed a Northern blot analysis of RNA collected from un-induced (-p63) and induced (+p63) cells (Fig. 1b). We found that the p63β isoform was the most potent activator of p53-target genes, followed by p63γ then p63α.

*TAp63 isoforms inhibit cell proliferation and induce apoptosis.*

To demonstrate the affect of p63 expression on overall cell viability a growth curve assay was performed over 5 day period. The results show that all p63 isoforms are competent to inhibit cell viability over time in culture (Fig. 2a). Surprisingly, we consistently observed a significant number of cells undergoing some form of cell death after only one day of induction. To confirm our suspicions that the cell death was due to apoptosis, live cells were stained with Annexin V-FITC and propidium iodide after 18 hrs of induction. When analyzed by FACS analysis, we found that the cell death induced by TAp63 isoforms was due to apoptosis (Fig. 2b). Furthermore, p63β possessed the greatest ability to induce apoptosis (25.3$\pm$4.6% and 24.9$\pm$6.2), followed by p63γ (18.3$\pm$1.3% and 20.4$\pm$0.7), and then by p63α (7.0$\pm$0.5% and 10.6$\pm$1.4%) (Fig. 2c).

*The TAp63 variant requires both the activation domain and the proline-rich domain to transactivate target gene promoters.*

The domains required for transcriptional activation by the TAp63 isoforms were mapped by transiently co-transfecting cDNA expressing different p63 isoforms into H1299 cells along with luciferase reporter constructs driven by sequences cloned from the *P21*, *FDXR*, and *GADD45* promoters. Figure 3a confirms that p63α, p63β, p63γ, ΔNp63β, and ΔNp63γ were competent to transactivate the exogenously transfected promoters.

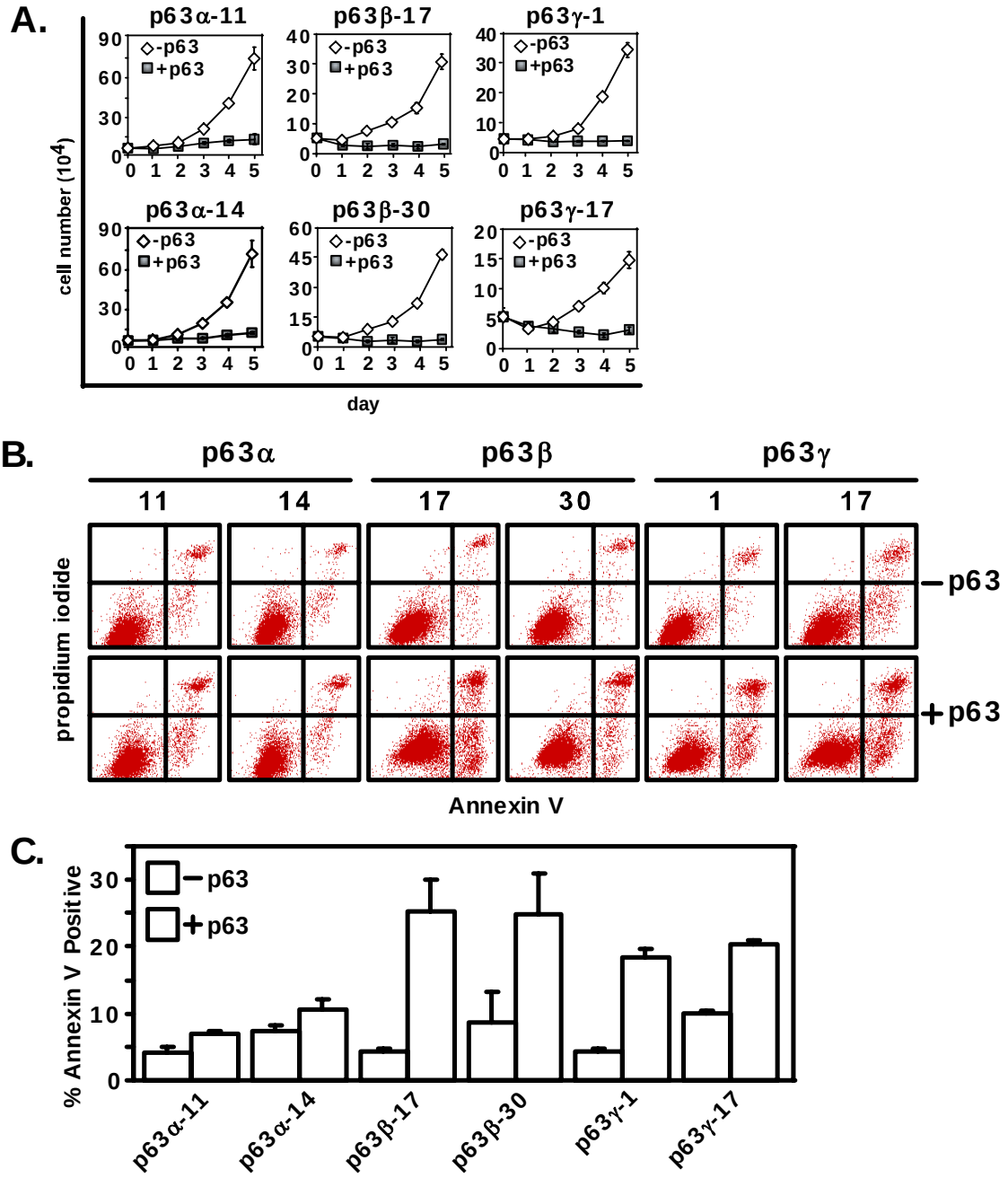


**Figure 2. TAp63 isoforms inhibit cell proliferation and induce apoptosis.** **(a) Growth curve analysis was performed on un-induced cells (-p63 and induced cells (+p63) over a 5-day period. (b) Apoptosis was measured following an 18 h induction (-p63 and +p63) using an Annexin V-FITC and propidium iodide staining kit (MBL; Tokyo, Japan). Cells in the upper right and lower right quadrants are Annexin V positive. (c) The percentage of apoptotic, Annexin-V positive cells from separately analyzed samples is represented in the histogram as the mean + standard deviation. The assay was performed in triplicate.**

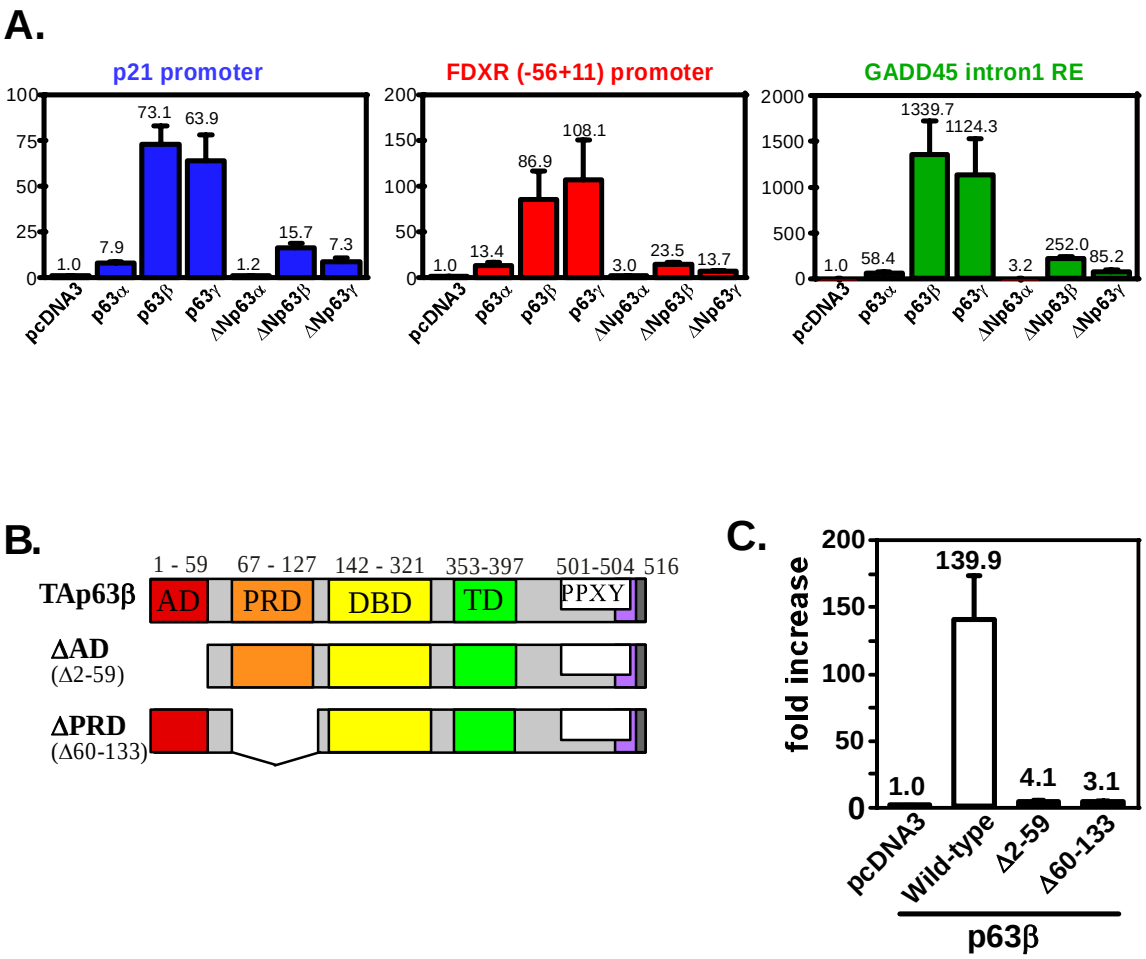


**Figure 3. The TAp63 variant requires both the activation domain and the proline-rich domain to transactivate target gene promoters.**
**(a) The ability of p63 isoforms to regulate the *P21*, *FDXR*, and *GADD45* promoters was measured by luciferase reporter assay. (b) Graphical representation of TAp63β constructs: wild-type p63β, p63β(ΔAD) deleted of residues 2 to 59 containing the acidic activation domain, and p63β(ΔPRD) deleted of residues 60 to 133 containing the proline-rich domain.**
**(c) p63β(ΔAD) and p63β(ΔPRD) are transcriptionally inert as measured by their inability to transactivate the *P21* promoter. Similar results were obtained with the *FDXR* and *GADD45* promoters (data not shown). Luciferase reporter assays were performed as described in Materials and methods. Experiments were performed in triplicate, with data reported as the fold increase in luciferase activity relative to that induced by an empty pcDNA3 control vector. Each data point was normalized for transcription efficiency using the *Renilla* luciferase activity as an internal.**

Furthermore, the experiment illustrates the different transcriptional activities inherent to the TA and ΔN variants and demonstrates the contributions of the α, β, and γ carboxy-termini. To examine other mechanisms that regulate TAp63 transactivation potential, we generated p63β(ΔTA) that does not encode the potent activation domain contained between residues 2 to 59 and p63β(ΔPRD) that does not encode the proline-rich domain between residues 60 to 133 (Fig. 3b). Here we show that deletion of the activation domain abolishes the transactivation potential of p63β (Fig. 3c). Interestingly, we also demonstrate that deletion of the proline-rich domain was also sufficient to make p63β transcriptionally inert (Fig. 3c). While the data represented in figure 3c was measured using the *P21* promoter, similar results were also obtained with the *FDXR* and *GADD45* promoters (data not shown). Altogether these data suggest that both the activation domain and proline-rich domain are required for transactivate by the TAp63 variant.

*Deletion of the proline-rich domain greatly attenuates the ability of p63β to induce endogenous gene expression in H1299 cell lines.*

To further explore the role of the proline-rich domain in p63 function, we generated multiple stable cells lines inducibly expressing p63β(ΔPRD). Two p63β(ΔPRD) expressing cell line clones, ΔPRD-8 and ΔPRD-12, were selected for further study. Western blot analysis confirms expression of the p63β(ΔPRD) construct in the absence of tetracycline (Fig. 4a). Furthermore, even though the p63β(ΔPRD) clones expressed at levels at or above that of the wild-type cell line, p63β-30, the western blot clearly demonstrates that deletion of the proline-rich domain greatly attenuates p63β transcriptional activity of p21, hdm2, and fdxr (Fig.4a.). To further illustrate the loss of

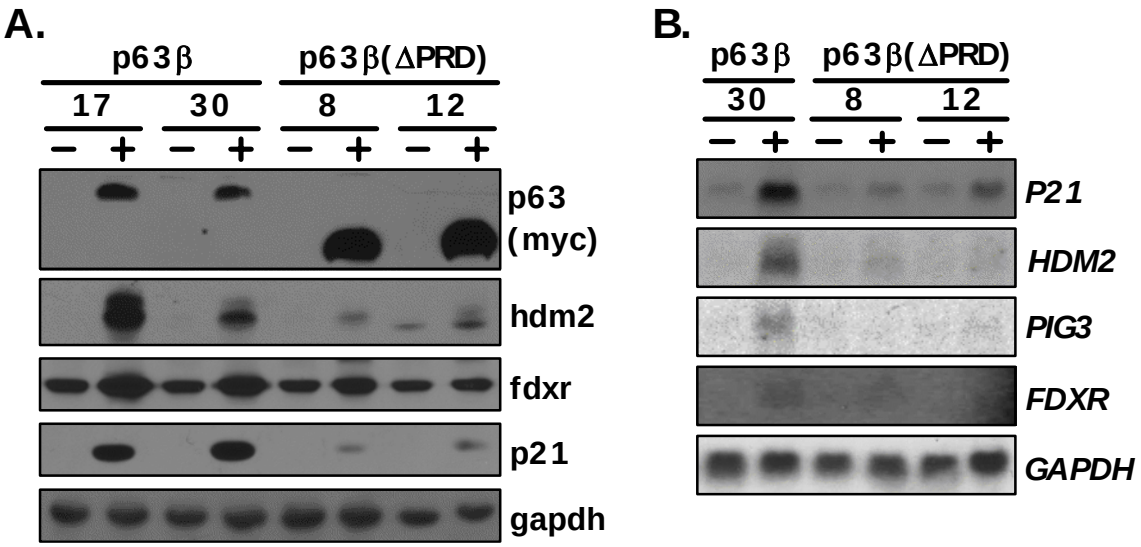


**Figure 4. Deletion of the proline-rich domain greatly attenuates the ability of p63 to induce endogenous gene expression in H1299 cell lines. (a) Western blot analysis demonstrates p63, hdm2, fdxr, and p21 protein levels at 18 h post-induction in wild-type p63β and p63β(ΔPRD) stable, inducible cell lines. Gapdh protein levels are utilized as a loading control. (b) Northern blot analysis of RNA extracted from stable, inducible cell lines following 18 h induction was performed as described in Materials and methods. Radio-labeled cDNA probes for *P21*, *HDM2*, *PIG3*, and *FDXR* genes were used to hybridize Northern blot membrane. GAPDH levels are used as a loading control.**

transcriptional activity brought about by deletion of the proline-rich domain, Northern blot analysis was performed. Again, we show that p63β(ΔPRD) fails to significantly induce transcription of *P21*, *HDM2*, *PIG3,* and *FDXR* genes (Fig. 4b). Next, we examined the affect that deletion of the proline-rich domain had on the ability of p63β(ΔPRD) to regulate overall cell viability.

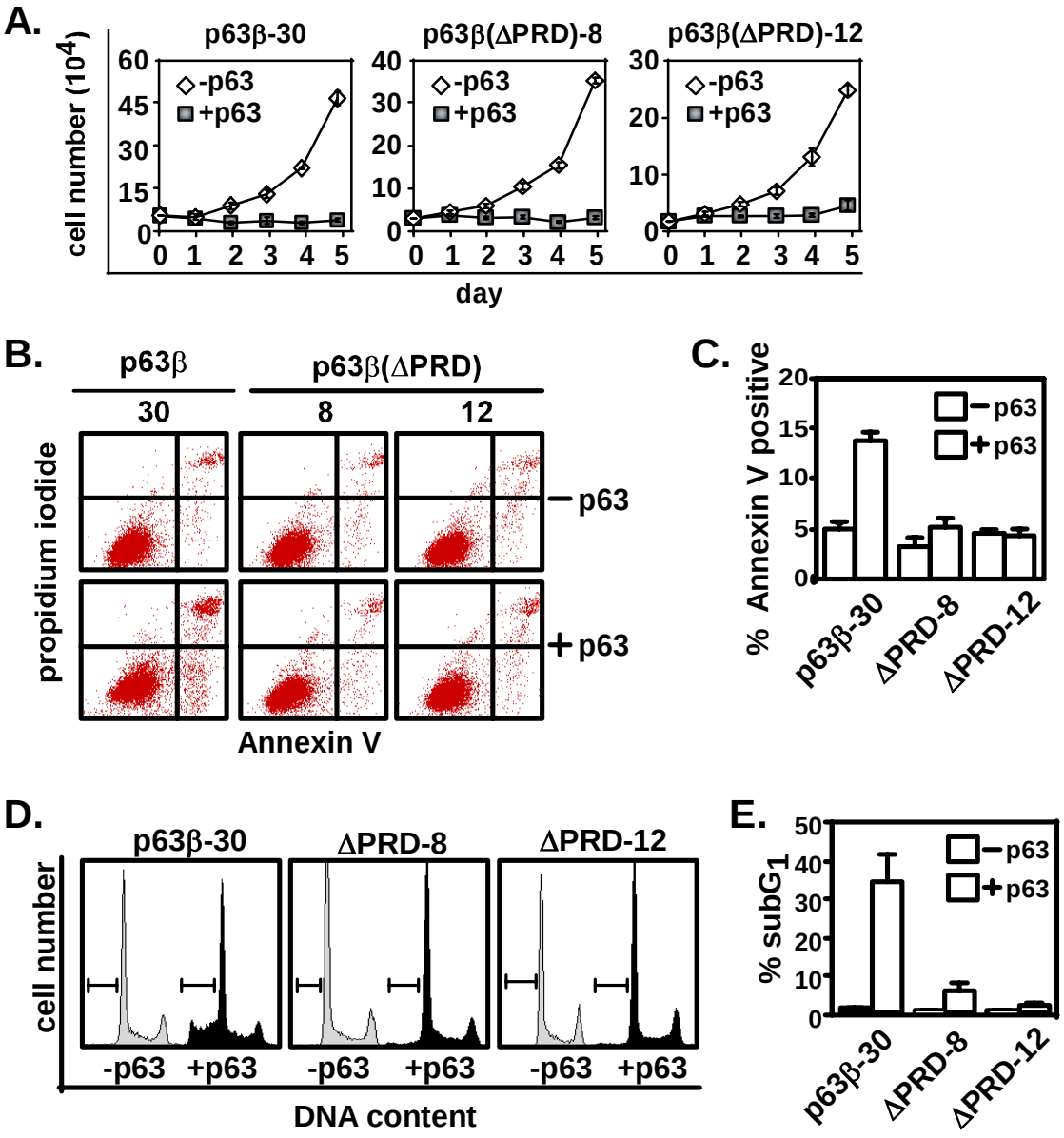


**Figure 5. p63 deleted of the proline-rich domain is competent to inhibit cell proliferation, but has greatly reduced pro-apoptotic function. (a) Growth curve analysis was performed over a 5-day period a described in Materials and methods. (b) Annexin V-FITC and propidium iodide staining was utilized as an indicator of early apoptosis in cells collected at 18 h post-induction. (c) The percentage of apoptotic Annexin-V positive cells from figure 5b are displayed in the histogram as the mean + standard deviation. (d) DNA histogram analysis was performed on un-induced cells (-p63) or induced cells (+p63) following a 48 h induction. (e) The percentage of cells accumulating a subG1 DNA content from figure 5d is depicted in the graph as the mean ± sd from three separately analyzed samples.**

*p63β deleted of the proline-rich domain is competent to inhibit cell proliferation, but has greatly reduced pro-apoptotic function.*

Interestingly, despite the loss of transcriptional activity (Fig. 3c, 4a, 4b) expression of p63β(ΔPRD) still resulted in significant growth inhibition (Fig 5a). To test whether the loss of cell viability was due to apoptosis, we performed an apoptosis assay. Figure 5b illustrates that the pro-apoptotic function of p63β(ΔPRD) is significantly attenuated at 5±0.9% and 4.6±0.2%, when compared to wild-type p63β at 15±0.9% (Fig. 5c). In addition, DNA histogram analysis revealed a significant subG1 accumulation indicative of apoptotic DNA fragmentation in wild-type 34±6.9%, but not ΔPRD at 6±1.9% and 2.1±0.3% expressing cell lines (Fig. 5d). Cell cycle analysis was unremarkable (data not shown).

*p63β(ΔPRD) inhibits wild-type p63β transactivation function in a dominant negative manner that does not compromise DNA-binding potential.*

To explore the mechanism of p63β(ΔPRD) loss of function a competitive luciferase assay was performed by co-transfecting varying amounts of cDNA expressing wild-type p63β and p63β(ΔPRD) along with the *P21* luciferase reporter. We found that p63β(ΔPRD) is a dominant negative inhibitor of wild-type p63β transactivation function in a dose dependent manner (Fig. 6a). To examine whether deletion of the proline-rich domain affected DNA binding, a chromatin immunoprecipitation assay was performed using cell lines p63β-30 and p63β(ΔPRD)-8. Following immunoprecipitation of protein-DNA complexes, we show that both wild-type p63β and p63β(ΔPRD) are found bound to the *P21* and *FDXR* promoters (Fig. 6b).

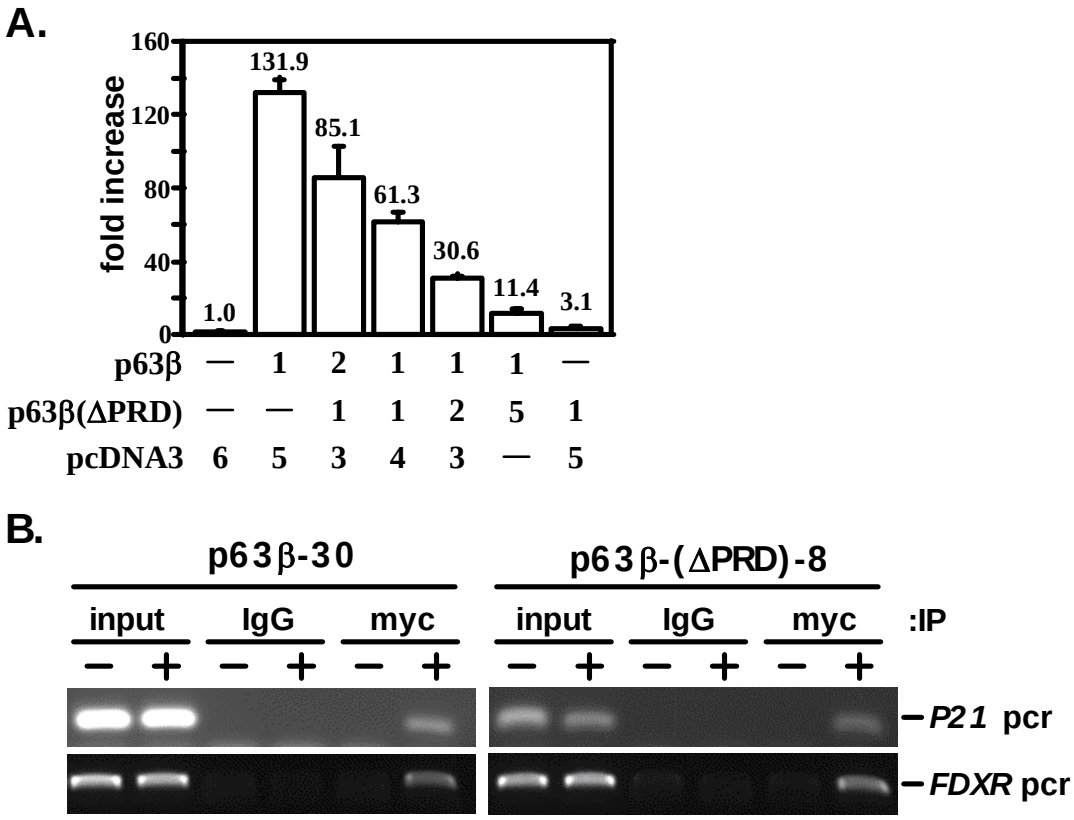


**Figure 6. p63β(ΔPRD) inhibits wild-type p63β transactivation function in a dominant negative manner that does not compromise DNA-binding potential. (a) Varying amounts of pcDNA3 expression vectors were transiently transfected into H1299 cells along with 100 ng pGL2-*P21* and 10 ng of *Renilla* control vector. Total DNA transfected was kept constant at 710ng per well using an empty pcDNA3 vector. Graph represents the mean + s.d. fold increase in luciferase activity from three separately analyzed samples. (b) Chromatin immunoprecipitation of p63β and p63β(ΔPRD) was carried using an anti-myc antibody (Ab9106). Rabbit IgG was used for a non-immune control. ChIP and PCR conditions are described in Materials and methods.**

*Mutational analysis of the proline-rich domain identifies a PXXP motif as a major contributor to p63β transactivation potential.*

Given that p63β(ΔPRD) loss of function was not due to a DNA-binding defect, we performed mutational analysis of the proline-rich domain to identify potential

sequences that might contribute to TAp63 transactivation potential. Knowing that proline-rich motifs can function as protein-protein interaction sites, we chose to disrupt the two PXXP motifs, where P is a proline and X is any amino acid, present in the proline-rich domain of the TAp63 variant by generating a point mutation converting the second proline to an alanine (Fig. 7a). When co-transfected with the *P21* luciferase reporter, we found that transactivation potential of p63β(P70A) was comparable to that of wild-type p63β. However, a significant loss of transactivation potential was consistently observed when the PXXP motif at residues 124 to 127 of p63β was disrupted separately, as with p63β(P127A), or in conjunction with the other PXXP motif at residues 67 to 70, as with p63β(P70A,P127A).

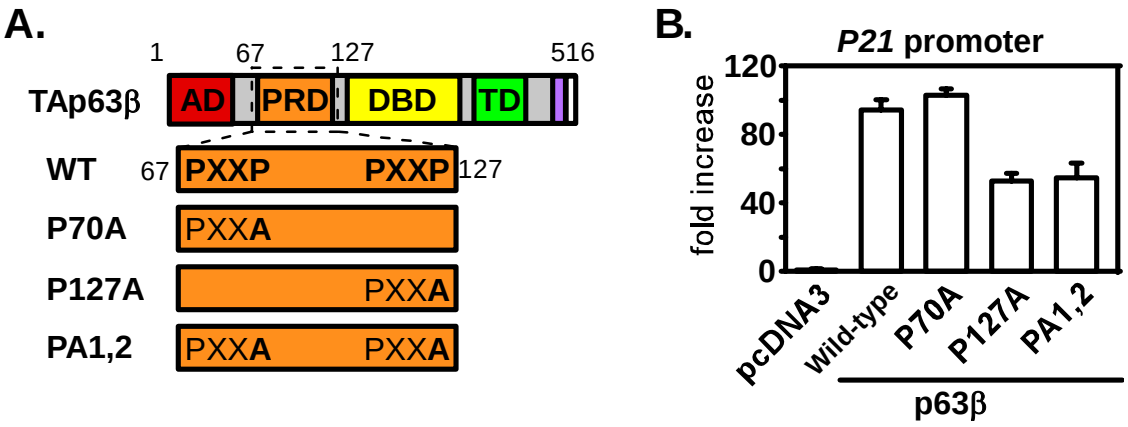


**Figure 7. Mutational analysis of the proline-rich domain identifies a PXXP motif as a major contributor to p63β transactivation potential. (a) Graphic representation of p63β constructs carrying point mutations in the proline-rich domain. (b) Luciferase reporter assay was carried out as described in materials and methods. Graph represents the fold increase measured from three separately measured samples.**

## Discussion

All p53 family members, that is p53, p63, and p73, have been shown to bind the consensus p53-response element, RRRC(A/T)(T/A)GYYY, where R is any purine and Y is any pyrimidine [11], and to regulate expression from p53-target genes [12-14]

In addition, recent studies have identified target genes that are specifically bound by p63 and 73, but not p53; these include the promoters of envoplakin (*ENV*) and *SMARCD3* [15], pigment epithelium-derived factor (*PEDF*) [16], the antioxidant glutathione reductase (*GPX2*) [17], and the vitamin D receptor (*VDR*) [18]. Significantly, regulation of unique target genes provides a mechanism whereby p63 and 73 functions can functionally diverge from that of p53. Still, knowledge of target genes bound by p63 and p73 only hints at the potential function of these transcription factors. Only after more is known about the pathways that regulate their expression, transcriptional activity, or stability can conclusions be drawn about p63 and p73 function during development and tumorigenesis.

Here we report that the proline-rich domain is critical for p63β to function as a transcriptional activator. Significantly, deletion of the proline-rich domain or deletion of the activation domain resulted in a similar loss of function as measured by the luciferase reporter assay (Fig. 3c). This loss of function was also seen with endogenous target gene activation, as p63β(ΔPRD) expressed in stable, inducible cell lines has drastically reduced transactivation potential when assayed by western blot or northern blot (Fig 4a, 4b). Interestingly, the loss of function phenotype reported here is similar to that described for p53 deleted of its PRD [19, 20]. For example, the ability of p53(ΔPRD), deleted of residues 62 to 91, to transactivate certain target genes was greatly attenuated,

while other target genes remained unchanged when compare to that seen with wild-type p53 induction [19-21]. Furthermore, similar to our results reported here, the p53(ΔPRD) was not competent to induce apoptosis, but retained the ability to inhibit cell proliferation [20]. The similarity of these results is surprising since p53 and p63 share little homology in their PRD, where human p53 PRD contains five PXXP motifs, the human p63 PRD only contains two PXXP motifs. Furthermore, while the proline-rich domain for p63 is highly conserved across species, the PRD of p53 is not. For instance the mouse *TP53* locus encodes a PRD with only two PXXP motifs that shares only 43% amino acid identity with its human counterpart [22]. Furthermore the p53 codon 72 polymorphism encoding a proline, instead of an arginine, reduces the pro-apoptotic function of p53 and in human but not mouse cells [23]. Altogether, these results suggest that the PRD for p53 family members contributes to their pro-apoptotic potential and may be differentially regulated by specific signaling pathways.

Given the lack of conservation seen with the p53 PRD, it is likely that modifications and protein-interactions with the p53 PRD differ significantly from species to species. Conversely, the p63 PRD is highly conserved suggesting that the sequences therein are critical for normal p63 function. Further analysis of this domain is required to identifying signaling pathways interacting with p63 and regulating p63 function through the modulation of the proline-rich domain.

## Materials and methods

### *Cell culture*

Stable, inducible cell lines were generated in H1299 cells using the tetracycline-off system as previously described [24]. Transfections were performed using the calcium phosphate precipitation method [25].

### Apoptosis assay

Following an 18 h induction, both floating and adhered cells were collected and washed in cold PBS. Staining was carried out using an Annexin V-FITC and propidium iodide staining kit (MBL, Japan) as suggested by the manufacturer. The experiment was performed in triplicate with data expressed as the mean number of Annexin V positive cells $\pm$ the standard deviation.

### *Chromatin immunoprecipitation assay (ChIP)*

H1299 cells were seeded at $3.0x10^6$ cells per 10cm dish and control (-p63) or induced (+p63) were incubated at 37ºC, 5%CO2. After indicated amount of time cells were removed from incubator, washed once with ice cold phosphate buffered saline at pH 7.4 (PBS), and protein was cross-linked to DNA by treating with 1% formaldehyde for 10 min at room temperature. Cell extracts were collected in RIPA buffer and sonicated to generate DNA fragments. Input DNA was saved ($1:50^{th}$). Protein-DNA complexes were immunoprecipitated with anti-Myc antibody (Abcam, Cambridge, UK) or a rabbit control IgG (Sigma, St. Louis, MO) conjugated to protein A-agarose beads. The beads were washed and the protein-DNA cross-linking was reversed by heating at 65ºC in high salt

overnight. DNA was extracted using a Qiagen DNA miniprep kit. PCR conditions and primer sequences were as previously described [26, 27].

*DNA histogram analysis*

Analysis was performed as previously described [10]. Briefly, $3 \times 10^5$ cells were plated per 10-cm dish. Cells were induced or un-induced to express p63 proteins for 48hrs Floating and adhered cells were collected and washed in cold PBS. Cells were resuspended in 100 ul of PBS and then fixed with 2 ml of 100% ethanol for at least 30 min at 4ºC. Cells were rehydrated in PBS then treated with RNase (Sigma) and stained with propidium iodide (Molecular Probes, Inc., Eugene, OR). FACS data was collected using a FACS-Caliber cell sorter (BD Biosciences). The percentage of cells in each phase of the cell cycle was analyzed using Cell Quest software. The percentage of sub-$G_1$ accumulation was used as a marker of apoptosis.

*Growth Curve Analysis*

The overall viability of each cell line was addressed by growth curve analysis, as previously described [10]. Briefly, cells were plated at a density of $5 \times 10^4$ cells per 6-cm dish and with or without tetracycline. Adherent cells were counted at 24 h intervals over a 5 day period using a Coulter cell counter. Medium was changed at day 3 for both control and experimental cell groups.

*Luciferase assay*

Transient transfections were carried out using the ESCORT V reagent (Sigma) as described by the manufacturer. Briefly, H1299 cells were plated at 5 x $10^4$ cells per well in 12-well plates and allowed to recover overnight. Cells were co-transfected with 100 ng of activator in pcDNA3 expression vector, 100 ng of pGL2 reporter vector, and 5 ng of pRL-CMV, as an internal control. Cells were washed with cold PBS and lysed in 150 µl of passive lysis buffer at 36 h post-transfection, Luciferase activity was measured using a Turner Designs luminometer. Dual luciferase assays were performed in triplicate according to the manufacturer's instruction and each reading was normalized to *Renilla* luciferase activity to correct for changes in transfection efficiency (Promega). The fold increase in relative luciferase activity was calculated as the product of luciferase activity induced by pcDNA3 constructs expressing p63 divided by that induced by a pcDNA3 empty control vector. The reporter constructs, pGL2-basic-p21 (full-length promoter), pGL2-Fos-GADD45 (intron 1 RE), and pGL2-basic-FDXR (–56 to +11 promoter) were described in previous studies.

*Northern blots analysis*

Cells were plated at a density of 2.5 x 106 cells/10-cm dish and incubated for indicated amounts of time. RNA was collected in 1.0 ml of Trizol reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. Northern blots were prepared using 10 or 20 µg of total RNA. The cDNA probes for *P21*, *HDM2*, *PIG3*, and *GAPDH* were prepared as previously described [20].

*Western blots analysis*

Western blot analysis was performed as previously described [10]. Blots were developed using the following antibodies and conditions: Myc (Ab9106) antibody at 1:3000 (Abcam), p21 (C19) antibody at 1:2000 (Santa Cruz Biotechnology, Inc; Santa Cruz, CA), fdxr antibody, anti-actin polyclonal antibody at 1:5000(Sigma), and secondary anti-rabbit-HRP at 1:5000 (Sigma).

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# CONCLUSION

p63 is a transcription factor that is homologous to the tumor suppressor p53. A requirement for p63 in epithelial development has been well documented; however, the role of p63 in tumorigenesis is highly controversial. Contributing to this debate, the *TP63* locus expresses multiple gene products with poorly characterized function. We found that both the TAp63 and ΔNp63 variant retain the potential to regulate gene expression and cell viability. We determined that the p63β isoform was the most potent p63 isoform, followed by p63γ then p63α. Furthermore, each TAp63 variant possessed the ability to inhibit proliferation, induce transcription, and promote apoptosis. In addition, we showed that amongst the ΔNp63 variants, ΔNp63β had the strongest potential to transactivate target genes, followed by ΔNp63α then ΔNp63γ. Interestingly, we observed that ΔNp63α and ΔNp63β are effective in suppressing cell proliferation whereas ΔNp63γ possesses a much weaker potential in growth suppression.

To determine the mechanisms that regulate p63 function, we performed mutational analysis of p63 domains and potential signaling motifs encoded by *TP63* and tested the ability of these constructs to transactivate exogenous target gene promoters and generated stable, inducible cell lines to examine their ability to regulate cell viability and transactivate endogenous target gene promoters. Previously, our laboratory was the first to identify a transcriptional activity for the ΔNp63 variant. Here, we defined the activation domain for the ΔNp63 variant to include the 14 unique ΔN residues and adjacent sequences within the proline-rich domain. We identified a PXXP motif in the proline-rich domain at residues 68 to 72 that significantly contributed to the transactivation potential of ΔNp63β. In addition, we showed that a PPXY motif common

to the α and β, but not γ, C-termini was a critical regulator of ΔNp63 transactivation potential.

The transcriptional activity of the TAp63 variant can also be regulated by the proline-rich domain. We found that deletion of the activation domain (residues 2 to 59) or proline-rich domain (residues 60 to 133) was sufficient to render the TAp63 variant transcriptionally inert. Furthermore, expression of p63β(ΔPRD) in inducible, stable cell lines demonstrated that the proline-rich domain was essential for transactivation of endogenous target genes and for induction of apoptosis by p63β. In addition, we identified a PXXP motif at residues 124 to 127 that was a significant contributor to p63 transactivation potential.

In summation, our data suggest that p63 activity can be regulated at the level of transcription or by post-translational modifications. Promoter selection determines the expression of either the stronger TAp63 activation domain or the weaker ΔNp63 activation domain. In addition, alternative splicing determines the selection of the α, β, or γ C-termini, each of which retain a unique ability to regulate transactivation by the N-terminal activation domains. The α C-terminus has previously been characterized as an inhibitor of p63 function [27]; here, we demonstrated that both the α and β C-termini encode a proline-rich region that can promote transactivation of the ΔNp63 variant. Furthermore, the transactivation potential of p63 is regulated by PXXP and PPXY motifs that serve as ligand-binding sites for SH3 and WW domain containing proteins, respectively. We concluded that these proline-rich motifs could serve as binding sites for signaling molecules regulating p63 function.

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