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p63 Expression Is Associated with p53 Loss in Oral-Esophageal Epithelia of p53-deficient Mice[1]

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ABSTRACT

The *p53* gene family, comprising *p53*, *p63*, and *p73*, has overlapping and distinctive functional roles. These members share structural similarities allowing for dynamic interplay in the activation of genes that are important in development and key cellular functions, such as the induction of apoptosis. Whereas *p53* is a classical tumor suppressor gene, *p63* and *p73* do not share this feature in cancer formation and progression. The compensation in the expression level of these members in a background that is deficient for one of them has not been examined previously. Given the importance of p63 in the development and differentiation of oral-esophageal stratified squamous epithelia and the absence of oral-esophageal tumors in p53-null mice, we postulated and describe herein that p63 expression is associated with the loss of p53 in a p53-deficient background. Both full-length and amino-truncated forms of p63 are expressed and increased in oral-esophageal epithelia of p53-null mice when compared with wild-type mice, and the induction of p21 may potentially be preserved through the increase of p63.

INTRODUCTION

The *p53* tumor suppressor gene is frequently mutated in human cancers (1). Additionally, in many cancers, p53 function is altered through binding to viral oncoproteins or abrogation of p53 degradation by mdm-2/hdm-2 in concert with $p19^{ARF}$/$p14^{ARF}$ (2). Generally speaking, most p53 mutations are missense, leading to stabilization of protein with gain-of-function. Some p53 mutants can inactivate WT p53 through hetero-oligomerization.

Recently, information has emerged about p53 homologues, such as p73 and p63 (3–5), with the emphasis on p63 in this study. Cloned through degenerate PCR, p63 is expressed in the squamous epithelium and the thymus (6) and other tissues as well (7). P63 has different transcripts attributable to alternative splicing (α,β, γ), and the use of different promoters results in retention of the TA[4] or ΔN (4, 8, 9). Thus, these p63 isoforms are referred to as TAp63α,β, γ and ΔNα,β, γ. All p63 isoforms contain DNA binding and hetero-oligomerization domains. However, the ΔNp63 versions lack the NH_2-terminal transactivation domain but can still bind to DNA and, thus, may function as dominant negative proteins.

The TAp63γ and TAp63β transactivate promoters at levels comparable with WT p53, but TA-63α does not contain this property (reviewed in Ref. 3). In particular, TAp53 can activate *in vitro* p53 responsive promoters such as p21, GADD45, Bax, and mdm2 (reviewed in Refs. 8 and 10). TAp63γ and TAp63β induce apoptosis in transient transfection experiments in contrast to TA-p63α (3, 4, 6, 11). TAp63γ can be induced after UV irradiation (12). By contrast, the ΔN isoforms block the functions of p53. This may be attributable to competition for DNA binding sites to prevent p53 or TAp63 from binding DNA. Alternatively, it is conceivable that p53 or TAp63 may be sequestered by ΔNp63 through the oligomerization domain or another domain (13). Precedence for interactions between p53 family members has been established with the observation that mutant p53 can down-regulate both p63 and p73 through a direct interaction with the p53 core domain (14).

There is little evidence to suggest that p63 acts as a tumor suppressor gene. Mutations of p63 in human tumors are exceedingly rare (3, 5, 8, 9, 15). Patients with germ-line mutations in the DNA binding domain of p63 result in developmental defects but not tumors (16). Additional insights into the functions of p63 have been gained through the generation and characterization of mice in which p63 has been ablated in embryonic stem cells through homologous recombination. p63-null mice are viable at birth but die several h later and are not susceptible to spontaneous tumorigenesis (17, 18). Mutant newborn mice and late stage embryos have craniofacial abnormalities, limb truncations, and a complete absence of epidermis and related appendages (17, 18). Histological analysis has revealed the absence of a stratified epithelium in the epidermis with a lack of the characteristic structure of basal, suprabasal, and cornified layers as well as hair follicles. Instead of an epidermis, $p63^{-/-}$ late stage embryos retain isolated patches of epithelial cells along the exposed dermis. Furthermore, the normally stratified squamous epithelium of tongue, esophagus, and forestomach, with the same characteristic structure of basal, suprabasal, and differentiated cells, was replaced by an unusual array of cuboidal, goblet-like epithelium.

Given that p63 appears to be important for the development and possibly also differentiation of the stratified squamous epithelium, we postulated that p63 may have a critical role in the maintenance of the oral-esophageal squamous epithelium by compensating for the loss of p53 in p53-deficient mice.

MATERIALS AND METHODS

DNA Constructs. Total RNA was extracted from the ME-180 cell line (American Type Culture Collection) using the TRIzol method (Life Technologies, Inc.). First-strand synthesis was then performed on 5 μg of RNA using the Superscript First-Strand Synthesis system for RT-PCR (Life Technologies, Inc.). The following primers were used to amplify ΔNp63:

ΔNp63α 5′ATGTTGTACCTGGAAAACAA and 5′CACTCCCCCTCCTCTTTGA

ΔNp63 γ 5′ATGTTGTACCTGGAAAACAA and 5′CTATGGGTACACTGATCGGT.

The ΔNp63α PCR product was 1761 bp, and the one for ΔNp63γ was 1182 bp. These fragments were amplified using the Elongaze enzyme mix (Life Technologies, Inc.). After denaturing at 94°C for 60 s, PCR consisted of 35 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s followed by 72°C for 5 min. PCR products were then analyzed on a 1% agarose gel. Nested PCR was

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[4] The abbreviations used are: TA, acidic NH_2 terminus; ΔN, truncated NH_2 terminus; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TAMRA, 6-carboxyl-N,N,N′,N′-tetramethylrhodamine; ABC, avidin-biotin peroxidase complex; WT, wild type.

Table 1 *Quantitative scoring of p63 immunohistochemical staining in tongue (T) or esophagus (E) in WT or p53-null ($p53^{-/-}$) mice*[a]

	WT mice % positive p63	p53-null mice % positive p63	*P*
TA p63 E	0.46 ± 0.15	0.83 ± 0.11	<0.05
TA p63 T	0.49 ± 0.10	0.75 ± 0.11	<0.05
ΔN p63 E	0.67 ± 0.07	0.88 ± 0.08	<0.05
ΔN p63 T	0.67 ± 0.10	0.89 ± 0.06	<0.05

[a] For comparisons of ΔN p63 immunohistochemistry, 5 WT and 5 $p53^{-/-}$ mice were used, and for full-length p63 immunohistochemistry, 5 WT and 5 $p53^{-/-}$ mice were used. Values are expressed as percentage ± standard deviation. $P < 0.05$ is considered statistically significant.

then performed using the above ΔNp63α and ΔNp63γ primers tagged with *Xho*I and the β-actin Kozak consensus motif at the 5′ prime end and *Not*I at the 3′ end. Nested PCR products were then subcloned into a pCIBA vector and purified by the alkaline lysis method. The p21 promoter-luciferase reporter gene constructs used were for WT p21 (WWP-Luc) and when the p53 DNA binding sites were mutated in the context of the full-length p21 promoter (6-Luc).

Cell Culture and Transient Transfection. Mouse oral epithelia from WT and p53-null mice were peeled off underlying tissues after incubation with 1.5 units/ml *Dispase I* (Boehringer Mannhem). Subsequently, the tissues were trypsinized. The cell suspension from the latter was plated and subcultivated in serum-free medium (Life Technologies, Inc.). The mouse oral epithelial cells or keratinocytes from WT or normal (Mokn) and p53-null mice (Mokp$53^{-/-}$) were transiently transfected at 80% confluence with 0.5–1 μg of plasmid mixtures preincubated with 12 μl of Plus Reagent and 16 μl of LipofectAMINE Reagent (Life Technologies, Inc.). Cells were transfected with either WWP-Luc or 6-Luc and β-Gal constructs as well as with the ΔNp63 γ or ΔNp63 α constructs. The cells were incubated for 36 h at 37°C and then washed with PBS and harvested with Reporter lysis buffer (Promega). Luciferase and β-Galactosidase assays were performed. All experiments were performed in triplicate, and at least three independent experiments were done (results expressed as mean +/− SD).

TaqMan RT-PCR Assay. TaqMan RT-PCR assay was conducted according to the manufacturer's instructions (PE Applied Biosystems). In brief, oligonucleotides (probes) for TaqMan RT-PCR were labeled with FAM(6-carboxyfluorescein; p21, bax) or VIC (GAPDH) and 3′ prime quencher, TAMRA. The following primer and probe sequences were used:

p21 primers: 5′-CGAGAACGGTGGAACTTTGAC-3′ and 5′-TCCCAGACGAAGTTGCCCT-3′

p21 probe: 6FAM-TCGTCACGGAGACGCCGCTG-TAMRA

Bax primers: 5′-GGAGCAGCTTGGGAGCG-3′ and 5′-AAAAGGCCCCTGTCTTCATGA-3′

Bax probe: 6FAM-CGGGCCCACCAGCTCTGAACA-TAMRA.

GAPDH primers and the Vic-labeled probe were obtained from PE Applied

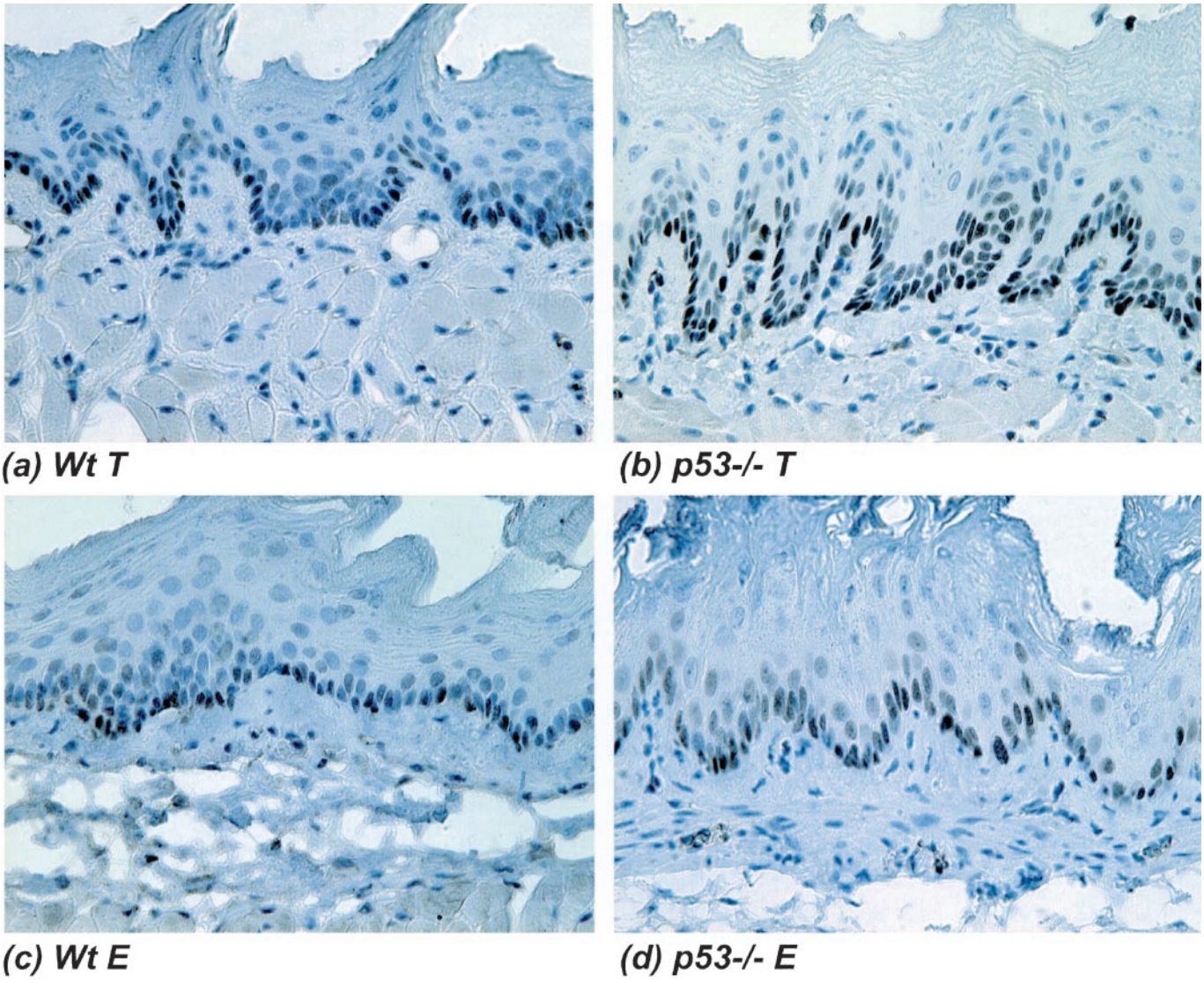

Fig. 1. Full-length or TAp63 immunohistochemistry (D20 antibody) immunohistochemical staining (×40) in WT mouse tongue (*A*), $p53^{-/-}$ mouse tongue (*B*), WT mouse esophagus (*C*), and $p53^{-/-}$ mouse esophagus (*D*). Note the basal cell nuclear staining in $p53^{-/-}$ tongue and esophagus.

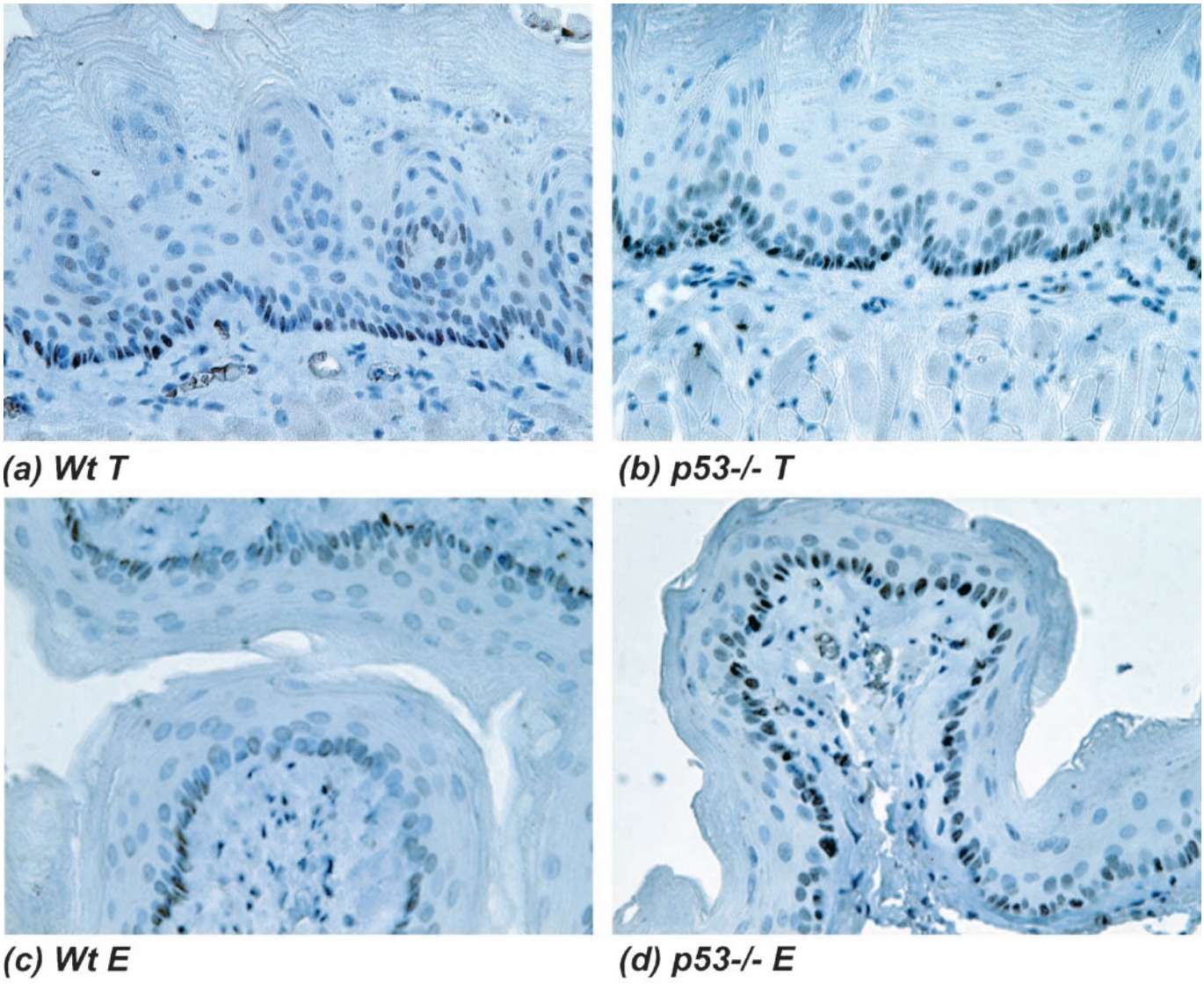

Fig. 2. ΔNp63 immunohistochemistry (N16 antibody) immunohistochemical staining (×40) in WT mouse tongue (*A*), $p53^{-/-}$ mouse tongue (*B*), WT mouse esophagus (*C*), and $p53^{-/-}$ mouse esophagus (*D*). Note the basal and suprabasal cell nuclear staining in $p53^{-/-}$ tongue and esophagus.

Biosystems. All primers and probes were designed with the use of Primer Express Version 1.0 (PE Applied Biosystems). Total RNA was isolated from tongue and esophageal epithelia of WT and p53-null mice using the TRIzol method. Total RNA (1 μg) was used for reverse transcription and amplification using TaqMan Reverse Transcription Reagents according to manufacturer's protocol (PE Applied Biosystems). A master mix of TaqMan reagents was prepared, and 10 ng of each reverse transcription sample was used in the TaqMan PCR reaction. Each tube contained both a gene probe and primers and a GAPDH control probe and primer. Each sample was done in quadruplicate. Reactions in which reverse transcriptase was not added to the reverse transcription reaction were used to control for genomic contamination. The increase in fluorescence was proportional to the concentration of template in the PCR. The standard curve method was used to quantitate amounts of each gene relative to the GAPDH amount in each reaction according to the manufacturer's protocol (PE Applied Biosystems). Reactions were carried out in 96-well plates using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems).

Histology and Immunohistochemistry. Age-matched (4–5 months), WT, and p53-deficient mice littermates from the same BL/6 background strain were sacrificed. Oral-esophageal tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and tissue sections were stained with H&E in a manner similar to our previous studies (19). Immunohistochemical staining was performed in mouse tongue and esophageal tissue sections by the ABC method

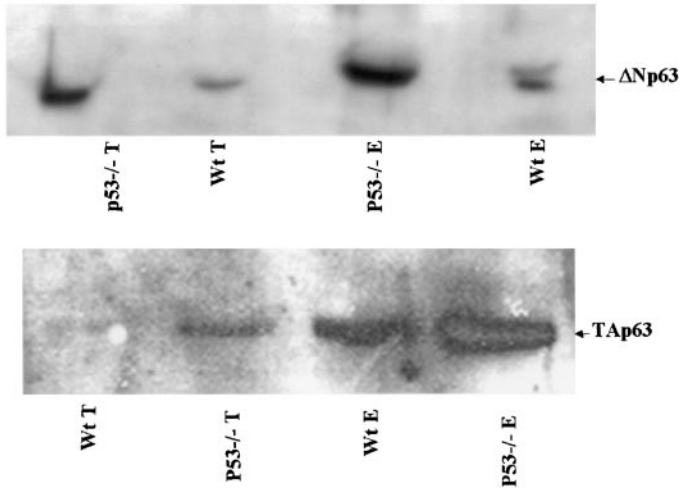

Fig. 3. Western blot analysis of WT and $p53^{-/-}$ mice tongues and esophagi for full-length TAp63 (*A*) and ΔNp63 (*B*). Note the increased level of full-length p63 and ΔNp63 in epithelial protein lysates derived from $p53^{-/-}$ mice. Equal loading and transfer of proteins were confirmed with Ponceau S staining of membranes and reprobing with an actin antibody (data not shown).

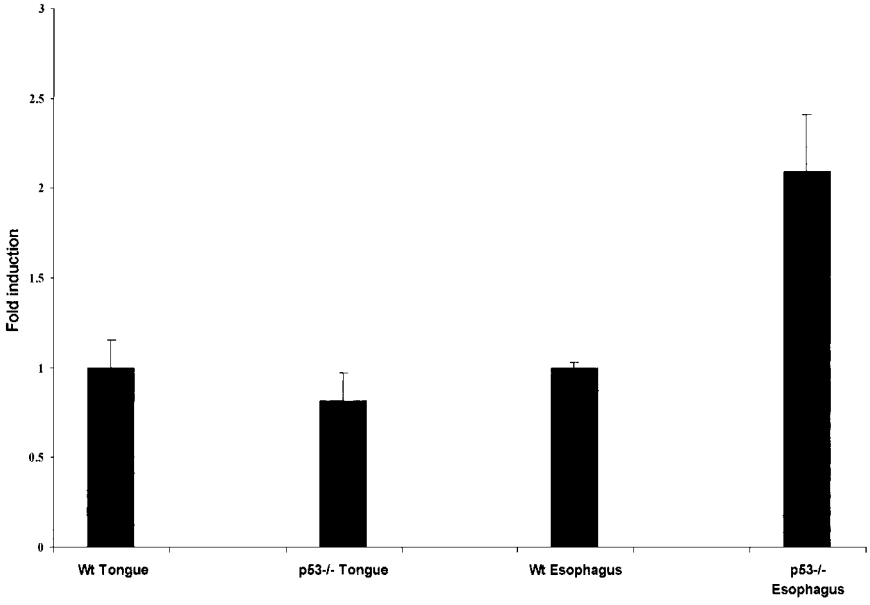

Fig. 4. TaqMan assay of p21 mRNA expression level in WT and p53-null tongue and esophagus. Note that p21 mRNA expression level in p53-null tongue is comparable with that of WT tongue. p21 mRNA expression level is increased in p53-null esophagus compared with WT esophagus.

using the Vectastain Elite ABC kit (Vector Laboratories) as described previously (20). Sections (3–5 μm) were mounted on adhesive-coated slides, deparaffinized, and rehydrated through xylene and alcohol. After rinsing in tap water and PBS, slides were placed in plastic Coplin jars containing 10 mM citrate buffer. Jars were covered with loose-filling caps and heated in the microwave oven for 20 min to unmask antigen. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 5 min. Sections were blocked with either 5% rabbit serum or protein blocking agent (Immunotech) for 15 min after being cooled. Slides were then incubated with primary antibody (4A4 for full-length p63 and Ab-1 for ΔNp63 from PharMingen and Oncogene Science, respectively) overnight at 4°C, washed in PBS, and incubated with the corresponding biotinylated secondary antibody for 60 min at room temperature. After PBS washes, sections were incubated with ABC Elite reagent for 5 min at room temperature, and reaction products were developed using diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) as chromogen and counterstained with hematoxylin.

Three ×40 power fields were counted on each slide. The positively stained nuclei were counted and divided by the total number of nuclei per high power field, and a mean was calculated. Of note, cytoplasmic staining was rare. Two independent scorers were blinded to the slide source when doing the evaluation. Student's t test was used for statistical analysis, and $P < 0.05$ was considered statistically significant. Additionally, staining intensity was designated from 1–3, where 1 is weak, 2 is moderate, and 3 is strong based upon similar qualitative approaches described previously (20).

Western Blot Analysis. The tongue or esophageal mucosa was immediately dissected away from the muscularis propria after incubation in 1.5 units/ml *Dispase I* (Boehringer Mannheim) overnight at 4°C. The epithelium was pealed off with forceps, minced, and lysed in ELB buffer [50 mM HEPES (pH 7.4), 0.1% NP-40, and 250 mM NaCl] with protease inhibitors (5 μg/ml aprotinin, 100 μg/ml phenylmethane sulfonyl fluoride, and 5 μg/ml leupeptin) and phosphatase inhibitors (5 μg/ml sodium vanadate and 10 mM sodium fluoride) for 45 min on ice. The lysates were centrifuged at 13,000 rpm at 4°C for 15 min, and supernatants were collected. Total protein (10 μg) of each sample was separated on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to Immobilon membranes (Millipore) at 100 V for 1 h at 4°C. Blocking was performed in 5% milk, 10 mM Tris pH 7.4, 150 mM NaCl, and 0.2% Tween 20 overnight at 4°C. Primary antibody against p63 (D20 for TAp63 and N16 for ΔNp63; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:2,000 dilution. Secondary antibody was peroxidase conjugated sheep antigoat immunoglobulin (1:2,000 dilution; Sigma Chemical Co.). The detection system was enhanced chemiluminescence (Amersham). Equal loading of proteins was confirmed by Ponceau S staining of the membranes and reprobing the membranes with an actin antibody.

RESULTS

Age-matched WT and p53-null mice were assessed for histopathological changes in the oral-esophageal squamous epithelium and not

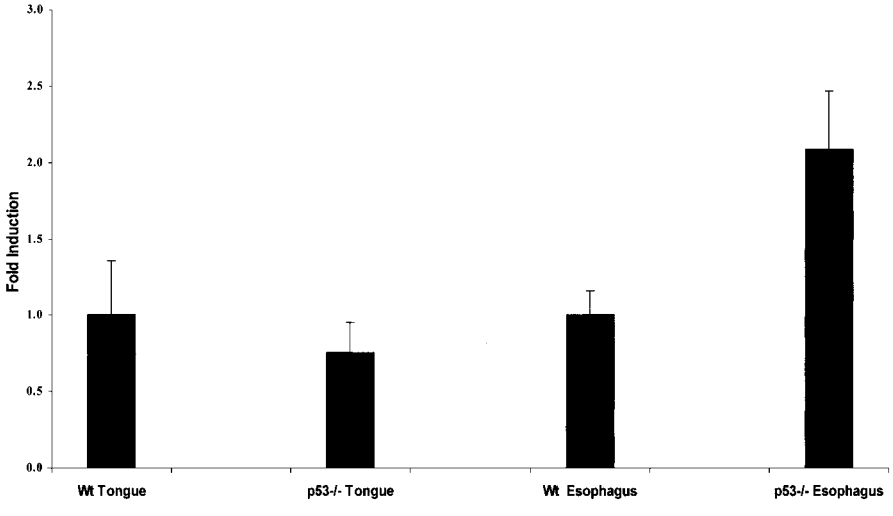

Fig. 5. TaqMan assay of Bax mRNA expression level in WT and p53-null tongue and esophagus. Note that bax mRNA expression in p53-null tongue is comparable with that of WT tongue. Bax mRNA expression level is increased in p53-null esophagus compared with WT esophagus.

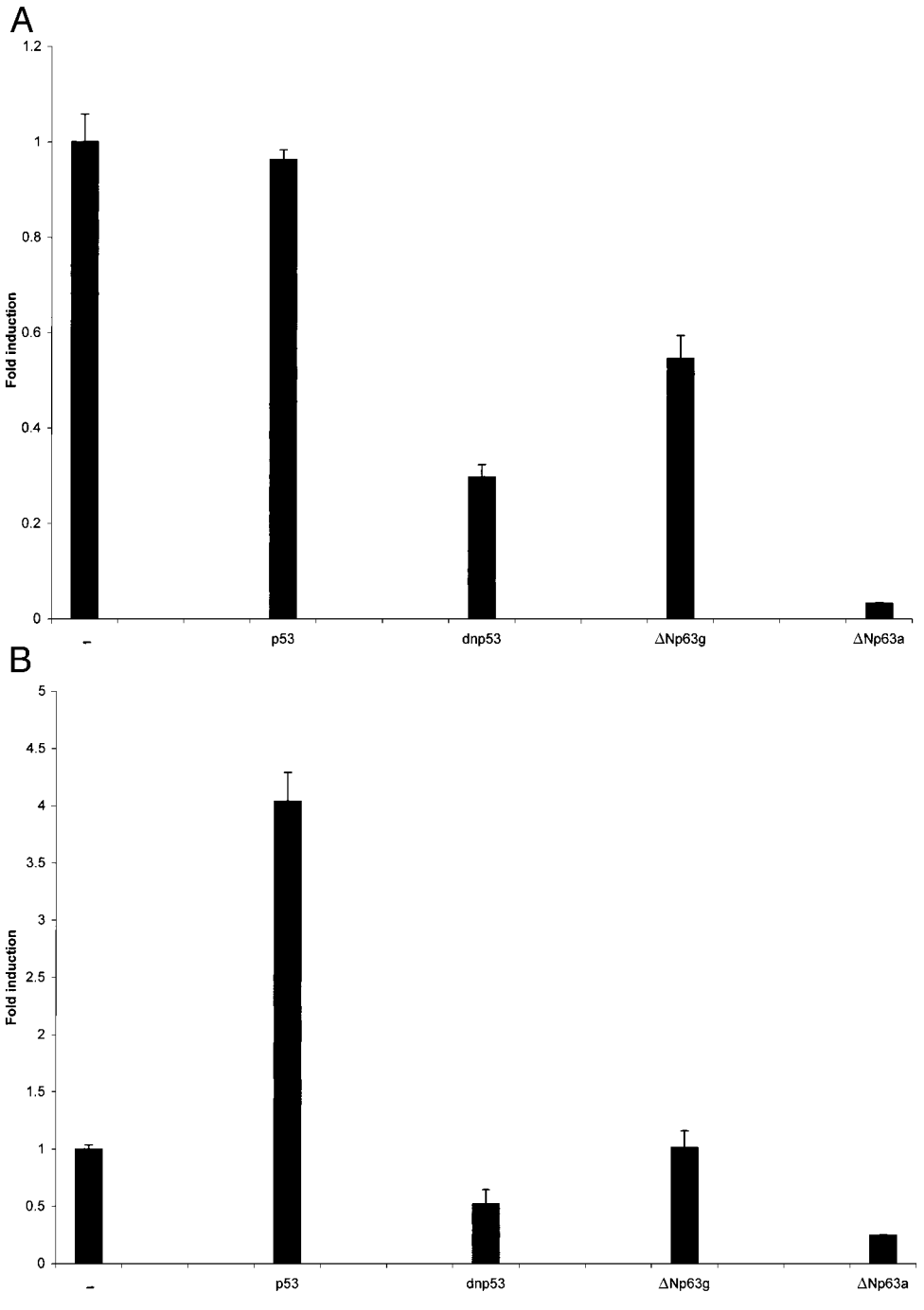

Fig. 6. Cotransfection of WT p21 promoter-luciferase reporter gene and WT p53, dominant negative p53, ΔNp63γ, or ΔNp63α into either WT or normal oral keratinocytes (*A*) *versus* p53-null oral keratinocytes (*B*). Luciferase activity was standardized to β-galactosidase activity and expressed as fold-induction. Transfections were carried out in triplicate and done in at least three independent experiments (mean +/− SD).

found to have any evidence of hyperplasia, dysplasia, carcinoma, or impaired squamous epithelial differentiation. These mice were then assessed for p63 expression using immunohistochemical approaches to detect both the full-length (TA) and amino-truncated (ΔN) versions of p63, respectively. The antibodies do not cross-react with each other but do recognize the α,β and γ isoforms for their respective TAp63 or ΔNp63 proteins. This analysis led to the determination that both TAp63 and ΔNp63 are expressed in oral-esophageal epithelia of WT and p53-null mice (Table 1; Figs. 1 and 2). Additionally, there is increased expression of both TAp63 and ΔNp63 in the oral-esophageal epithelia of p53-null mice when compared with their age-matched WT littermates, in a statistically significant fashion (Table 1). There also appears to be predominance of TAp63 in basal cells compared with ΔNp63 in this compartment (Figs. 1 and 2). Furthermore, the intensity of p63 staining, a qualitative parameter, appears to be enhanced in p53-null mice compared with their WT counterparts (Figs. 1 and 2). ΔNp63 staining intensity was moderate to high in tongues and esophagi of p53-null mice compared with weak in WT mice. TAp63 showed a moderate to high staining intensity in the same tissues of p53-null mice compared with a weak to moderate staining intensity in WT mice.

As further and independent corroboration of p63 expression, tongue and esophageal epithelial cells from both WT and p53-null mice were isolated from which protein lysates were used for Western blot analysis. This revealed that TAp63 protein is expressed at a higher level in oral (8.5×) and esophageal (1.3×) epithelial cells from p53-null mice compared with WT mice (Fig. 3). It is possible that the molecular mass corresponding to p63 represents posttranslational modifica-

tions and/or recognition of different isoforms as observed by others (7). Np63 expression was also increased in oral (4×) and esophageal (3×) esophageal epithelial cells from p53-null mice compared with WT mice (Fig. 3).

Because it is known that p21 and Bax are targets of p53 *in vitro*, we postulated that p21 and Bax may still be expressed in the oral-esophageal epithelia of p53-null mice, possibly as the result of the associated increase of TAp63 noted by immunohistochemical and Western blot analyses. Indeed, quantitative measurement of p21 and Bax mRNA levels by the TaqMan assay revealed that p21 and Bax mRNA expression levels in tongues of p53-null mice is comparable with that in WT mice (Figs. 4 and 5). Additionally, the expression level of p21 as well as Bax mRNA in p53-null esophagus was 2-fold higher when compared with that of WT esophagus (Figs. 4 and 5).

To investigate further the functional consequences of p63-mediated transactivation of a p53-responsive promoter, we successfully established oral epithelial cells in culture from WT and p53-null mice. These cells have the expected cytokeratin profile (keratins 5, 14, 4, and 13), and the p53-null cells do not express p53 (data not shown). As expected, dominant-negative p53, ΔNp63γ, and ΔNp63α suppress p21-mediated transactivation in WT or normal oral keratinocytes, in contrast to WT p53 (Fig. 6). However, in p53-null oral keratinocytes, WT p53 transactivates the p21 promoter, but ΔNp63γ no longer suppresses the p21 promoter (Fig. 6). Dominant-negative p53 and ΔNp63α retain their abilities to suppress the p21 promoter in a p53-null background. These results suggest there may be functional differences between ΔNp63γ and ΔNp63α in transactivation of the p21 promoter.

DISCUSSION

The evidence for *p63* as a tumor suppressor gene is not compelling as that for p53. P63 mutations are extremely rare in human cancers and derived cell lines (15), although germ-line mutations in p63 are associated with the ectrodactyly, ectodermal dysplasia and cleft lip/palate (EEC) syndrome (16). This would appear to suggest that p63 is important in different types of cellular functions, and there are differences in the physiological roles of p53 family members, which may, in part, reflect differences in interactions with each other (16).

Recently, analysis of p73-null mice revealed unique roles for p73 in neurogenesis, sensory pathways, and homeostatic control (13, 14). p63 is relatively tissue restricted, and targeted disruption through homologous recombination in mice leads to a dramatic phenotype, part of which is consistent with a critical role in squamous epithelial cell development (17, 18). Functionally, p63 can bind to p53-DNA binding sites *in vitro* and can activate transcription of p53-responsive promoters.

Whereas p53 family members have distinctive roles, they also have physiological and functional overlapping features. To elucidate how p63 and p53 may be complementary in oral-esophageal squamous epithelial cells, we used a comparison of p53-null and WT mice. Both TAp63 and ΔNp63 are present in the oral-esophageal epithelia with increased expression and staining intensity in p53-null mice compared with age-matched WT littermates. Our data provide evidence for the coexistence of TAp63 and ΔNp63 in basal and suprabasal cells, suggesting a dynamic interplay in their homeostatic control of differentiation (21). It is possible that TAp63 serves as a positive regulator of the switch from proliferating basal cells to differentiating suprabasal cells through the transactivation of p21 and bax. Indeed, p63 has been suggested to be a "marker" of stem or progenitor cells in the basal cell compartment (22). By contrast, ΔNp63 may contribute to the equilibrium between proliferation and differentiation by virtue of its potential dominant-negative function in modulating promoters such as p21 and bax. Interestingly, this may not be true for all ΔNp63 isoforms in that our data suggest differences between ΔNp63γ and ΔNp63α in modulating p21 promoter activity in oral keratinocytes derived from WT and p53-null mice. Additionally, whereas we find that p21 and bax are expressed in a p53-null background by the TaqMan assay, we can only infer their transactivation *in vivo* by TAp63 which is either not effectively opposed by ΔNp63, or alternatively, ΔNp63 acts to counterbalance even greater TAp63-mediated transactivation of p21 and bax than is detectable. In either context, the expression of p21 and bax in the oral-esophageal epithelia in a p53-null background may help to explain the lack of dysplasia or cancer in these tissues.

In summary, the p53-null mice provide an excellent model in which to study the role of p63 in oral-esophageal squamous epithelia given the importance of p63 in the development and differentiation of squamous epithelia.

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