The p53 Homologue ΔNp63α Interacts with the Nuclear Factor-κB Pathway to Modulate Epithelial Cell Growth

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Abstract

The p53 homologue ΔNp63α is overexpressed and inhibits apoptosis in a subset of human squamous cell carcinomas (SCC). Here, we report that in normal keratinocytes overexpressing ΔNp63α and in human squamous carcinoma cells, ΔNp63α physically associates with phosphorylated, transcriptionally active nuclear c-Rel, a nuclear factor-κB family member, resulting in increased c-Rel nuclear accumulation. This accumulation and the associated enhanced proliferation driven by elevated ΔNp63α are attenuated by c-Rel small interfering RNA or overexpression of mutant IκBαM, indicating that c-Rel–containing complex formation is critical to the ability of elevated ΔNp63α to maintain proliferation in the presence of growth arresting signals. Consistent with a role in growth regulation, ΔNp63α-c-Rel complexes bind a promoter motif and repress the cyclin-dependent kinase inhibitor p21WAF1 in both human squamous carcinoma cells and normal keratinocytes overexpressing ΔNp63α. The relationship between ΔNp63α and activated c-Rel is reflected in their strong nuclear staining in the proliferating compartment of primary head and neck SCC. This is the first report indicating that high levels of ΔNp63α interact with activated c-Rel in keratinocytes and SCC, thereby promoting uncontrolled proliferation, a key alteration in the pathogenesis of cancers. [Cancer Res 2008;68(13):5122–31]

Introduction

Considerable debate has focused on the role of the p53 homologue p63 in human cancer pathogenesis (1). Dysregulation of p63 is observed in most squamous cancers, with *p63* gene amplification and/or overexpression reported in squamous cell cancers of the head and neck (HNSCC), lung, cervix, and skin (2–4). Functional determination of the consequences of p63 overexpression is complicated by the existence of multiple protein variants of p63, which show overlapping and opposing functions.

The *p63* gene is transcribed as two classes: TA and ΔN (5). TAp63 isoforms contain an NH_2-terminal p53-like transactivation domain and are capable of transactivating known p53-responsive genes as well as distinct sequences (5–7). In contrast, ΔN isoforms lack this domain due to alternate promoter usage and can block transactivation by either p53 or TAp63 isoforms while still harboring direct transactivation potential (8–10). p63 overexpression in human cancers has been predominantly associated with ΔNp63 isoforms (1, 2, 4). Additional complexity within each class of isoform is derived from COOH-terminal alternative splicing, giving rise to TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ. Unlike *p53*, the *p63* gene is critical for normal development of stratified squamous epithelium (11, 12), and several studies have indicated a requirement for temporal regulation of individual p63 isoforms in both development and maintenance of mature epidermis (13–15). However, the specific contribution of each of the known isoforms remains a subject of active investigation.

Previously, we used primary murine epidermal keratinocytes and adenoviral vectors to mimic ΔNp63α overexpression observed in human SCCs. We showed that overexpressed ΔNp63α maintains keratinocyte proliferation and blocks morphologic and biochemical differentiation despite the presence of signals that induce growth arrest and differentiation (10, 16). ΔNp63α overexpression was subsequently shown by others to promote survival in a subset of HNSCCs by physical association with and blockade of transcription of apoptosis genes by another p53 family member, p73 (17). To gain mechanistic insight into the altered growth regulation of murine keratinocytes associated with elevated ΔNp63α expression, we profiled extracts from keratinocytes overexpressing ΔNp63 or β-galactosidase (β-gal) for differential transcription factor binding, which provided evidence for a novel form of regulation of nuclear factor-κB (NF-κB) by ΔNp63.

NF-κB is widely expressed, with effects that are cell type and context dependent. Dysregulation of NF-κB activity is associated with multiple human diseases including cancer (18), and therapeutics targeting constitutive NF-κB activity are the subject of clinical trials in oncology (19). The NF-κB family consists of five subunits, which function as homodimers and heterodimers. Rel-A, Rel-B, and c-Rel contain a transactivation domain, whereas p50/105 and p52/100 do not. Within the normal epidermis, NF-κB plays an important role in regulating homeostasis (20, 21). During the development and progression of SCC, the NF-κB_1-Rel-A (p50/p65) heterodimer has been implicated in promotion or repression of the malignant phenotype dependent on the context (22, 23).

Here, we show that murine keratinocytes overexpressing ΔNp63α accumulate transcriptionally active c-Rel in their nuclei and that nuclear c-Rel accumulation is required to maintain ΔNp63α-mediated proliferation in the presence of signals that normally induce growth arrest. Accumulation of c-Rel is also seen in the nuclei of tumor specimens and cell lines of human HNSCCs expressing endogenous ΔNp63α. Additionally, ΔNp63α and c-Rel physically interact. Their association is observed *in vitro* in both human and murine cells and has been confirmed in murine cells *in vivo* on the promoter of the cyclin-dependent kinase (CDK) inhibitor p21WAF1. These findings provide a mechanism whereby

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c-Rel contributes to the altered growth regulation of ΔNp63α-overexpressing keratinocytes. This is the first report showing ΔNp63α-mediated regulation of active c-Rel, which is known for its oncogenic propensity (24, 25), and implicates ΔNp63α-c-Rel complexes in human HNSCC.

Materials and Methods

Cell culture. Primary keratinocytes isolated from C57BL/6NCr mice were cultured in 0.05 mmol/L Ca^{2+}-containing medium to maintain proliferation, and induced to differentiate by elevating Ca^{2+} levels to 0.12 mmol/L (10). HNSCC cell lines UM-SCC-11A, UM-SCC-22B, and UM-SCC-38 have been described previously (26). *N*-ethylmaleimide (NEM), a thiol modifier, was added to the culture medium immediately following adenoviral transduction to block *in vivo* phosphorylation (27).

Gene transfer. Adenoviruses (ΔNp63α, $\Delta Np63^{p40}$, IκBαM, and β-gal) and transduction methodology were described previously (2, 10, 16, 28).

Reporter constructs, NF-κB (29) or p21WAF1 (30), were transfected using Lipofectamine Reagent System (Life Technologies), or using Lipofectamine 2000 for cotransfections with small interfering RNA (siRNA). Activity relative to protein concentration was determined via the Luciferase Assay System (Promega Corp.).

Reporter assay-only transfections. Keratinocytes were transfected 17 h after adenoviral transduction with the NF-κB reporter construct (3 μg) and harvested 24 h after transfection.

c-Rel siRNA and reporter assay transfections. Keratinocytes were transfected with a siRNA pool (c-Rel or nontargeting, 200 pmol; Dharmacon) plus NF-κB reporter construct (1.5 μg) 24 h before adenoviral introduction of ΔNp63α or β-gal. Samples were harvested 24 h later.

Transcription factor binding assay. Nuclear extracts from ΔNp63α-overexpressing, $\Delta Np63^{p40}$-overexpressing, or β-gal–overexpressing keratinocytes (31) were used to screen the Panomics DNA Array I.

Western blot analysis. Primary antibodies used were the following: Rel-A (F-6), Rel-B (C-19), c-Rel (C), p100/52 (447), p105/50 (E-10), IκBα (C21), IκBβ (C20), IκBε (M121), p63 DNA-binding domain (4A4), and p63 α-domain (H129; all from Santa Cruz Biotechnology); actin (AC-15; Sigma Immuno Chemicals); and keratin 10 and filaggrin (Babco). Signal was detected using horseradish peroxidase–linked anti-mouse, anti-goat, or anti-rabbit secondary antibodies.

Phosphatase assay. Nuclear extracts were incubated in 1× SAP buffer ± 10 units shrimp alkaline phosphatase (SAP; Promega) for 3 h at 37°C followed by inactivation at 65°C.

Bromodeoxyuridine incorporation analysis. Fluorescence-activated cell sorting (FACS) analysis was performed as described previously (16). Seventeen hours after adenoviral infection (ΔNp63α or β-gal), cells were maintained in fresh 0.05 mmol/L Ca^{2+}-containing medium or switched to 0.12 mmol/L for 24 h, with addition of 10 μmol/L bromodeoxyuridine (BrdUrd) for the final 4 h. siRNA experiments were performed as described except that keratinocytes were transfected with the siRNA pools as noted 12 h before adenoviral transduction.

Coimmunoprecipitation analysis. Lysates were precleared with appropriate antibody and beads and then incubated overnight at 4°C with c-Rel (4A4), p63 (H129), Rel-A (F-6), or control antibody. Protein A/G Plus beads were added for the final hour, and samples were washed four times with PBS, resolved by SDS-PAGE, and analyzed. The ExactaCruz F reagent system (Santa Cruz Biotechnology) was used for cases where the same species was used to generate the primary antibody for immunoprecipitation and Western blot analysis.

Reverse transcription-PCR. Primary murine keratinocytes were transfected with siRNA pools (c-Rel or nontargeting, 200 pmol) 8 h before adenoviral transduction (ΔNp63α or β-gal). Seventeen hours later, cultures were exposed to 0.12 mmol/L Ca^{2+} for 15 h or maintained in 0.05 mmol/L Ca^{2+}-containing medium. RNA was harvested via the Qiagen RNeasy Plus Mini and reverse transcribed (1 μg) using the AccuScript High Fidelity First-Strand cDNA Synthesis kit (Stratagene) with an oligo(dT) primer. Target sequences were amplified from cDNA pool aliquots in 1× reaction buffer [10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$], 400 μmol/L of each deoxynucleotide triphosphate, 5 units AmpliTaq DNA polymerase (Applied Biosystems), and 250 ng of each primer. Following a 3-min hot start, the reaction profile was the following: denaturation, 94°C, 30 s; annealing, 30 s [p21WAF1: 57°C, 22 cycles, 478 bp product; hypoxanthine phosphoribosyltransferase (HPRT): 51°C, 25 cycles, 526 bp product]; elongation, 72°C, 45 s. The primer sequences were as follows: p21WAF1, 5′-AATCCTGGTGATGTCCGACCTGTT-3′ (forward) and 5′-AGACCAATCTGCGCTTGGAGTGAT-3′ (reverse); HPRT, 5′-CGTCGTGATTAGCGATGATGA-3′ (forward) and 5′-TTCAAATCCAACAAACTCTGGC-3′ (reverse). PCR products were quantified using Spot densitometry software on an Alpha Innotech imaging system.

Electrophoretic mobility shift assays. The LightShift Chemiluminescent electrophoretic mobility shift assay (EMSA) kit was used (Pierce) with oligonucleotides from the p21WAF1 promoter p63-binding site #1 (p63BS#1; 32): 5′-TGGCCATCAGGAACATGTCCCAACATGTTGAGCTCTGGCA-3′ (forward) and 5′-TGCCAGAGCTCAACATGTTGGGACATGTTCCTGATGGCCA-3′ (reverse). Oligonucleotides were end labeled using the 3′ Biotin end-labeling kit (Pierce) and incubated with nuclear extract (6 μg/reaction) before resolution (4% acrylamide). For radioactive EMSAs, oligonucleotides were 5′ end labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]dATP. Nuclear extracts (6 μg/reaction) were incubated at room temperature with 1 μL of labeled probe (20,000 cpm) and resolved by gel electrophoresis.

Chromatin immunoprecipitation. ΔNp63α-overexpressing or β-gal–overexpressing keratinocytes were fixed in 1% formaldehyde solution for 10 min. The reaction was stopped by the addition of 1× glycine buffer. Following washing, cells were scraped into PBS. Chromatin was isolated and sheared enzymatically for 10 min (ChIP-IT Express kit, Active Motif). Samples were immunoprecipitated overnight at 4°C with antibodies to c-Rel, p63α H129, or IgG control. The chromatin was eluted and cross-links were reversed before proteinase K digestion. Following a 3-min hot start, the PCR profile was the following: denaturation, 94°C, 30 s; annealing, 58°C, 30 s; elongation, 72°C, 30 s for 35 cycles; 220 bp product. The primer sequences were as follows: p21-binding site #1, 5′-ACTAGCTTTCTGGCCTTCAGGAAC-3′ (forward) and 5′-CCTGATACATGTCACAAGATACATACCACC-3′ (reverse).

Immunostaining. Patient-matched carcinoma and normal stratified squamous epithelium biopsies were obtained under Institutional Review Board–approved NIH protocol 04-C-0141 in the outpatient clinic. Frozen sections (10 μm) on silanated glass were fixed with 4% paraformaldehyde/PBS at 4°C for 5 min. Nonspecific binding was blocked with 5.5% serum/TBS, and endogenous tissue peroxidase was quenched with 0.6% H_2O_2/TBS before incubation with primary antibodies, c-Rel (C), ΔNp63 (N-16), or isotype control (diluted 1:100 in 3% bovine serum albumin/TBS) overnight at 4°C. Samples were then incubated with biotinylated secondary antibody and then avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories) and 3,3′-diaminobenzidine (1–5 min depending on target antigen) to reveal immune complexes. Sections were counterstained with Gill's formula hematoxylin (Vector Laboratories), dehydrated, cleared, and mounted using Permount (Fisher).

Results

ΔNp63α overexpression promotes nuclear c-Rel accumulation. ΔNp63α expression is associated with the proliferative compartment of normal stratified squamous epithelium and this protein is overexpressed in SCCs (2, 3, 5). Previously, we showed that elevated exogenous ΔNp63α maintains primary murine keratinocytes in an undifferentiated proliferative state in the presence of signals that normally induce growth arrest and differentiation (10, 16). To identify downstream targets of elevated ΔNp63α that may mediate these effects in squamous epithelium, nuclear extracts prepared from keratinocytes overexpressing ΔNp63 or β-gal were screened for differential transcription factor regulation. Differential binding to a NF-κB consensus sequence was

observed in samples overexpressing ΔNp63 compared with β-gal controls (data not shown), indicating the potential involvement of NF-κB in the altered growth regulation associated with ΔNp63 overexpression.

Five NF-κB subunits form heterodimers/homodimers that display differential binding affinity for multiple NF-κB consensus sequences (33). To confirm the altered NF-κB–binding activity observed with elevated ΔNp63 and to determine which subunits were involved, we profiled nuclear extracts from ΔNp63α-overexpressing versus β-gal–overexpressing keratinocytes by Western blotting. c-Rel was enhanced in nuclei of keratinocytes overexpressing ΔNp63α relative to β-gal control, whereas nuclear levels of the other NF-κB subunits were unaffected (Fig. 1*A*).

The c-Rel detected in nuclear extracts from ΔNp63α-overexpressing keratinocytes resolved into two species, potentially reflecting posttranslational modifications (Fig. 1*A* and *B*). To address whether the two species of c-Rel observed in the nuclei of ΔNp63α-overexpressing keratinocytes reflected differences in phosphorylation status, keratinocytes were incubated with NEM, a thiol modifier that had been previously shown to block c-Rel phosphorylation *in vivo* (27), for 21 h immediately following adenoviral transduction. Culturing with NEM resulted in loss of the upper species, indicating that it is a phosphorylated form of c-Rel (Fig. 1*B*). As confirmation, nuclear extracts isolated from keratinocytes overexpressing ΔNp63α were treated with 10 units SAP for 3 h at 37°C. Incubation with SAP eliminated the upper species, confirming that it is a phosphorylated form of c-Rel (Fig. 1*B*).

Nuclear c-Rel accumulated in response to elevated ΔNp63α expression in keratinocytes is transcriptionally active. Phosphorylation of c-Rel is known to positively affect its transactivation capacity (34). To assess whether c-Rel enhancement resulting from ΔNp63α elevation affects NF-κB transcriptional activity, keratinocytes were transfected with a NF-κB–responsive luciferase reporter construct (29) following adenoviral introduction of ΔNp63α or β-gal. This revealed a 6- to 17-fold increase in NF-κB reporter activity in ΔNp63α-overexpressing keratinocytes relative to β-gal controls (Fig. 1*C*). Using this assay, we also compared keratinocytes overexpressing $\Delta Np63^{p40}$, a truncated form of ΔNp63 lacking the entire α-COOH terminus that is present in ΔNp63α (Fig. 1*C*). In contrast to ΔNp63α, no reporter gene activity was observed in keratinocytes overexpressing $\Delta Np63^{p40}$, indicating a requirement

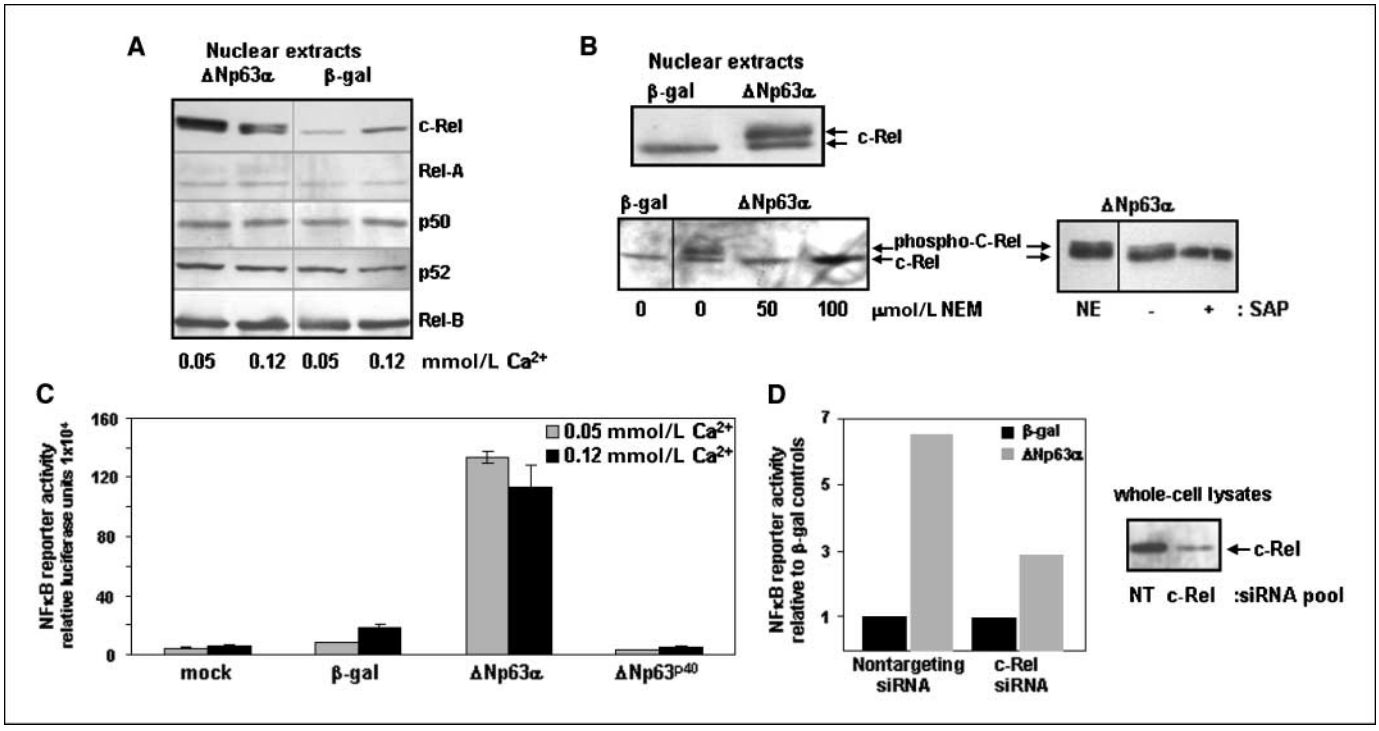

Figure 1. ΔNp63α overexpression results in increased nuclear levels of transcriptionally active phosphorylated c-Rel in murine keratinocytes. *A,* elevated ΔNp63α expression specifically enhances nuclear levels of the NF-κB subunit c-Rel. Western blots of nuclear extracts from primary murine keratinocytes harvested 21 h after adenoviral introduction of human ΔNp63α or β-gal. Cultures were maintained in medium containing 0.05 mmol/L Ca^{2+} throughout or exposed to 0.12 mmol/L Ca^{2+} for the final 4 h. c-Rel levels are increased in the nuclei of ΔNp63α-overexpressing keratinocytes. *B,* c-Rel is phosphorylated in response to elevated ΔNp63α. Western blot analysis of nuclear extracts from keratinocytes overexpressing ΔNp63α or β-gal cultured under control conditions (*top*) or in the presence of the thiol modifier NEM at concentrations noted for 21 h following adenoviral introduction to block *in vivo* phosphorylation (*bottom left*). The upper species is eliminated in the presence of NEM. *Bottom right,* phosphorylation was confirmed by Western blot analysis of nuclear extracts derived from keratinocytes overexpressing ΔNp63α incubated in the presence or absence of SAP for 3 h at 37°C. A nonincubated control nuclear extract (*NE*) is included for visual reference. SAP treatment results in the loss of the upper phosphorylated species. *C,* ΔNp63α overexpression results in NF-κB–mediated transactivation; α-domain of ΔNp63α is required. NF-κB–responsive reporter gene activity in keratinocytes overexpressing ΔNp63α, $\Delta Np63^{p40}$ (ΔNp63 lacking α-domain), or β-gal. Samples were harvested 24 h after transfection. *Columns,* mean of triplicate samples of a representative experiment performed thrice; *bars,* SD. *D,* c-Rel is required for NF-κB–mediated transactivation following ΔNp63α overexpression. Keratinocytes were cotransfected with NF-κB reporter construct and c-Rel–targeting siRNA or control siRNA 24 h before adenoviral infection with ΔNp63α or β-gal. Samples were harvested 24 h after adenoviral introduction. *Right,* Western blot reveals depletion of c-Rel in whole-cell lysates at time of harvest. *NT,* nontargeting siRNA. *Left,* fold increase in NF-κB reporter gene activity in ΔNp63α-overexpressing cultures compared with β-gal control cultures in the presence or absence of c-Rel siRNA. Triplicate wells were averaged and are presented as fold increase relative to β-gal controls that are normalized to 1.0. Experiment was performed twice with consistent results; representative experiment is shown.

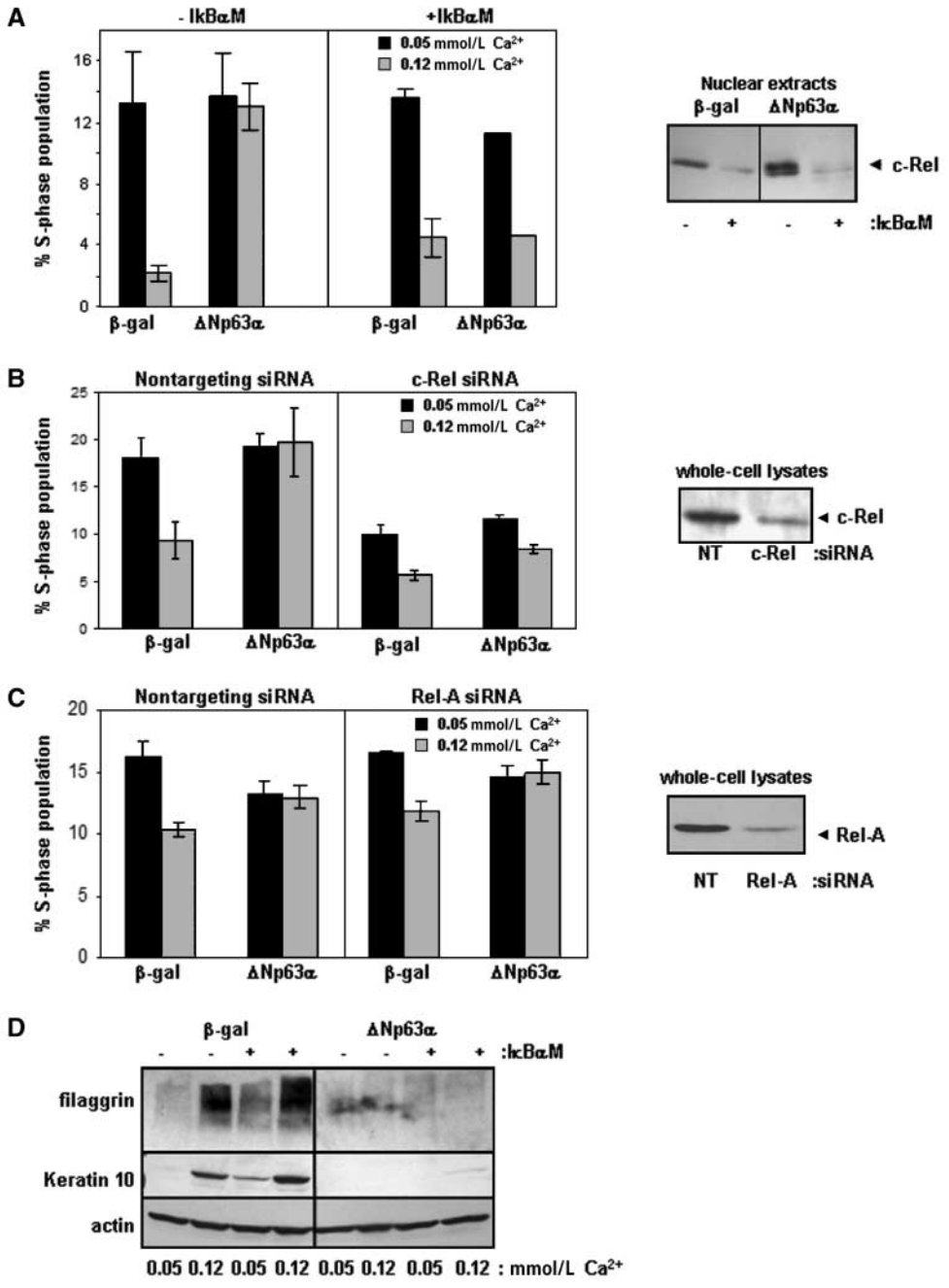

Figure 2. Enhanced nuclear c-Rel is required for ΔNp63α-mediated loss of keratinocyte growth regulation but not differentiation defects. *A* to *C,* flow cytometry analysis of BrdUrd incorporation in keratinocytes overexpressing ΔNp63α or β-gal under proliferating (0.05 mmol/L Ca^{2+}) or differentiating (0.12 mmol/L Ca^{2+}) conditions. *A,* blocking NF-κB nuclear translocation with the IκBαM superrepressor restores normal Ca^{2+}-mediated growth regulation to ΔNp63α-overexpressing keratinocytes. Primary murine keratinocytes (1.5 d after plating) were coinfected with adenovirus encoding ΔNp63α or β-gal in combination with IκBαM superrepressor or empty vector control. Seventeen hours after infection, the medium was changed; cells were maintained for a further 24 h in 0.05 or 0.12 mmol/L Ca^{2+} and pulsed with BrdUrd (10 μmol/L) for the final 4 h. *Columns,* mean of triplicate samples from a representative experiment performed thrice; *bars,* SD. *Right,* Western blot of corresponding nuclear extracts confirms that coinfection with the IκBαM superrepressor effectively reduces levels of nuclear c-Rel in ΔNp63α-overexpressing keratinocytes. *B,* c-Rel siRNA knockdown partially restores normal growth arrest in ΔNp63α-overexpressing keratinocytes. Keratinocyte cultures were transfected with c-Rel or nontargeting siRNA 12 h before introduction of ΔNp63α or β-gal by adenovirus. Decreasing c-Rel levels by siRNA results in an overall reduction of proliferation in all cultures (*right panel* versus *left panel of histogram*) and partially restores Ca^{2+}-mediated growth arrest to ΔNp63α-overexpressing keratinocytes (*right side* and *right panel of histogram*). *Columns,* mean of triplicate samples from a representative experiment; *bars,* SD. *Right,* Western blot of whole-cell lysates confirms that c-Rel expression is reduced in keratinocytes transfected with c-Rel siRNA. *C,* Rel-A does not contribute to the aberrant growth arrest response observed in ΔNp63α-overexpressing keratinocytes. Keratinocytes were transfected with Rel-A–targeted siRNA to deplete Rel-A levels or with nontargeting siRNA as control. Depleting Rel-A by siRNA has no effect on keratinocyte proliferation under these conditions. *Columns,* mean of triplicate samples from a representative experiment; *bars,* SD. *Right,* Western blot of whole-cell lysates confirms that Rel-A siRNA effectively reduces Rel-A expression in these cultures. *D,* blocking NF-κB nuclear translocation does not restore induction of markers of terminal differentiation in ΔNp63α-overexpressing keratinocytes. Western blot of whole-cell lysates from keratinocytes overexpressing ΔNp63α or β-gal ± IκBαM superrepressor and exposed to 0.12 mmol/L Ca^{2+} for 24 h. Blocking NF-κB nuclear translocation does not restore the Ca^{2+}-mediated induction of the early marker of keratinocyte differentiation, keratin 10, or the late marker, filaggrin.

for the α-tail of p63 in mediating this NF-κB transactivation activity. Repetition of the NF-κB reporter assay in keratinocytes in which c-Rel had been reduced using siRNA before the adenoviral introduction of ΔNp63α or β-gal revealed a >50% reduction in fold increase of reporter activity relative to samples in which c-Rel was not targeted (Fig. 1*D*). The siRNA silencing of c-Rel was incomplete (Fig. 1*D*, Western blot); therefore, this degree of reduction in activity underscores the critical contribution of c-Rel to ΔNp63α-induced NF-κB–mediated transactivation in keratinocytes.

Enhanced nuclear NF-κB levels are required for sustained proliferation mediated by ΔNp63α. c-Rel is critical for antigen-dependent B-cell proliferation and T-cell receptor–mediated T-cell proliferation and has been implicated in the maintenance of normal keratinocyte proliferation (21, 35, 36). A substantial block in nuclear accumulation of c-Rel was achieved by hindering NF-κB nuclear translocation through the introduction of an adenovirus encoding the IκBαM superrepressor (Fig. 2*A*, Western blot; ref. 28). This approach reduced ΔNp63α-induced nuclear accumulation of c-Rel to levels approximating those in β-gal control cultures. FACS analysis revealed that β-gal control cultures underwent normal Ca^{2+}-induced growth arrest in both the presence and absence of the IκBαM superrepressor (note decrease in S-phase fraction; Fig. 2*A*, histogram). Consistent with previous results (10), ΔNp63α-overexpressing keratinocytes do not arrest in response to 0.12 mmol/L Ca^{2+} (Fig. 2*A*, histogram, −IκBαM). Blocking NF-κB subunit translocation with the IκBαM superrepressor restored responsiveness to Ca^{2+}-induced growth arrest in ΔNp63α-overexpressing keratinocytes (Fig. 2*A*, histogram, +IκBαM).

A siRNA approach was used to further dissect the requirement for specific NF-κB subunits in the aberrant proliferation observed in conjunction with ΔNp63α overexpression (10). Overall levels of proliferation were decreased in both β-gal–overexpressing and ΔNp63α-overexpressing cultures following c-Rel depletion compared with control cultures with nontargeting siRNA (Fig. 2*B*). However, the relative growth arrest response in β-gal control cultures remained similar (48.9% S-phase reduction with nontargeting siRNA versus 43.8% S-phase reduction with c-Rel siRNA; Fig. 2*B*, histogram). These results suggest that normal constitutive levels of c-Rel do not significantly affect Ca^{2+}-mediated growth arrest in normal cells. Consistent with previous findings (10), ΔNp63α-overexpressing keratinocytes that had been transfected with nontargeting siRNA abnormally continued proliferating following exposure to 0.12 mmol/L Ca^{2+} (0% reduction in the S phase; Fig. 2*B*). In contrast, transfecting c-Rel–targeting siRNA into ΔNp63α-overexpressing keratinocytes partially restored growth arrest response (26.4% reduction in the S phase; Fig. 2*B*, histogram, right side). The use of c-Rel siRNA did not entirely block c-Rel protein expression (Western blot); thus, it is likely that some c-Rel protein remained available for nuclear accumulation and contributed to the remaining abnormal proliferation observed. As c-Rel was the only NF-κB subunit detectably altered in ΔNp63α-overexpressing keratinocytes (Fig. 1*A*), these siRNA results taken together with the IκBαM superrepressor data (Fig. 2*A*) support a requirement for enhanced nuclear c-Rel in the mediation of enhanced proliferation by ΔNp63α.

Rel-A has been implicated in the maintenance of normal keratinocyte proliferation (21); therefore, we also used Rel-A–targeting siRNA to assess the contribution of Rel-A to growth regulation in ΔNp63α-overexpressing keratinocytes. In contrast to studies using c-Rel siRNA, depleting Rel-A did not restore normal growth arrest to ΔNp63α-overexpressing keratinocytes (Fig. 2*C*). Based on these findings, and our observation that ΔNp63α overexpression does not alter nuclear levels of Rel-A (Fig. 1*A*), we conclude that Rel-A does not participate in the aberrant growth arrest response in keratinocytes overexpressing ΔNp63α.

In conjunction with loss of Ca^{2+}-mediated growth regulation, elevation of ΔNp63α protein expression blocks the onset of squamous morphology as well as the induction of keratinocyte differentiation-specific gene expression (10, 16). Blocking c-Rel translocation with the IκBαM superrepressor did not restore

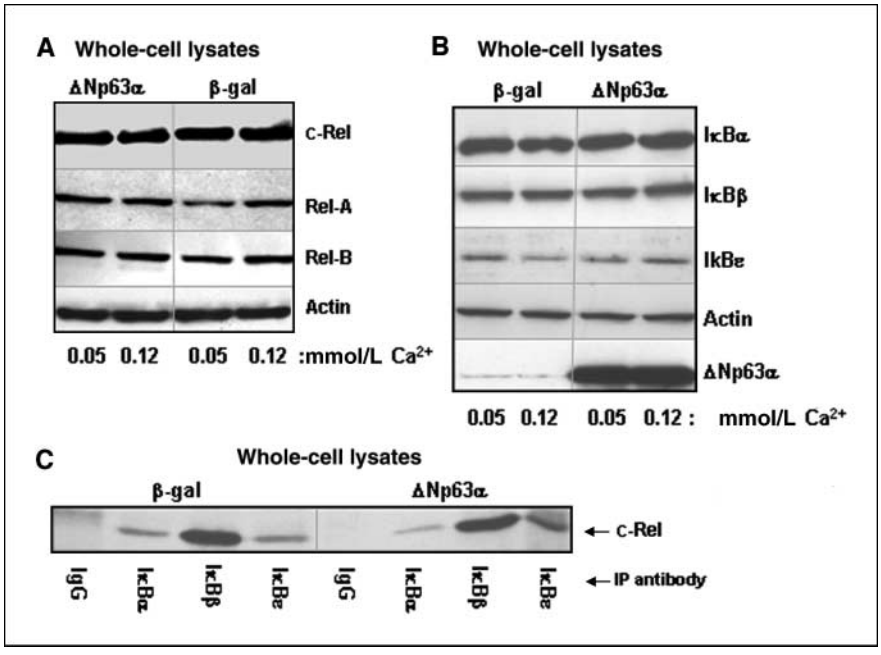

Figure 3. Mechanism of nuclear c-Rel enhancement is not dependent on disruption of cytoplasmic IκB-c-Rel interactions. *A,* ΔNp63 overexpression does not alter total levels of cellular NF-κB. Western blots of whole-cell lysates from primary mouse keratinocytes harvested 21 h after adenoviral introduction of human ΔNp63α or β-gal. Cultures were maintained in medium containing 0.05 mmol/L Ca^{2+} or exposed to 0.12 mmol/L Ca^{2+} for the final 4 h. *B,* levels of IκB regulatory proteins are not decreased in ΔNp63α-overexpressing keratinocytes that accumulate nuclear c-Rel. Western blot of whole-cell lysates of keratinocytes overexpressing β-gal or ΔNp63α. *C,* ΔNp63α overexpression does not inhibit normal cytoplasmic interactions between c-Rel and the IκB proteins. Coimmunoprecipitation analysis of whole-cell lysates from keratinocytes overexpressing ΔNp63α or β-gal.

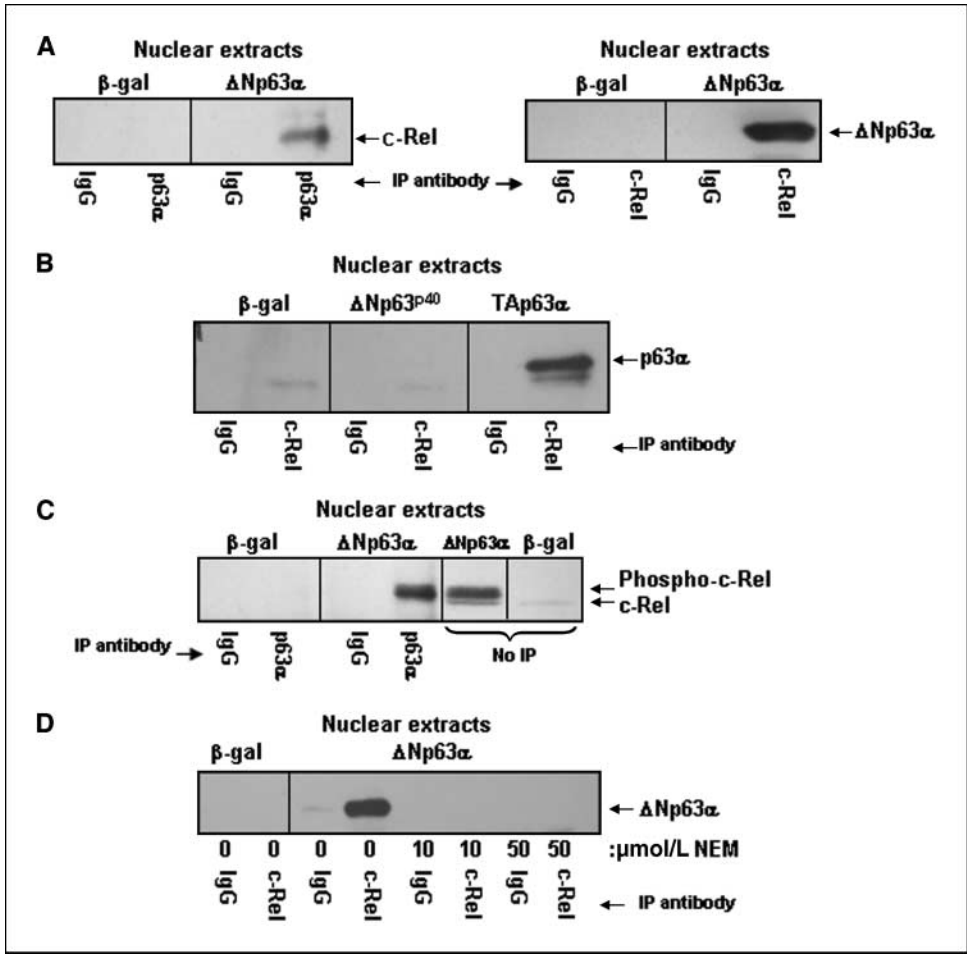

Figure 4. ΔNp63α and c-Rel physically interact in a phosphorylation, p63 α-domain–dependent manner. *A,* ΔNp63α and c-Rel physically interact in the nuclei of ΔNp63α-overexpressing keratinocytes. Coimmunoprecipitation analysis of nuclear extracts from keratinocytes overexpressing ΔNp63α or β-gal. Nuclear extracts were immunoprecipitated with antibody to p63 (*left*) or c-Rel (*right*) and probed for c-Rel or p63, as noted. *B,* the α-domain but not the ΔN-domain of p63 is required for the interaction between p63 and c-Rel. Nuclear extracts from keratinocytes overexpressing $\Delta Np63^{p40}$ (a truncated form of ΔNp63 lacking the α-COOH terminus), TAp63α, or β-gal were immunoprecipitated with antibody to c-Rel and probed for p63. *C,* ΔNp63α associates with phosphorylated c-Rel. Coimmunoprecipitation analysis of nuclear extracts from keratinocytes overexpressing ΔNp63α or β-gal resolved alongside ΔNp63α or β-gal nuclear extracts (*No IP*). Comigration and Western blot reveal that the upper, phosphorylated species of c-Rel interacts with ΔNp63α. *D,* protein phosphorylation is required for association between ΔNp63α and c-Rel. Coimmunoprecipitation analysis of nuclear extracts from keratinocytes overexpressing ΔNp63α or β-gal. Culture with NEM for 1 d following adenoviral infection to block *in vivo* phosphorylation eliminates the interaction between ΔNp63α and c-Rel.

expression of differentiation markers to ΔNp63α-overexpressing keratinocytes, indicating that NF-κB subunits do not participate in this aspect of ΔNp63α biological activity (Fig. 2*D*).

Enhanced nuclear levels of c-Rel in response to elevated ΔNp63α result from altered intracellular localization without altering cytoplasmic IκB:c-Rel interactions. Both ΔN and TAp63 isoforms modulate gene transcription (8, 10, 37). To address whether the enhanced nuclear NF-κB levels reflect the activity of ΔNp63α as a transcription factor, total levels of each subunit in whole-cell lysates were assessed. No changes in total cellular expression of c-Rel, Rel-A, or Rel-B were observed between ΔNp63α-overexpressing and control cultures (Fig. 3*A*), indicating that enhanced nuclear c-Rel levels resulted from altered intracellular localization. p50/105 and p52/100 were undetectable under these conditions (data not shown).

Control of NF-κB localization within the cell is largely mediated by the IκB family of proteins, which sequester NF-κB in an inactive state in the cytoplasm. On phosphorylation, the IκBs are degraded, freeing NF-κB heterodimers/homodimers to translocate to the nucleus (38). Western blot analysis of whole-cell lysates derived from keratinocytes overexpressing ΔNp63α versus β-gal showed no reduction in IκB protein levels in keratinocytes harboring elevated ΔNp63α (Fig. 3*B*).

As IκB levels are maintained in ΔNp63α-overexpressing keratinocytes, we speculated that ΔNp63α might perturb normal cytoplasmic interactions between NF-κB and the IκBs. We focused on c-Rel, as this is the only subunit altered in ΔNp63α-overexpressing keratinocytes. Coimmunoprecipitation analyses of whole-cell lysates revealed that the normal associations between IκBα, IκBβ, or IκBε and c-Rel remain intact in the presence of overexpressed ΔNp63α (Fig. 3*C*).

ΔNp63α and c-Rel physically associate in the nuclei of keratinocytes expressing high levels of ΔNp63α. In addition to sequestering NF-κB in the cytoplasm, IκBα and IκBε can disrupt NF-κB-DNA interactions in the nucleus and, through nuclear export signals, can actively shuttle NF-κB back into the cytoplasm (39, 40). IκBα/IκBε shuttling is mediated by CRM1, which when blocked results in enhanced nuclear NF-κB accumulation. A failure in shuttling, potentially due to a blocked association between c-Rel and IκBα/IκBε, could result in nuclear accumulation of c-Rel in ΔNp63α-overexpressing keratinocytes. Unlike our findings in whole-cell lysates (Fig. 3*C*), we detected no association between c-Rel and IκBα, IκBβ, or IκBε in nuclear extracts derived from either β-gal–overexpressing or ΔNp63α-overexpressing keratinocytes (data not shown). However, coimmunoprecipitation analyses revealed a physical interaction between ΔNp63α and c-Rel in nuclear extracts of ΔNp63α-overexpressing keratinocytes (Fig. 4*A*). In contrast to c-Rel, interaction was not seen between ΔNp63α and Rel-A in ΔNp63α-overexpressing keratinocytes (data not shown).

Association between ΔNp63α and phospho-c-Rel requires the p63 α-domain and phosphorylation. To assess if the

α-COOH terminus of ΔNp63α is required for the physical interaction between ΔNp63α and c-Rel, coimmunoprecipitation was performed with nuclear extracts from keratinocytes overexpressing $\Delta Np63^{p40}$, which lacks the α-tail, as well as keratinocytes overexpressing TAp63α, which differs from ΔNp63α only at the NH_2 terminus. No interaction was seen between $\Delta Np63^{p40}$ and c-Rel (Fig. 4*B*), but interaction was observed between c-Rel and TAp63α, suggesting that the α-tail of p63 contributes to this interaction.

To address which c-Rel species interacts with ΔNp63α, coimmunoprecipitation reactions were resolved on a gel next to nonimmunoprecipitated nuclear extracts from keratinocytes overexpressing ΔNp63α or β-gal (Fig. 4*C*). This revealed that the upper, phosphorylated form of c-Rel is the predominant species that interacts with ΔNp63α in the nuclei of keratinocytes overexpressing ΔNp63α. To determine if phosphorylation is necessary for this physical interaction, keratinocytes overexpressing ΔNp63α were cultured in the presence of 10 μmol/L NEM and then subjected to coimmunoprecipitation. Blocking protein phosphorylation by NEM treatment abrogated the interaction between ΔNp63α and c-Rel (Fig. 4*D*).

Overexpressed ΔNp63α physically associates with c-Rel on the p21WAF1 promoter. Previously, we showed (16) that overexpression of ΔNp63α in primary murine keratinocytes blocks induction of the CDK inhibitor p21WAF1 in response to elevated extracellular Ca^{2+}. Others have shown that ΔNp63α binds to and acts as a transcriptional repressor for the p21WAF1 promoter (32). Because enhanced levels of c-Rel are critical to the ability of ΔNp63α to maintain proliferation under conditions that normally induce growth arrest (Fig. 2*A* and *B*), we asked whether c-Rel also regulates p21WAF1. Cotransfection assays using a luciferase reporter under control of the p21WAF1 promoter confirmed that, like ΔNp63α, c-Rel negatively regulates the p21WAF1 promoter (Fig. 5*A*). Keratinocytes cotransfected with the p21WAF1 promoter construct in combination with a human c-Rel cDNA exhibited a >50% decrease in luciferase activity relative to the control samples (*left*), whereas use of siRNA to decrease endogenous c-Rel levels resulted in enhanced p21WAF1 promoter activity compared with control (*right*).

We also evaluated the effect of depleting c-Rel on endogenous p21WAF1 induction. siRNA-mediated depletion of c-Rel in β-gal control keratinocytes did not affect the induction of p21WAF1 mRNA expression following exposure to 0.12 mmol/L Ca^{2+} (Fig. 5*B*). Consistent with previous results, ΔNp63α overexpression blocks normal Ca^{2+}-mediated induction of p21WAF1. As silencing of c-Rel by siRNA transfection is incomplete (Fig. 2*B*), some c-Rel remains available for nuclear accumulation. Despite this, depletion of c-Rel in keratinocytes overexpressing ΔNp63α resulted in a small but reproducibly detectable induction of p21WAF1 in response to 0.12 mmol/L Ca^{2+} (Fig. 5*B*).

The p21WAF1 promoter contains two known p53 response elements, which have been previously shown to bind p63 (32). EMSAs performed using "p63BS#1" (32), which corresponds to the p53/p63 consensus site in the reporter constructs used in Fig. 5*A*, revealed that nuclear extracts derived from ΔNp63α-overexpressing keratinocytes produced a DNA-protein complex that could be supershifted with a c-Rel antibody, showing a physical association *in vitro* between ΔNp63α and c-Rel on the p21WAF1 promoter (Fig. 5*C*) consistent with a role for c-Rel in regulating p21WAF1. In addition to the p53/p63 consensus binding sites, promoter analysis of the p21WAF1 promoter sequence revealed the presence of one potential c-Rel/p65–binding sequence,[3] but EMSAs performed with oligonucleotides to this sequence did not reveal binding (data not shown). The association between ΔNp63α and c-Rel on p63BS#1 of the p21WAF1 promoter was confirmed *in vivo* by chromatin immunoprecipitation (ChIP) analysis of ΔNp63α-overexpressing versus β-gal control keratinocytes using antibodies to either c-Rel or p63. As shown in Fig. 5*D*, a c-Rel-ΔNp63α complex in ΔNp63α-overexpressing keratinocytes occupies this p53/p63 consensus site *in vivo*.

ΔNp63α and c-Rel are strongly expressed throughout HNSCCs. To determine whether the association between ΔNp63α and c-Rel extends to normal and malignant human squamous epithelia, immunostaining was performed on human squamous mucosa and HNSCC tumor samples. Nuclear expression of both p63 and c-REL is associated with the basilar proliferative compartment of normal human mucosa, as defined by Ki67 immunostaining (Fig. 6*A*). Nuclear colocalization shown by strong nuclear staining of both proteins is diffusely seen throughout SCC tissue samples (Fig. 6*A*). Increased, diffuse nuclear costaining of ΔNp63 and c-REL was observed in the malignant squamous epithelia of 13 of 16 (81%) of HNSCC specimens examined, indicating that such nuclear colocalization is common in HNSCC.

Endogenous ΔNp63α and c-Rel physically associate in nuclei of HNSCC cell lines. Next, we addressed whether a physical association between endogenous ΔNp63α and c-REL occurs in cells of human cancers known to express high levels of ΔNp63α. Western blotting of nuclear extracts from the UM-SCC-11A, UM-SCC-22B, and UM-SCC-38 SCC lines revealed that all of these lines express both ΔNp63α and a form of c-REL that comigrates with the phosphorylated species seen in keratinocytes with elevated ΔNp63α (Fig. 6*B*). Coimmunoprecipitation analysis of nuclear extracts isolated from these cell lines revealed a physical association between ΔNp63α and c-REL (Fig. 6*C*), consistent with our findings in primary mouse keratinocytes. EMSAs performed with nuclear extracts from the HNSCC line UM-SCC-46 revealed that a protein-p63BS#1 DNA complex is also formed in this cell background that can be partially supershifted with a c-Rel antibody (Fig. 6*D*). As in murine keratinocytes overexpressing ΔNp63α, ChIP assay confirmed association of both p63 and c-REL with the same p21WAF1 promoter site in UM-SCC-46.[4] This confirms the presence of endogenous ΔNp63α-c-Rel complexes that exhibit DNA-binding activity in HNSCC on a relevant target gene *in vitro*.

Discussion

We show that overexpressing ΔNp63α in primary murine keratinocytes leads to the nuclear accumulation of phosphorylated, transcriptionally active c-Rel, which is required to maintain aberrant proliferation mediated by overexpressed ΔNp63α. In these cells, and in human SCC cells endogenously expressing these proteins, ΔNp63α and phospho-c-Rel physically associate in the nuclei and on the p21WAF1 promoter.

c-Rel was originally identified as the cellular counterpart of the v-Rel oncogene, known to cause lymphomas. c-Rel plays an important role in normal cellular homeostasis (21, 35, 36), including that of the epidermis (21), and enhanced nuclear c-Rel has been associated with solid and hematopoietic cancers (41, 42).

[3] B. Yan et al., unpublished data.
[4] H. Lu, unpublished observations.

In contrast to numerous studies of the NF-κB heterodimer p50-p65, the role of c-Rel in transformation of squamous epithelium remains largely unexplored. However, several studies point to the oncogenic propensity of dysregulated c-Rel expression in other systems. Retroviral overexpression of full-length wild-type c-Rel can transform primary spleen cells *in vitro* (25). Furthermore, forced overexpression of c-Rel *in vivo* under control of the mouse mammary tumor virus long terminal repeat promoter resulted in mammary tumorigenesis and correlated with induction of NF-κB target genes, including *c-myc* and *cyclin D1* (24). Treatment of these c-Rel–transformed mammary tumor cells with dimethylbenzanthracene *in vitro* resulted in epithelial to mesenchymal transition (43).

The transforming ability of c-Rel both *in vitro* and *in vivo* is dependent on the presence of its transactivation domain (44, 45). The transformation capacity of c-Rel can be enhanced by mutations and deletions within the transactivation domain, suggesting that the strength of transactivation activity can determine the potency of c-Rel (44, 46). The transactivation domain of c-Rel contains multiple phosphorylation sites and variable levels of phosphorylation have been shown to influence transactivation of distinct sets of target genes (47). In this report, we show that, in addition to being phosphorylated, the c-Rel that is modulated by ΔNp63α has transcriptional NF-κB reporter-enhancing and p21 gene-repressing activity. Future studies will aim to identify the effect of sustained ΔNp63α elevation on c-Rel target gene expression.

Regulation of NF-κB is a dynamic process (38–40). In the classic paradigm of NF-κB regulation, cytoplasmic IκB proteins retain NF-κB in an inactive state, with NF-κB nuclear translocation following IκB degradation. Once within the nucleus, NF-κB induces resynthesis of IκBs, and IκBα and IκBε can dissociate NF-κB from DNA and usher it to the cytoplasm via their nuclear export functions (39, 40). IκBβ can function in its phosphorylated form to dissociate NF-κB from DNA, whereas unphosphorylated IκBβ forms a ternary complex with NF-κB and DNA and can protect it from dissociation by IκBα or IκBε (48). Our data support a model whereby enhanced ΔNp63α expression results in nuclear accumulation of c-Rel without disrupting IκB-c-Rel cytoplasmic interactions or causing degradation of the IκBs (Fig. 3). We have shown

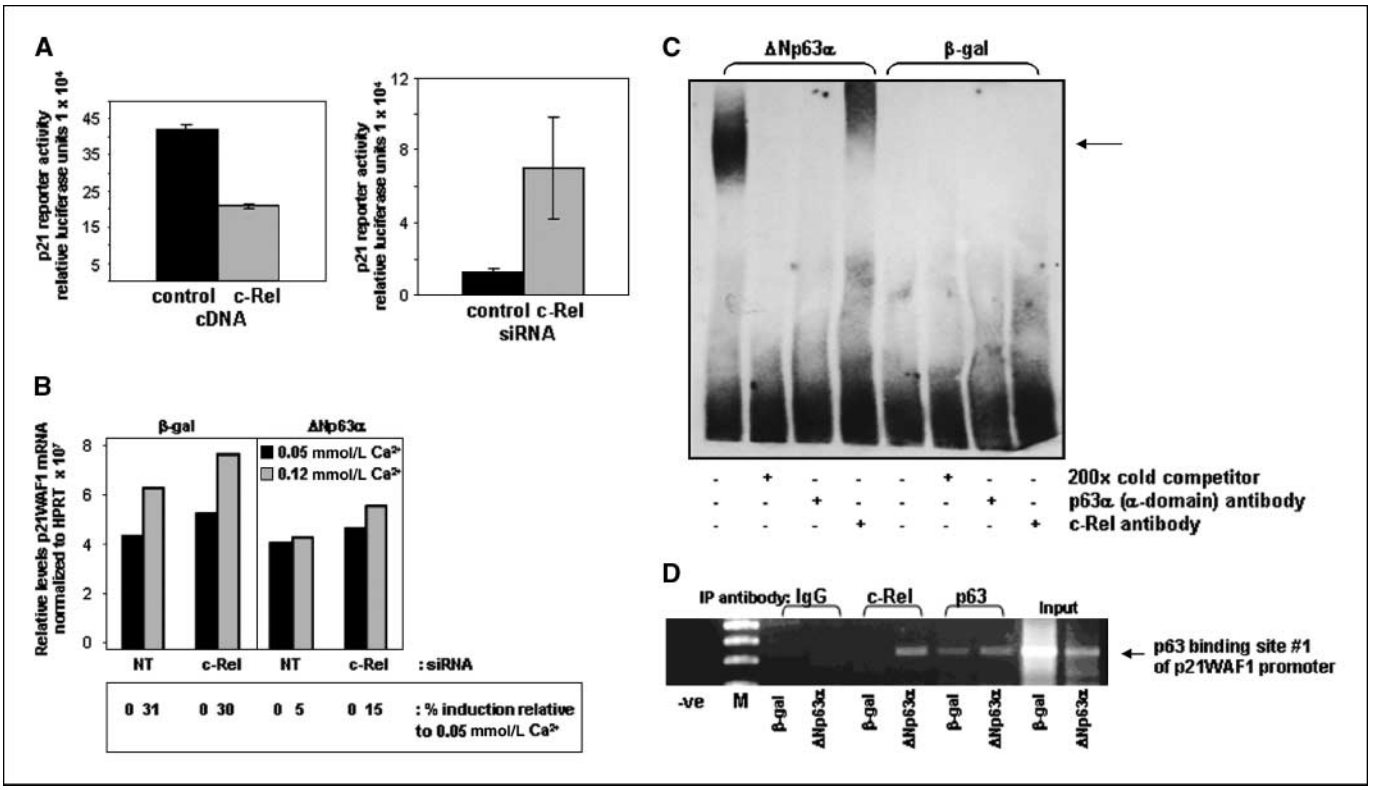

Figure 5. ΔNp63α and c-Rel both negatively regulate the CDK inhibitor p21WAF1 and interact *in vitro* and *in vivo* at a p63-binding site on the p21WAF1 promoter. *A,* modulating c-Rel levels alters p21WAF1 reporter gene activity. p21WAF1 reporter gene activity following cotransfection in combination with a human c-Rel cDNA construct, c-Rel–targeted siRNA, or controls. Overexpression of c-Rel represses p21WAF1 reporter gene activity (*left*), whereas reducing c-Rel expression levels with targeted siRNA enhances expression of a p21WAF1 luciferase reporter construct (*right*). *B,* incomplete silencing of c-Rel by targeted siRNA allows slight restoration of induction of endogenous p21WAF1. Semiquantitative reverse transcription-PCR analysis of p21WAF1. Ca^{2+}-mediated induction of p21WAF1 is unaffected by siRNA knockdown of c-Rel in control keratinocytes overexpressing β-gal. Consistent with previous results (16, 32), Ca^{2+}-mediated p21WAF1 induction is blocked by the overexpression of ΔNp63α. siRNA knockdown of c-Rel results in a small but reproducible induction of p21WAF1 in ΔNp63α-overexpressing keratinocytes in response to 0.12 mmol/L Ca^{2+} (15% in c-Rel–targeted versus 5% induction in nontargeted siRNA controls), as determined by spot densitometry analysis using an Alpha Innotech imaging system. Experiment was repeated with consistent results. *C,* ΔNp63α and c-Rel physically associate on the p21WAF1 promoter *in vitro*. EMSA analysis of nuclear extracts from keratinocytes overexpressing ΔNp63α or β-gal. The p63BS#1 from the p21WAF1 promoter used in the reporter gene assays was biotin labeled and used in the binding reactions. A protein-DNA complex seen only in the presence of overexpressed ΔNp63α is supershifted with a c-Rel antibody and interrupted with a p63-specific antibody. The experiment was performed four times with consistent results; representative experiment is presented. *D,* ΔNp63α and c-Rel physically associate on the p21WAF1 promoter *in vivo*. ChIP analysis was performed on samples derived from keratinocytes overexpressing ΔNp63α or β-gal using the antibodies noted. PCR primers were designed to flank the p63BS#1 from the p21WAF1 promoter. Association of the c-Rel-ΔNp63α complex with p63BS#1 is observed. Input DNA: PCR products generated using DNA template from total genomic DNA. Lane labeled "-ve" indicates an absence of DNA in the PCR reaction. *M,* molecular weight marker. Results shown are representative of two independent experiments.

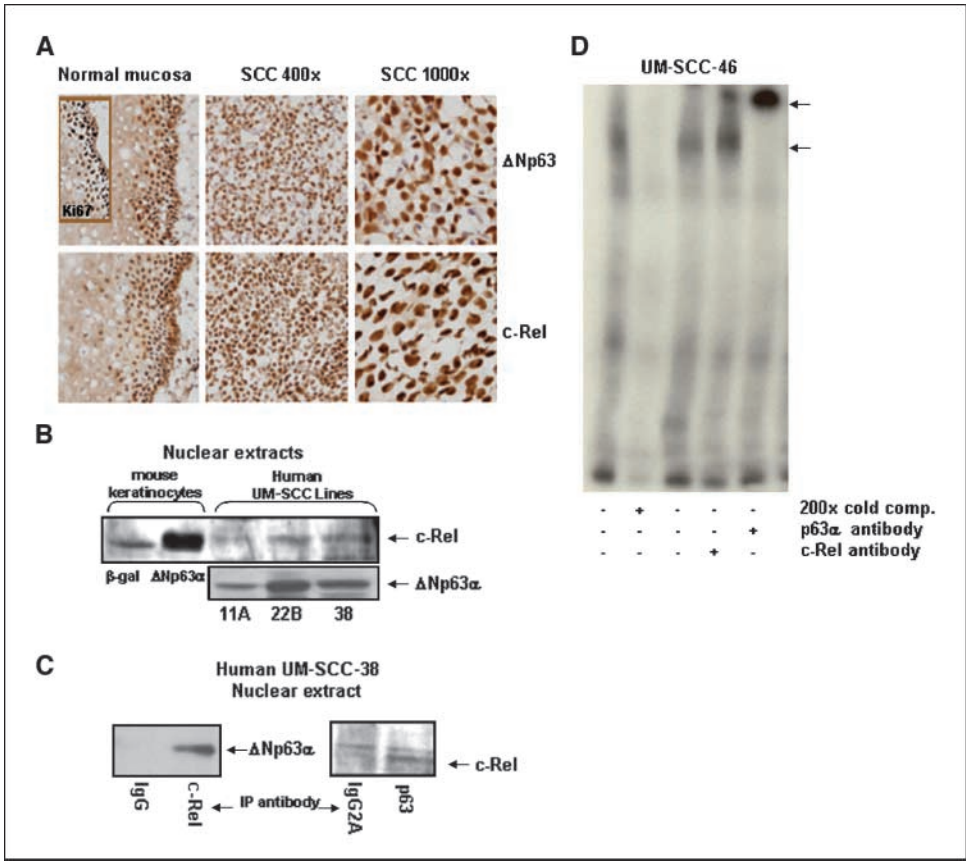

Figure 6. Endogenous ΔNp63α and c-Rel expression are correlated and expanded in primary human cancers and associate in the nuclei of human SCC cells. *A,* nuclear expression patterns of ΔNp63α and c-Rel are expanded and associated in primary human SCCs. Immunostaining of normal mucosa and SCC tissue sections with p63 and c-Rel. The proliferative compartment of normal mucosa is identified by Ki67 immunoreactivity. *B,* endogenous ΔNp63α and c-Rel are present in nuclei of human HNSCC lines. Western blots of nuclear extracts prepared from SCC lines. Mouse keratinocytes overexpressing ΔNp63α or β-gal are included as controls. *C,* endogenous nuclear ΔNp63α and c-Rel physically interact in SCC cells. Coimmunoprecipitation analysis of UM-SCC-38 nuclear extracts. Nuclear extracts were immunoprecipitated with antibody to c-Rel (*left*) or p63 (*right*) and probed for c-Rel or p63, as noted. *D,* endogenous nuclear ΔNp63α and c-Rel derived from squamous carcinoma cell lines are associated on the p21WAF1 promoter *in vitro*. EMSA analyses of nuclear extracts derived from the HNSCC cell line UM-SCC-46. A ^{32}P-labeled probe using the p63BS#1 from the p21WAF1 promoter was used in these reactions. A protein-DNA complex is seen and can be partially supershifted with a c-Rel antibody. Use of the smaller ^{32}P tag in the HNSCC experiments allowed for a supershift band to be seen with the p63 antibody as well.

that c-Rel physically interacts with ΔNp63α in the cell nucleus and propose that this association inhibits nuclear, but not cytoplasmic, interaction of c-Rel with the IκB proteins by blocking binding. This results in enhanced nuclear accumulation of c-Rel due to the inability of IκBα and IκBε to interact with and remove c-Rel. The c-Rel that accumulates in the nuclei of ΔNp63α-overexpressing cells is phosphorylated and transcriptionally active, as determined by reporter gene assay, and can interact in a complex with ΔNp63α on the p21WAF1 promoter to block promoter activity.

The physical association between ΔNp63 and phosphorylated c-Rel requires the α-COOH terminus of ΔNp63α; like ΔNp63α, TAp63α also physically associates with c-Rel, whereas ΔNp63^{p40} does not (Fig. 4*B*). Although less is understood about the role of TAp63 in cancer development, dysregulated TAp63α has been reported to influence the development and progression of chemically induced skin tumors (49). Whether the downstream effects of TAp63α in this context are mediated by c-Rel remains to be determined.

It was initially proposed that overexpression of ΔNp63 in human cancers blocks the tumor suppressor activity of p53 (50). It has recently been shown that the ability of ΔNp63α to repress p73-dependent apoptosis enhances the survival of a subset of SCC cells (17). The data presented herein support a novel mechanism whereby overexpression of ΔNp63α induces dysregulation of the proto-oncogene c-Rel via physical association, resulting in loss of normal keratinocyte growth regulation. Enhancement of transcriptionally active c-Rel and activation of downstream effectors could be a means whereby ΔNp63α influences the growth and phenotypic characteristics of human cancers. Consistent with our model, a recent clinical trial targeting constitutively active NF-κB in HNSCC via a proteasome inhibitor was found to block nuclear localization of Rel-A but not c-Rel.[5] The findings presented here suggest that distinct NF-κB complexes can promote proliferation of keratinocytes, act in concert with other NF-κB dimers to promote an aggressive cancer phenotype, and offer novel targets and useful biomarkers for optimizing therapeutic efficacy in this subset of poorly responsive cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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