Tumor and Stem Cell Biology

ΔNp63 Versatilely Regulates a Broad NF-κB Gene Program and Promotes Squamous Epithelial Proliferation, Migration, and Inflammation

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Abstract

Head and neck squamous cell carcinoma (HNSCC) and many other epithelial malignancies exhibit increased proliferation, invasion, and inflammation, concomitant with aberrant nuclear activation of TP53 and NF-κB family members ΔNp63, cRel, and RelA. However, the mechanisms of cross-talk by which these transcription factors coordinate gene expression and the malignant phenotype remain elusive. In this study, we showed that ΔNp63 regulates a cohort of genes involved in cell growth, survival, adhesion, and inflammation, which substantially overlaps with the NF-κB transcriptome. ΔNp63 with cRel and/or RelA are recruited to form novel binding complexes on p63 or NF-κB/Rel sites of multitarget gene promoters. Overexpressed ΔNp63- or TNF-α-induced NF-κB and inflammatory cytokine interleukin-8 (IL-8) reporter activation depended on RelA/cRel regulatory binding sites. Depletion of RelA or ΔNp63 by small interfering RNA (siRNA) significantly inhibited NF-κB–specific, or TNF-α–induced IL-8 reporter activation. ΔNp63 siRNA significantly inhibited proliferation, survival, and migration by HNSCC cells in vitro. Consistent with these data, an increase in nuclear ΔNp63, accompanied by increased proliferation (Ki-67) and adhesion (β4 integrin) markers, and induced inflammatory cell infiltration was observed throughout HNSCC specimens, when compared with the basilar pattern of protein expression and minimal inflammation seen in nonmalignant mucosa. Furthermore, overexpression of ΔNp63c in squamous epithelial cells in transgenic mice leads to increased suprabasilar cRel, Ki-67, and cytokine expression, together with epidermal hyperplasia and diffuse inflammation, similar to HNSCC. Our study reveals ΔNp63 as a master transcription factor that, in coordination with NF-κB/Rel, orchestrates a broad gene program promoting epidermal hyperplasia, inflammation, and the malignant phenotype of HNSCC. Cancer Res; 71(10); 3688–700. ©2011 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) and other epithelial cancers display coordinated changes in cell proliferation, migration, and inflammatory cell infiltration, as well as frequent abnormalities of the TP53 and NF-κB transcription factor families (1–5). HNSCC and epidermal malignancies are known to carry a high mutation rate for TP53, but TP53 mutation alone is insufficient to account for cancer development and differences in prognosis. This is attributable, in part, to the diversity of mutations or other mechanisms of inactivation that affect TP53 functions (6, 7). In addition, more recent studies have pointed to redundant and distinct functions of other TP53 family members, such as p63, in the modulation of gene expression and the malignant phenotype (8–10). p63 consists of 6 isoforms differing in the N- or C-terminal region as a result of alternative promoter usage or splicing (11). TAp63 isoforms contain full-length N-terminal transactivating domains, whereas ΔNp63 has a truncated N-terminus. The p63 isoforms share high sequence homology with the TP53 DNA-binding domain (12) and, thus, enable TA and ΔNp63 isoforms to transactivate or inhibit TP53 target genes, respectively. ΔNp63 is the most abundant isoform detected in the basal layer of mucosa, skin, other epithelial tissues (8, 13), and in HNSCC (14). Furthermore, p63 isoforms regulate a broad range of target genes (13, 15, 16), although the
different partners and mechanisms by which they coordinate such gene activation remain incompletely defined.

Recently, we have shown that ΔNp63 can form a novel complex with the transcriptionally functional NF-κB family member cRel to bind a p63 regulatory site and repress expression of p21Cip1, thus enhancing proliferation of murine keratinocytes (17). The NF-κB/Rel family includes 5 functionally and structurally related proteins, including RelA and cRel, which are often classified together as components of the canonical NF-κB pathway (18). Furthermore, aberrant activation of RelA is observed in the majority of HNSCCs and many other malignancies and has been shown to regulate a broad gene program to promote cancer progression, inflammation, metastasis, and resistance to therapies (1, 4, 19). However, RelA, cRel, and other members and transcription factors of the NF-κB family were found to be variably activated and sensitive to proteasome inhibition in different subsets of HNSCC lines and specimens obtained from patients resistant to therapy (20, 21). These findings suggested that cRel, RelA, and other transcription factors might contribute to coregulation of NF-κB target gene expression and the malignant phenotype in HNSCC and epidermal tumorigenesis.

The finding that ΔNp63 and cRel can bind and downregulate a classical TP53 target gene suggested a new paradigm by which these 2 transcription factors can interact and coregulate target gene(s). In the present study, we explored the hypothesis that ΔNp63, RelA, and cRel members of the NF-κB family can interact together to more broadly regulate transcription of TP53/p63 and NF-κB/Rel target genes that are known to be important in pathologic alterations in HNSCC and squamous epithelial cells.

**Materials and Methods**

**Cell lines**

The UM-SCC cell lines used were originally obtained from Dr. Thomas E. Carey, University of Michigan, in 2000. TP53 sequence analysis for exons 4 to 9 was done in our laboratory as previously reported (22). DNA was sent for sequence genotyping in 2008 and Fall 2010 to compare and verify their unique origin from original stocks, as recently described (23). The 9 loci analyzed included D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA, and amelogenin. Primary human epidermal keratinocytes (HEKA) were purchased and cultured in accordance with the manufacturer’s protocol (Invitrogen) and used within 5 passages. HEKA and human oral mucosal keratinocytes (HOK) exhibit similar global gene expression patterns for ΔNp63 and other genes presented in this study (data not shown).

**Immunohistochemical analysis**

Frozen HNSCC tissues were obtained from the Cooperative Human Tissue Network. Detailed immunohistochemistry (IHC) protocol is presented in the Supplementary Information. The staining patterns and morphology were observed under an Olympus BX51 microscope, and images were captured by using an Olympus DP70 camera and DP70 Image Manager software. De-identified formalin-fixed and paraffin-embedded HNSCC and mucosal tissue arrays were obtained from Cybrdi. Images were acquired using an Aperio ScanScope, and the staining intensity was quantified using the Aperio Cell Quantification Software (Aperio).

**Short interfering RNA**

The short interfering RNA (siRNA) targeting the unique exon in the N-terminal of ΔNp63 was made by Integrated DNA Technologies (Supplementary Table S1), based on the previous publication (24). siRNAs targeting TAp63, total p63, or other genes were purchased from Dharmacon or Qiagen.

**Real-time reverse transcriptase PCR**

The RNA isolated was subjected to reverse transcription and PCR as described (25). PCR primers for ΔNp63 and TAp63 were synthesized (Supplementary Table S1), and other primers were purchased (Applied Biosystems). The gene expression was normalized to 18S rRNA as an internal control.

**Reporter gene assays**

Reporter gene assays were assayed using the Dual-Light System Kit (Applied Biosystems) and measured by Wallac VICTOR2 1420 Multilabel Counter (Perkin Elmer). Differences in reporter activity due to nonspecific transfection or gene-related antiproliferative or cytotoxic effects were normalized using either β-Gal or WST-1 Cell Proliferation Reagent (Roche Diagnostics) according to the manufacturer’s instructions.

**Prediction of transcription binding sites**

The binding sites in the promoter regions of NF-κB, p63, or TP53 target genes were identified using the published binding and experimental information after querying National Center for Biotechnology Information and other databases. The novel p63 and NF-κB binding sites were predicted using Genomatix and position-weighted matrices (PWM) built on published information (see Supplementary Methods).

**Chromatin immunoprecipitation assay**

Protein–DNA complexes were precipitated using the antibodies indicated, and PCR was carried out using specific primers as described in Supplementary Methods.

**Images of cultured cell morphology, wound healing, and cell migration through Matrigel**

Images of cultured cells and wound-healing experiments were taken under an Olympus IX70 inverted microscope at room temperature using the SPOT RT Color Digital Camera (Diagnostic Instrument, Inc.) and the SPOT Advanced version 4.0.1 software program. Images of cells migrating through Matrigel were acquired using an Olympus BX51 microscope and quantification was done using the Aperio Cell Quantification Software program (Aperio).

ΔNp63α transgenic mouse model and immunofluorescence

A Tet-responsive ΔNp63α transgenic (TG) mouse model has been developed by crossing HA-ΔNp63α with Tet-OFF TG
animals under bovine K3 promoter (K5-tTA; ref. 26). Expression of ΔNp63z in TG mouse was induced after birth, and immunofluorescence staining was done as described in Supplemental Methods. Immunofluorescence images were observed under a Nikon FXA fluorescence microscope, captured using a Nikon digital camera, and analyzed using ImageJ, Adobe Photoshop, and Adobe Illustrator software programs.

Statistical analysis
Data are presented as mean ± SD, and the statistical analysis relative to the control is calculated (t-test, *P < 0.05).

Results
ΔNp63 regulates a broad gene program that overlaps with NF-κB targets
Nuclear ΔNp63 staining of intermediate and strong intensity was observed throughout malignant epithelia in 18 of 21 (~85%) frozen HNSCC specimens when compared with the basilar, localized, staining pattern seen in squamous mucosa (Supplementary Fig. S1A). Proliferating human keratinocytes (HEKA) and HNSCC (UM-SCC) lines predominantly expressed ΔNp63 mRNA and protein, with most HNSCC lines possessing mutant (mt) TP53 expressing higher levels than those with wild-type (wt) TP53 (Supplementary Fig. S1B). To investigate the broad role of ΔNp63 in regulating gene expression, we selected UM-SCC1 and UM-SCC6 lines with wtTP53 and intermediate ΔNp63 expression, as well as UM-SCC22B and UM-SCC38 lines with mtTP53 and higher ΔNp63 expression level (Supplementary Fig. S1B). p63 was knocked down by specific siRNA targeting ΔNp63, total p63 (Supplementary Fig. S2A; Supplementary Table S1), or TAp63 (not shown), confirming distinctive specificity of ΔNp63 knockdown. A broad gene panel, including NF-κB and TP53 family targets, was tested (Fig. 1A; Supplementary Fig. S2B; Supplementary Table S2). In UM-SCC1 cells, ΔNp63 knockdown led to significant modulation of genes critical in inflammation, including members of the interleukin-1 (IL-1) family, CX32, IL-6, and IL-8; in growth regulation and apoptosis, such as PDGFA, TGF-α, TP53, p73, MDM2, Bcl-2, and Bcl-xL, and in adhesion, such as ITGA6, ITGB4, and ICAM1 (Fig. 1A). Similar results were obtained for most genes in UM-SCC6, UM-SCC22B, and UM-SCC38 cells, although some differed between wtTP53 and mtTP53 lines (Supplementary Fig. S2B; Supplementary Table S2). Together, these results suggested that ΔNp63 could coregulate a broad gene program, including several known NFκB and TP53 target genes.

Systems biology analyses predict transcription binding motifs and network between ΔNp63- and NF-κB–regulated genes
Next, we compared 80 genes modulated by ΔNp63 or RelA knockdown, or treatment with NF-κB inducer TNF-α, and found a broad overlap of 26 genes (Fig. 1B). We then predicted NF-κB binding motifs on the proximal promoters (~500 ± 100 bp) of these genes using the Genomatix software program and PWMs based on published data (B. Yan, unpublished data; Supplementary Methods). RelA and cRel sites were highly represented in the proinflammatory cytokine and NF-κB gene clusters, whereas NF-κB1 sites predominated in the adhesion gene cluster (Fig. 1B). Literature review and annotated Ingenuity Pathway Analysis (IPA) linked many of the modulated genes via related physical, signal, or transcriptional interactions to ΔNp63 and NF-κB (Fig. 1C) or other pathways (Supplementary Table S3). These findings supported the hypothesis that ΔNp63 regulates a broad gene program that partially overlaps with the NF-κB transcriptome.

ΔNp63, cRel, and RelA binding to predicted sites of target gene promoters detected by chromatin immunoprecipitation
We searched for putative p63 and/or NF-κB–specific regulatory sequences in the promoter regions of p63 target genes identified in this study using Genomatix (B. Yan, unpublished data; Supplementary Methods). Many of the ΔNp63 target genes contained predicted p63 and/or NF-κB binding sites (Fig. 2A; Supplementary Table S4), consistent with coregulation and a potential functional interaction between the 2 factors (Fig. 1A and C; Supplementary Table S3). To test this hypothesis, chromatin immunoprecipitation (ChIP) assays were conducted in cell lines that express ΔNp63 at relatively high (UM-SCC46, Fig. 2B) or lower levels (UM-SCC1, not shown). We detected significant basal p63 binding activity on 13 sites of 8 promoters, including a known p63 binding site in the p21Cip1 gene, which served as a positive control (Supplementary Table S4; refs. 17, 27). IL-8, CSF2, PDGFA, and YAPI promoters were additionally selected for ChIP analyses with multiple antibodies, without or with TNF-α treatment (Fig. 2B). Antibodies included for ChIP assay were selected on the basis of previous findings that, in HNSCC, ΔNp63 can form a protein complex with cRel (17), canonical RelA/p65 is often activated (18, 28), and IkB kinase alpha (IKKα) may bind to ΔNp63 (13, 29). ChIP results revealed that p63 and cRel exhibited higher basal binding activities at most predicted binding sites, and TNF-α potently induced cRel binding to all sites. In addition, as expected, TNF-α greatly induced RelA/p65 binding activity in the previously defined proximal p65/cRel site in the IL-8 promoter (30). Minimal basal and TNF-α–induced IKKα-binding activities were detected (Fig. 2B).

Coordinate binding of p63, cRel, and/or RelA protein complexes to p63 and NF-κB regulatory elements
To investigate whether ΔNp63, cRel, and RelA bind to the same cognate elements present in these promoters, electrophoretic mobility shift assays (EMSA) were carried out with nuclear extracts from UM-SCC1 (Fig. 3) and UM-SCC46 (not shown). Multiple protein complexes were bound to the oligonucleotide containing the sequence of RelA/cRel site of the IL-8 promoter [main bands (MB); Fig. 3A, lanes 2 and 4]. Remarkably, supershift with an anti-RelA antibody (lane 6), or an antibody against the p63 C-terminus (H129; lane 7), attenuated most MBs. Furthermore, lesser supershift of MB activity was detectable using 2 different cRel C-terminal antibodies (lanes 10 and 11). A protein complex was bound to the oligonucleotide containing the sequence of the p63 site
Figure 1. Knockdown of ΔNp63 regulates a broad gene expression program that overlaps with the NF-κB transcriptome and network. A, relative fold-change in mRNA expression after ΔNp63 siRNA transfection of UM-SCC1 for 24, 48, or 72 hours (white, gray, or black bars); *, P < 0.05 versus control siRNA. B, heat map for up- (red) or downregulated (green) mRNA expression after ΔNp63, RelA siRNA, or TNF-α treatment. Dots indicate predicted RelA, NF-κB, and cRel binding sites in the proximal promoter regions (−500 bp to +100 bp of transcription start site). Gene categories are as follows: blue, growth and apoptosis; pink, cytokines; black, adhesion; brown, NF-κB. C, IPA illustrates important functional networks between ΔNp63-modulated genes and RelA, cRel, or NF-κB.

Of IL-8 promoter, and was attenuated by both p63-specific antibodies (Fig. 3B, lanes 15 and 16) and shifted with anti-p63 H137 antibody (lane 16). Although the RelA antibody had no significant effect (lane 14), the cRel antibody attenuated MB binding activity, indicative of cRel co-binding to the predicted p63 binding site (lane 18). A predicted CSF2 promoter p63 site oligonucleotide-bound complex was shifted or attenuated with anti-p63 antibodies but not with RelA antibody (Fig. 3C, lanes 20–22). For the predicted CSF2 promoter Rel site, RelA and p63 were both detected by supershift (lanes 24–26), and, furthermore, the anti-cRel antibody significantly decreased the MBs (lanes 27 and 28). We obtained similar results for the YAP promoter p63 site (Fig. 3D). Both anti-p63 antibodies attenuated MBs (lanes 31 and 32), with H137 detecting 2 supershifted bands (lane 32). Although no supershift was detected with anti-RelA (lane 30), anti-cRel attenuated MB
binding activity (lane 34). These data provide evidence for coordinate and differential binding of distinct p63, cRel, and/or RelA complexes to p63 and NF-κB regulatory elements of multiple genes.

**p63 and TNF-α regulate NF-κB, TP53, p21Cip1, or IL-8 transcription activity**

Thereafter, we investigated how p63 modulated NF-κB or TP53-mediated transcriptional activity (Fig. 4; Supplementary Fig. S3). ΔNp63 knockdown markedly decreased specific NF-κB or RelA/cRel site–dependent IL-8 reporter gene activities (construct: −133 bp; ref. 30); however, the effects were less potent than with RelA knockdown (Fig. 4A, top row). Conversely, overexpression of ΔNp63 increased NF-κB and IL-8 reporter gene activities more potently than TAp63 (Supplementary Fig. S3, left column). In contrast, overexpressed ΔNp63 weakly stimulated TP53 while repressing TP53 target p21Cip1 reporter activity (Fig. 4A, bottom row).

Figure 2. Distinct ΔNp63, cRel, and RelA binding activities on p63- or NF-κB–responsive elements. A, p63 and NF-κB/Rel binding sites [with base pairs from transcription start site (TSS)] on gene promoters were predicted by Genomatix by including user-defined p63 PWM as described in Supplementary Methods. B, ChIP assay conducted using anti-p63, cRel, IKKα, RelA, and isotype antibodies without [control (Ctrl)] or with TNF-α treatment, followed by real-time PCR. Mean relative binding activity ± SD for triplicates; *P < 0.05 compared with isotype (*) or between control and TNF-α treatment (#).

**IL-8**

p63 (−1,425) c-Rel/p65 (−83)

p63 (−1,415)

p63 (−1,485)

p50 (−1,609)

p63 (−2,324) c-Rel/p65/p50

p63 (−2,314)

p50 (−2,261)

**CSF2**

p50cRel

cRel/p65
cRel/p65/p50

cRel (−702)

**PDGFA**

p50 (−845 bp)

p50 (−845 bp)

p63 (−1,495 bp)

p63 (−1,486 bp)

**YAP1**

p63 (−2,324 bp)

p63 (−2,314 bp)

p63 (−2,218 bp)

**Figure 2.** Distinct ΔNp63, cRel, and RelA binding activities on p63- or NF-κB–responsive elements. A, p63 and NF-κB/Rel binding sites [with base pairs from transcription start site (TSS)] on gene promoters were predicted by Genomatix by including user-defined p63 PWM as described in Supplementary Methods. B, ChIP assay conducted using anti-p63, cRel, IKKα, RelA, and isotype antibodies without [control (Ctrl)] or with TNF-α treatment, followed by real-time PCR. Mean relative binding activity ± SD for triplicates; *P < 0.05 compared with isotype (*) or between control and TNF-α treatment (#).
Transcriptional regulation of the IL-8 gene by ΔNp63 was further examined in UM-SCC1 cells using luciferase reporter constructs with serial deletions and point mutations (Fig. 4B; ref. 30). Consistent with observations discussed in the preceding sections, overexpression of ΔNp63 exhibited a stimulatory pattern for most of the promoter constructs that was similar to that of the NF-κB–inducing cytokine TNF-α. Deleting the distal predicted p63-binding site (−1,424 bp) had a minimal effect, whereas increased reporter activity was observed using the minimal −133-bp promoter construct containing the RelA/cRel binding site, for which we established p63, cRel, and RelA co-binding activities in the preceding paragraphs (Fig. 3A, lanes 5–12). Furthermore, deletion or point mutation of this RelA/cRel site (−83 bp) or nearby AP-1 site (−128 bp) had a profound impact on both TNF-α– and ΔNp63– induced reporter activity (Fig. 4B, bottom panel), indicating that ΔNp63– and TNF-α– induced IL-8 activation are both dependent on these sites.
To further test the dependency of IL-8 promoter activity on ΔNp63, RelA, or cRel transcriptional components, IL-8 reporter activity was measured under combinatorial knockdown or inducing conditions (Fig. 4C). ΔNp63 depletion partially inhibited basal IL-8 reporter activity and almost completely suppressed TNF-α–induced IL-8 reporter activation (2 of the left panels). Conversely, basal level (control vector) IL-8 reporter activity was more strongly suppressed by deletion of RelA than cRel, whereas ΔNp63 overexpression enhanced the relative contribution of cRel (2 of the right panels). These results are consistent with the relatively greater binding of RelA and ΔNp63 relative to cRel to the IL-8 RelA/cRel binding site as shown by EMSA (Fig. 3A), and previous evidence indicating RelA is the major NF-xB family subunit responsible for binding and transactivation of the IL-8 promoter in HNSCC (28).

ΔNp63 modulates proliferation, differentiation, cell cycle, survival, and migration in HNSCC cell lines

Knockdown of ΔNp63, but not TAp63, markedly inhibited cell proliferation, increased sub-G0 DNA fragmentation and G0-G1 blockade, and decreased S-phase and G2-M cell-cycle phases in UM-SCC1 and UM-SCC6 cell lines (Fig. 5A and B; Supplementary Fig. S4A and B). Furthermore, ΔNp63 depletion induced development of larger and flatter cells of differentiated cell morphology (Fig. 5C), reduced wound-healing motility in scratch assay (Fig. 5D; Supplementary Fig. S4C), and cell migration in classical Matrigel assay by up to 35% (P < 0.05; Supplementary Fig. S4D). Thus, the effect of modulation of ΔNp63 upon multiple vital biological functions is consistent with its role in the regulation of a broad gene program.

Increase in ΔNp63, Ki-67, and β4 integrin immunostaining and inflammation in HNSCC compared with mucosa

We further examined the pattern of expression of ΔNp63, markers of proliferation (Ki-67) and adhesion (β4 integrin), and cellular inflammation in human HNSCC and mucosa specimens. An increase in nuclear, localized, ΔNp63 staining was observed throughout the malignant epithelia in most HNSCC samples from independent series of 21 frozen (e.g. Fig. 6A; Supplementary Fig. S1) and 20 fixed tumor tissues (e.g., Fig. 6B). In contrast, nuclear ΔNp63 was predominantly...
localized in the basal layers of 2 patient-matched mucosa available from the series of frozen tissues (Fig. 6A; Supplementary Fig. S1), and 6 mucosal specimens from the series of fixed tissues (Fig. 6B). Interestingly, we observed similar patterns of staining for the Ki-67 proliferative marker and β4 integrin adhesion marker throughout HNSCC tumor specimens, and a basilar pattern of expression for these markers in mucosa (Fig. 6A). We found a positive and statistically significant correlation between ΔNp63 and Ki-67 IHC histoscores for 19 of 20 (95%) HNSCC and 6 mucosal tissue specimens from the tissue array (Fig. 6B). One outlier among the HNSCC samples exhibited higher Ki-67 staining but low ΔNp63 IHC staining, possibly related to a different mechanism of Ki-67 protein expression. Among 3 mucosa samples with relatively higher ΔNp63 and intermediate Ki-67 staining, epithelial hyperplasia with expansion of basilar nuclear layers was observed (e.g., Fig. 6A, top vs. bottom panel of mucosa). Additionally, increased nuclear ΔNp63 in HNSCC was accompanied by adjacent focal or diffuse infiltration of inflammatory cells in 9 of the 20 specimens (45%; P = 0.04 through Fisher exact test, not shown). Thus, the ability of ΔNp63 to broadly regulate gene expression and critical biological processes in tumor cells in vitro was supported by an increase in nuclear ΔNp63, proliferation and adhesion markers, and inflammatory cell infiltration in HNSCC specimens when compared with mucosa in vivo.

**ΔNp63 TG mice exhibit epithelial hyperplasia and inflammation**

To determine the effects of ΔNp63 overexpression in squamous epithelia in vivo, we examined a recently established TG mouse model in which ΔNp63Δα was conditionally induced in skin under the control of a keratin-5 promoter (26, 31). ΔNp63 TG mice began to develop skin lesions and erythema within 1 month of induction (Fig. 7A) and showed hyperplastic and hyperproliferative epidermis with diffuse infiltration of inflammatory cells in subadjacent stroma (Fig. 7B). In TG mice, nuclear ΔNp63 expression extended to suprabasilar layers of the hyperplastic epidermis, whereas the endogenous ΔNp63 expression was restricted in the innermost basal layer of the epidermis and hair follicles in wild-type mice (Fig. 7C, upper left). Furthermore, cRel expression was increased and colocalized together with ΔNp63 in the epidermis, and induced in stromal infiltrating cells (Fig. 7C, top right). As in hyperplastic human mucosal specimens (Fig. 6A and B), ΔNp63 and proliferation marker Ki-67 were increased in suprabasilar layers of the thickened TG epidermis exhibiting cytokeratin-K14 (CK) staining (Fig. 7C, bottom panels). The altered gene-expression profile expressed in the skin of TG mice includes inflammatory cytokines, adhesion molecules, and other genes expressed in UM-SCC cell lines (Fig. 7D). Together, these results indicate that overexpression of ΔNp63 in 2 independent biological systems, HNSCC and TG mice, promotes...
epithelial hyperproliferation, NF-kB/Rel-regulated cytokine gene expression, and inflammation.

**Discussion**

In this article, we provide evidence for a new mechanism and model of interaction between members of the TP53 and NF-kB families in coregulating the transcriptome and phenotype in squamous epithelia of HNSCC and ΔNp63 TG mice (Fig. 7E). ΔNp63, the predominant p63 isoform overexpressed in most HNSCC and in the basilar layers of squamous epithelia, shows a versatile ability to coordinately regulate expression of a broad program of genes that overlap the NF-kB/Rel and TP53/p63 transcriptomes. Furthermore, ΔNp63 displays novel interactions with NF-kB family members, cRel and RelA, through co-binding to newly predicted and known p63 and NF-kB promoter regulatory sites of NF-kB/Rel target genes. ΔNp63 promoted proliferation, cell cycle, migration, and inflammatory cytokine expression by HNSCC in vitro. These observations are consistent with studies showing an increase in ΔNp63 accompanied by proliferative and integrin adhesion protein markers, as well as inflammatory cell infiltration throughout the tumor microenvironment in vivo. In addition, the overexpression of ΔNp63α in TG mice led to suprabasilar modulation of cRel protein and cytokine genes as well as the development of hyperplasia and inflammation in the epidermis. This model supports the causal function of ΔNp63 as a key transcriptional regulator of proliferation and inflammation in squamous epithelia.

Our results show a novel bifunctional role of ΔNp63 in coordinating known NF-kB or TP53 downstream genes important in proliferation and cell survival. We showed that ΔNp63 siRNA inhibited gene expression of known NF-kB target genes CCND1, Bcl-2, and Bcl-xL that, upon individual knockdown, were previously shown to inhibit proliferation and survival of UM-SCC in vitro (25, 32–35). ΔNp63 reciprocally repressed expression and transactivation activity of TP53 family members TP53, TAp63, and p73, as well as target genes such as p21Cip1 and PUMA, which mediate growth arrest and apoptosis. ΔNp63 was previously reported as a repressor of TP53 and p73 target genes p21Cip1, NOXA, and PUMA in squamous epithelial and HNSCC (14, 17, 27). Furthermore, predominant nuclear expression of ΔNp63 and deficiency of TAp63 were observed in a subset of non-SCC tumors in mice heterozygous for p63 and TP53 alleles (36), similar to observations in human HNSCC with altered p63 and TP53 (14). Variability in the...
Figure 7. Skin from ΔNp63α TG mice shows hyperplasia, cellular inflammation, and increased proinflammatory cytokine expression. ΔNp63α was induced in skin of TG animals for 2 months after birth. A, gross morphology, erythematous lesions. B, histology, hematoxylin and eosin stain. Top, epidermal hyperplasia and cellular inflammation in dorsal skin sections (×100; scale bar, 300 μm). Bottom, infiltrating inflammatory cells in the dermis highlighted by yellow arrowheads (×600; scale bar, 50 μm). C, dorsal skin sections from ΔNp63α TG and wild-type mice were stained with anti-ΔNp63, cRel, Ki-67, and K14 (CK) antibodies and DAPI counterstain (scale bar, 75 μm). D, gene expression in dorsal skin 16 days (top) or 1 month (bottom) following ΔNp63α induction in neonatal TG mice, versus wild-type littermates. E, model of ΔNp63 and NF-κB/Rel interactions mediating regulation of a broad gene program. Overexpressed ΔNp63 following carcinogenesis and cRel or RelA, activated by TNF-α from inflammation in the tumor microenvironment, interact via p63 or NF-κB sequences of target gene promoters. p63/Rel protein complexes regulate a broad gene program that overlaps with NF-κB transcriptome and promotes the malignant phenotype.
effects of ΔNp63-modulated gene expression in the UM-SCC cell line overexpressing mtTP53, compared with UM-SCC deficient for wtTP53, suggests that effects of ΔNp63 may potentially be modified by TP53 status (22, 25, 32). ΔNp63 and TP53 share high homology of the transactivation and DNA-binding domains and differentially bind to ΔNp63/TP53 sites; furthermore, these may thereby competitively inhibit or alter one another’s transactivation function (8, 9). Consistent with this finding, we previously found that the cross-talk between TP53 and NF-kB in differentially modulating Bcl-xL and Bax expression is dampened in the subset of UM-SCC cells overexpressing mtTP53 (25, 32). The relationship between ΔNp63 and Ki-67 proliferation-marker histoscores in HNSCC and containing in suprabasilar layers of a few hyperplastic mucosal samples in tissue array, as well as in skin samples from ΔNp63 TG mice, provides additional evidence for a relationship between increased nuclear ΔNp63 and proliferation in vivo.

A novel and unexpected finding from this study is the demonstration that ΔNp63 promotes a broad NF-kB program that includes multiple inflammatory cytokine genes. The inflammatory cytokines modulated by ΔNp63 in HNSCC cells and ΔNp63 TG mice largely overlap with the NF-kB-regulated cytokine repertoire, which we previously identified in HNSCC culture supernatants, patient serum, and tumor specimens (37, 38). Furthermore, many of these ΔNp63 and NF-kB coregulated inflammatory factors have been individually shown to promote the aggressive malignant behavior that leads to poor prognosis of HNSCC (4, 19, 22, 25, 37–42). Proinflammatory cytokines have been shown to attract infiltrating neutrophils and macrophages that enhance angiogenesis, and they greatly promote cancer cell survival and metastasis (41). Consistent with this, we observed that increased nuclear ΔNp63 level is linked with infiltrating inflammatory cells in HNSCC, and in the dermis of ΔNp63 TG mice (Figs. 6C and 7B). The clinical importance of our findings that ΔNp63 promotes inflammation and aggressive malignant phenotypes is further supported by a recent report showing that oral leukoplakias with increased ΔNp63 expression and inflammatory cell infiltration exhibit a higher rate of cancer development and worse prognosis (42).

The role of ΔNp63 in modulation of multiple cell adhesion genes, such as α6β4 integrin and laminins, migration by wound healing, and Matrigel assays in vitro, and an association with suprabasilar β4 integrin expression in HNSCC tumors in situ suggest it may also function in promoting cell migration during invasion and metastasis in tumor progression. Consistent with this, we have previously shown that increased suprabasilar α6β4 expression occurs in more than 70% of HNSCC with higher invasive–metastatic potential in a prospective clinical study, and that this integrin promotes laminin-mediated adhesion and migration (43, 44). In addition, a causal role for ΔNp63 in modulating a broad adhesion gene program, including β4 integrin, was independently shown in breast cancer epithelial cells (39). Knockdown of ΔNp63 by short hairpin RNA (shRNA) downregulated adhesion by 24 and 48 hours, and adhesion could be partially restored by expression of α6β4. The finding that ΔNp63 promotes a similar broad adhesion and survival gene program in HNSCC and breast cancer cell lines suggests that such dysregulation may be of broader importance in epithelial cancers.

Remarkably, we found that the promoters of multiple ΔNp63- and NF-kB–modulated genes contain nearby or overlapping p63 and NF-kB binding sites (Fig. 2A), providing the potential for members of the 2 families to coordinately or physically interact on the same or nearby sites. Indeed, family members ΔNp63, cRel, and RelA showed cobinding to the same or nearby promoter sites of several of these genes by ChIP and EMSA supershift analyses (Figs. 2 and 3). Interestingly, although ΔNp63 cobinding with cRel was detected on both p63 and Rel regulatory sites, RelA binding was not detected on the p63 sites of IL-8, CSE2, and YAP promoters. Furthermore, we obtained evidence for binding of ΔNp63 and cRel, but not RelA, to the p63 site of p21cip1 promoter in primary murine keratinocytes and HNSCC by ChIP and EMSA (17, 45). In contrast, ΔNp63 cobinding with RelA was principally detected on Rel sites by supershift analysis. Thus far, it appears that ΔNp63 regulates NF-kB target genes through interaction with cRel, on p63 binding sites, and cRel and RelA via Rel sites.

Supporting a novel functional role of ΔNp63 in coregulating NF-kB gene activation, knockdown or overexpression of ΔNp63-modulated activation of NF-kB–specific reporter and IL-8 promoter was dependent on NF-kB regulatory sites. ΔNp63α bound to the NF-kB/Rel site on the proximal promoter was required for ΔNp63α- and TNF-α–induced IL-8 promoter transactivation (Figs. 3 and 4). Knockdown of RelA exhibited a more potent inhibitory effect than knockdown of cRel on transactivation of the basal IL-8 reporter activity, whereas the relative contribution of cRel increased when ΔNp63 was overexpressed (Fig. 4C). These data are consistent with the differential contribution of the 3 transcription factors to gene regulation under different conditions of activation and binding specificity. As with ΔNp63 in promoting IL-8 transcription, a recent report implicates p63 in IL-6 expression through a NF-kB promoter binding site in tonsillar crypt epithelium in palmoplantar pustulosis (46).

The broad role of ΔNp63, both in promoting NF-kB and antagonizing TP53, is consistent with its ancestral coevolution with NF-kB as a multifunctional transcriptional coordinator (47–49). With the mutation or inactivation of TP53, aberrant activation of ΔNp63 together with NF-kB/Rel s appears to mediate a broad primordial gene program and pro survival response. In that context, it may not be surprising that ΔNp63 serves as a master control to versatilely coregulate NF-kB, TP53 and other gene programs to promote proliferation, survival, migration, and inflammation in cancer. Based on evidence that ΔNp63-overexpressing HNSCC and other cancers are more sensitive to cisplatin-chemotherapy–induced ΔNp63 degradation (29, 50), ΔNp63 and target gene signatures may warrant further investigation as potential biomarkers for selection of cisplatin and other therapies that target ΔNp63, cRel, and RelA activation in cancer.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Correction: ΔNp63 Versatilely Regulates a Broad NF-κB Gene Program and Promotes Squamous Epithelial Proliferation, Migration, and Inflammation

In this article (Cancer Res 2011;71:3688–700), which was published in the May 15, 2011, issue of Cancer Research (1), the graph in Fig. 7D was mislabeled because of a production error. The corrected figure appears below.

Figure 7.

Reference


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