TNF-α Promotes c-REL/ΔNp63α Interaction and TAp73 Dissociation from Key Genes That Mediate Growth Arrest and Apoptosis in Head and Neck Cancer

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Abstract

Inflammation-induced activation of proto-oncogenic NF-κB/REL and dysfunction of tumor suppressor TP53/p63/p73 family transcription factors are key events in cancer progression. How inflammatory signaling coordinates dysregulation of these two transcription factor families during oncogenesis remains incompletely understood. Here, we observed that oncoprotein c-REL and tumor suppressor TAp73 are coexpressed and complex with ΔNp63α in the nucleus of a subset of head and neck squamous cell carcinoma (HNSCC) cell lines with mutant (mt)TP53. TNF-α, a proinflammatory cytokine, promoted c-REL nuclear translocation, c-REL/ΔNp63α interaction, and dissociation of TAp73 from ΔNp63α and the nucleus to the cytoplasm, whereas c-REL siRNA knockdown attenuated this effect. Overexpression of c-REL or a c-REL κb site DNA-binding mutant enhanced protein interaction with ΔNp63α and TAp73 dissociation, implicating c-REL/ΔNp63α-specific interactions in these effects. We discovered that TNF-α or genetic alteration of c-REL expression inversely modulates ΔNp63α/TAp73 interactions on distinct p63 DNA-binding sites, including those for key growth arrest and apoptotic genes p21WAF1, NOXA, and PUMA. Functionally, c-REL repressed these genes and the antiproliferative effects of TNF-α or TAp73. Conversely, c-REL siRNA depletion enhanced TAp73 promoter interaction and expression of genes mediating growth arrest and apoptosis. Similar to TNF-α-treated HNSCC lines, human HNSCC tumors and hyperplastic squamous epithelia of transgenic mice overexpressing ΔNp63α that exhibit inflammation also show increased nuclear c-REL/ΔNp63α and cytoplasmic TAp73 localization. These findings unveil a novel and reversible dynamic mechanism whereby proinflammatory cytokine TNF-α-induced c-REL/ΔNp63α interactions inactivate tumor suppressor TAp73 function, promoting TNF-α resistance and cell survival in cancers with mtTP53. Cancer Res; 71(21): 6867–77. ©2011 AACR.

Introduction

TP53, a tumor suppressor that mediates growth arrest and apoptosis of critically damaged cells, is the most frequently mutated gene in cancer, including head and neck squamous cell carcinomas (HNSCC; refs. 1, 2). The TP53 family also includes p63 and p73, which have overlapping functions in cell growth and apoptosis, as well as development of squamous epithelia of the skin and mucosa (3, 4). When TP53 is mutated or inactivated, transactivating (TA) isoforms TAp63 and TAp73 can potentially replace the tumor suppressor function of TP53. TAp63 and TAp73 have full-length N-terminal domains which share homology, transactivating, and tumor suppressor functions with TP53. Although TAp63 and TAp73 are rarely mutated, expression of alternatively transcribed ΔN isoforms can differentially affect their transactivating and tetramerization. Recent evidence suggests that the ΔNp63α isoform is predominantly overexpressed together with TAp73 in major subsets of HNSCC, breast, and other cancers (5). Nuclear interactions between ΔNp63α and TAp73 proteins on regulatory promoters have been implicated in repression of genes mediating growth arrest and apoptosis (4, 5). However, what factor(s) regulate these interactions between overexpressed ΔNp63α and TAp73, and how their interaction leads to inactivation of TAp73 in cancers with mtTP53, remains unclear.

Among candidate regulatory factors, TNF-α is a cytotoxic cytokine which is expressed by cancer and infiltrating
inflammatory cells in the tumor microenvironment of many cancers, including HNSCC (6, 7). Interestingly, we found that HNSCC are paradoxically resistant to TNF-α-mediated growth arrest and apoptosis, and that such resistance involves aberrant activation of NF-κB/REL transcription factors (8). Among these, TNF-α has been shown to signal via a canonical pathway to promote nuclear translocation and transactivation of RELA (p65) and c-REL (9). RELA (p65) promotes expression of genes that enhance cell proliferation and survival of cancers, including HNSCC (10–12). However, inhibition of RELA and these pro-survival genes by siRNA in vitro or by proteasome inhibitor in a phase I clinical trial in vivo showed limited cytotoxic and clinical activity (11–13). Amplification and nuclear localization of c-REL has also been previously detected and found to be relatively unaffected by proteasome inhibition in HNSCC (13, 14). These observations raised the question whether TNF-α-regulated or overexpressed c-REL may contribute to inhibition of growth arrest and apoptosis of HNSCC by a distinct mechanism.

We recently discovered that overexpressed ΔNp63α colocalizes and forms novel nuclear complexes with c-Rel in murine keratinocytes or ortholog c-REL in human HNSCC (15). Herein, we explored the hypothesis that TNF-α and c-REL activation is linked to modulation of the aforementioned ΔNp63α/TAp73 interactions and dysregulation of growth arrest and apoptosis in cancer. We reveal a dynamic and reversible mechanism whereby TNF-α promotes c-REL nuclear translocation and interaction with ΔNp63α, dissociation of TAp73 from distinct p65 promoter sites of key growth arrest and apoptotic genes, and TAp73 translocation to the cytoplasm. These results can help explain how inflammatory factor TNF-α and c-REL, through ΔNp63α, inhibit the compensatory ability of TAp73 to activate genes that mediate growth arrest and apoptosis in HNSCC with mutant TP53.

Materials and Methods

Cell lines
The characteristics, TP53 genotype, and culture of HNSCC (UMSCC) cell lines obtained from the University of Michigan were previously described (16, 17). UMSCC lines used herein were obtained in 2008. Authentication was done at the University of Michigan by DNA genotyping of alleles for 9 loci; D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA, and the amelogenin locus in 2008 and 2010, as recently described (18). Cell line stocks were preserved at −80°C and cultured for fewer than 6 months before use.

Western blot
Western blot analysis was done as previously (17). Primary antibodies are listed in Supplementary Methods.

Real-time RT-PCR
RNA isolation and cDNA synthesis were done as previously (19). Real-time PCR primers and probes for ΔNp63α and TAp63 validated previously (20) were synthesized by Applied Biosystems. Primers to amplify TAp73 and ΔNp73 are listed in Supplementary Methods.

Plasmid, siRNA transfection, and reporter gene assays
These experiments were conducted as described previously (19). ΔNp63α-specific siRNA (21) was synthesized by Integrated DNA Technologies (IDT). TAp73-specific siRNA was purchased from Dharmacon. The 2.6-kb p21-luc reporter was provided by Dr. Bert Vogelstein laboratory (22). Each sample was assayed in triplicate and data were presented as mean ± SD.

Coimmunoprecipitation analysis
Coimmunoprecipitation (co-IP) was done as described previously (15). Antibodies used are listed in Supplementary Methods.

Electrophoretic mobility shift assay
Oligonucleotides covering the p21WAF1 promoter p63-binding site (−2,283 bp, 5’-TGGCCGTCAAGAATGTCCCAA-CATGTTGACGCTGGCA-3’; Supplementary Fig. S3A) were [γ-32P]-end-labeled with 10U of T4 polynucleotide kinase (New England Biolabs), and electrophoretic mobility shift assay (EMSA) was done as previously described (23).

Chromatin immunoprecipitation analyses
Chromatin immunoprecipitation (ChIP) assays were done using the Magna ChIP G kit (Upstate) as previously (24). For quantitative PCR primers see Supplementary Methods.

DNA-based cell-cycle and apoptotic flow cytometric analysis
DNA flow cytometric analysis was done as previously (12). Samples were run on fluorescence-activated cell analyzer (FACS Canto, BD Biosciences, San Jose, CA) within 1 hour and analyzed using DIVA flow cytometric analysis software.

Additional experimental detail, methods, and figures are included in Supplementary Information.

Results

Overexpression and protein interactions among c-REL, ΔNp63α, and TAp73 in a subset of head and neck cancer cell lines with mutant TP53
Expression of c-REL, p63, p73, and TP53 was characterized in a panel of cell lines that included 9 HNSCC (UMSCC) and normal primary human epidermal keratinocytes (HEK) (Supplementary Fig. S1). The UMSCC lines selected were derived from primary and metastatic tumors exhibiting an aggressive clinical course (survival <15 months) and representative genetic abnormalities, including inactivation or mutation of TP53 (16–18), c-REL, ΔNp63α, TAp73, and TP53 protein were detected by Western blots and qRT-PCR (Supplementary Fig. S1A–F). Total c-REL protein was increased, as were c-REL and ΔNp63α protein in most UMSCC, compared with normal HEK (H) cells (Supplementary Fig. S1A, left, right panels). Interestingly, even greater c-REL expression was detected in 3 of 4 cell lines (UMSCC-22B, -38, and -46) with elevated ΔNp63α, TAp73, and mtTP53 (UMSCC-22A, -22B, -38, -46; Supplementary Fig. S1A, left panel and Supplementary Fig. S1B–E). Thus, we find that c-REL, ΔNp63α, and TAp73 are upregulated with mtTP53 in an overlapping subset of HNSCC lines.
Both c-REL and TAp73 exhibited basal interaction with ΔNp63α in whole-cell extracts from 2 independent cell lines by co-IP analysis (Fig. 1A; UM-SCC-22A or -46). However, c-REL and TAp73 exhibited weak or negligible interaction with one another, seen only with supersensitive substrate and prolonged exposure. Furthermore, c-REL, but not RELA(p65), interacted with ΔNp63α (although RELA formed an independent complex with c-REL; Fig. 1B), providing evidence for distinct interactions by these RELs. The complexes between c-REL, ΔNp63α, and TAp73, identified in whole-cell extracts above, were found in nuclear, but not cytoplasmic extracts (Fig. 1C). No interaction with miTP53 was observed (Fig. 1B and C). In the absence of further stimulation, a significant portion of nuclear ΔNp63α co-IPed with either c-REL or TAp73, when compared with input (Fig. 1D).

**TNF-α or genetic modulation of c-REL affects nuclear localization and interactions between c-REL, ΔNp63, and TAp73**

The inflammatory cytokine TNF-α is a canonical inducer of c-REL and is expressed in the HNSCC microenvironment in vivo, but not by these cell lines in vitro (7, 25). We examined the effect of exogenous TNF-α on the cellular distribution of c-REL, ΔNp63α, and TAp73 in the UM-SCC-22A cell line, which has lower endogenous c-REL expression, making it amenable to TNF-α or genetic modulation (Supplementary Fig. S1). TNF-α stimulation increased nuclear localization of c-REL within 30 minutes (Fig. 2A, left panel). Remarkably, TNF-α not only induced c-REL but also decreased TAp73, without significantly altering ΔNp63α expression in nuclear extracts (Fig. 2A, right panel). Moreover, TNF-α stimulation increased the interaction between nuclear c-REL and ΔNp63α and a corresponding dissociation of nuclear ΔNp63 and TAp73 in co-IP (Fig. 2B). This dissociation corresponded with a decrease in TAp73 in the nuclear fraction and appearance in the cytoplasmic fraction (Fig. 2C). Conversely, c-REL knockdown by siRNA enhanced ΔNp63α/TAp73 interaction without or with TNF-α (Fig. 2D).

Similar to TNF-α–induced c-REL, overexpressing c-REL-Flag in UMSCC-22A resulted in increased interaction between c-REL and ΔNp63α in the nuclear fraction (Fig. 3A, top panels). Concurrently, TAp73 dissociated from ΔNp63α in the nuclear fraction and appeared in the cytoplasmic fraction (Fig. 3A, bottom panels). Further supporting that c-REL and TAp73 form alternative complexes with ΔNp63α, no significant interaction between c-REL and TAp73 was detected in co-IP. Overall, the above findings indicate that TNF-α–induced or overexpressed c-REL is involved in the dissociation of TAp73 from ΔNp63α in the nucleus to the cytoplasm.

Previous studies have indicated that c-REL can bind κB motifs bound by other NF-κB/REL members, as well as distinct promoter sites and synthetic oligonucleotides (26). We compared the effect of overexpressing a wild-type (wt)c-REL and a N-terminal RxxRxR to AxxAxA mutant reported to abrogate c-REL binding to classical REL/κB DNA motifs (27). Overexpression and TNF-α enhanced nuclear translocation of exogenous wt and AxxAxA mutant c-REL (Fig. 3B). Interestingly, both wt and AxxAxA mutant c-REL reduced total nuclear TAp73 and ΔNp63-TAp73 interaction, and this reduction was preferentially enhanced with TNF-α–stimulated AxxAxA mutant c-REL (Fig. 3C and D). These results suggested that TNF-α–induced...

**Figure 1.** Interactions among c-REL, ΔNp63α, TAp73, and RELA(p65) in HNSCC with miTP53. A, left, whole-cell lysates from UM-SCC 22A cells or, right, 46 cells, were immunoprecipitated, then immunoblotted with antibodies as indicated. B, whole-cell lysates of UM-SCC 22A were immunoprecipitated with c-REL or p65 antibodies, then immunoblotted with the antibodies as indicated. C, cytoplasmic and nuclear fractions were immunoprecipitated with IgG control or c-REL antibody, then probed for c-REL, ΔNp63α, TAp73, and TP53. D, whole-cell lysates of UM-SCC 22A were immunoprecipitated with c-REL, p63 and TAp73 antibodies, and immunoprecipitated proteins and total lysates (input) were then immunoblotted with p63 antibody. IB, immunoblotting; IP, immunoprecipitation.
or overexpressed c-REL interacts with ΔNp63α and modulates bound TAp73 in a manner distinct from those previously shown to mediate c-REL binding to xB DNA recognition sequences.

**TNF-α or genetically modulated c-REL reciprocally affects binding of TAp73 with ΔNp63α on p63 sites of p21WAF1, NOX4, and PUMA gene promoters**

To explore whether TNF-α modulates interactions between c-REL, ΔNp63α, and TAp73 on p63/p73 regulatory sites, we examined their binding to an oligonucleotide encoding a well-characterized p63/p73-binding site (−2,283 to −2,273; Supplementary Fig. S2A) from the promoter of the p63/p73-regulated growth arrest gene p21WAF1 (15, 23). Using this p63 site-specific radiolabeled oligonucleotide probe, a major band was revealed by EMSA in human UMSCC-22A cells (Fig. 4A, left panel, MB). Binding specificity was confirmed by unlabeled probe competition, and the complex contained c-REL, ΔNp63α, or TAp73, shown by supershift with specific antibodies. Similar to our findings in nuclear extracts, TNF-α stimulated c-REL binding, while decreasing binding of TAp73, without significantly affecting ΔNp63α.

Next, we examined the endogenous binding of c-REL, ΔNp63α, and TAp73 to p21WAF1 promoter DNA using ChIP assays. All 3 factors exhibited basal binding to the p63-binding site region of the p21WAF1 promoter in UM-SCC 22A, -46, or -1 cell lines (Fig. 4A, right panel; Supplementary Fig. S2B–D). TNF-α induced c-REL binding and decreased TAp73 binding, without significantly affecting ΔNp63α binding to the p63 site in 2 different cell lines (Fig. 4B; Supplementary Fig. S2C). Similarly, c-REL overexpression also induced the same effects in either UMSCC-22A or -1 lines (Fig. 4A, right panel; Supplementary Fig. S2D). Conversely, c-REL depletion by siRNA significantly decreased c-REL and ΔNp63α binding, and reciprocally enhanced TAp73-binding activity in UMSCC-22A cells (Supplementary Fig. S3E). Importantly, ΔNp63α siRNA knockdown attenuated basal and TNF-α inducible binding of c-REL, ΔNp63α, and TAp73, implicating ΔNp63α as a key anchor for c-REL- and TAp73-binding activity (Fig. 4C). Together, these findings in 3 different UMSCC lines indicate that TNF-α or genetic modulation of c-REL results in reciprocal modulation of TAp73 binding, with ΔNp63α on the established p63 regulatory site of the p21WAF1 promoter.

To examine the broader role of TNF-α–induced c-REL, ΔNp63α, and TAp73 interactions, we carried out ChIP assays for p63-binding sites identified by bioinformatic analysis (28; Supplementary Methods) on the promoters of 2 key proapoptotic genes, NOXA4 and PUMA (Supplementary Fig. S2A, arrows). As with p21WAF1, stimulation with TNF-α increased c-REL and decreased TAp73 binding without affecting ΔNp63α binding to the predicted p63 sites in the PUMA and NOXA4 promoters (Fig. 4D). No significant binding by RELAp65 or TP53 was detected on the p63 promoter sites of any of these 3 genes (Fig. 4A, right panel; D), consistent with co-IP results above. Together, our results indicate that TNF-α or genetically modulated c-REL reciprocally affects binding of ΔNp63α with TAp73 on p63 sites of multiple gene promoters.

**c-REL differentially modulates p21WAF1, NOX4, and PUMA gene expression in UMSCC cell lines differing in expression of TP53 family members**

We next examined how c-REL–modulated ΔNp63α–TAp73 interactions affect expression of growth arrest mediator p21WAF1. The UMSCC cell subsets express lower or higher baseline levels p21WAF1 mRNA and protein (Supplementary Fig. S3A and B), similar to relative differences in expression of wt or mt TP53, ΔNp63α, and TAp73 shown above (Supplementary Fig. S1A–D). Knockdown of endogenous c-REL by siRNA significantly reduced levels of c-REL mRNA in 2 cell lines, UMSCC-1 and UMSCC-22A (Fig. 5A, left panel), from those subsets that differ in baseline expression of p21WAF1 and TP53 family proteins. However, c-REL depletion in UMSCC-22A cells with abundant mtTP53, ΔNp63α, and TAp73 resulted in a strong increase in p21WAF1 mRNA and protein.
levels, when compared with UMSSC-1 cells lacking wtTP53 and TAp73 (Fig. 5A, right panel; Fig. 5B, left panel). Conversely, overexpression of c-REL by transient transfection repressed p21WAF1 expression in UMSSC-22A cells, but not in UMSSC-1 cells (Fig. 5B, right panel). Similarly, TNF-α induced nuclear c-REL, although inhibiting expression of TAp73 target protein p21WAF1 (Supplementary Fig. S3C). Increased p21WAF1 expression after c-REL depletion in UMSSC-22A was attenuated by cotransfection with TAp73 siRNA (Supplementary Fig. S3D), indicating the contribution of TAp73. Transfection of TAp73-deficient UMSSC-1 cells with TAp73 increased, whereas cotransfection with c-REL suppressed an increase in p21WAF1 expression (Supplementary Fig. S3E). Depletion of c-REL by siRNA also enhanced PUMA and NOXA expression in the mtTP53 cell line UMSSC-22A, but not in UMSSC-1 cells deficient in TP53 and TAp73 (Fig. 5C). Together, these results indicate that c-REL represses 3 important growth arrest and proapoptotic genes in HNSCC expressing ΔNp63 and TAp73 with mtTP53.

**c-REL promotes cellular proliferation and survival in UMSSC cells expressing ΔNp63, TAp73 with mtTP53**

We further examined the function of c-REL in proliferation and survival of HNSCC cells in vitro. c-REL depletion with siRNA had no significant effect on proliferation of UMSSC-1 cells that express lower levels of wtTP53 and other family members, but inhibited proliferation and density of UMSSC-22A cells that exhibit high protein levels of ΔNp63α, TAp73 and mtTP53 and inducible p21WAF1 (Fig. 6A and B; Supplementary Fig. S4A), c-REL siRNA depletion also increased sub-G0 DNA-positive dead cells in UMSSC-22A, but not in UMSSC-1 cells (Supplementary Fig. S4B), whereas c-REL overexpression reduced the sub-G0 fraction in UMSSC-22A cells (Supplementary Fig. S4C). The results showing that c-REL promotes cell proliferation and survival are consistent with the effect of c-REL in repressing ΔNp63α and TAp73. c-REL enhanced and TAp73 inhibited proliferation, whereas cotransfection of c-REL with TAp73 abrogated the inhibitory function of TAp73 (Fig. 6C). As HNSCC are relatively resistant to TNF-α–mediated inhibition of growth and survival (8), we examined the functional effect of TNF-α treatment or c-REL transfection, alone or in combination, on UM-SCC-22A cells (Fig. 6D). TNF-α alone weakly inhibited and c-REL enhanced proliferation, but the combination of TNF-α and c-REL overexpression further enhanced proliferation. Together, these data support the role of endogenous or exogenously overexpressed c-REL in repressing the antiproliferative function of TNF-α and TAp73.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Overexpressed c-REL- and c-RELΔ-binding mutant interacts with ΔNp63α and modulates TAp73. A, after transfection with increasing amount of the expression plasmid for Flag-c-REL, interaction between exogenous c-REL and endogenous ΔNp63α was increased (top), and interaction between endogenous ΔNp63α and TAp73 was decreased, with redistribution of TAp73 from the nucleus to the cytoplasm (bottom). B, detection of endogenous c-REL 48 hours after transfection with empty pCDNA3 vector, recombinant Flag-c-REL wild-type, and Flag-c-REL with AxxAxA mutations of the RoxBdx N-terminal αB DNA-binding residues, in cytoplasmic and nuclear fractions (CF, NF). β-Actin and Oct-1 were used as loading controls. C, nuclear TAp73 expression detected 48 hours after pCDNA3 and c-REL plasmid transfection. TNF-α treatment (20 ng/mL, 60 minutes). D, overexpression of recombinant c-REL wild-type and c-RELΔ-binding mutant enhanced interaction between c-REL and ΔNp63α, and decreased interaction between ΔNp63α and TAp73. TNF-α treatment enhanced the effect of wild-type and AxxAxA mutant c-REL in decreasing ΔNp63α-TAp73 interactions. IP, immunoprecipitation.
Aberrant expression and localization of c-REL, Δp63α, and TAp73 in HNSCC and Δp63α transgenic mice exhibiting squamous hyperplasia

To further confirm the biological significance in human HNSCC, we compared the expression and cellular distribution of these proteins in human HNSCC and squamous epithelia. Human HNSCC tumors displayed increased (2+ to 3+) nuclear c-REL, Δp63α, and TAp73 staining in comparison with a basilar pattern of nuclear staining for these proteins in nonmalignant squamous mucosae (5/5; 100%; Fig. 7A; Supplementary Fig. S5A and B). Interestingly, greater nuclear c-REL and cytoplasmic TAp73 were detected in HNSCC tumors than in unstimulated UMSCC cultured in vitro (Supplementary Fig. S1A), consistent with overexpression of inflammatory factors such as TNFα, previously shown in the HNSCC tumor environment (7).

We also examined a recently developed transgenic mouse model in which overexpression of Δp63α targeted to skin under a tet-K5 promoter results in inflammation, overexpression of TNF-α, other proinflammatory cytokines, and squamous hyperplasia (28, 30, J. Du and Z. Chen, unpublished data). By 1 month, these mice developed cutaneous hyperplasia, with...
increased suprabasilar nuclear c-Rel and ΔNp63α, and both cytoplasmic and nuclear staining for TAp73 in squamous epithelia (Fig. 7B). Thus, overexpression of ΔNp63α results in suprabasilar enhancement of nuclear c-REL and ΔNp63α, and cytoplasmic/nuclear distribution of TAp73, similar to that of orthologous proteins throughout malignant human squamous epithelia.

Discussion

Here we unveil evidence for a new paradigm (Fig. 7C), whereby an inflammatory factor, TNF-α, can promote nuclear interaction of c-REL with ΔNp63α, and TAp73 translocation to the cytoplasm, inhibiting the compensatory ability of TAp73 to activate key genes that mediate growth arrest and apoptosis in HNSCC with mutant TP53. Our findings establish a novel link between TNF-α expression and resistance (6–8, 10), nuclear activation of proto-oncogene c-REL (13–15), and dysregulation of p63/p73 family transcription factors (4, 5, 23, 28, 29), which have been independently implicated in cancer progression. TNF-α–induced c-REL/ΔNp63α interactions and TAp73 dissociation were shown at unique p63 DNA regulatory sites within the promoters of important growth arrest and proapoptotic genes. The role of TNF-α induced c-REL in these specific interactions with ΔNp63α/TAp73 did not seem to involve RELA, nor c-REL transactivation domain residues involved in binding to κB enhancers shared with other NF-κB/REL family members. These findings suggest that TNF-α–induced c-REL has a distinct function in inactivating TAp73 in p63/p73 promoter-regulated proapoptotic gene expression, complementing that previously shown for RELA in promoting κB-regulated prosurvival genes important in the malignant...
phenotype (10, 31). Furthermore, our findings suggest that targeting TNF-α signaling, c-REL, or other regulators of these c-REL–ΔNp63α–TAp73 interactions could enhance TAp73 function, potentially helping in prevention or treatment of cancers with altered TP53 status.

A novel finding of this study is the demonstration of the role of TNF-α in coordinating interactions between nuclear c-REL/ΔNp63α and reciprocal dissociation of ΔNp63α/TAp73 complexes, linking these previously reported components and interactions into a common dynamic mechanism. Previously, we discovered nuclear interactions between murine c-Rel and overexpressed ΔNp63α in keratinocytes and human c-REL and ΔNp63α in HNSCC (15). In murine keratinocytes, c-Rel and ΔNp63α were shown to prevent growth arrest, suggesting that corresponding c-REL/ΔNp63α complexes in HNSCC may also contribute to loss of growth control and promote the malignant phenotype. Independently, overexpressed ΔNp63α was shown to interact with TAp73 and promote survival in HNSCC (29). However, the relationship between these components, factor(s) modulating them, and basis for TAp73 inactivation were unknown. Here, we found that either c-REL or TAp73 interact with ΔNp63α, but we detected minimal interaction between c-REL and TAp73, suggesting that c-REL or TAp73 alternately complex with ΔNp63α. Supporting this model, we further established that TNF-α dynamically promotes nuclear c-REL/ΔNp63α interaction and reciprocal dissociation of ΔNp63α/TAp73. This is accompanied by nuclear–cytoplasmic translocation of TAp73, thus providing a basis for the inactivation of TAp73. TNF-α–induced c-REL was an important component of this mechanism, as c-REL siRNA attenuated dissociation of ΔNp63α/TAp73, although c-REL overexpression had a similar promoting effect on the dissociation and cytoplasmic translocation of TAp73. These effects of TNF-α or c-REL in promoting increased nuclear c-REL/ΔNp63α and cytoplasmic TAp73 in a subset of HNSCC lines may help explain the similar distribution we observed in HNSCC tumors, in which increased TNF-α expression as well as amplification and nuclear localization of c-REL have been detected (7, 13, 14).

These dynamic nuclear c-REL–ΔNp63α–TAp73 interactions, modulated by TNF-α or c-REL, were mirrored on established and novel predicted p63 regulatory sites in the promoters of several growth arrest and apoptotic genes. EMSA results indicated TNF-α induces reciprocal modulation between c-REL or TAp73 in binding to an established p63 site sequence of the p21WAF1 promoter (15, 23). ΔNp63α binding remained relatively unchanged by TNF-α, but ΔNp63α knockout inhibited ChIP binding of both c-REL and TAp73 to the p21WAF1 promoter, indicating that ΔNp63α bound to this p63 site is a key anchor for c-REL and TAp73 transcription factors. Although NOXA and PUMA were also previously identified as p63/p73 target genes (29), specific binding sites for p63/p73 were not previously resolved. Using a bioinformatics approach, we predicted p63-binding sites in these genes. We observed similar specificity in binding and modulation by TNF-α of c-REL and TAp73 bound with ΔNp63α on p21WAF1, NOXA, and PUMA promoters by ChIP assay. We have recently shown the capability of TNF-α to modulate c-REL/ΔNp63α interaction on p63 sites of additional genes by ChIP and EMSA.
and obtained further evidence for the specificity of c-REL in interacting with ΔNp63α at p63 regulatory sites. Preliminary ChIP sequencing results indicate that c-REL, p63, and/or p73 factors bind at many additional loci (H. Lu, unpublished data). Together, these findings suggest that the dynamic modulation of nuclear interactions involving these transcription factors observed in co-IP analysis are likely related to their specific interactions on p63/p73 sites of multiple gene promoters.

Our results further revealed the important and reversible function of nuclear c-REL in attenuating the compensatory ability of TAp73 to promote expression of these key growth arrest and apoptotic genes. Knockdown of c-REL potentiated the expression of p21WAF1, NOXA, and PUMA, further supporting the biological and potential therapeutic relevance of c-REL–ΔNp63α–TAp73 interactions observed on their promoters. Moreover, the modulation of these growth arrest and apoptotic genes was specifically observed in UMSCC-22A from a subset overexpressing TAp73 and ΔNp63α with mtTP53. This effect was not seen in UMSCC-1 from a subset with attenuation of expression and function of both TAp73 and wtTP53, which we have shown can result from other therapeutically reversible mechanisms (17, 32). Consistent with these findings, c-REL modulation affected proliferation and apoptosis in UMSCC-22A but not UMSCC-1. Conversely, overexpressed c-REL inhibited the expression of p21WAF1 and antiproliferative effects when TAp73 was reexpressed in TAp73-deficient UMSCC-1, supporting an important role for c-REL in inhibiting the compensatory ability of TAp73. Together, these results highlight the functional importance and potentially reversible nature of c-REL–mediated inhibition of TAp73 function in HNSCC overexpressing TAp73, ΔNp63α, and mtTP53.

Our findings in UMSCC lines are likely to be of broader relevance in HNSCC and other cancers. Increased nuclear c-REL/ΔNp63α and nuclear and cytoplasmic TAp73 was observed in a majority of HNSCC tumor specimens. Similarly, increased ΔNp63α and TAp73 was previously seen in an independent panel of HNSCC tumors and lines and linked with inactivation of TAp73 (5, 29). Breast cancer specimens...
also show increased ΔNp63 and TAp73 immunostaining, and this is most often seen in specimens with mtTP53 status (33). Subsets exhibiting increased expression of ΔNp63 in HNSCC and breast cancer have also been reported to be more sensitive to the chemotherapeutic drug cisplatin (33, 34), underscoring the potential clinical relevance of identifying and selecting agents active in these tumor subsets.

Inflammatory mediator TNF-α is identified as a key modulator of dynamic interactions of c-REL with ΔNp63α and inactivation of TAp73. This mechanism could therefore contribute to the acquired resistance and promoting effects of TNF-α produced by inflammatory cells during tumorigenesis and metastatic tumor progression of HNSCC and other cancers (6–8, 10). Supporting this hypothesis, we show here that combining TNF-α with overexpression of c-REL not only negated the inhibitory effect of TNF-α but also enhanced proliferation over that observed with either alone. Furthermore, human HNSCC tumors and epithelia of K5-ΔNp63α transgenic mice that exhibit increased TNF-α expression, inflammation, and epithelial proliferation (7, 28, J. Du and Z. Chen, unpublished observations) showed greater nuclear c-REL/ΔNp63α and distribution of TAp73 between the nucleus and the cytoplasm. Previous findings support targeting TNF-α or the canonical signal pathway which activates c-REL, for prevention or therapy of SCC. Knockout of TNF-α or TNF receptor-1, or TNF-α inhibitors, have been shown to reduce chemical carcinogenesis and malignant progression of SCC of the skin and other epithelial cancers (6). We previously showed that blocking canonical pathway signaling increased TNF-α cytotoxicity in HNSCC in vitro and induced apoptosis and regulation of established murine and human SCC in vivo (8). However, early-phase clinical trials with TNF-α or proteasome inhibitors, which inhibit canonical pathway activation, have shown limited potential to slow disease progression in patients with advanced cancers (6, 13).

Consequently, other molecular requirements for coordinated modulation of c-REL, ΔNp63αt, and TAp73 interactions on the promoters of target genes merit investigation as targets for therapy. Unique structural characteristics of c-REL have previously been implicated in TNF-α–induced, TBK and IKKe/p75 or IL-κB activation (35, 36). One of our laboratories found that the ΔNp63α domain, critical for oligomerization (5), is necessary for interaction with c-Rel (15). Cisplatin-induced IKKe activation and β activation have been reported to promote degradation of ΔNp63α and stabilization of p73, suggesting DNA damaging agents, and these kinases may be important modulators of these important components of the mechanism described herein (37, 38).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Consequently, other molecular requirements for coordinated modulation of c-REL, ΔNp63αt, and TAp73 interactions on the promoters of target genes merit investigation as targets for therapy. Unique structural characteristics of c-REL have previously been implicated in TNF-α–induced, TBK and IKKe/p75 or IL-κB activation (35, 36). One of our laboratories found that the ΔNp63α domain, critical for oligomerization (5), is necessary for interaction with c-Rel (15). Cisplatin-induced IKKe activation and β activation have been reported to promote degradation of ΔNp63α and stabilization of p73, suggesting DNA damaging agents, and these kinases may be important modulators of these important components of the mechanism described herein (37, 38).

Disclosure of Potential Conflicts of Interest

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