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## TNF- $\alpha$ promotes c-REL/ $\Delta$ Np63 $\alpha$ 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- $\kappa$ B/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 co-expressed and complex with  $\Delta$ Np63 $\alpha$  in the nucleus of a subset of head and neck squamous cell carcinoma (HNSCC) cell lines with mutant (mt)TP53. TNF- $\alpha$  a pro-inflammatory cytokine, promoted nuclear translocation and c-REL/ $\Delta$ Np63 $\alpha$  interaction and dissociation of TAp73 from nuclear  $\Delta$ Np63 $\alpha$  to the cytoplasm, while c-REL siRNA depletion attenuated this effect. Overexpression of c-REL or a c-REL  $\kappa$ B-site DNA binding mutant enhanced protein interaction with  $\Delta$ Np63 $\alpha$  and TAp73 dissociation, implicating c-REL/ $\Delta$ Np63 $\alpha$ -specific interactions in these effects. We discovered TNF- $\alpha$ - or genetic alteration of c-REL expression inversely modulates  $\Delta$ Np63 $\alpha$ /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 anti-proliferative effects of TNF- $\alpha$  or TAp73. Conversely, c-REL siRNA depletion enhanced TAp73 promoter interaction, and expression of genes mediating growth arrest and apoptosis. Similar to TNF- $\alpha$  treated HNSCC lines, human HNSCC tumors and hyperplastic squamous epithelia of transgenic mice overexpressing  $\Delta$ Np63 $\alpha$  that exhibit inflammation, also demonstrate increased nuclear c-REL/ $\Delta$ Np63 $\alpha$  and cytoplasmic TAp73 localization. These findings unveil a novel and reversible dynamic mechanism whereby pro-inflammatory cytokine TNF- $\alpha$ -induced c-REL/ $\Delta$ Np63 $\alpha$  interactions inactivate tumor suppressor TAp73 function, promoting TNF- $\alpha$  resistance and cell survival in cancers with mtTP53.

### Keywords

c-REL;  $\Delta$ Np63; TAp73; TNF- $\alpha$ ; squamous cell carcinoma

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## 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) (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 function with TP53. While TAp63 and TAp73 are rarely mutated, expression of alternatively transcribed  $\Delta N$  isoforms can differentially affect their transactivation and tetramerization, respectively. Recent evidence suggests that the  $\Delta Np63\alpha$  isoform is predominantly overexpressed together with TAp73 in major subsets of HNSCC, breast, and other cancers (5). Nuclear interactions between  $\Delta Np63\alpha$  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  $\Delta Np63\alpha$  and TAp73, and how their interaction leads to inactivation of TAp73 in cancers with mtTP53, remains unclear.

Among candidate regulatory factors, Tumor Necrosis Factor (TNF)- $\alpha$  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- $\alpha$ -mediated growth arrest and apoptosis, and that such resistance involves aberrant activation of NF- $\kappa$ B/REL transcription factors (8). Among these, TNF- $\alpha$  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 prosurvival genes by siRNA *in vitro* or by proteasome inhibitor in a phase I clinical trial *in vivo* demonstrated limited cytotoxic and clinical activity (11–13). Amplification and nuclear localization of c-REL has also been previously detected, and to be relatively unaffected by proteasome inhibition in HNSCC (13, 14). These observations raised the question whether TNF- $\alpha$  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  $\Delta Np63\alpha$  co-localizes and forms novel nuclear complexes with c-Rel in murine keratinocytes or orthologue c-REL in human HNSCC (15). Herein, we explored the hypothesis that TNF- $\alpha$  and c-REL activation is linked to modulation of the aforementioned  $\Delta Np63\alpha$ /TAp73 interactions and dysregulation of growth arrest and apoptosis in cancer. We reveal a dynamic and reversible mechanism whereby TNF- $\alpha$  promotes c-REL nuclear translocation and interaction with  $\Delta Np63\alpha$ , dissociation of TAp73 from distinct p63 promoter sites of key growth arrest and apoptotic genes, and TAp73 translocation to the cytoplasm. These results can help explain how inflammatory factor TNF- $\alpha$  and c-REL, through  $\Delta Np63\alpha$ , 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 performed at the University of Michigan by DNA genotyping of alleles for 9 loci, D3S1358, D5S818, D7S820, D8S1179, D13S317,

D18S51, D21S11, FGA, vWA and amelogenin locus in 2008 and 2010, as recently described (18). Cell line stocks were preserved at  $-80$  degrees C and cultured for fewer than 6 months before use.

### Western Blot

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

### Real time RT-PCR

RNA isolation and cDNA synthesis were performed as previously (19). Real time PCR primers and probes for  $\Delta$ Np63 and TAp63 validated previously (20) were synthesized by Applied Biosystems. Primers to amplify TAp73 and  $\Delta$ Np73 are listed in Supplemental Methods.

### Plasmid, siRNA Transfection and Reporter gene assays

These experiments were performed as described previously (19).  $\Delta$ Np63 specific siRNA (21) was synthesized by Integrated DNA Technologies (IDT). TAp63 specific siRNA was purchased from Dharmacon. The 2.6kb p21-luc reporter was provided by Dr. Bert Vogelstein's laboratory (22). Each sample was assayed in triplicate and data were presented as mean  $\pm$  SD.

### Coimmunoprecipitation analysis (IP)

Co-IP was performed as described previously (15). Antibodies used are listed in Supplemental Methods

### Electrophoretic mobility shift assay

Oligonucleotides covering the p21WAF1 promoter p63-binding site ( $-2283$ bp, 5'-TGGCCGTCAGGAACATGTCCCAACATGTTGAGCTCTGGCA-3', Fig. S3A) were [ $\gamma$ - $^{32}$ P]-end-labeled with 10 U of T4 polynucleotide kinase (New England Biolabs), and EMSA was performed as previously described (23).

### Chromatin Immunoprecipitation Analyses (ChIP)

ChIP assays were performed using the Magna ChIP<sup>TM</sup> G kit (Upstate) as previously (24). For QRT-PCR primers see Supplemental Methods.

### DNA based cell cycle and apoptotic flow analysis

Flow analysis was performed as previously (12). Samples were run on FACS Canto machine within 1 hour and analyzed using DIVA flow cytometric analysis software.

Additional experimental detail, methods and figures are included in Supplemental Information

## RESULTS

### Overexpression and protein interactions among c-REL, $\Delta$ Np63 $\alpha$ 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) (Fig. S1). The UMSCC lines selected were derived from primary and metastatic tumors exhibiting an aggressive clinical course (survival  $<15$  mos.), and representative genetic

abnormalities, including inactivation or mutation of TP53 (16–18). c-REL,  $\Delta$ Np63 $\alpha$ , TAp73, and TP53 protein were detected by Western blots, and quantitative RT-PCR (Fig. S1A–F). Total c-REL protein was increased, as were nuclear c-REL and  $\Delta$ Np63 $\alpha$  protein in most UMSSC compared with normal HEK (H) cells (Fig. S1A, *Left, Right panels*). Interestingly, even greater c-REL expression was detected in 3 of 4 cell lines (UMSSC 22B, 38, 46) with elevated  $\Delta$ Np63 $\alpha$ , TAp73 and mtTP53 (UMSSC 22A, 22B, 38, 46) (Fig. S1A, *Left panel; S1B–E*). Thus, we find that c-REL,  $\Delta$ Np63 $\alpha$ , TAp73 are overexpressed with mtTP53 in an overlapping subset of HNSCC lines.

Both c-REL and TAp73 exhibited basal interaction with  $\Delta$ Np63 $\alpha$  in whole cell extracts from two independent cell lines by co-IP analysis (Fig. 1A; UMSSC-22A or -46). However, c-REL and TAp73 exhibited weak or negligible interaction with one another, seen only with supersensitive substrate and prolonged exposure. Further, c-REL, but not RELA(p65), interacted with  $\Delta$ Np63 $\alpha$  (although RELA formed an independent complex with c-REL) (Fig. 1B), providing evidence for distinct interactions by these RELs. The complexes between c-REL,  $\Delta$ Np63 $\alpha$ , and TAp73, identified in whole cell extracts above, were found in nuclear, but not cytoplasmic extracts (Fig. 1C). No interaction with mtTP53 was observed (Fig. 1B, C). In the absence of further stimulation, a significant portion of nuclear  $\Delta$ Np63 $\alpha$  co-IPed with either c-REL or TAp73, when compared with input (Fig. 1D).

### TNF- $\alpha$ or genetic modulation of c-REL affects nuclear localization and interactions between c-REL, $\Delta$ Np63 and TAp73

The inflammatory cytokine TNF- $\alpha$  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- $\alpha$  on the cellular distribution of c-REL,  $\Delta$ Np63 $\alpha$  and TAp73 in the UMSSC 22A cell line, which has lower endogenous c-REL expression, making it amenable to TNF- $\alpha$  or genetic modulation (Fig. S1). TNF- $\alpha$  stimulation increased nuclear localization of c-REL within 30' (Fig. 2A, *left panel*). Remarkably, TNF- $\alpha$  not only induced c-REL, but also decreased TAp73, without significantly altering  $\Delta$ Np63 $\alpha$  expression in nuclear extracts (Fig. 2A, *right panel*). Moreover, TNF- $\alpha$  stimulation increased the interaction between nuclear c-REL and  $\Delta$ Np63 $\alpha$ , and a corresponding dissociation of nuclear  $\Delta$ Np63 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 depletion by siRNA enhanced  $\Delta$ Np63 $\alpha$ /TAp73 interaction without or with TNF- $\alpha$  (Fig. 2D).

Similar to TNF- $\alpha$ -induced c-REL, overexpressing c-REL-Flag in UMSSC-22A resulted in increased interaction between c-REL and  $\Delta$ Np63 $\alpha$  in the nuclear fraction (Figure 3A, *upper panels*). Concurrently, TAp73 dissociated from  $\Delta$ Np63 $\alpha$  in the nuclear fraction, and appeared in the cytoplasmic fraction (Fig. 3A, *lower panels*). Further supporting that c-REL and TAp73 form alternative complexes with  $\Delta$ Np63 $\alpha$ , no significant interaction between c-REL and TAp73 was detected in co-IP. Overall, the above findings indicate that TNF- $\alpha$ -induced or overexpressed c-REL is involved in the dissociation of TAp73 from  $\Delta$ Np63 $\alpha$  in the nucleus to the cytoplasm.

Previous studies have indicated that c-REL can bind  $\kappa$ B motifs bound by other NF- $\kappa$ B/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 RxxRxxR to AxxAxA mutant reported to abrogate c-REL binding to classical REL/ $\kappa$ B DNA motifs (27). Overexpression and TNF- $\alpha$  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  $\Delta$ Np63-TAp73 interaction, and this reduction was preferentially enhanced with TNF- $\alpha$ -stimulated AxxAxA mutant c-REL (Fig. 3C, D). These results

suggested that TNF- $\alpha$ -induced or overexpressed c-REL interacts with  $\Delta$ Np63 and modulates bound TAp73 in a manner distinct from those previously shown to mediate c-REL binding to  $\kappa$ B DNA recognition sequences.

### **TNF- $\alpha$ or genetically modulated c-REL reciprocally affects binding of TAp73 with $\Delta$ Np63 $\alpha$ on p63 sites of *p21WAF1*, *NOXA* and *PUMA* gene promoters**

To explore whether TNF- $\alpha$  modulates interactions between c-REL,  $\Delta$ Np63 $\alpha$  and TAp73 on p63/p73 regulatory sites, we examined their binding to an oligonucleotide encoding a well characterized p63/p73 binding site (-2283 to -2273; 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 electrophoretic mobility shift assay (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,  $\Delta$ Np63 $\alpha$  or TAp73, demonstrated by supershift with specific antibodies. Similar to our findings in nuclear extracts, TNF- $\alpha$  stimulated c-REL binding, while decreasing binding of TAp73, without significantly affecting  $\Delta$ Np63 $\alpha$ .

Next, we examined the endogenous binding of c-REL,  $\Delta$ Np63 and TAp73 to *p21WAF1* promoter DNA using chromatin immunoprecipitation (ChIP) assays. All three factors exhibited basal binding to the p63 binding site region of the *p21WAF1* promoter in UM-SCC 22A, -46 or -1 cells (Fig. 4A, right panel; S2B, C, D). TNF- $\alpha$  induced c-REL binding and decreased TAp73 binding, without significantly affecting  $\Delta$ Np63 $\alpha$  binding to the p63 site in two different cell lines (Fig. 4B, S2C). Similarly, c-REL overexpression also induced the same effects in either UMSCC 22A or -1 lines (Fig. 4B, S2D). Conversely, c-REL depletion by siRNA significantly decreased c-REL and  $\Delta$ Np63 binding, and reciprocally enhanced TAp73 binding activity in UM-SCC 22A cells (Fig. S3E). Importantly,  $\Delta$ Np63 $\alpha$  siRNA knockdown attenuated basal and TNF- $\alpha$  inducible binding of c-REL,  $\Delta$ Np63 $\alpha$  and TAp73, implicating  $\Delta$ Np63 $\alpha$  as a key anchor for c-REL and TAp73 binding activity (Fig. 4C). Together, these findings in 3 different UMSCC lines indicate that TNF- $\alpha$  or genetic modulation of c-REL results in reciprocal modulation of TAp73 binding with  $\Delta$ Np63 $\alpha$  on the established p63 regulatory site of the *p21WAF1* promoter.

To examine the broader role of TNF- $\alpha$ -induced c-REL,  $\Delta$ Np63 and TAp73 interactions, we performed ChIP assays for p63 binding sites identified by bioinformatic analysis (28; suppl methods) on the promoters of two key pro-apoptotic genes, *NOXA* and *PUMA* (Fig. S2A, arrows). As with *p21WAF1*, stimulation with TNF- $\alpha$  increased c-REL and decreased TAp73 binding without affecting  $\Delta$ Np63 bound to the predicted p63 sites in the *PUMA* and *NOXA* promoters (Fig. 4D). No significant binding by RELA(p65) 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- $\alpha$  or genetically modulated c-REL reciprocally affects binding of  $\Delta$ Np63 $\alpha$  with TAp73 on p63 sites of multiple gene promoters.

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

We next examined how c-REL modulated  $\Delta$ Np63 $\alpha$ -TAp73 interactions affect expression of growth arrest mediator *p21WAF1*. The UM-SCC cell subsets express lower or higher baseline levels *p21WAF1* mRNA and protein (Fig. S3A,B), similar to relative differences in expression of wt or mt TP53,  $\Delta$ Np63 $\alpha$ , and TAp73 shown above (Fig. S1A,B,D). Depletion of endogenous c-REL by siRNA significantly reduced levels of c-REL mRNA in two cell lines, UMSCC 1 and UMSCC 22A (Fig. 5A, left panel), from these subsets that differ in baseline expression of *p21WAF1* and TP53 family proteins. However, c-REL depletion in

UMSCC 22A cells with abundant mtTP53,  $\Delta$ Np63 and TAp73 resulted in a strong increase in p21WAF1 mRNA and protein levels, when compared to UMSCC 1 cells lacking wtTP53 and TAp73 (Fig. 5A, right panel; 5B, left panel). Conversely, overexpression of c-REL by transient transfection repressed p21WAF1 expression in UMSCC 22A cells, but not in UMSCC 1 cells (Fig. 5B, right panel). Similarly, TNF- $\alpha$  induced nuclear c-REL, while inhibiting expression of TAp73 target protein p21WAF1 (Fig. S3C). Increased p21WAF1 expression after c-REL depletion in UMSCC 22A was attenuated by co-transfection with TAp73 siRNA (Fig. S3D), indicating the contribution of TAp73. Transfection of TAp73-deficient UMSCC 1 cells with TAp73 increased, while co-transfection with c-REL suppressed an increase in p21WAF1 expression (Fig. S3E). Depletion of c-REL by siRNA also enhanced PUMA and NOXA expression in the mtTP53 cell line UMSCC 22A, but not in UMSCC 1 cells deficient in TP53 and TAp73 (Fig. 5C). Together, these results indicate that c-REL represses three important growth arrest and pro-apoptotic genes in HNSCC expressing  $\Delta$ Np63 and TAp73 with mtTP53.

### **c-REL promotes cellular proliferation and survival in UMSCC cells expressing $\Delta$ Np63, 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 UMSCC 1 cells that express lower levels of wtTP53 and other family members, but inhibited proliferation and density of UMSCC 22A cells that exhibit high protein levels of  $\Delta$ Np63 $\alpha$ , TAp73 and mtTP53 and inducible p21WAF1 (Fig. 6A,B; Fig. S4A). c-REL siRNA depletion also increased sub-G0 DNA-positive dead cells in UMSCC 22A, but not in UMSCC 1 cells (Fig. S4B), whereas c-REL overexpression reduced the sub-G0 fraction in UMSCC 22A cells (Fig. S4C). The results showing that c-REL promotes cell proliferation and survival are consistent with the effect of c-REL in repressing p21WAF1, NOXA and PUMA gene expression (Fig. 5), which mediate growth arrest and apoptosis in HNSCC (29).

To further examine the effects of c-REL on the suppressor function of TAp73, UMSCC 1 lacking TAp73 was transfected with control vector, c-REL, TAp73, or a combination of c-REL and TAp73. c-REL enhanced, and TAp73 inhibited proliferation, while co-transfection of c-REL with TAp73 abrogated the inhibitory function of TAp73 (Fig. 6C). As HNSCC are relatively resistant to TNF- $\alpha$ -mediated inhibition of growth and survival (8), we examined the functional effect of TNF- $\alpha$  treatment or c-REL transfection alone, or in combination, on UMSCC 22A cells (Fig. 6D). TNF- $\alpha$  alone weakly inhibited and c-REL enhanced proliferation, but the combination of TNF- $\alpha$  and c-REL overexpression further enhanced proliferation. Together, these data support the role of endogenous or exogenously overexpressed c-REL in repressing the anti-proliferative function of TNF- $\alpha$  and TAp73.

### **Aberrant expression and localization of c-REL, $\Delta$ Np63 $\alpha$ , and TAp73 in HNSCC and $\Delta$ Np63 $\alpha$ 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+~3+) nuclear co-staining for c-REL (24/24; 100%) and  $\Delta$ Np63 $\alpha$  (20/24; 83%), and decreased TAp73 staining distributed in the cytoplasm and nucleus throughout the malignant epithelia, when compared with a basilar pattern of nuclear staining for these proteins in nonmalignant squamous mucosae (5/5; 100%) (Figs. 7A, S5A,B). Interestingly, greater nuclear c-REL and cytoplasmic TAp73 were detected in HNSCC tumors than in unstimulated UMSCC cultured *in vitro* (Fig. S1A), consistent with overexpression of inflammatory factors such as TNF $\alpha$  previously demonstrated in the HNSCC tumor environment (7).

We also examined a recently developed transgenic mouse model in which overexpression of  $\Delta Np63\alpha$  targeted to skin under a tet-K5 promoter results in inflammation, overexpression of TNF- $\alpha$ , other proinflammatory cytokines, and squamous hyperplasia (28, 30). By one month, these mice developed cutaneous hyperplasia, with increased suprabasilar nuclear c-Rel and  $\Delta Np63\alpha$ , and both cytoplasmic and nuclear staining for TAp73 in squamous epithelia (Fig. 7B). Thus, overexpression of  $\Delta Np63\alpha$  results in suprabasilar enhancement of nuclear c-REL and  $\Delta Np63\alpha$ , 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- $\alpha$ , can promote nuclear interaction of c-REL with  $\Delta Np63\alpha$ , 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- $\alpha$  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- $\alpha$ -induced c-REL/ $\Delta Np63\alpha$  interactions and TAp73 dissociation were demonstrated at unique p63 DNA regulatory sites within the promoters of important growth arrest and pro-apoptotic genes. The role of TNF- $\alpha$  induced c-REL in these specific interactions with  $\Delta Np63\alpha$ /TAp73, did not appear to involve RELA, nor c-REL transactivation domain residues involved in binding to  $\kappa B$  enhancers shared with other NF- $\kappa B$ /REL family members. These findings suggest that TNF- $\alpha$ -induced c-REL has a distinct function in inactivating TAp73 in p63/p73 promoter-regulated proapoptotic gene expression, complementing that previously demonstrated for RELA in promoting  $\kappa B$ -regulated prosurvival genes important in the malignant phenotype (10, 31). Furthermore, our findings suggest that targeting TNF- $\alpha$  signaling, c-REL, or other regulators of these c-REL- $\Delta Np63\alpha$ -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- $\alpha$  in coordinating interactions between nuclear c-REL/ $\Delta Np63\alpha$  and reciprocal dissociation of  $\Delta Np63\alpha$ /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  $\Delta Np63\alpha$  in keratinocytes, and human c-REL and  $\Delta Np63\alpha$  in HNSCC (15). In murine keratinocytes, c-Rel and  $\Delta Np63\alpha$  were shown to prevent growth arrest, suggesting that corresponding c-REL/ $\Delta Np63\alpha$  complexes in HNSCC may also contribute to loss of growth control and promote the malignant phenotype. Independently, overexpressed  $\Delta Np63\alpha$  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  $\Delta Np63\alpha$ , but we detected minimal interaction between c-REL and TAp73, suggesting that c-REL or TAp73 alternately complex with  $\Delta Np63\alpha$ . Supporting this model, we further established that TNF- $\alpha$  dynamically promotes nuclear c-REL/ $\Delta Np63\alpha$  interaction and reciprocal dissociation of  $\Delta Np63\alpha$ /TAp73. This is accompanied by nuclear-cytoplasmic translocation of TAp73, thus providing a basis for the inactivation of TAp73. TNF- $\alpha$ -induced c-REL was an important component of this mechanism, as c-REL siRNA attenuated dissociation of  $\Delta Np63\alpha$ /TAp73, while c-REL overexpression had a similar promoting effect on the dissociation and cytoplasmic translocation of TAp73. These effects of TNF- $\alpha$  or c-REL in promoting increased nuclear c-REL/ $\Delta Np63\alpha$  and cytoplasmic TAp73 in a subset of HNSCC lines may help explain the similar distribution we observed in HNSCC tumors,

where increased TNF- $\alpha$  expression, as well as amplification and nuclear localization of c-REL have been detected (7, 13, 14).

These dynamic nuclear c-REL- $\Delta$ Np63 $\alpha$ -TAp73 interactions modulated by TNF $\alpha$  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- $\alpha$  induces reciprocal modulation between c-REL or TAp73 in binding to an established p63 site sequence of the *p21WAF1* promoter (15, 23).  $\Delta$ Np63 $\alpha$  binding remained relatively unchanged by TNF- $\alpha$ , but  $\Delta$ Np63 $\alpha$  knockdown inhibited ChIP binding of both c-REL and TAp73 to the *p21WAF1* promoter, indicating that  $\Delta$ Np63 $\alpha$  bound to this p63 site is a key anchor for c-REL and TAp73 transcription factors. While *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- $\alpha$  of c-REL and TAp73 co-bound with  $\Delta$ Np63 $\alpha$  on *p21WAF1*, *NOXA*, and *PUMA* promoters by ChIP assay. We have recently demonstrated the capability of TNF- $\alpha$  to modulate c-REL/ $\Delta$ Np63 $\alpha$  interaction on p63 sites of additional genes by ChIP and EMSA (28), and obtained further evidence for the specificity of c-REL in interacting with  $\Delta$ Np63 $\alpha$  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 biologic and potential therapeutic relevance of c-REL- $\Delta$ Np63 $\alpha$ -TAp73 interactions observed on their promoters. Moreover, the modulation of these growth arrest and apoptotic genes was specifically observed in UMSSC 22A from a subset overexpressing TAp73 and  $\Delta$ Np63 $\alpha$  with mtTP53. This effect was not seen in UMSSC 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 UMSSC22A but not UMSSC 1. Conversely, overexpressed c-REL inhibited the expression of p21WAF1 and anti-proliferative effects when TAp73 was re-expressed in TAp73-deficient UMSSC 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,  $\Delta$ Np63 $\alpha$  and mtTP53.

Our findings in UMSSC lines are likely to be of broader relevance in HNSCC and other cancers. Increased nuclear c-REL/ $\Delta$ Np63 $\alpha$  and nuclear and cytoplasmic TAp73 was observed in a majority of HNSCC tumor specimens. Similarly, increased  $\Delta$ Np63 $\alpha$  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  $\Delta$ Np63 and TAp73 immunostaining, and this most often seen in specimens with mtTP53 status (33). Subsets exhibiting increased expression of  $\Delta$ Np63 in HNSCC and breast cancer have also been reported to be more sensitive to the chemotherapy drug cisplatin (33, 34), underscoring the potential clinical relevance of identifying and selecting agents active in these tumor subsets.

Inflammatory mediator TNF- $\alpha$  is identified as a key modulator of dynamic interactions of c-REL with  $\Delta$ Np63 $\alpha$ , and inactivation of TAp73. This mechanism could therefore contribute



to the acquired resistance and promoting effects of TNF- $\alpha$  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- $\alpha$  with overexpression of c-REL not only negated the inhibitory effect of TNF- $\alpha$ , but enhanced proliferation over that observed with either alone. Further, human HNSCC tumors and epithelia of K5- $\Delta$ Np63 $\alpha$  transgenic mice that exhibit increased TNF- $\alpha$  expression, inflammation, and epithelial proliferation (7, 28), showed greater nuclear c-REL/ $\Delta$ Np63 $\alpha$ , and distribution of TAp73 between the nucleus and the cytoplasm. Previous findings support targeting TNF- $\alpha$ , or the canonical signal pathway which activates c-REL, for prevention or therapy of SCC. Knockout of TNF- $\alpha$  or TNF receptor-1, or TNF- $\alpha$  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- $\alpha$  cytotoxicity in HNSCC *in vitro*, and induced apoptosis and regression of established murine and human SCC *in vivo* (8). However, early phase clinical trials with TNF- $\alpha$  or proteasome inhibitors, which inhibit canonical pathway activation, have demonstrated limited potential to slow disease progression in patients with advanced cancers (6, 13).

Consequently, other molecular requirements for coordinated modulation of c-REL,  $\Delta$ Np63 $\alpha$  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- $\alpha$ -induced, TBK and IKKepsilon phosphorylation, dimerization, DNA binding, or transactivation (35, 36). One of our laboratories found that the  $\Delta$ Np63  $\alpha$ -domain, critical for oligomerization (5), is necessary for interaction with c-Rel (15). Cisplatin induced IKK $\alpha$  and  $\beta$  activation have been reported to promote degradation of  $\Delta$ Np63 $\alpha$  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).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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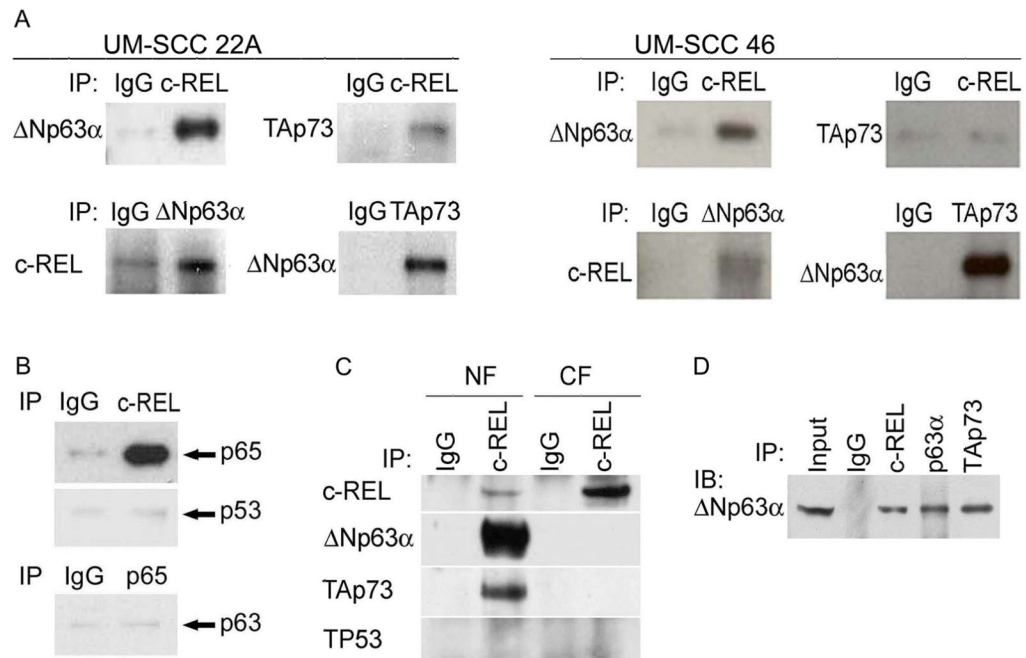
The authors thank Drs. James F. Battey (NIDCD/NIH), Victor Zhurkin (NCI/NIH), and Cheng-Ming Chiang (University of Texas, Southwestern Medical Center) for serving as readers and for helpful comments.

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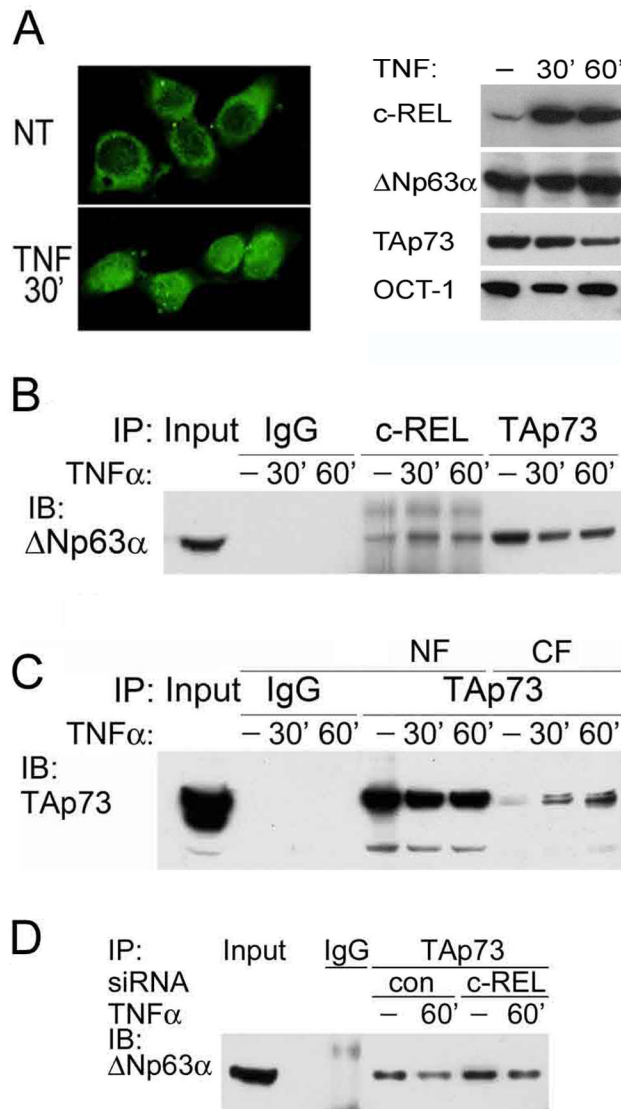
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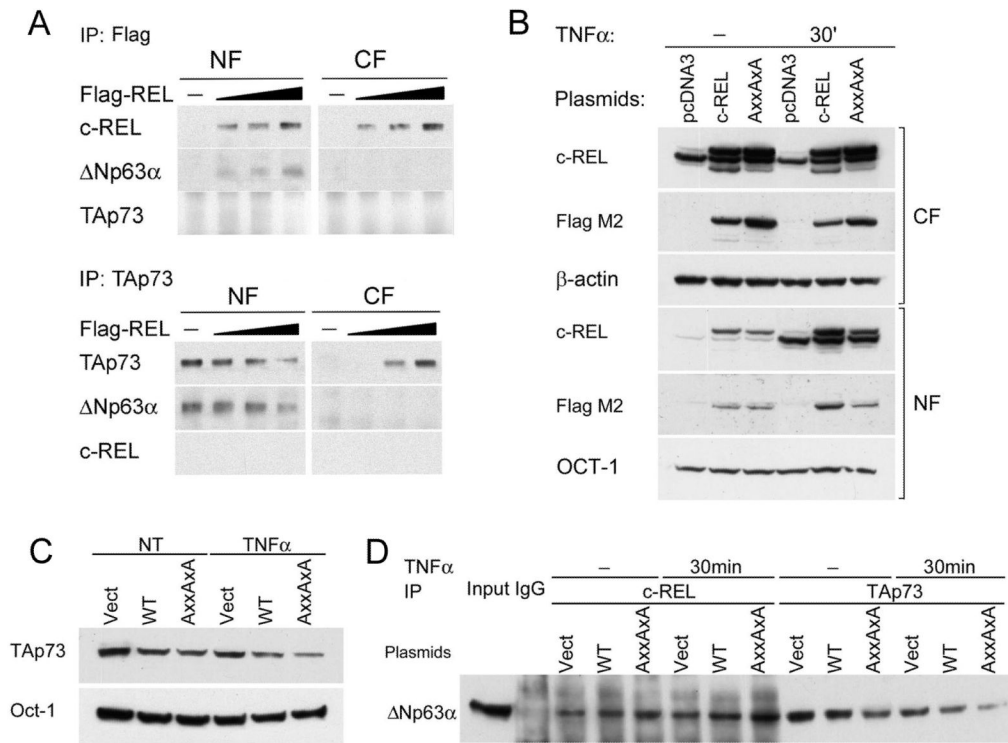
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**Figure 1.**

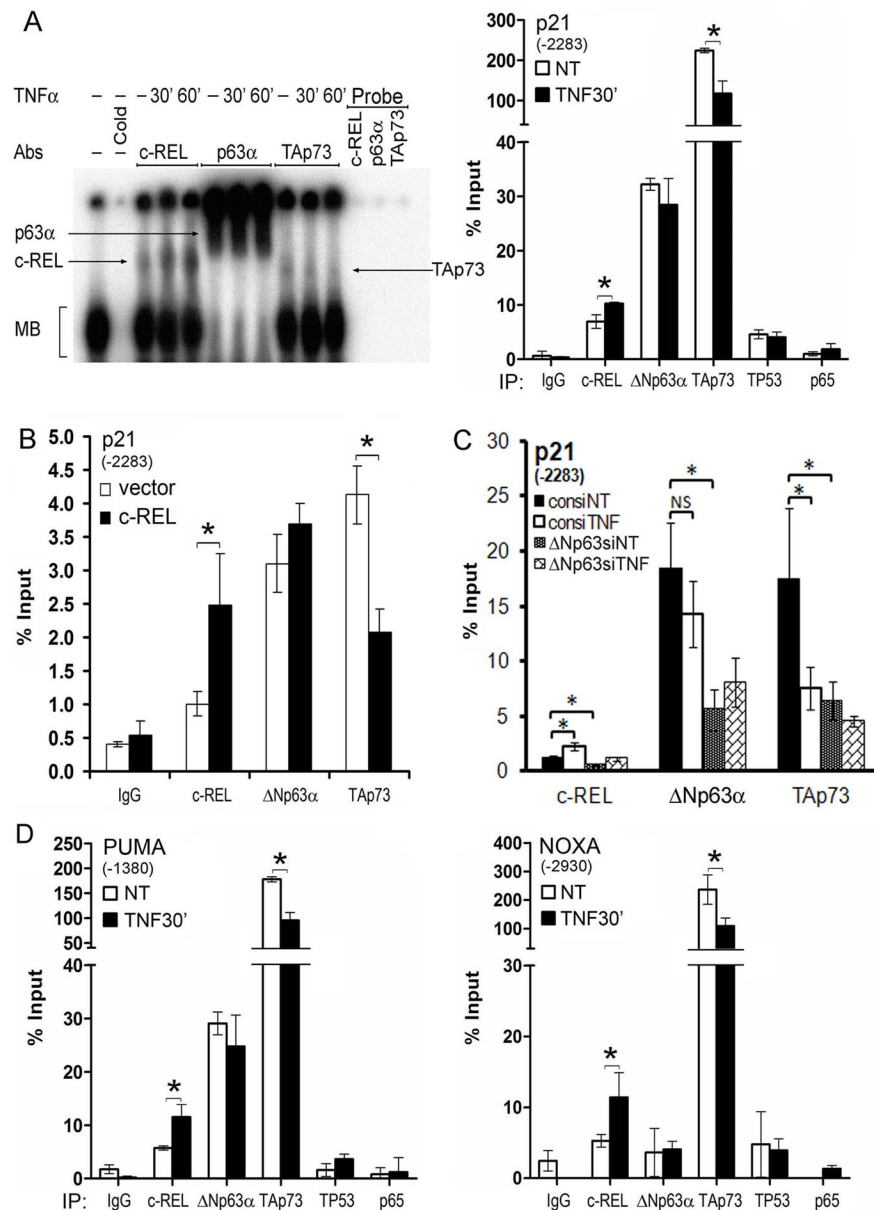
Interactions among c-REL,  $\Delta$ Np63 $\alpha$ , TAp73, and REL(p65) in HNSCC with mTP53. A, left, Whole cell lysates from UM-SCC 22A cells or, right, 46 cells, were immunoprecipitated, then immunoblotted with antibodies as indicated. B, Whole cell lysates of UM-SCC 22A were immunoprecipitated with 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,  $\Delta$ Np63 $\alpha$ , 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.



**Figure 2.** TNF- $\alpha$ -induced and overexpressed c-REL interacts with  $\Delta$ Np63 $\alpha$  and modulates TAp73. A, Left, Distribution of c-REL detected by IF staining in nuclear and cytoplasmic cellular counterparts, non-treated (NT), or with 20 ng/ml TNF- $\alpha$  for 30 min (200X). Right, nuclear c-REL,  $\Delta$ Np63 $\alpha$  and TAp73 proteins were detected before and after 20ng/ml TNF- $\alpha$  treatment for 30 and 60 minutes. OCT-1:loading control. B, Increased nuclear interaction between c-REL and  $\Delta$ Np63 $\alpha$ , and decreased nuclear interaction between TAp73 and  $\Delta$ Np63 $\alpha$  were observed 30 and 60 min after TNF- $\alpha$  treatment. C, Decreased nuclear and increased cytoplasmic TAp73 were observed 30 and 60 min after TNF- $\alpha$  treatment. D, Knockdown of c-REL increased the interaction between TAp73 and  $\Delta$ Np63 $\alpha$  without and with TNF- $\alpha$  stimulation.

**Figure 3.**

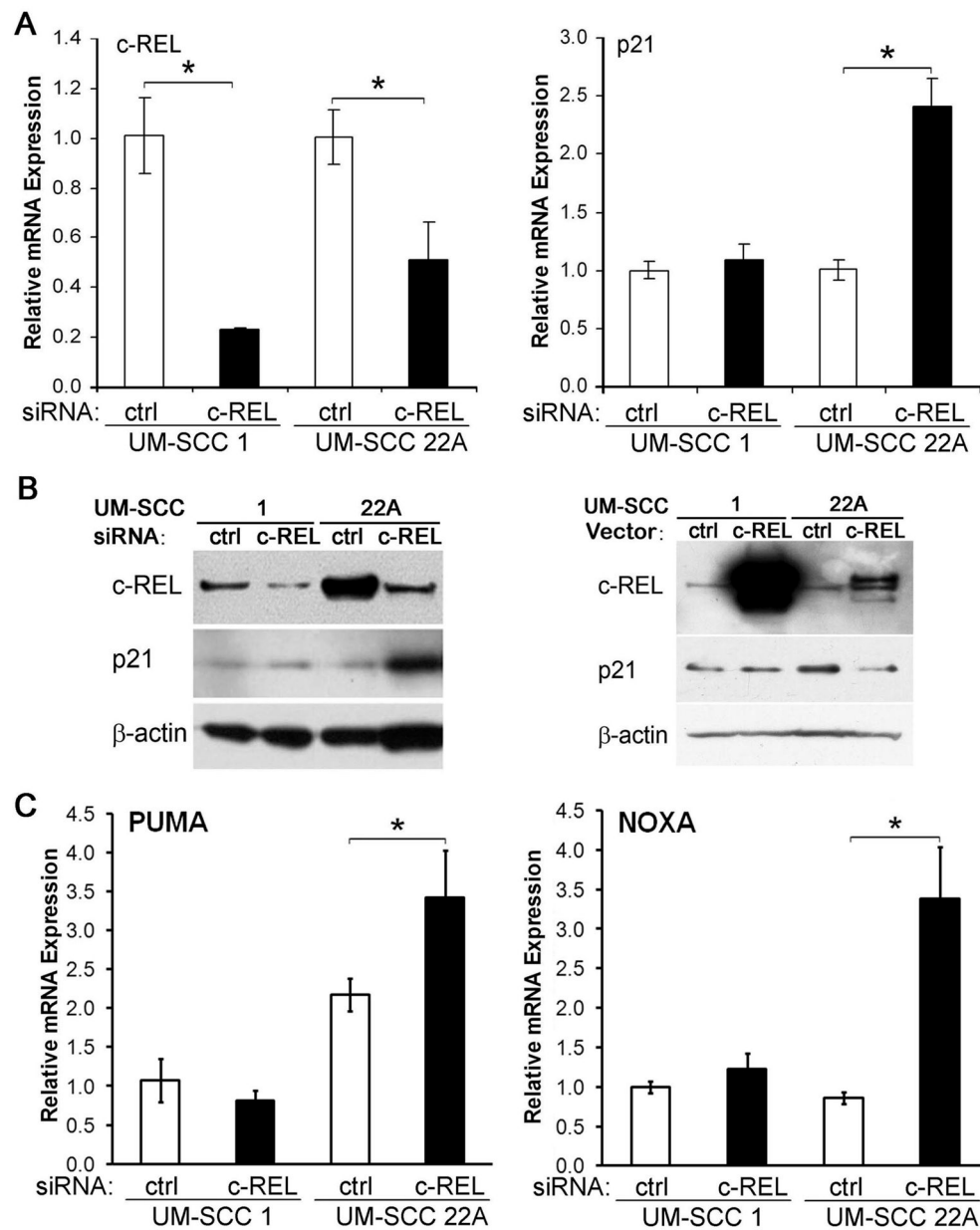
Overexpressed c-REL and c-REL κB binding mutant interacts with ΔNp63α and modulates TAp73. A, After transfection with increasing amount of the expression plasmid for Flag-c-REL, interaction between exogenous c-REL and endogenous ΔNp63α was increased (Upper), and interaction between endogenous ΔNp63α and TAp73 was decreased, with redistribution of TAp73 from the nucleus to the cytoplasm (Lower). B, Detection of endogenous c-REL 48h after transfection with empty pCDNA3 vector, recombinant Flag-c-REL wild-type, and Flag c-REL with AxxAxA mutations of the RxxRxxR N-terminal κB 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 48h after pCDNA3 and c-REL plasmid transfection +/- TNF-α treatment (20ng/ml, 60min). D, Overexpression of recombinant c-REL wild type and c-REL AxxAxA 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.



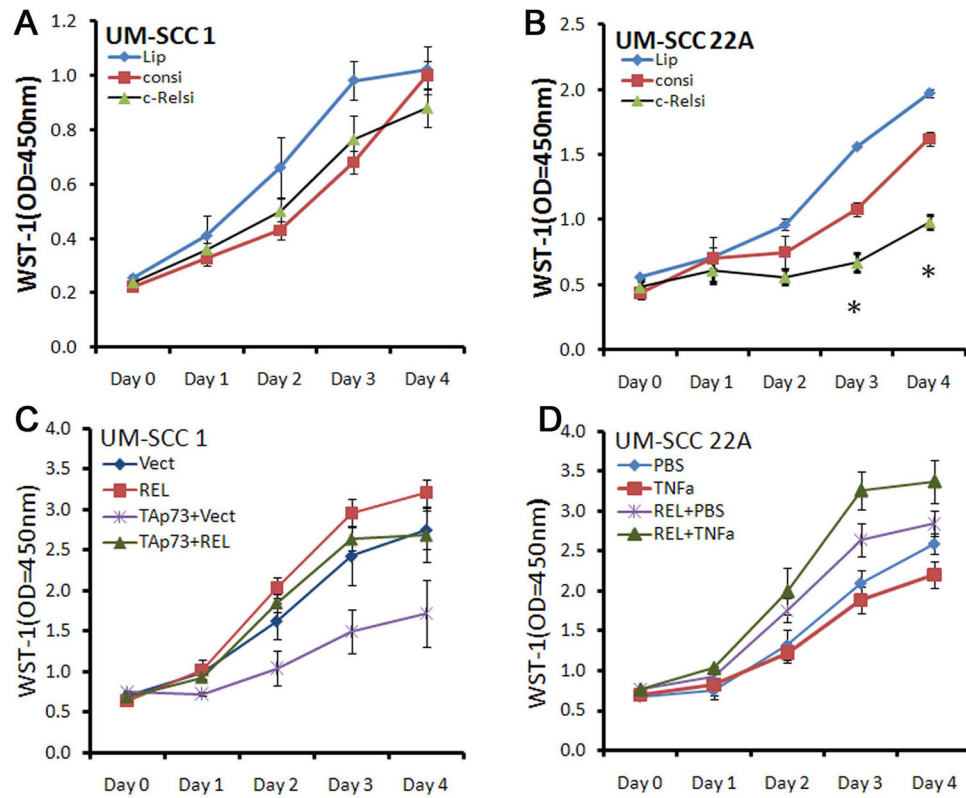
**Figure 4.** c-REL,  $\Delta$ Np63 $\alpha$  and TAp73 complex binding to the p21WAF1, NOXA and PUMA gene promoters A, Left, EMSA with nuclear extracts from UM-SCC 22A cells showing protein bound to oligonucleotide containing the established p63 binding site sequence (-2283bp) for the p21WAF1 promoter. MB: Main band. Cold: unlabeled oligonucleotide competition (200X); Arrows, protein supershifts using antibodies against  $\Delta$ Np63 $\alpha$ , TAp73 or c-REL. c-REL binding gradually increased while TAp73 binding decreased after TNF- $\alpha$  treatment (20ng/ml for 30' and 60'). Probe only (no extract), indicates the antibodies lack inherent probe shifting activity. A, Right, ChIP assay indicates c-REL,  $\Delta$ Np63 $\alpha$ , TP53, and TAp73 bound to (-2283bp) p63 site of p21WAF1 gene promoter, while p65 showed no significant binding activity. TNF- $\alpha$  (20ng/ml for 30') increased the binding of c-REL, but decreased the binding of TAp73. B, Overexpression of c-REL increased c-REL but decreased TAp73 binding to the p21WAF1 promoter. C,  $\Delta$ Np63 siRNA decreased the binding activities of c-

REL,  $\Delta$ Np63 $\alpha$ , and TAp73. D, Left, *PUMA* and Right, *NOXA* p63 site ChIP binding for antibodies indicated,  $-/+$  TNF- $\alpha$  20ng/ml for 30' (\*  $p<0.05$ ).

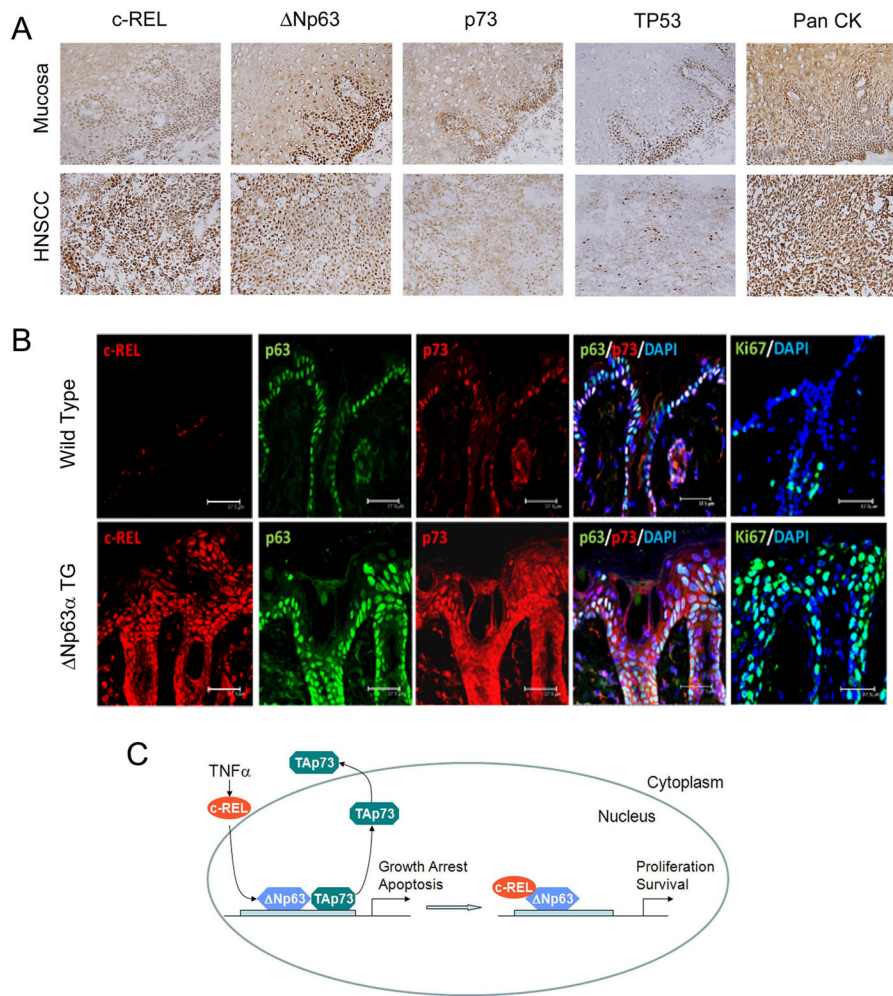




**Figure 5.** c-REL modulates p21WAF1, PUMA, NOXA expression in HNSCC lines. A, Left, c-REL siRNA significantly decreased c-REL mRNA expression in UM-SCC 1 (deficient wtTP53) and 22A (mtTP53) cells. A, Right, significant induction of *p21WAF1* mRNA was detected only in UM-SCC 22A cells. B, Western blots, Left, c-REL protein expression decreased after c-REL siRNA knockdown in UM-SCC 1 and 22A cells, but p21WAF1 protein increased only in UM-SCC 22A. B, Right, Transfection with plasmid to overexpress c-REL decreased p21WAF1 protein expression in UM-SCC 22A cells. C, Left, *PUMA* and Right, *NOXA*, mRNA expression after c-REL siRNA depletion in UM-SCC 1 and 22A cells, with significant induction of *PUMA* and *NOXA* in UM-SCC 22A cells. The mean  $\pm$  SD is shown. \*  $P < 0.05$ .

**Figure 6.**

c-REL differentially affects cellular density of UM-SCC lines exhibiting differences in TP53 family protein expression. A, UM-SCC 1 (deficient for wtTP53 and TAp73), or B UM-SCC 22A (increased mtTP53, TAp73) following knockdown of c-REL by siRNA. C, UM-SCC 1, over-expression of TAp73 decreased proliferation, while co-transfection of c-REL restored cell proliferation. D, UM-SCC 22A proliferation after overexpression of c-REL was increased by TNF- $\alpha$ . Lip: Lipofectamine alone; consi, control siRNA; c-RELSi, c-REL siRNA; REL, c-REL vector; TAp73, TAp73 vector; TNF $\alpha$ , 20ng/ml. Proliferation data were normalized against Day 0 WST-1 optical density (OD450nm) value.



**Figure 7.** Immunohistochemical staining and protein expression of TP53, p63, p73 and c-REL in HNSCC and  $\Delta$ Np63 $\alpha$  transgenic mice. Proposed model for interaction of c-REL,  $\Delta$ Np63 $\alpha$ , and TAp73. A, Immunostaining for c-REL,  $\Delta$ Np63, p73, and TP53 in representative matched squamous mucosa and HNSCC tumor specimens. Pancytokeratin staining was used as a positive control to highlight epithelia. Original, 200X. B, Dorsal skin sections from adult transgenic (TG) K5- $\Delta$ Np63 $\alpha$  mice were stained with specific antibodies for c-REL,  $\Delta$ Np63 $\alpha$ , and TAp73 by immunofluorescence as indicated. Ki67, marker of proliferation and Keratin 14, marker of epidermal differentiation. Bars, 37.5  $\mu$ M. C, Model of TNF- $\alpha$  modulated c-REL,  $\Delta$ Np63 $\alpha$ , and TAp73 interactions in HNSCC. In response to TNF- $\alpha$  c-REL undergoes nuclear translocation, binding with  $\Delta$ Np63 $\alpha$ , while TAp73 dissociates from the promoters of growth arrest and apoptotic genes, and the nucleus, enhancing cell proliferation and survival. c-REL mediated effects are pronounced in the subset the HNSCC with increased expression of c-REL,  $\Delta$ Np63 $\alpha$ , and TAp73 with mtTP53.