

# Attenuated Transforming Growth Factor $\beta$ Signaling Promotes Nuclear Factor- $\kappa$ B Activation in Head and Neck Cancer

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## Abstract

**Although constitutively activated nuclear factor- $\kappa$ B (NF- $\kappa$ B), attenuated transforming growth factor  $\beta$  (TGF $\beta$ ) signaling, and TP53 mutations frequently occur in human cancers, how these pathways interact and together contribute to malignancy remains uncertain. Here, we found an association between overexpression of NF- $\kappa$ B-related genes, reduced expression of TGF $\beta$  receptor (T $\beta$ R) subunits and downstream targets, and TP53 genotype in head and neck squamous cell carcinoma (HNSCC). In response to recombinant TGF $\beta$ 1, both growth inhibition and TGF $\beta$  target gene modulation were attenuated or absent in a panel of human HNSCC lines. However, in HNSCC cells that retained residual TGF $\beta$  signaling, TGF $\beta$ 1 inhibited both constitutive and tumor necrosis factor  $\alpha$ -stimulated NF- $\kappa$ B activity. Furthermore, HNSCC lines overexpressing mutant (mt) TP53 and human tumor specimens with positive TP53 nuclear staining exhibited reduced T $\beta$ RII and knocking down mtTP53 induced T $\beta$ RII, increasing TGF $\beta$  downstream gene expression while inhibiting proinflammatory NF- $\kappa$ B target gene expression. Transfection of ectopic T $\beta$ RII directly restored TGF $\beta$  signaling while inhibiting inhibitor  $\kappa$ B $\alpha$  degradation and suppressing serine-536 phosphorylation of NF- $\kappa$ B p65 and NF- $\kappa$ B transcriptional activation, linking these alterations. Finally, experiments with T $\beta$ RII conditional knockout mice show that abrogation of TGF $\beta$  signaling promotes the sustained induction of NF- $\kappa$ B and its proinflammatory target genes during HNSCC tumorigenesis and progression. Together, these findings elucidate a regulatory framework in which attenuated TGF $\beta$  signaling promotes NF- $\kappa$ B activation and squamous epithelial malignancy in the setting of altered TP53 status. [Cancer Res 2009;69(8):3415–24]**

## Introduction

The development and progression of cancer are the result of sequential genetic and biological events, including loss of tumor suppressor genes and gain of function in proto-oncogenes. These multiple defects lead to self-sufficient proliferation with limitless replicative potential, evasion of apoptosis, tissue invasion and

metastasis, and sustained angiogenesis (1). Mounting evidence indicates that nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a critical mediator in many of these processes through transcriptional regulation of several hundred downstream genes, the products of which promote the malignant phenotype (2).

In regulating this broad genetic program, NF- $\kappa$ B transcription factors are assembled through dimerization of five subunits: RelA(p65), p50(NF- $\kappa$ B1), p52(NF- $\kappa$ B2), c-Rel, and RelB. Each dimer is bound in the cytoplasm by an inhibitor  $\kappa$ B (I $\kappa$ B), which prevents its nuclear translocation (3). Cell stimulation transiently activates the I $\kappa$ B kinase (IKK) complex, which is composed of two catalytic subunits (IKK $\alpha$  and IKK $\beta$ ) and a regulatory subunit (IKK $\gamma$ ). Activated IKK phosphorylates I $\kappa$ B proteins, resulting in their proteosomal degradation and subsequent translocation of NF- $\kappa$ B dimers to the nucleus. NF- $\kappa$ B is constitutively activated in diverse human malignancies, including head and neck squamous cell carcinoma (HNSCC), wherein numerous activating signals have been elucidated, including proinflammatory cytokines tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin-1 (IL-1); growth factors epidermal growth factor (EGF) and hepatocyte growth factor; and intermediate kinases phosphoinositide 3-kinase (PI3K), casein kinase 2, and IKK (4). Whereas previous studies have focused on the positive signals contributing to NF- $\kappa$ B activation in HNSCC, signals that negatively regulate NF- $\kappa$ B activity remain poorly understood (5).

The transforming growth factor  $\beta$  (TGF $\beta$ ) signaling pathway performs an essential regulatory role in maintaining normal epithelial homeostasis (6). TGF $\beta$  signals through three TGF $\beta$  receptor (T $\beta$ R) subunits (T $\beta$ RI, T $\beta$ RII, and T $\beta$ RIII), resulting in phosphorylation of Smad2 and Smad3, which, with Smad4, enter the nucleus and regulate transcription. TGF $\beta$  potently inhibits epithelial proliferation by up-regulation of cyclin-dependent kinase inhibitor genes *p15<sup>INK4b</sup>*, *p21<sup>Cip1</sup>*, and *p57<sup>Kip2</sup>* and down-regulation of *c-MYC* and *ID1* expression (6). Thus, TGF $\beta$  signaling shows potent tumor suppression, and transcriptional inactivation or mutations of *T $\beta$ RI* and *T $\beta$ RII* have been reported in human epithelial malignancies (7). Whereas mutation of *T $\beta$ RII* is uncommon in HNSCC development, decreased T $\beta$ RII expression occurs frequently and leads to a less differentiated, more aggressive phenotype (8, 9). However, the relationship between alterations in TGF $\beta$  signaling and NF- $\kappa$ B activation, and the mechanisms contributing to reduced *T $\beta$ RII* expression in HNSCC have not been elucidated.

The TP53 tumor suppressor represents another commonly altered target underlying the development of cancer, including HNSCC. Defects in TP53 function contribute to loss of cell cycle regulation, genomic instability, and therapeutic resistance, promoting the survival of malignant cells. Mutation of *TP53* in HNSCC occurs with a frequency of ~50%, resulting in altered *TP53* expression and function (10). In HNSCCs that retain a wild-type

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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(wt) *TP53* genotype, approximately one-half exhibit deficient *TP53* expression by immunohistochemistry (11, 12). Finally, *TP53* mutations are not restricted to neoplastic cells but frequently affect adjacent, normal-appearing keratinocytes, implicating *TP53* mutation in the initiation of HNSCC (13).

We recently identified distinct molecular signatures in HNSCCs differing in *TP53* status, wherein genome-wide microarray and bioinformatic analysis revealed activation of NF- $\kappa$ B target gene expression with concomitant down-regulation of TGF $\beta$  pathway genes (14, 15). These observations prompted us to hypothesize that a potential relationship exists among TGF $\beta$  signaling, NF- $\kappa$ B activation, and *TP53* status, which we define here using HNSCC-derived cell lines, human tissue specimens, and an animal model of HNSCC generated by *T $\beta$ R11* deletion from murine head and neck epithelia (16).

## Materials and Methods

**Cell lines and mice.** HNSCC cell lines from University of Michigan squamous cell carcinoma (UM-SCC) series were obtained from Dr. T.E. Carey (University of Michigan). UM-SCC cell lines were previously characterized and found to possess molecular and phenotypic alterations expressed *in situ* and important in the pathogenicity of HNSCC (15). The tumor and outcome characteristics of patients providing UM-SCC cell lines are shown in Supplementary Table S1. The *TP53* mutation status of these cell lines was analyzed by bidirectional DNA sequencing of exons 2 to 9 (Supplementary Table S2). UM-SCC lines and primary human epidermal keratinocytes (HeKa) were cultured as previously described (12). All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the Oregon Health and Science University. *T $\beta$ R11*-floxed mice and *K5.CrePR1* transgenic mice were established and maintained as described (16).

**Reagents and transfection.** Recombinant TNF $\alpha$  and TGF $\beta$ 1 were from R&D Systems. Primary antibodies against the following proteins were used: T $\beta$ R11 (Santa Cruz), p-Smad2 (Cell Signaling), Smad2/3 (BD Biosciences), TP53 (Santa Cruz), serine-536 and serine-276 phosphorylated p65 (Cell Signaling), p65 (Santa Cruz), p50 (Santa Cruz), Oct-1 (Cell Signaling), and  $\beta$ -actin (Cell Signaling). All transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transient transfection was performed using constructs for T $\beta$ R11 and pcDNA3 control vector. The expression vector containing the human TP53 open reading frame (pORF-hTP53) and the empty control vector (pORF-mcs) were obtained from InvivoGen. Control small interfering RNA (siRNA) was from Qiagen and *TP53* siRNA was from Dharmacon.

**Real-time quantitative reverse transcription-PCR.** RNA isolation and cDNA synthesis were performed as previously described (12, 16). cDNA products were subjected to real-time quantitative reverse transcription-PCR (QRT-PCR) using TaqMan Assays-on-Demand probes (Applied Biosystems). An 18S rRNA probe was used as an internal control. Each sample was examined in triplicate. The relative mRNA expression levels were determined by normalizing with the 18S transcripts, the values of which were calculated using the comparative  $C_t$  method. Further details are available in the Supplementary Materials and Methods.

**Immunohistochemistry and immunofluorescence.** Formalin-fixed and paraffin-embedded HNSCC tissue arrays were obtained from Cybrdi as previously described (17). Each array contained HNSCC tumor tissues from 20 individuals spotted in triplicate plus normal mucosa tissues from six normal subjects spotted in duplicate. Immunohistochemistry and immunofluorescence analysis are described in Supplementary Materials and Methods.

## Results

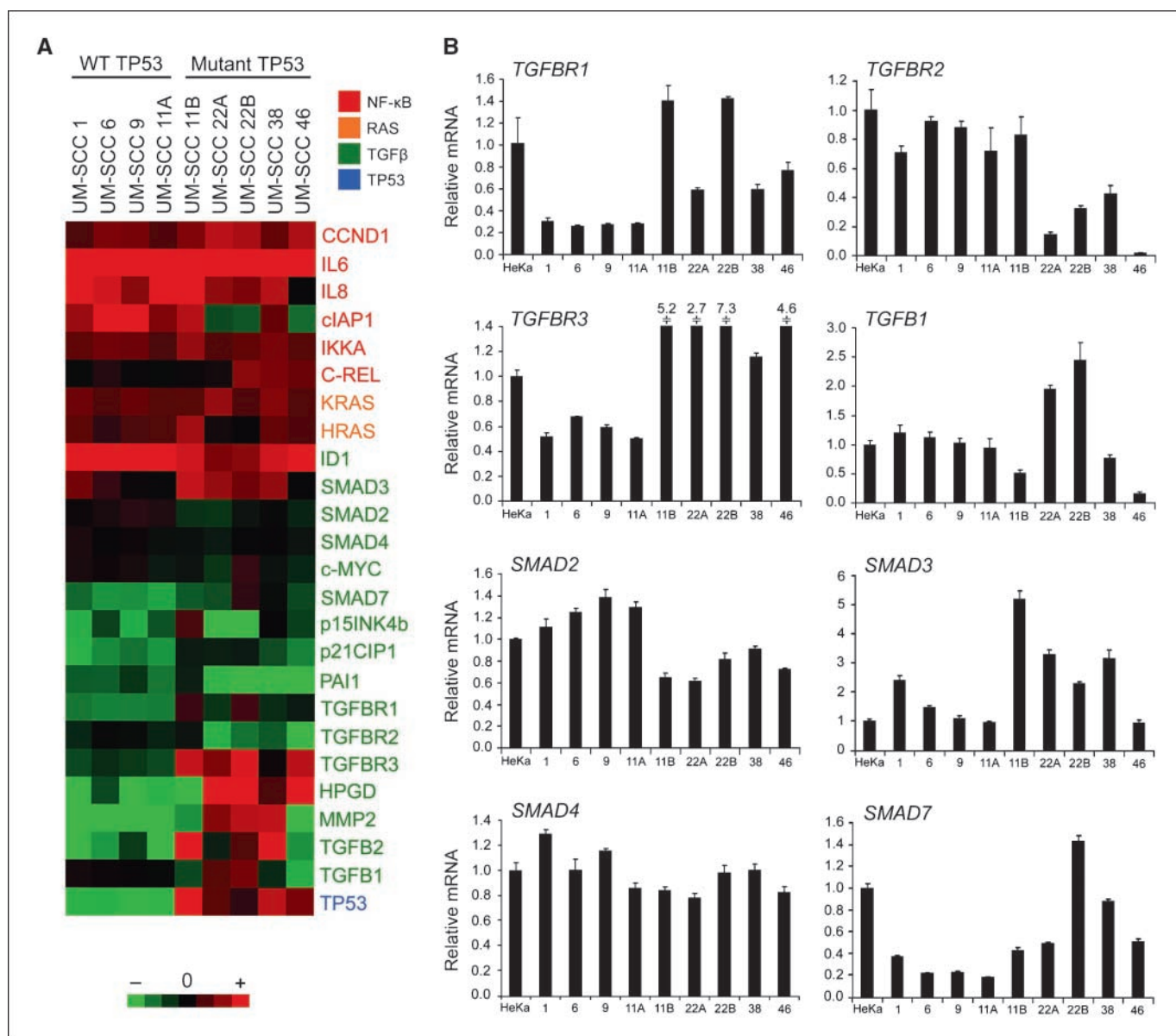
**Aberrant expression of TGF $\beta$  and activated NF- $\kappa$ B genes in HNSCC cells with distinct TP53 status.** Previous microarray

profiling and bioinformatic analyses of gene expression in a panel of nine HNSCC cell lines (Supplementary Table S1; ref. 18) and primary HeKa cells predicted potential cross talk among NF- $\kappa$ B, TGF $\beta$ , and TP53 signaling pathways (14). We performed real-time QRT-PCR to assess the expression of critical components of these pathways and downstream effector genes generating a cluster-based heatmap normalized to the expression pattern in primary keratinocytes (HeKa; Fig. 1A).

Increased basal expression of an NF- $\kappa$ B activating kinase, subunit (*IKKA* and *C-REL*) and target proto-oncogenes and proinflammatory genes (*CCND1*, *cIAP1*, *IL-6*, and *IL-8*) were detected (Fig. 1A). Conversely, attenuated TGF $\beta$  signaling was indicated by down-regulation of at least one of three essential TGF $\beta$  receptor subunits and other downstream targets (Fig. 1A and B). Strikingly, reduction in receptor expression clustered closely with the TP53 genotype and expression signature (Supplementary Table S2; Fig. 1A, bottom row). HNSCC cells deficient in wtTP53 transcript and protein (UM-SCC-1, UM-SCC-6, UM-SCC-9, and UM-SCC-11A) underexpressed *T $\beta$ R1* and *T $\beta$ R11*, whereas most lines expressing mutant (mt) *TP53* (UM-SCC-11B, UM-SCC-22A, UM-SCC-22B, UM-SCC-38, and UM-SCC-46) showed reduced *T $\beta$ R11* levels. Expression of downstream signaling components *SMAD2*, *SMAD3*, and *SMAD4* varied but was not markedly reduced. Additionally, TGF $\beta$  target genes *p15<sup>INK4b</sup>*, *p21<sup>Cip1</sup>*, *SMAD7*, *HPGD*, *PAIL*, and *MMP2* were strongly down-regulated in most UM-SCC lines. By contrast, *ID1*, previously implicated in metastatic aggressiveness of human SCC, was up-regulated in all UM-SCC lines (19). *KRAS* or *HRAS* overexpression was also detected in this panel of human HNSCC cell lines, as previously observed in ~80% of human HNSCC tissue samples (16). Together, these data suggest that HNSCC exhibit aberrant NF- $\kappa$ B gene signatures in the context of multiple deficiencies in the TGF $\beta$  signaling network, which are linked to deficient wtTP53 or mtTP53 status.

**HNSCC cells exhibit deficient TGF $\beta$ -induced growth inhibition and transcriptional activity.** To determine if reduced expression of TGF $\beta$  receptor subunits affects TGF $\beta$  signal-induced growth arrest in HNSCC cells, we measured cell proliferation by a 5-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using five UM-SCC lines of differing TP53 status (UM-SCC-6, UM-SCC-11B, UM-SCC-22A, UM-SCC-22B, and UM-SCC-46) and HeKa control cells under varying concentrations of rTGF $\beta$ 1. TGF $\beta$ 1 significantly inhibited HeKa but not HNSCC cell proliferation (Fig. 2A and Supplementary Fig. S1A for UM-SCC-11B and UM-SCC-22A). UM-SCC-11B, UM-SCC-22A, and UM-SCC-46 exhibited complete resistance to TGF $\beta$ -induced cytostatic effects. UM-SCC-6 and UM-SCC-22B showed a partial reduction in cell proliferation, suggesting attenuated TGF $\beta$  signaling and cytostatic activity.

We next examined whether a reduction in cellular response to TGF $\beta$ 1 affects expression of TGF $\beta$  downstream target genes important in growth arrest and other biological functions. In HeKa, TGF $\beta$ 1 exposure strongly induced mRNA expression of *PAIL*, *MMP2*, *p15<sup>INK4b</sup>*, *p57<sup>Kip2</sup>*, and *SMAD7* and suppressed expression of *c-MYC*, an NF- $\kappa$ B target gene (ref. 20; Fig. 2B and Supplementary Fig. S1B for UM-SCC-11B and UM-SCC-22A; data not shown for *p57<sup>Kip2</sup>*). By contrast, all HNSCC lines exhibited diminished sensitivity to TGF $\beta$ -modulated gene expression. Collectively, these findings show that HNSCC cells with deficient expression of component(s) of the TGF $\beta$  signaling pathway exhibit functional attenuation of TGF $\beta$  signaling through loss of TGF $\beta$ -mediated growth arrest and transcriptional activity.



**Figure 1.** Aberrant NF- $\kappa$ B and deficient TGF $\beta$  pathway gene expression signatures in HNSCCs with distinct TP53 status. **A**, gene expression heatmap of important genes related to TGF $\beta$ , NF- $\kappa$ B, TP53, and RAS signaling pathways in nine HNSCC (UM-SCC) cell lines, normalized to normal primary HeKa. *Red*, increased expression; *green*, decreased expression. Clustering was associated with TP53 genotype and mRNA levels of wtTP53 and mtTP53. **B**, reduced TGF $\beta$  pathway component expression in HNSCC cells. Quantitative comparison of relative mRNA levels for eight TGF $\beta$  signal pathway genes. Expression levels of individual genes in HeKa cells were set to the value of 1 arbitrary unit. *Columns*, mean between triplicate samples; *bars*, SD.

**Induction of residual TGF $\beta$  signaling inhibits NF- $\kappa$ B activation.** As our previous bioinformatic analysis (14) and results in Fig. 1 suggested potential cross talk and an inverse relationship between the TGF $\beta$  and NF- $\kappa$ B gene expression, we examined if TGF $\beta$  could inhibit NF- $\kappa$ B activation. We selected the two HNSCC lines (UM-SCC-6 and UM-SCC-22B) that exhibited residual TGF $\beta$ -induced growth inhibition and UM-SCC-46 as a negative control line because of its deficient TGF $\beta$  signaling (Fig. 2). In Fig. 3A, UM-SCC-6 and UM-SCC-22B exhibited significant detectable TGF $\beta$ 1-induced TGF $\beta$  luciferase reporter gene at 12 hours, whereas UM-SCC-46 was not responsive to TGF $\beta$ 1 treatment. UM-SCC-6, UM-SCC-22B, and UM-SCC-46 were transfected with a NF- $\kappa$ B reporter plasmid and treated  $\pm$ TGF $\beta$ 1 and  $\pm$ TNF $\alpha$ . TGF $\beta$ 1

inhibited constitutive and/or TNF $\alpha$ -induced NF- $\kappa$ B transcriptional activity in UM-SCC-6 and 22B, whereas UM-SCC-46 was unresponsive to TGF $\beta$ 1 treatment (Fig. 3B). Taken together, these findings show that TGF $\beta$  is able to suppress NF- $\kappa$ B activation in some UM-SCC cells.

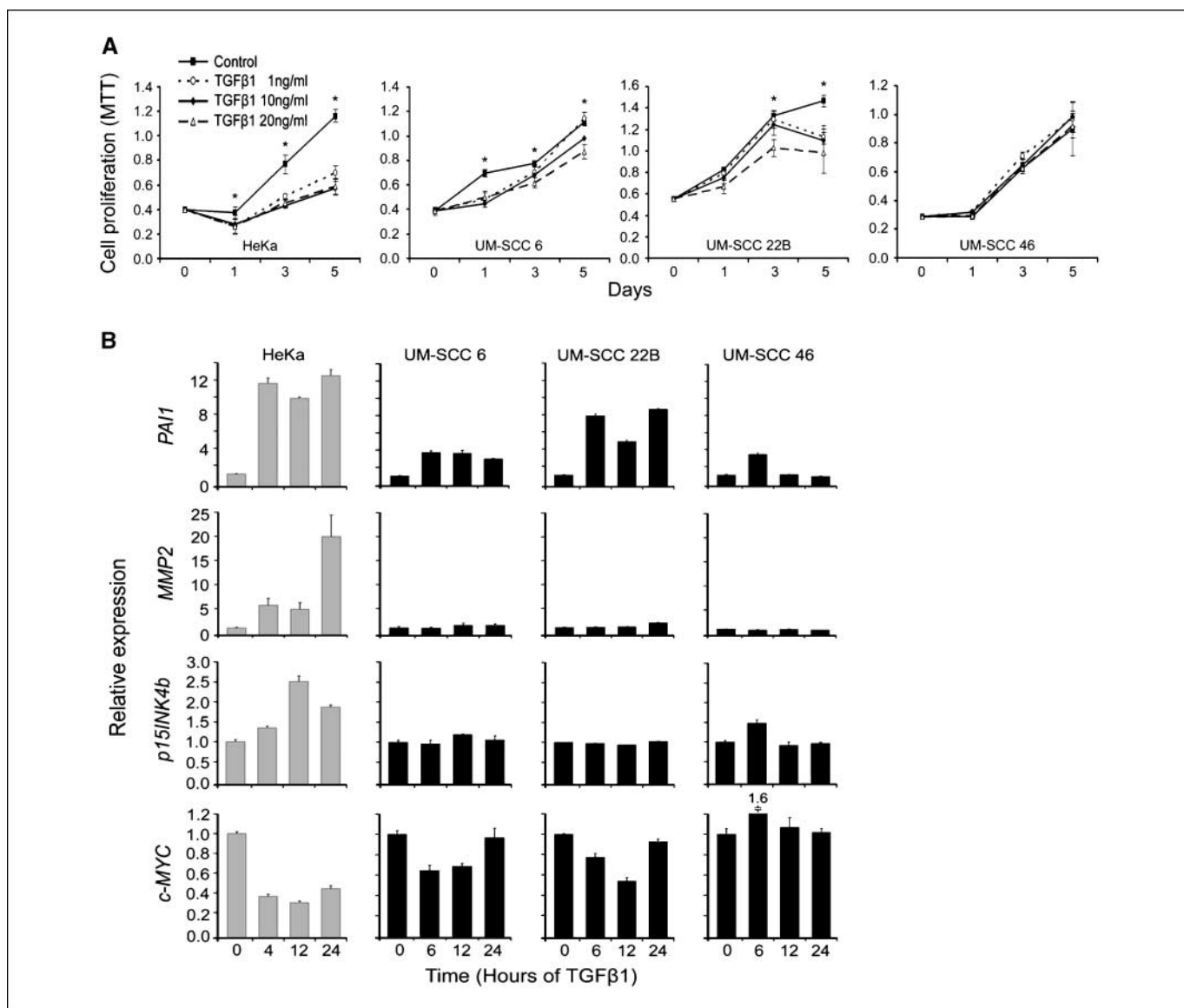
**Linkage between diminished T $\beta$ RII expression and signaling, mtTP53 accumulation, and NF- $\kappa$ B target gene activation in a subset of HNSCC.** Given that loss of T $\beta$ RII gene expression and signaling was observed in a subset of HNSCC cell lines expressing mtTP53 (Fig. 1; Supplementary Table S1), we examined if a similar subset is found in HNSCC tumors *in situ*. Immunostaining for T $\beta$ RII, phosphorylated (activated) Smad2, and TP53 protein expression was performed using a tissue array with 20 HNSCC

specimens and six normal oral mucosa specimens (Fig. 4A). Sixteen of the 20 HNSCC samples (80%) displayed absent (–) or low T $\beta$ RII protein, with 11 of these 16 showing absent (–) or low phospho-Smad2. Of nine scored completely negative for T $\beta$ RII, eight showed decreased p-Smad2 staining (Supplementary Fig. S2A). Conversely, all normal oral mucosa samples exhibited both strong T $\beta$ RII and activated p-Smad2 staining. Together, these observations confirm that deficient T $\beta$ RII expression and p-Smad2 signaling is prevalent in human HNSCC (16).

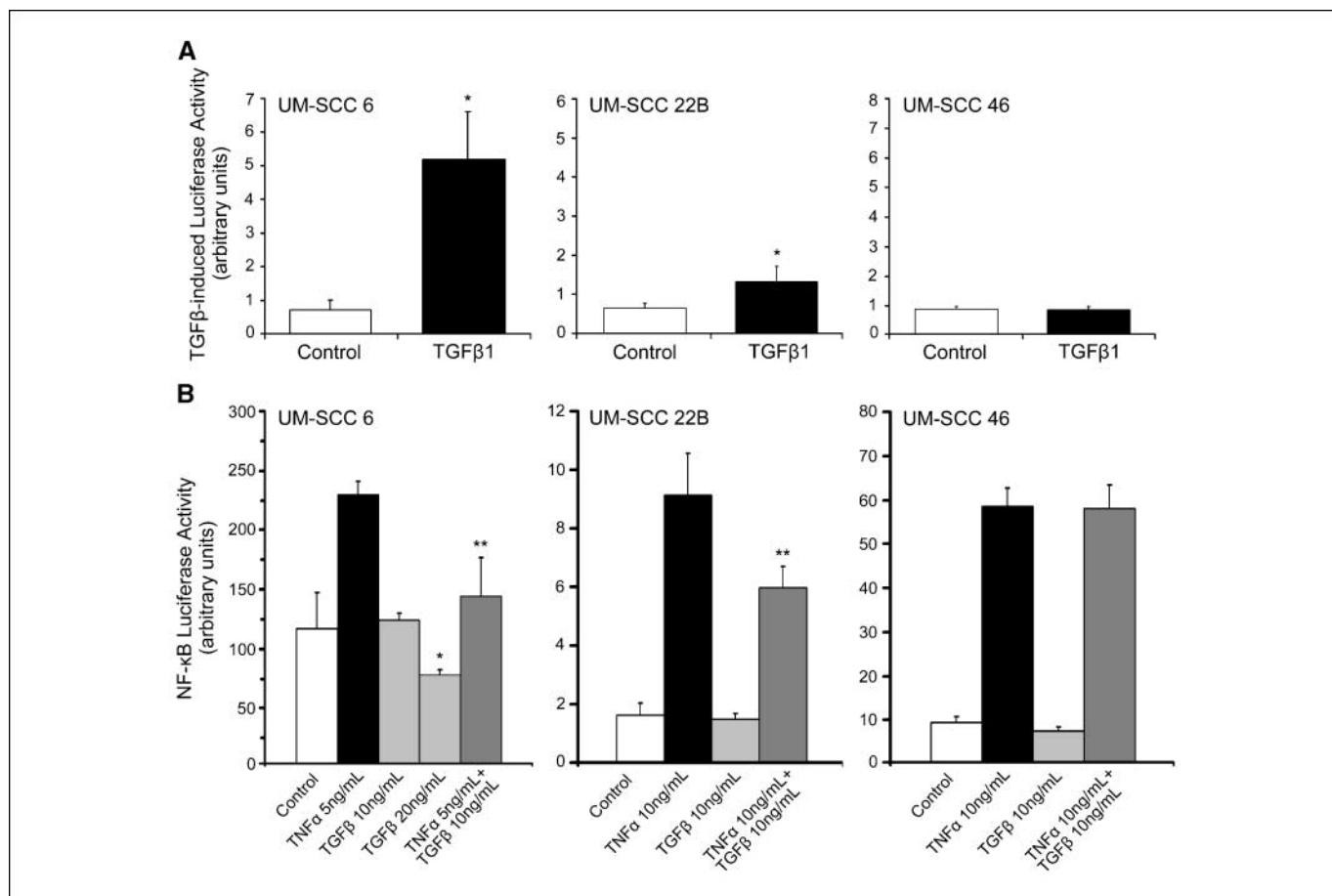
As TP53 mutations often result in proteins highly resistant to degradation, detection of TP53 nuclear accumulation by immunohistochemistry is strongly associated with mutations in the TP53 gene (21). Consistent with an expected mutation rate of ~50% in HNSCC (10), 11 of the 20 tumors (55%) showed nuclear

staining for TP53. Nine of these 11 (82%) exhibited concomitantly reduced or absent T $\beta$ RII staining (Supplementary Fig. S2A). Of the six HNSCC samples that were most strongly positive for TP53 nuclear staining, five (83%) showed a corresponding loss of T $\beta$ RII staining within the tumor tissue. None of the normal mucosa specimens exhibited significant TP53 staining. Thus, decreased T $\beta$ RII expression is seen in a subset of HNSCCs exhibiting increased accumulation of TP53 protein, as well as in a subset without TP53 staining.

We next investigated whether mtTP53 may have a suppressive role in T $\beta$ RII expression by comparing TP53 and T $\beta$ RII expression in HeKa with wtTP53, two cell lines with a defect in wtTP53 expression (UM-SCC-9 and UM-SCC-11A), and four cell lines expressing missense mtTP53s (UM-SCC-11B, UM-SCC-22A/B, and



**Figure 2.** Attenuated TGF $\beta$ -induced growth inhibition and target gene expression in HNSCC cells. **A**, functional attenuation of TGF $\beta$ -mediated growth arrest in UM-SCC lines. Cell proliferation was measured in a 5-d MTT assay in HeKa cells and UM-SCC lines of differing TP53 status. Cells were treated with 1, 10, or 20 ng/mL of rTGF $\beta$ 1. Cell growth rates were analyzed in quadruplicate. Points, mean; bars, SD. \*,  $P < 0.05$ . **B**, attenuated TGF $\beta$ -induced activation of PAI1, MMP2, and p15INK4b and suppression of c-MYC in UM-SCC. UM-SCC lines and HeKa cells were stimulated with 10 ng/mL for indicated time points, and total RNA was isolated and assayed for TGF $\beta$  target gene mRNA levels by QRT-PCR. Expression of individual genes at 0 h was set to the value of 1 arbitrary unit. Columns, means between triplicate samples; bars, SD.



**Figure 3.** Stimulation of residual TGF $\beta$  signaling suppresses NF- $\kappa$ B activation in UM-SCC cells. **A**, TGF $\beta$  reporter gene activity in UM-SCC-6, UM-SCC-22B, and UM-SCC-46 cotransfected with a TGF $\beta$ -induced reporter plasmid and  $\beta$ -galactosidase reporter plasmid and cultured  $\pm$ TGF $\beta$ 1 (10 ng/mL) for 12 h. **B**, NF- $\kappa$ B reporter gene activity in the same three lines cotransfected with NF- $\kappa$ B and  $\beta$ -galactosidase reporter plasmids and treated with different concentrations of TNF $\alpha$ , TGF $\beta$ 1, or the combination for 12 h. Luciferase values are normalized to  $\beta$ -galactosidase activity. *Columns*, means between triplicate samples; *bars*, SD. \*,  $P < 0.05$  versus untreated control cells; \*\*,  $P < 0.05$  versus TNF $\alpha$ -stimulated cells.

UM-SCC-46; Fig. 4B; Supplementary Table S2). Whereas similar levels of T $\beta$ RII mRNA and protein expression were detected in either HeKa expressing or UM-SCC deficient for wtTP53 protein (Figs. 1B and 4B), diminished T $\beta$ RII mRNA and protein levels were seen in most UM-SCC lines expressing mtTP53, consistent with the pattern observed in a subset of HNSCC tumors above (Figs. 1B and 4A and B). Transfection of wtTP53 into UM-SCC-11A cells deficient in endogenous wtTP53 promoted T $\beta$ RII and target gene expression (Supplementary Fig. S3), suggesting that expression of wtTP53 has an enhancing effect on T $\beta$ RII expression whereas endogenous mtTP53 in the other subset may have a dominant-negative effect.

To further establish if the mtTP53 in UM-SCC lines contributes to deficient T $\beta$ RII expression, we tested the effect of TP53 siRNA to inhibit TP53 mRNA expression in UM-SCC lines with mtTP53. TP53 siRNA efficiently inhibited endogenous TP53 expression in UM-SCC-11B and UM-SCC-22A over 96 hours (Fig. 4C; Supplementary Fig. S2B). Knockdown of mtTP53 resulted in a progressive increase in T $\beta$ RII gene expression relative to control siRNA (Fig. 4C and Supplementary Fig. S2B). Similar results were seen in UM-SCC-22B and UM-SCC-46 (Fig. 4D; data not shown). Concomitant with enhanced T $\beta$ RII expression, restoration of TGF $\beta$  signaling was evident by increased transcription of TGF $\beta$

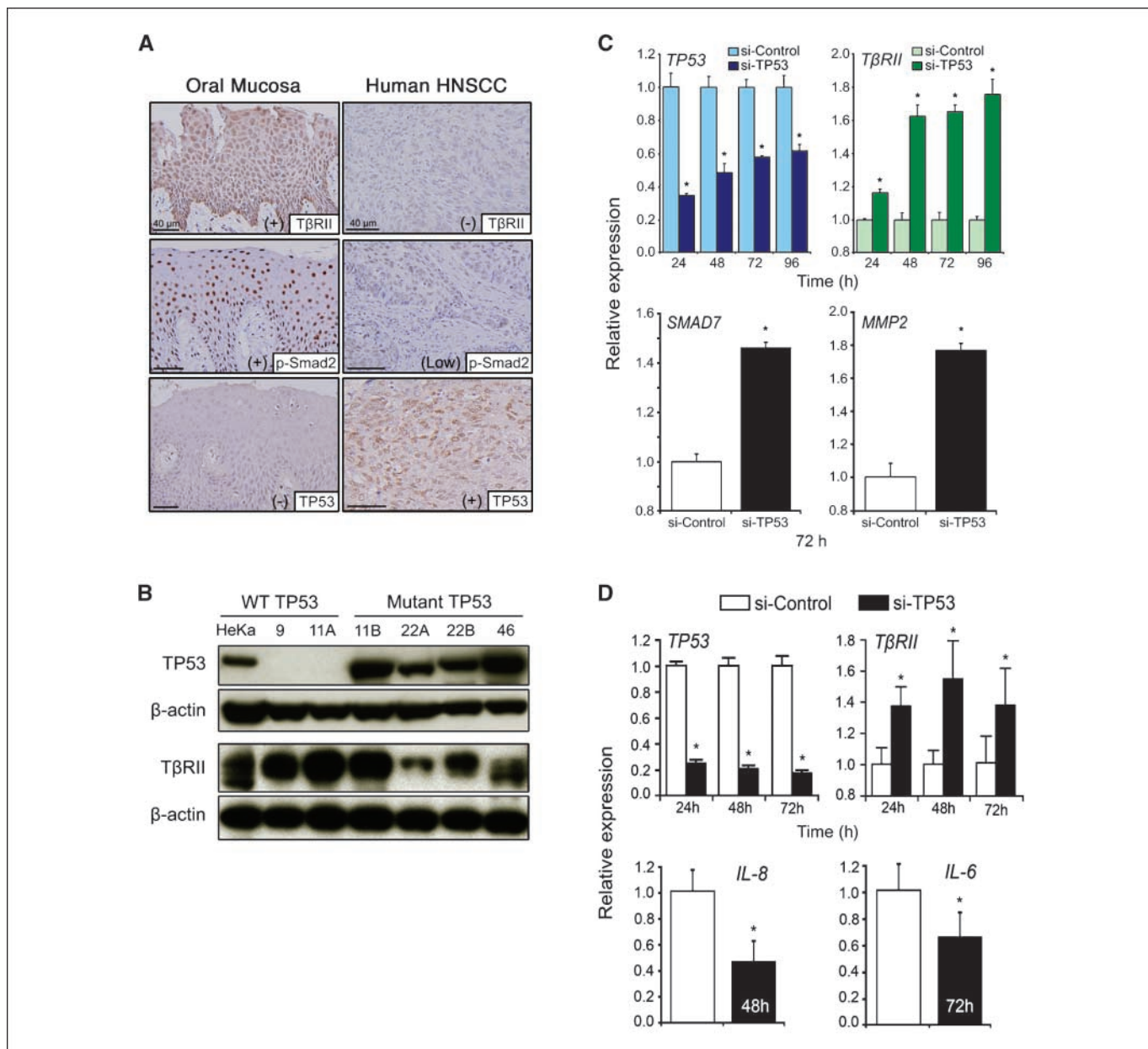
downstream genes, SMAD7 and MMP2, at 72 hours (Fig. 4C). We further compared effects of TP53 siRNA on the expression of TP53, T $\beta$ RII, and NF- $\kappa$ B REL(p65)-dependent proinflammatory genes IL-6 and IL-8 (14). Figure 4D shows that, after TP53 siRNA knockdown and increase in T $\beta$ RII, there is a significant reduction in both IL-8 and IL-6 expression in the mtTP53 line UM-SCC-22B. Collectively, the results above suggest that mtTP53 may be a mechanism contributing to attenuated T $\beta$ RII gene expression, TGF $\beta$  signaling, and enhanced NF- $\kappa$ B inflammatory gene expression in HNSCCs.

**Ectopic T $\beta$ RII expression restores TGF $\beta$  signaling and suppresses NF- $\kappa$ B activation in HNSCC cells.** To directly examine the role of T $\beta$ RII expression and signaling in inhibition of NF- $\kappa$ B, we determined if transient transfection of a plasmid expressing exogenous T $\beta$ RII protein could restore TGF $\beta$  activity and suppress NF- $\kappa$ B signaling in HNSCC lines that showed deficient T $\beta$ RII. We selected UM-SCC-46 because it had previously exhibited marked attenuation of TGF $\beta$  signaling (Fig. 2). Upon transfection, ectopic T $\beta$ RII was successfully transiently expressed between 48 and 96 hours (Fig. 5A). Expression of T $\beta$ RII dramatically induced TGF $\beta$  signaling in UM-SCC-46, illustrated by enhanced levels of activated phosphorylated Smad2 at 48 hours (Fig. 5B). This implicated T $\beta$ RII

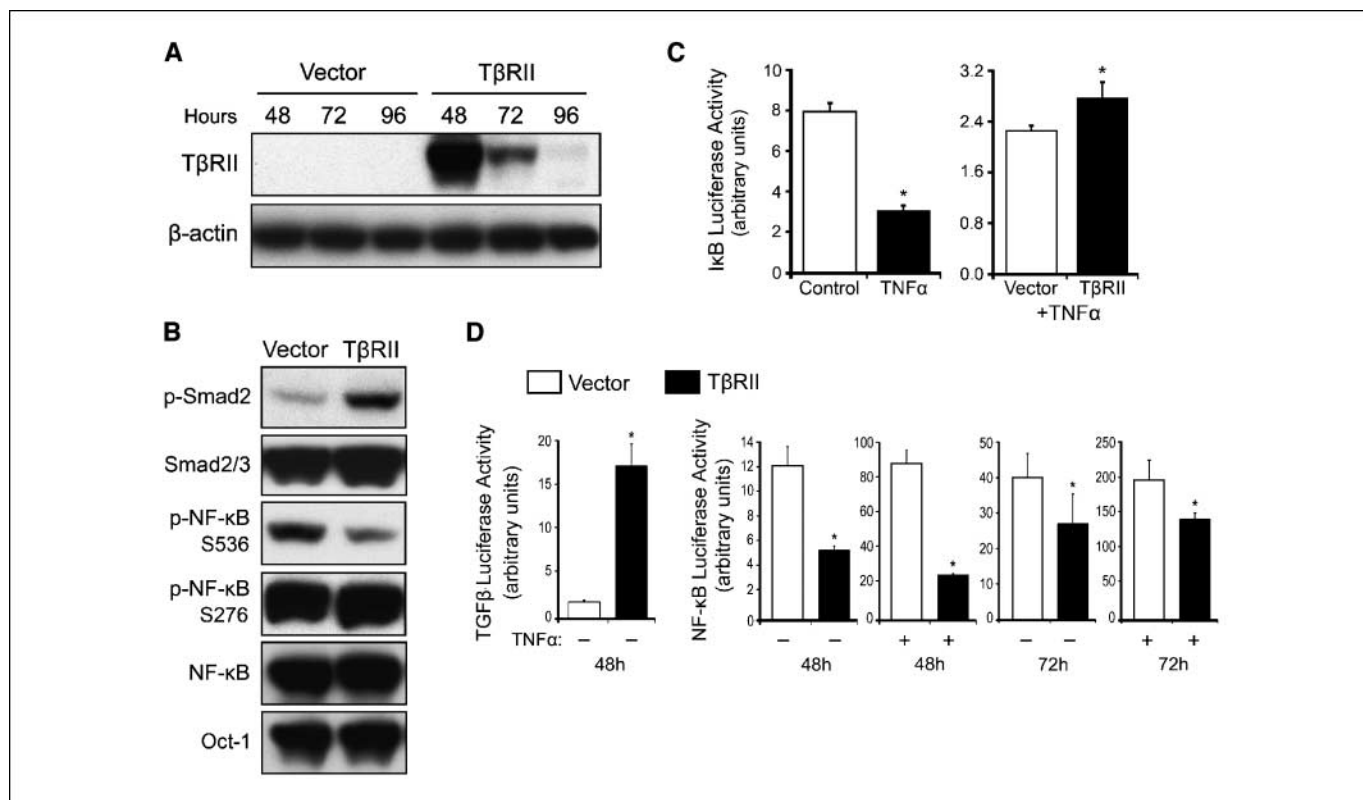
expression as a critical defect in TGF $\beta$  signaling in these HNSCC cells.

We further examined the effect of restored T $\beta$ RII signaling on established mechanisms required for NF- $\kappa$ B target gene transactivation. IKK-dependent signal phosphorylation of p65(RelA) serine-536 and PKA-inducible phosphorylation of serine-276 have been shown to enhance NF- $\kappa$ B gene transactivation after stimulation with TNF $\alpha$  (22). Increased expression of T $\beta$ RII strongly suppressed TNF $\alpha$ -induced p65(RELA) serine-536 phosphorylation,

but not serine-276 phosphorylation, or expression of total nuclear p65 or Oct-1, an unrelated control (Fig. 5B and Supplementary Fig. S4A). Together, these data are consistent with the hypothesis that T $\beta$ RII in these HNSCC inhibits NF- $\kappa$ B transactivation by a signal-dependent mechanism at or above IKK. To further examine if T $\beta$ RII modulates IKK-dependent I $\kappa$ B $\alpha$  degradation involved in upstream signal activation and nuclear translocation of NF- $\kappa$ B (2, 23), we used a plasmid expressing an I $\kappa$ B-luciferase fusion protein (24), which can serve as a nontranscriptional reporter of



**Figure 4.** Linkage between diminished T $\beta$ RII expression and signaling, mtTP53 accumulation, and NF- $\kappa$ B target gene activation in a subset of HNSCC. **A**, photomicrographs show diminished T $\beta$ RII and phosphorylated (activated) Smad2 with TP53 accumulation in a representative HNSCC tumor compared with the opposite pattern in normal oral mucosa. Semiquantitative scoring and classification of T $\beta$ RII, p-Smad2, and TP53 protein staining of 20 tumors is described in Supplementary Materials and Methods and Supplementary Fig. S2: -, negative; Low, reduced; +, strong staining. **B**, diminished T $\beta$ RII expression is detected in a subset of UM-SCC lines expressing mtTP53. Western blot analysis of T $\beta$ RII, TP53, and  $\beta$ -actin in HeKa, wtTP53, and mtTP53 UM-SCC cell lines. **C**, TP53 siRNA knockdown enhances T $\beta$ RII and target genes SMAD7 and MMP2 mRNA expression in mtTP53 UM-SCC-22A. **D**, TP53 siRNA knockdown enhances T $\beta$ RII and inhibits NF- $\kappa$ B target gene IL-8 and IL-6 mRNA expression in UM-SCC-22B. UM-SCC lines were transfected with either control or TP53 siRNA. Quantitation of mtTP53 inhibition and target genes was assessed using QRT-PCR at the indicated time points. The expression level in each control siRNA-treated sample is designated as 1.0. Columns, mean between triplicate samples; bars, SD. \*,  $P < 0.05$ .

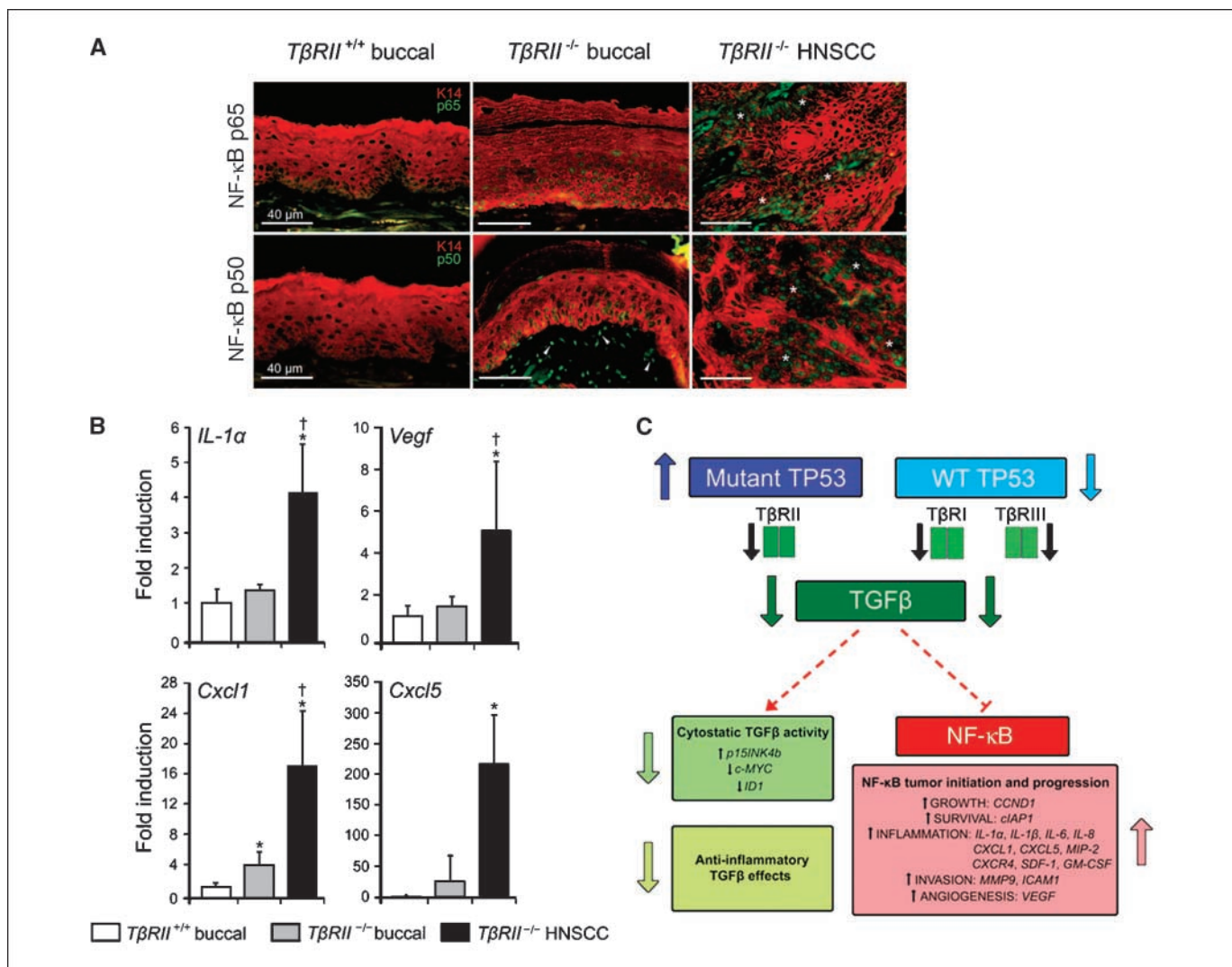


**Figure 5.** T $\beta$ RII transfection restores TGF $\beta$  signaling and inhibits NF- $\kappa$ B activation in HNSCC. **A**, transient transfection with *TβRII* expression vector enhances expression of T $\beta$ RII protein. Western blots were performed using whole-cell lysates from UM-SCC-46 cells at the indicated times. **B**, Western blot analysis of nuclear extracts from UM-SCC-46 showing reexpression of T $\beta$ RII restores TGF $\beta$  phosphorylated Smad2 signaling and suppresses IKK-dependent serine-536 but not PKA-mediated serine-276 phosphorylation of RELA(p65) NF- $\kappa$ B subunit after TNF $\alpha$  (10 ng/mL). UM-SCC-46 cells were transfected with a *TβRII* expression plasmid or control vector, and nuclear extracts were harvested after 48 h for immunoblotting. *Oct-1*, loading control. **C**, T $\beta$ RII attenuates TNF $\alpha$ -induced degradation of I $\kappa$ B $\alpha$ -luciferase fusion protein. UM-SCC-46 cells transfected with an I $\kappa$ B-luciferase fusion protein  $\pm$ control or *TβRII* expression vector for 24 h and  $\pm$ TNF $\alpha$  (50 ng/mL) for 30 min. **D**, T $\beta$ RII induces TGF $\beta$  and attenuates NF- $\kappa$ B reporter gene transactivation in UM-SCC-46. UM-SCC-46 line was cotransfected with the respective reporter plasmid plus a *TβRII* expression or control vector and cultured  $\pm$ TNF $\alpha$  (10 ng/mL) for 12 h before the indicated time points. All luciferase values are normalized to  $\beta$ -galactosidase. Columns, mean of triplicate samples; bars, SD. \*,  $P < 0.05$ .

kinase activation and degradation. As expected, TNF $\alpha$ -enhanced degradation of the I $\kappa$ B $\alpha$ -luciferase fusion protein (Fig. 5C). Upon transfection of ectopic T $\beta$ RII, restoration of TGF $\beta$  signaling resulted in a significant attenuation of TNF $\alpha$ -induced I $\kappa$ B-luciferase protein degradation ( $\sim 25\%$ ;  $P < 0.05$ ), suggesting that a mechanism upstream of IKK-mediated I $\kappa$ B degradation could partially contribute to the attenuating effect of TGF $\beta$  on NF- $\kappa$ B signaling.

Once we showed that exogenous T $\beta$ RII expression could inhibit NF- $\kappa$ B phosphorylation and I $\kappa$ B degradation, we sought to confirm if transient T $\beta$ RII expression and signaling suppressed aberrant basal and TNF $\alpha$ -enhanced NF- $\kappa$ B transcriptional activity, using three UM-SCC lines expressing mtTP53. UM-SCC-46 exhibited the strongest restoration of TGF $\beta$ -induced luciferase activity at 48 hours upon ectopic expression of T $\beta$ RII, followed by UM-SCC-22B and UM-SCC-22A (Fig. 5D and Supplementary Fig. S4B). Restoration of T $\beta$ RII signaling inhibited both constitutive and TNF $\alpha$ -induced NF- $\kappa$ B transcriptional activity in UM-SCC-46 (Fig. 5D). UM-SCC-22A and UM-SCC-22B also exhibited a significant reduction in NF- $\kappa$ B transactivation, but this was most clearly shown after exposure to TNF $\alpha$  (Supplementary Fig. S4B). These findings show that restoration of T $\beta$ RII signaling not only reduces activated RELA(p65) serine-536 phosphorylation but can also suppress NF- $\kappa$ B reporter gene transactivation in HNSCC lines expressing different mtTP53s.

**Loss of TGF $\beta$  signaling promotes NF- $\kappa$ B activation in murine HNSCCs with conditional deletion of *TβRII*.** To further examine the role of attenuated TGF $\beta$  signaling in the aberrant activation of NF- $\kappa$ B, we investigated mucosa and tumors from a murine model in which K5-targeted genetic deletion of *TβRII* from mucosal stratified epithelia, in combination with 7,12-dimethylbenz (*a*)anthracene (DMBA) initiation caused the development of primary and metastatic HNSCCs histologically and molecularly similar to human HNSCC (16). Tissue samples were collected from buccal mucosa of five *TβRII*<sup>+/+</sup>, *TβRII*<sup>-/-</sup>, and HNSCCs from five *TβRII*<sup>-/-</sup> mice and compared. Representative H&E-stained tumor sections showed atypical hyperproliferative epithelia by 3 months and HNSCC by 6 months in *TβRII*<sup>-/-</sup> mice when compared with their wt littermates (ref. 16; Supplementary Fig. S5). Immunostaining of *TβRII*<sup>-/-</sup> mucosa and HNSCCs confirmed the loss of T $\beta$ RII protein and the absence of phosphorylated Smad2, showing abrogation of TGF $\beta$  signaling compared with wt controls (Supplementary Fig. S5). As classical NF- $\kappa$ B signal activation involves the nuclear translocation of p65 and p50, we examined the aforementioned tissues for the presence and localization of these subunits using immunofluorescence microscopy (Fig. 6A). As in human HNSCC lines above, concomitant with loss of TGF $\beta$  signaling, a corresponding increase was seen in detectable nuclear RELA(p65) phosphorylated serine-536, as well as p50 staining in five of five



**Figure 6.** Abrogation of TGF $\beta$  signaling promotes NF- $\kappa$ B activation and proinflammatory target gene expression in HNSCCs of  $T\beta RII^{-/-}$  mice. **A**, NF- $\kappa$ B p65 and p50 subunit immunofluorescence staining of buccal squamous mucosa from  $T\beta RII^{+/+}$ ,  $T\beta RII^{-/-}$ , and HNSCC from  $T\beta RII^{-/-}$  mice. Buccal tissues representative of five  $T\beta RII^{+/+}$  and  $T\beta RII^{-/-}$  mice at 3 mo and HNSCC from five  $T\beta RII^{-/-}$  mice at 6 mo after DMBA initiation. Green, nuclear phosphorylated p65 and p50; red, epithelial K14; arrowheads, p50+ nuclei in  $T\beta RII^{-/-}$  buccal stroma; asterisks, NF- $\kappa$ B in regions with reduced K14. **B**, QRT-PCR analysis of NF- $\kappa$ B proinflammatory gene mRNA expression from  $T\beta RII^{+/+}$  and  $T\beta RII^{-/-}$  buccal and  $T\beta RII^{-/-}$  HNSCC tissue samples. Expression of genes in  $T\beta RII^{+/+}$  buccal tissue was set to 1 arbitrary unit. Columns, mean on triplicate samples of mRNA from five mice per group; bars, SE. \*,  $P < 0.05$  versus DMBA-initiated  $T\beta RII^{-/-}$  preneoplastic buccal tissue; †,  $P < 0.05$  versus DMBA-initiated  $T\beta RII^{-/-}$  preneoplastic buccal tissue. **C**, proposed model for enhanced tumorigenesis and malignant progression resulting from loss of TGF $\beta$  signaling and resultant NF- $\kappa$ B and gene activation. mTP53 represses  $T\beta RII$  expression, whereas deficient wtTP53 is associated with diminished  $T\beta RI$  and  $T\beta RIII$  expression, attenuating TGF $\beta$  signaling in HNSCC subsets. Deficient TGF $\beta$  signaling renders epithelia resistant to TGF $\beta$  cytotatic and antiinflammatory programs while enhancing aberrant activation of NF- $\kappa$ B target pro-survival and inflammatory gene programs.

$T\beta RII^{-/-}$  buccal mucosa and five of five  $T\beta RII^{-/-}$  basilar or HNSCC specimens. In contrast, normal  $T\beta RII^{+/+}$  mucosa revealed basilar or no NF- $\kappa$ B activation in the five specimens studied. Interestingly, regions in HNSCCs with the strongest NF- $\kappa$ B activation exhibited reduced K14 expression, consistent with previous studies implicating aberrant NF- $\kappa$ B activity and down-regulation of K14 in the epithelial-to-mesenchymal transition of high-risk HNSCCs (25).

Given the critical role of NF- $\kappa$ B in inflammation and our findings in Fig. 6A, we investigated the expression of NF- $\kappa$ B-regulated proinflammatory and angiogenic genes in these tissues by QRT-PCR (Fig. 6B).  $T\beta RII^{-/-}$  HNSCCs exhibited a significant induction of NF- $\kappa$ B-inducible genes *IL-1 $\alpha$* , *Vegf*, *Cxcl1/Gro1*, and *Cxcl5/Ena-78* when compared with both  $T\beta RII^{-/-}$  buccal mucosa and normal  $T\beta RII^{+/+}$  mucosa ( $P < 0.05$ ). Increased expression levels of *Cxcl1* and *Cxcl5* was also detected in  $T\beta RII^{-/-}$  preneoplastic lesions

compared with  $T\beta RII^{+/+}$  buccal tissues (Fig. 6B). Potentially further reflective of an inflammatory state, we observed previously (16) and here in the  $T\beta RII^{-/-}$  underlying stroma the presence of numerous infiltrating cells, which are strongly positive for nuclear NF- $\kappa$ B (p50) immunofluorescence (Fig. 6A). Together, these data strongly support a model where abrogation of TGF $\beta$  signaling in stratified epithelia promotes the aberrant activation of NF- $\kappa$ B, target cytokines, enhanced inflammation, and development and malignant progression of HNSCC (Fig. 6C).

## Discussion

Herein, we show that altered expression of TGF $\beta$  receptor subunits and deficient TGF $\beta$  signaling are common in human HNSCC lines and tissues, which exhibit enhanced activation of



NF- $\kappa$ B. We show that restoration of TGF $\beta$  signaling inhibits such aberrant NF- $\kappa$ B signaling and transcriptional activity, indicating that deficient TGF $\beta$  signaling is a critical event leading to the activation of NF- $\kappa$ B in HNSCC. Our data further suggest that TP53 mutation may be one mechanism that contributes to attenuated TGF $\beta$  signaling in a subset of HNSCCs through its ability to suppress *T $\beta$ R2* expression. Finally, *T $\beta$ R2*<sup>-/-</sup> conditional knockout mice that develop HNSCC with enhanced inflammation exhibited increased NF- $\kappa$ B activation and proinflammatory gene expression with tumor development, marking a significant connection between deficient TGF $\beta$  signaling and NF- $\kappa$ B activation in tumorigenesis. Whereas we have previously shown that sustained induction of NF- $\kappa$ B plays a critical role in the pathogenesis of HNSCC, we now implicate loss of a negative regulatory signaling pathway, TGF $\beta$ , as an integral step in this activation.

We previously reported that NF- $\kappa$ B-regulated gene signatures are differentially up-regulated in HNSCCs with distinct TP53 status and that overexpression of either *K-RAS* or *H-RAS* at the transcriptional level frequently occurs in human HNSCC tissues with concomitant reduction of *T $\beta$ R2* mRNA levels (14, 16, 17). Here, we found reduced *T $\beta$ R2* gene expression with concurrent *RAS* overexpression in a panel of human HNSCC cell lines and report additional TGF $\beta$  receptor and biological alterations, including those involving the NF- $\kappa$ B and TP53 pathways (Fig. 1A and B). Notably, expression of certain genes clustered according to TP53 status, including the TGF $\beta$  receptor subunits, *SMAD3*, *SMAD7*, *TGFB1*, *TGFB2*, *HPGD*, *p21*<sup>Cip1</sup>, and *MMP2*, as well as the NF- $\kappa$ B-related genes *C-REL*, *IL-8*, and *cIAP1*, suggesting that differential regulatory mechanisms that exist are directly or indirectly affected by TP53 genotype. Furthermore, the aforementioned genes serve as critical effectors commonly involved in the progression of aggressive HNSCCs (4, 16, 26, 27).

Although it has been suggested that inactivation of *T $\beta$ R1* and *T $\beta$ R2* expression occurs more frequently than genetic disruption in human HNSCC tissues (16, 28), the mechanisms by which this inhibition takes place in HNSCC have not been well defined. Our findings provide evidence that overexpression of mtTP53 can contribute to decreased expression of *T $\beta$ R2* and signaling in a subset of HNSCC. Other mechanisms independent of mtTP53 accumulation may contribute to the deficient expression of *T $\beta$ R2* in other HNSCC, such as those detected in tumor tissue arrays that lack TP53 staining. Here, we also detected reduced *T $\beta$ R1* or *T $\beta$ R3* expression in a subset of HNSCC tumor lines deficient in wtTP53, representing other potential defects that merit further investigation. Down-regulation of *T $\beta$ R3* transcript levels has been recently reported in breast and prostate cancers corresponding to more aggressive phenotypes and metastatic dissemination (29).

A connection between TP53 and TGF $\beta$  signaling was suggested in thyroid epithelial cells, wherein inactivation of wtTP53 induced a loss of responsiveness to TGF $\beta$ 1 treatment, accompanied by a partial reduction in TGF $\beta$  receptor expression (30). Subsequent studies revealed that ectopic overexpression of mtTP53 proteins induced resistance of squamous carcinoma cells to TGF $\beta$ -mediated growth inhibition (31, 32). Here, we provide further evidence for these relationships in distinct subsets of the same tumor type. Although *T $\beta$ R2* expression is not solely dependent on nor significantly attenuated in the UM-SCC cell lines deficient in endogenous wtTP53, transfection of wtTP53 into these cells further enhanced *T $\beta$ R2* expression and TGF $\beta$  downstream signaling (Supplementary Fig. S3) whereas, in UM-SCC lines with mtTP53, RNA interference-mediated TP53 inhibition enhanced *T $\beta$ R2* and

downstream *SMAD7* gene expression, supporting a dominant-negative role of mtTP53 in *T $\beta$ R2* signaling (Fig. 4C).

Mutant TP53 knockdown and enhanced *T $\beta$ R2* expression was also followed by attenuation in expression of prototypical NF- $\kappa$ B target genes *IL-6* and *IL-8* (Fig. 4D). In a murine squamous cell carcinoma model, we previously observed increased expression of mtTP53 protein accompanied by a corresponding reduction in *T $\beta$ R2* expression, TGF $\beta$ 1 responsiveness, and increased activation of NF- $\kappa$ B and target genes as tumors progressed to a metastatic phenotype (33, 34). Furthermore, in a TP53-null cell system, a recent study showed that ectopic mtTP53 can interact with the promoter of *T $\beta$ R2*, contributing to diminished *T $\beta$ R2* gene expression (35). Together, these observations suggest that mtTP53 represents one mechanism that can reduce the expression of *T $\beta$ R2* and TGF $\beta$  signaling in a subset of SCCs and other cancers. However, as there are likely genetic and epigenetic mechanisms contributing to reduced or absent TP53 and *T $\beta$ R2* expression in HNSCCs, it is not surprising that certain HNSCC specimens summarized in Supplementary Fig. S2, which exhibit reduced *T $\beta$ R2* expression, do not also exhibit nuclear TP53 staining.

Although this is the first demonstration of a relationship between TGF $\beta$  and NF- $\kappa$ B signaling in HNSCC, these findings are supported by other studies showing cross talk. In human intestinal mononuclear cells, pretreatment with TGF $\beta$ 1 diminished TNF $\alpha$ -induced activation of p65 (36), whereas in a transgenic mouse model of renal inflammation, TGF $\beta$  target gene *Smad7* promoted induction of *I $\kappa$ B $\alpha$* , inhibiting an NF- $\kappa$ B-driven inflammatory response (37). Here, we show that restored TGF $\beta$  signaling partially reduces degradation of *I $\kappa$ B $\alpha$*  and strongly inhibits IKK $\beta$ -dependent serine-536 phosphorylation of p65, localizing such cross talk at or above the level of IKK. In HNSCC, IL-1-IL-1R, TNF-TNFR1, TGF $\alpha$ -EGFR, and PI3K-CK2-IKK signaling pathways have been shown to promote IKK and NF- $\kappa$ B activation (38-40), and these pathways have been reported to include components that may be modulated by TGF $\beta$  signaling, such as TGF $\beta$  activating kinase (TAK1), TAK1 binding kinase, CK2, and ID1-mediated PI3K-Akt activation (23, 41, 42). Studies are underway to determine if these or other mechanisms enhance cytokine and growth factor activation of NF- $\kappa$ B and inflammation in HNSCC.

The potential relationship between reduced expression of *T $\beta$ R2* and NF- $\kappa$ B activation was of particular interest because of our previous demonstration that conditional targeting of *T $\beta$ R2* in murine aerodigestive epithelia results in HNSCCs exhibiting dramatic inflammation (16). Here, we show that both *T $\beta$ R2*<sup>-/-</sup> preneoplastic mucosa and HNSCCs show sustained induction of nuclear activation of classic NF- $\kappa$ B1/RelA (p50/p65) and its proinflammatory target genes (Fig. 6A and B). Most of these factors are significant mediators of the human NF- $\kappa$ B-regulated genetic program, critical in inflammation, angiogenesis, tumorigenesis, and progression of human cancers (2, 26, 43-46). Together, our findings suggest a novel regulatory framework whereby abrogation of TGF $\beta$  signaling in epithelial cells enhances NF- $\kappa$ B activation, tumorigenesis, and a deleterious inflammatory signaling circuit through recruitment of infiltrating immune and angiogenic cells. Furthermore, we show that mtTP53 may serve as an upstream repressor of *T $\beta$ R2* expression, contributing to TGF $\beta$  inhibition (Fig. 6C). It is notable that alterations in TP53, abrogated TGF $\beta$  signaling, and activation of NF- $\kappa$ B are facets of many cancers, raising the possibility that the mechanisms we have uncovered may extend beyond HNSCCs. Indeed, agents such as heat shock protein 90 inhibitors, which simultaneously inhibit both

NF- $\kappa$ B and mtTP53, are under investigation and show promise in numerous preclinical models (47). Thus, NF- $\kappa$ B and mtTP53 may represent concomitant pharmacologic targets in tumors wherein compromised TGF $\beta$  signaling leads to enhanced NF- $\kappa$ B activation and resultant malignant progression.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Attenuated Transforming Growth Factor $\beta$ Signaling Promotes Nuclear Factor- $\kappa$ B Activation in Head and Neck Cancer

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