# Aberrant IKKα and IKKβ cooperatively activate NF-κB and induce EGFR/AP1 signaling to promote survival and migration of head and neck cancer

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## Abstract

The Inhibitor-κB Kinase-Nuclear Factor-κB (IKK-NF-κB) and Epidermal Growth Factor Receptor-Activator Protein-1 (EGFR-AP-1) pathways are often co-activated and promote malignant behavior, but the underlying basis for this relationship is unclear. Resistance to inhibitors of IKKβ or EGFR is observed in head and neck squamous cell carcinomas (HNSCC). Here, we reveal that both IKKα and β contribute to nuclear activation of canonical and alternate NF-κB/REL family transcription factors, and overexpression of signal components enhancing co-activation of the EGFR-AP1 pathway. We observed that IKKα and IKKβ exhibit increased protein expression, nuclear localization and phosphorylation in HNSCC tissues and cell lines. Individually, IKK activity varied amongst different cell lines, but overexpression of both IKKs induced the strongest NF-κB activation. Conversely, siRNA knockdown of both IKKs significantly decreased nuclear localization and phosphorylation of canonical RELA and IκBα, and alternative p52 and RELB subunits. Knockdown of both IKKs more effectively inhibited NF-κB activation, broadly modulated gene expression, and suppressed cell proliferation and migration. Global expression profiling revealed that NF-κB, cytokine, inflammatory response, and growth factor signaling are among the top pathways and networks regulated by IKKs. Importantly,

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**Conflicts of interests**

IKKα and IKKβ together promoted the expression and activity of TGFα, EGFR, and AP1 transcription factors cJun, JunB, and Fra1. Knockdown of AP1 subunits individually decreased 8/15 (53%) of IKK-targeted genes sampled, and similarly inhibited cell proliferation and migration. Mutations of NF-κB and AP1 binding sites abolished or decreased IKK-induced *IL-8* promoter activity. Compounds such as wedelactone with dual IKK inhibitory activity, and geldanomycins that block IKKα/β and EGFR pathways were more active than IKKβ-specific inhibitors in suppressing NF-κB activation and proliferation, and inducing cell death. We conclude that IKKα and IKKβ cooperatively activate NF-κB and EGFR/AP1 networks of signaling pathways, and contribute to the malignant phenotype and the intrinsic or acquired therapeutic resistance of HNSCC.

## Keywords

IKKα; IKKβ; NF-κB; AP1; head and neck cancers

## Introduction

The inhibitor-kappaB kinases (IKKs) mediate signal phosphorylation-induced degradation of inhibitor kappaBs (IκBs), a critical step in releasing the NF-kappaB (NF-κB) family of transcription factors for cytoplasmic-nuclear translocation, DNA binding, and target gene transactivation (1, 2). The activation of the canonical NF-κB pathway is triggered by pro-inflammatory signals through a heterotrimeric complex of the IKKα and IKKβ catalytic subunits and the non-catalytic subunit, IKKγ. This complex can phosphorylate and degrade IκBs and promote nuclear translocation and phosphorylation of RELA/NK-κB1 (p65/p50) (1, 2). An alternative pathway, regulated by IKKα homodimers, induces processing of the precursor p100 to NF-κB2 (p52) to translocate with RELB (1, 2).

Aberrant activation of IKKs, NF-κB subunits, and their regulated gene programs have been implicated in the pathogenesis of many cancers, including head and neck squamous cell carcinomas (HNSCC) (3, 4). Blocking IKK-mediated IκBα degradation and NF-κB activation has been shown to inhibit aberrant gene expression, malignant phenotypes, and therapeutic resistance in pre-clinical HNSCC models (5, 6). Earlier experiments suggested that the IKKβ subunit was essential for canonical NF-κB activation through mediation of IκB degradation (1, 2), while IKKα was dispensable for IκB phosphorylation (7). The importance of IKKβ and proteasome in the canonical NF-κB pathway lead to drug development of bortezomib, a proteasome inhibitor that blocks IκB degradation and RELA activation, and MLN-120b, an inhibitor of IKKβ kinase activity (8, 9). However clinical trials using bortezomib in HNSCC showed limited clinical efficacy, due to incomplete targeting of other NF-κB/REL subunits, and other signaling pathways such as EGFR and AP1, which were shown to contribute to the resistance to bortezomib and radiation in HNSCC (10–12). Similarly, our preliminary studies showed that IKKβ inhibitors had modest effects *in vitro* and in pre-clinical HNSCC xenograft models (J. Ricker and J. Friedman, unpublished observations). Together, these findings suggested that drugs targeting IKKβ-mediated activation of NF-κB alone are insufficient, and the hypothesis that IKKα may contribute to canonical and/or alternative NF-κB/REL activation, and promotion

of the malignant phenotype. The few prior studies of IKKα in SCC have emphasized its potential role as a tumor suppressor, reporting increased malignant phenotype with decrease in its expression in poorly differentiated human SCC and in IKKα KO mice (13, 14). However, we have observed that increased IKKα with IKKβ are prevalent in the majority of differentiated HNSCC. How both IKKs together contribute to activation of the NF-κB pathway(s) and promoting the malignant phenotype in these cancers, remain incompletely understood.

Concurrent with NF-κB activation, the EGFR/AP1 signaling pathway is another important contributor of HNSCC pathogenesis. EGFR and its ligand TGFα are overexpressed in more than 90% HNSCC (15–17). The *EGFR* and *TGFα* genes may be amplified, but are more often overexpressed at the transcriptional level, by unknown mechanism(s) in HNSCC (18). Together, EGFR and TGFα form an autocrine signaling loop with the MAPK/ERK pathway and activate AP1 transcription factors to promote malignancy (15, 19). The AP1 transcription factor family of Jun and Fos contains 7 members: cJun, JunB, JunD, cFos, FosB, FosL(Fra1) and FosL2(Fra2) (19). We previously observed that co-activation of AP1 in HNSCC, either constitutively or induced through upstream signaling by TNF-α, EGFR, and MAPK/ERK, or tobacco carcinogens, is mediated through cJun, JunB and Fra1 (15, 16, 20). Nuclear co-activation of NF-κB/RELs, and overexpression of EGFR-AP1, was also subsequently linked in oral premalignant lesions and HNSCC tumor tissues by immunostaining (21), but the basis for this association remains undefined. Evidence for cross-talk and co-activation between NF-κB and AP1 signaling has been observed in skin, breast, and other solid tumors (22–24). To date, the role of IKKα and β in mechanisms of activation of the classical and alternative NF-κB pathways, and EGFR/AP1 signaling in human HNSCC remain unclear.

In this study, we observed that both IKKα and IKKβ are overexpressed and activated in most HNSCC tumors and cell lines. Cooperatively, they activated NF-κB/REL family members, cross-regulated the expression and activities of EGFR/AP1, and promoted proliferation and migration of HNSCC. Consistent with defined genetic studies, dual chemical inhibitors of IKKs, or IKKs and EGFR/AP1 more effectively inhibited NF-κB activation, cellular proliferation, and survival. Together, our data suggest the critical roles of IKKs as co-regulators of both the canonical and alternative NF-κB pathways, and mediators of NF-κB and EGFR/AP1 signaling cross-talk that promote pathogenesis of HNSCC.

## Results

### Aberrant expression and phosphorylation of IKKα and IKKβ in HNSCC tumor specimens and cell lines

IKKα and IKKβ expression, phosphorylation and localization in HNSCC were examined by immunohistochemical staining (IHC) of frozen sections of 18 HNSCC and 5 matched mucosa samples (Fig. 1A). Increased staining of IKKα, IKKβ and phospho-IKKα/β was observed throughout malignant epithelia compared to mucosa, which exhibited limited IKK staining in basilar layers (Fig. 1A). The staining intensity and localization of IKKs in tumors were quantified by histoscore. Over 90% exhibited predominantly nuclear IKKα staining, IKKβ was detected in both nucleus and cytoplasm, and phosphorylated-IKK staining was

more intense in the cytoplasm (Fig. 1B). The staining specificity of the antibody for p-IKKα/β was confirmed by immunofluorescence staining after knockdown of IKKα, IKKβ or both, in HNSCC line UMSCC1 (data not shown). Furthermore, IKKα and IKKβ protein expression and phosphorylation was increased in the whole cell and nuclear extracts of 9 HNSCC lines (UM-SCC) derived from patients with aggressive clinical courses [survival 15 months,](25), compared to primary human keratinocytes (HeKa, Fig. 1C). Thus, these HNSCC cell lines exhibited both IKKα and β expression and p-IKKα/β, consistent with most HNSCC specimens.

## Overexpression and activation of IKKα or IKKβ enhance IκB degradation and NF-κB signaling in HNSCC cell lines

To study the functional roles of IKKs, we overexpressed wild type (WT) or genetic mutant constructs of IKKs in UM-SCC lines. IKKα and IKKβ can be phosphorylated at the Serine phospho-acceptor sites, S176/S180 or S177/181, which were substituted with Alanines (SS->AA) to prevent IKK activation, or with Glutamate (SS->EE) to produce constitutively active IKKs. Catalytically inactive kinases (KA mutants) were produced by Lysine44 substitution with Alanine (K44A, Fig. 2A, upper panels) (26, 27). Transfection of all IKK expression vectors increased corresponding IKK protein expression (Fig. 2A, lower panels). Overexpression of IKKα or β EE increased kinase activity and decreased IκBα luciferase fusion protein reporter by >90% (Fig. 2B). Conversely, overexpression of kinase-inactive KA plasmids increased IκBα, with IKKβ KA mutant exhibiting a stronger dominant negative effect (Fig. 2B). A higher basal level of NF-κB activity was observed in UM-SCC11B compared to UM-SCC1. IKK WT and IKK EE individually increased NF-κB activation, with IKKβ having a greater effect in UM-SCC1 and IKKα in UM-SCC11B (Fig 2C, D). Co-expression of IKKα and IKKβ WT or EE resulted in the highest NF-κB activation in UM-SCC1, but not in UM-SCC11B (Fig. 2C, D). In both cell lines, IKKα AA had a greater effect in attenuation of NF-κB activity, whereas IKKβ KA inhibited NF-κB more than IKKα KA (Fig. 2C, D). Together, the data suggest differential but combinatory roles of IKKα and IKKβ in mediating IκBα degradation and NF-κB activation in different HNSCC lines.

## Knockdown of IKKα or IKKβ alone or in combination by siRNA inhibits NF-κB subunit expression, IκBα degradation and NF-κB activation

To define the roles of endogenous IKKα and IKKβ on NF-κB/REL activation, we knocked down both IKK subunits individually and in combination using siRNAs. We first examined the effects of IKK knockdown in UM-SCC1 cells treated with TNF-α, an inducer of canonical signaling which is detected in human HNSCC tumor specimens (28). The efficacy and specificity of the knockdowns were verified in cytoplasmic and nuclear fractions in UM-SCC1 (Fig. 3A). Canonical subunit RELA was modestly inhibited by IKKα or β knockdown, and strongly inhibited by dual IKK knockdown in both the cytoplasm and nucleus. A similar stepwise reduction from individual to dual IKK inhibition was observed for cytoplasmic phospho-RELA (p-RELA) at Serine536, while individual IKK knockdown enhanced nuclear p-RELA relative to basal or dual knockdown. The NF-κB1 subunit involved in the canonical pathway, p105 and its processed product p50, were also reduced after IKKα or β individual knockdown, while the strongest effects were observed after

knockdown of both. We detected expression of alternate NF-κB2 p100 protein predominantly localized in the cytoplasm, while processed p52 and RelB predominantly localized in the nuclear fraction (Fig. 3A). Interestingly, expression of the NF-κB subunit p100, and its processed product, p52, were suppressed more by IKKβ knockdown, while dual knockdown was the most effective in inhibiting the nuclear fraction. These protein expression data for p100/p52 can be largely explained by the effects of IKK siRNAs on *p100/p52* mRNA, as blocking IKKβ exhibited a stronger inhibitory effect on p100/p52 mRNA expression than knockdown of IKKα, while knockdown of both IKKs exhibited the strongest effects (Fig. 3B). Knockdown of IKKs did not significantly affect *RELA* gene expression (Fig. 3B). These data are consistent with previous observations that the canonical pathway is reported to regulate transcription of alternate pathway subunits NF-κB2/p100 precursor of p52 (29, 30). In addition, IKKα knockdown preferentially inhibited nuclear RelB along with p-p52 at Ser222 (Fig. 3A), a site implicated in p52 dimer and heterodimer formation recently identified by Perkins and colleagues (31, 32). Further, under TNFα stimulation, we did not detect significant induction of p-p100 (ser866/870), a marker for alternate IKKα pathway signal mediated processing, in the cytoplasm or nucleus (data not shown). p-IκBα, the downstream target of canonical IKK activation, was more strongly inhibited by IKKβ–specific or combinatory knockdowns in the cytoplasmic or nuclear fraction. Together, the greater effects of dual inhibition upon the cytoplasmic-nuclear translocation and phosphorylation of NF-κB subunits and IκBα are consistent with predominantly canonical IKKα/β-dependent activation of these subunits in response to inflammatory signaling by TNF-α.

We next examined the functional effects of IKK knockdown under Lymphotoxin β (LTβ) stimulation (Fig. 3C and D), which is a major upstream signal for alternative NF-κB pathway activation, and detected in lymph nodes to which HNSCC metastasize (33). LTβ induced increased p-p100 in both cytoplasm and nucleus in UM-SCC1. As a consequence, LTβ increased p52 in the nucleus. Interestingly, knockdown of IKKβ decreased p-p100 and p52 more significantly than knockdown of IKKα, while knockdown of both IKKs blocked p-p100 induced with LTβ treatment in both cytoplasm and nucleus. These observations are consistent with above findings, where knockdown of *IKKβ* exhibited a stronger inhibitory effect on p100/p52 mRNA and total protein expression than knockdown of *IKKα*, and knockdown of both IKKs exhibited the strongest effects. Together, our results provide evidence that in HNSCC, the upstream signaling from both IKKs regulate downstream NF-κB subunits. The effect of IKKβ on the p100/p52 is predominantly through its regulatory function controlling *p100/p52* gene expression and processing, and is not solely dependent on IKKα-mediated phosphorylation of p-100, consistent with previous findings in other cell types (34–36).

To assay the functional effect of IKK knockdowns on IκB and NF-κB activity, we used IκBα-Luciferase fusion protein (Fig. 3E) and NF-κB reporter constructs (Fig. 3F). Dual IKK knockdown had a greater effect than individual knockdowns in different HNSCC cell lines UM-SCC1 (Fig. 3 E, F), UM-SCC11B (Fig. 3E) and UM-SCC6 (data not shown). Together, these data indicate that both IKKα and IKKβ individually and together contribute to IκBα degradation and NF-κB reporter activation. We examined the relative contribution of

canonical and alternate NF-κB/REL subunits on NF-κB reporter activity, which demonstrated significant effects of p65, p105/p50 and RELB knockdown (Nottingham L, unpublished observations).

## Dual knockdown of IKKs suppress a broader gene expression program involving NF-κB, cytokines and growth factor signaling pathways

Dual knockdown of IKKs showed significant down-regulation of genes compared to individual IKK knockdown at 48 hours using ~23K human gene profiling (Fig. 4A). Representative down-regulated genes are shown in Supplemental Fig. 1. IPA annotation indicates the genes enriched in cytokine-, immune response-, and NF-κB related pathways (top), and growth factor signaling pathways (bottom, Fig. 4B, Supplemental Table 2). The interactive networks, established using gene components in cytokines and NF-κB related pathways (Fig. 4C), or growth factor signaling pathways (Fig. 4D), reveal potential interactive relationships amongst different molecules and pathways.

Among the knockdown targets, we validated 20 genes by real-time RT-PCR, with the strongest effects were observed by dual IKK knockdown (Fig. 5A). These include known canonical NF-κB modulated genes such as *IκBα, IL-1* and *IL-8,* and the non-canonical *NF-κB2/p52* subunit gene, established above. Several genes involved in the EGFR/AP1 pathways were suppressed, prompting knockdown experiments with *FOSL1, cJun,* or *JunB* (Fig 5B). Eight of fifteen (53%) of IKK target genes were co-modulated by AP1 family members, suggesting overlapped as well as distinct gene targets between IKKs and AP1 signaling pathways. Many of the IKK or AP1 target genes are implicated in cell proliferation and migration, according to Ingenuity Pathway Analysis annotations (Supplemental Table 2). Genes in the ERK/p38/MAPK and PI3K/AKT pathways involved in cell proliferation, include *TGFα, HBEGF, EGFR, Jun, JunB, FOSL, EGR1, cyclinD1*, and *IGFBP*s. Genes involved in cell migration included those in the HGF and TGFβ signaling pathways, such as *EGR1*, *ETS*, *Jun*, *SERPINE1, MMPs, SMADs* and their family members. Together, these signaling pathways regulated by IKKs and AP1 family members have previously been implicated as individually important molecules in promoting the malignant phenotype of HNSCC and other cancers.

## Knockdown of IKKs and RELA inhibit EGFR/AP1 expression as well as AP1 and IL-8 promoter activities

To further investigate the specific effects of IKKs and NF-κB signaling on the EGFR/AP1 pathway, we examined the effects of IKK knockdowns on EGFR/AP1 protein expression by Western blot (Fig. 6A). Individual and dual IKK knockdown, affected expression of EGFR, cJun, JunB and Fra-1/FOSL proteins in whole cell lysates, consistent with that observed for mRNA expression shown in Fig. 5A. To further investigate the role of canonical IKK mediated regulation of transcription factor RELA in EGFR/AP1 gene regulation, we knocked down RELA by siRNA. A significant decrease in EGFR, JunB and FOSL(Fra1) expression were observed (Fig. 6B), establishing an important role for RELA in co-expression of these EGFR/AP-1 pathway components. Consistent with the above observations, knockdown of IKKs suppressed AP1 reporter activity at 48 and 72 hours (Fig. 6C and data not shown). Further, wild-type IKK-induced proximal IL-8 promoter activity

(−133bp from the transcription starting site) was completely abolished by a point mutation at the NF-κB site (−83bp), and partially blocked by a point mutation at the AP1 site (−128bp, Fig. 6D).

### Knockdown of IKKs and target AP1 genes inhibits cell proliferation and migration

The biological roles of IKKα and IKKβ on proliferation and migration were examined, and demonstrated that dual knockdown of IKKs more significantly inhibited cellular proliferation (Fig. 7A). Knockdown of IKKβ exhibited a greater inhibition of cellular migration than IKKα, while the dual knockdown demonstrated the strongest effect (Fig. 7B and C). We examined the effect of knockdown of IKK target AP1 subunits on cell proliferation and migration. Knockdown of FOSL and JunB individually, but not cJun, significantly inhibited cell proliferation (Fig. 7D; data not shown). Knockdown of all AP1 subunits individually suppressed cell migration, with cJun exhibiting the strongest effects (Figure 7E and F). The data suggest that knockdown of both IKKs and target AP1 subunits differentially affect cell growth and migration.

### Drugs targeting IKKβ, dual IKK, and IKK/EGFR/AP1 components differentially inhibit NF-κB activity and cell survival

To explore the therapeutic implications of the above findings, we examined the effects of small molecules available for preclinical or clinical studies capable of inhibiting IKKβ alone (MLN120b, SC-514) (37), both IKKs (Wedelactone), (38), or IKKα and EGFR/AP1 signaling (Hsp90 inhibitor and geldanamycin derivatives, 17-AAG, 17-DMAG) (39). We found that 17-AAG preferentially inhibited IKKα expression in UM-SCC1, and reduced a slower migrating IKKβ band in both cell lines (Fig. 8A). In contrast, MLN-120b most selectively inhibited IKKβ in UM-SCC11B cells. Pan-IKK inhibitor Wedelolactone attenuated IKKα expression in both cell lines, and the slower migrating band of IKKβ and phospho-p65 in UM-SCC11B cells (Fig. 8A). Hsp90 inhibitor, 17-DMAG, more potently inhibited the NF-κB reporter than MLN-120b in UM-SCC1 cells, while UM-SCC11B cells were sensitive to both drugs (Fig. 8B). 17-DMAG and Wedelolactone more potently inhibited cell proliferation in both cell lines, compared to IKKβ selective inhibitors, MLN-120b and SC-514 (Supplemental Fig. 2). Both 17-AAG and Wedelolactone are also strong inducers for cell death as demonstrated by the increased sub-G0 DNA fragmentation and cell cycle arrest at the G2M phase, decreased cell density and increased cell blebbing and detachment (Fig. 8C, and Supplemental Fig. 3A, B). MLN-120b and SC-514 showed cytostatic effects and an increased vacuolization (Supplemental Fig. 3B). We confirmed that IKKα wt and EE mutants rescued NF-κB reporter activation in cells treated with IKKβ selective inhibitor MLN120b, when compared to broader inhibitors such as 17AAG (data not shown). Altogether, these data suggest that inhibitors with dual IKKα/β or broader activity exhibit more potent effects on IKK and NF-κB protein expression, NF-κB activation, and induction of cell cycle arrest and cell death, compared to selective-IKKβ inhibitors.

## Discussion

In this study, we provide evidence supporting a model whereby both IKKα and IKKβ cooperatively activate NF-κB, as well as enhance EGFR/AP1 signaling, to promote the malignant phenotype of HNSCC (Fig. 8D). IKKα and IKKβ were aberrantly overexpressed in most tumor tissues and cell lines, indicating their co-expression is prevalent in oncogenesis in differentiated HNSCC (Fig. 1). IKKα was previously implicated as a tumor suppressor of differentiation and IKKβ-mediated NF-κB and EGFR/AP-1 activation in a smaller subset of undifferentiated human HNSCC and knockout mice (13, 24, 40). However, when co-expressed, modulating IKKα and IKKβ in combination induced the strongest effects on NF-κB activity, although either were found to dominantly modulate NF-κB in different cell lines (Fig. 2). IKKα and IKKβ were both shown to mediate nuclear translocation and activation of canonical NF-κB/REL subunits (Fig. 3A). While IKKα predominately affected nuclear RELB and p-p52 as expected, IKKβ predominantly modulated overall expression of p100/p52 mRNA and protein available for nuclear activation (Fig. 3A, B). Pathway and network analyses indicated a crosstalk between IKK/NF-κB and the EGFR/AP1 signaling pathways (Fig. 4), which was shown to be modulated through the expression and functions of NF-κB targets and EGFR/AP1 molecules (Fig 5–7). Knockdown of AP1 subunits affected an overlapping subset of IKK target genes, and inhibited cell proliferation and migration (Fig. 5 and 7). Dual targeting of IKKs either by siRNA or small molecule inhibitors with broader activities, showed greater inhibitory effects on cell proliferation, migration, cell cycle arrest, and apoptosis (Fig. 7 and 8). Our data strongly suggest that both IKKα/β are important modulators of signaling upstream of NF-κB activation, and cross-talk promoting co-activation of the EGFR/AP1 pathway, important in pathogenesis and therapeutic targeting of HNSCC.

Previously, the IKKβ subunit of the canonical IKK complex was believed to be the predominant kinase responsible for IκB-dependent NF-κB activation (7), and promotion of tumorigenesis and metastasis. By utilizing IKK genetic mutant IKK constructs, we demonstrate that the specific impacts and variable potencies of IKKα and IKKβ activity differ, but cooperate in NF-κB activation in HNSCC. Consistent with the previous paradigm, overexpressing kinase dead mutant IKKβ KA had a relatively greater effect compared with IKKα KA in blocking IκBα degradation or NF-κB activation, but constitutively active IKKα or β EE mutants both promoted IκB degradation and NF-κB activation (Fig. 2B–D), indicating IKKα potentiates activation. The activating effects of overexpression of IKKα WT and EE mutants, or inhibitory effects of IKKα AA mutants, together further support the role of signal activated IKKα in potentiating IKKβ activation of NF-κB. These results are consistent with previous reports that IKKα can enhance IKKβ activity, though IKKβ is the stronger, direct kinase for downstream canonical NF-κB activation (1, 26). Interestingly, UMSCC11B demonstrated a stronger effect of IKKα WT or EE overexpression (Fig. 2C, D), but greater sensitivity to IKKβ inhibitor MLN-120b (Fig. 8A and B), consistent with its constitutively predominant IKKβ activation.

Further results modulating IKKα, IKKβ, or both by siRNA support the hypothesis that endogenous IKKα and IKKβ cooperatively regulate nuclear canonical and alternative NF-κB/REL subunits and functional NF-κB reporter activation. As expected, either IKKα or β

partially attenuated canonical nuclear RELA and NF-κB reporter activity, while dual inhibition had the most potent effects (Fig. 3A, E, F). TNF-α and LTβ are often expressed by infiltrating cells in HNSCC and lymph nodes, where HNSCC preferentially metastasize (28, 41). Interestingly, while overall nuclear RELA was partly inhibited by knockdown of either IKK under TNF-α treatment, IKK-dependent phosphorylation of nuclear RELA ser536 was enhanced unless both were knocked down, a phenomenon observed when IKKα or β compensate upon knockout of either kinase in mice and human cell lines (40, 42). Overall and nuclear p100 and p52 were also inhibited with IKKβ siRNA (Fig. 3A), which is consistent with prior findings that IKKβ strongly regulates TNF-α-induced p100/p52 gene expression (Fig. 3B) (29, 43). LTβ, as an alternate pathway inducer, was shown to induce detectable p-p100 and nuclear p52, but IKKβ and dual knockdown still attenuated p-p100 and nuclear p52 protein (Fig. 3C, D). Recent observations suggest that expression, processing and nuclear localization of p100/p52 may be more dependent on canonical IKK activation and IκBα degradation than previously appreciated (34–36, 43–45). With canonical signal activation, Scheidereit and colleagues found that p105 and p100 interact and are co-processed to p50 and p52, and that canonically regulated IκB can co-modulate nuclear translocation of RELB, potentially modifying previous paradigms separating canonical and alternative pathways (33). Consistent with this possibility, our data show similar effects of IKK knockdowns on p105/p50 and p100/p52 (Fig 3A). Together, our results indicate that dual inhibition of IKKα and β has greatest effects on expression, processing, nuclear localization, and functional activation of canonical and alternate NF-κB/RELs in HNSCC.

Although our data clearly demonstrate that both IKKα and IKKβ co-modulate the NF-κB pathway and expression of many genes, IKKα contains a nuclear localization signal, and is capable of modulating gene expression through histone H3 modification, important for NF-κB dependent, and independent gene expression, such as Fos after stimulation with EGF (46). We found that IKKα was localized in both nucleus and cytoplasm of human HNSCC, and IKKα, β and p65 co-activated EGFR and AP-1 subunit gene expression (Fig. 5). This is in apparent contrast with non-malignant epidermis of IKKα knockout mice, where IKKα can repress an autocrine loop of EGFR, ERK, and EGFR ligands, potentially through NF-κB independent mechanisms (24). Further studies suggested that where IKKα is decreased, this may promote carcinogenesis primarily through loss of its dual function as a tumor suppressive component of an otherwise intact TGFβ pathway (14). However, for the majority of HNSCC tumors and cell lines, we have recently shown that TGFβ function is attenuated via decreased TGFβ receptor expression or downstream responsiveness, and IKKα is overexpressed together with enhancement of NF-κB, as well as EGFR expression and pathway activation (47–49).

Through global gene analysis, we identified a broad gene program regulated by IKKs individually or in combination (Fig. 4, 5, Supplemental Fig. 1). Interestingly, IKK modulated genes were enriched in several growth factor signaling pathways, including EGFR/AP1 signaling often co-activated and important in the malignant phenotype in HNSCC (Fig. 4B, lower panel, Fig. 5A). Knockdown of AP1 subunits revealed that AP1 co-modulates a major subset of the IKK regulated genes. Previously, IKK or NF-κB-modulated expression of AP1 family members was suggested by studies in LPS stimulated B cells,

monocytes, and dendritic cells (50–52), as well as in IKKα knockout MEFs (46, 53). However, the role and means by which IKK modulates the EGFR/AP1 pathway in HNSCC tumorigenesis has not previously been well elucidated. Our data in established HNSCC cell lines support a model, whereby activated IKKs and RELA can induce co-expression of TGF-α, EGFR and downstream Jun and FosL(Fra1) family members, and co-activation of AP1. Interestingly, the three AP1 subunits identified in this study are the same ones we had previously shown to be co-activated with NF-κB in HNSCC (20). In addition, our data showed that AP1 family members exhibited differential functions to modulate cellular functions, such as proliferation and migration (Fig 7D–F). Our data suggest that FosL(Fra1) cooperates with cJun or JunB to promote migration and inflammatory cytokine production (54, 55), extending findings beyond a previous study highlighting cJun's role in promoting cellular proliferation (19). Knockdown of IKKβ and cJun exhibited strong effects on cell migration (Fig. 7), consistent with knockdown of IKKβ affecting cJun mRNA and protein expression more significantly than IKKα (Fig. 5 and 6). Our data are also supported by recent evidence, that activated NF-κB signaling contributes to resistance of EGFR-targeted therapy (56). Together with our study, this suggests that concurrent suppression of both IKK/NF-κB and EGFR/AP1 signaling could enhance therapeutic targeting of HNSCC.

We found that small molecule inhibitors with broader activity against both IKKs (wedelactone), or IKKs and EGFR (Hsp90 inhibitors), had more potent anti-proliferative and cytotoxic activity than IKKβ specific inhibitors (MLN120b and SC-514; Fig. 7C, Supplemental Fig. 2 and 3), consistent with the combined targeting of IKKα and IKKβ with siRNA. Geldanamycin derivatives inhibit the heat shock protein 90kDa (HSP90), a molecular chaperone preferentially targeting IKKα degradation over IKKβ, and inducing apoptosis in different cancers (39). Although the chemical drugs, 17-AAG/17-DMAG have broader ranges of targets compared to specific siRNA targeting, inhibition of IKKα activity was confirmed by the significant suppression of NF-κB activation induced by transfection with WT and EE IKKα mutants, while IKKα rescued NF-κB inhibitory effects of IKKβ selective inhibitor MLN120 (data not shown). Also, 17AAG significantly decreased IKKα protein levels (Fig 8A), consistent with the drug's known ability to destabilize IKKα and increase its degradation rate. Wedelolactone also inhibits both IKKα and IKKβ through modulation of caspases and IκB phosphorylation (38). In contrast, IKKβ specific inhibitors MLN-120b and SC514 exhibited limited efficacy when evaluated in preclinical studies of multiple cancers (37, 57), consistent with our observations (Fig. 8C, Supplemental Fig. 2 and 3, and data not shown). Our data have implications for dual IKKα and β inhibition in chemo- or radiation sensitization. IKK knockdown may sensitize cancer cells to chemotherapy-induced apoptosis (58), while Hsp90 inhibitors analogous to 17-AAG/17-DMAG increase radiation sensitivity both *in vitro* and *in vivo* in HNSCC (manuscript in preparation). Our current study challenges the conventional idea of focusing heavily on IKKβ-mediated signaling for development of anti-tumor drugs, which have not lead to expected efficacies in preclinical studies. The current inhibitors targeting NF-κB signaling are based on previously observed roles of IKKβ and the classical NF-κB pathway in transgenic or knock-out mouse models (7). However, the lack of efficacy of these inhibitors in solid epithelial tumors suggest that blockade of IKKβ maybe be insufficient. Our data suggest the underlying reasons for the lack of therapeutic efficacy, and support our

hypothesis that blocking both classical NF-κB pathway mediated by IKKα/β and the alternative pathway mediated by IKKα produces an additive and more complete inhibition of down stream genes and malignant phenotypes.

## Materials and methods

### Immunohistochemical analysis of HNSCC tissue specimens, Western blot, Reporter gene assay, MTT assay, Flow cytometry, Wound healing assay

See Supplemental Materials and Methods.

### Cell lines and culture

University of Michigan head and neck squamous cell carcinoma (UM-SCC) cell lines were from T.E. Carey (University of Michigan, Ann Arbor, MI), and cultured under standard growth conditions. Cell DNA was sent for sequence genotyping in 2008 and fall 2010 to compare and verify their unique origin from original stocks, as recently described (25). The 9 loci analyzed included D3S1358, D5S818, D7S820, D8S1179, D13S317, D18S51, D21S11, FGA, vWA and amelogenin. Human epidermal keratinocytes were obtained from (Invitrogen, Carlsbad, CA) and harvested at less than passage 5.

### siRNAs and plasmids

siRNAs were obtained from IDT (Coralville, IA), multiple siRNAs were individually tested, and three duplexes of siRNAs with best knockdown efficiency and specifity for each target were selected and pooled for the experiments (Supplemental Table 1). IKK expression and mutant constructs were kindly provided by Dr. Ulrich Siebenlist (NIH/NIAID, Bethesda, MD) and Dr. Michael Karin (UC San Diego), consisting of IKKα and IKKβ wild type (WT), phosphoacceptor-mutant (SS->AA, Serine to Alanine), constitutively activated (SS->EE, Serine to Glutamic Acid), and kinase dead (K44A, Lysine to Alanine) vectors (26, 27).

### Microarray and gene expression analysis

Microarray expression profiling was performed using Illumina Beadchip Array, and data were normalized and summarized in GenomeStudio software (Illumina). Genes with more than 50% decreased expression after IKKα and/or IKKβ siRNA knockdown were selected and compared to scramble siRNA treated cells, and analyzed in statistical software "R". To display the common and unique down-regulated genes in different treated samples, 3-way Venn diagram was made using VennDiagram package from Bioconductor (http://www.bioconductor.org). Ingenuity Pathway Analysis (IPA) was used to detect the significantly enriched signaling pathways within down-regulated genes, and two networks were established using genes involved in depicted pathways.

### Chemotherapeutic drugs

17-AAG (17-allylamino 17-demethoxygeldanamycin, tanespimycin) and 17-DMAG (17-Dimethylaminoethylamino-17-demethoxygeldanamycin, alvespimycin), exhibit very similar *in vitro* activity, but have different solubility and toxicity profiles *in vivo* (59). The drugs were synthesized by the Radiation Oncology Branch at NCI/NIH, and kindly provided by

Dr. David Guis. SC-514 is a selective and reversible inhibitor of IκB kinase 2 (IKK2) (IC$_{50}$ = 3–12 μM) that displays greater than 10-fold selectivity over 28 other kinases (Cayman Chemical). Wedelolactone (a natural compound isolated from the herb *E. prostrata*) was obtained from Biomol (Plymouth Meeting, PA). MLN-120b was provided by Millennium Pharmaceuticals, Inc under a Material Transfer Agreement.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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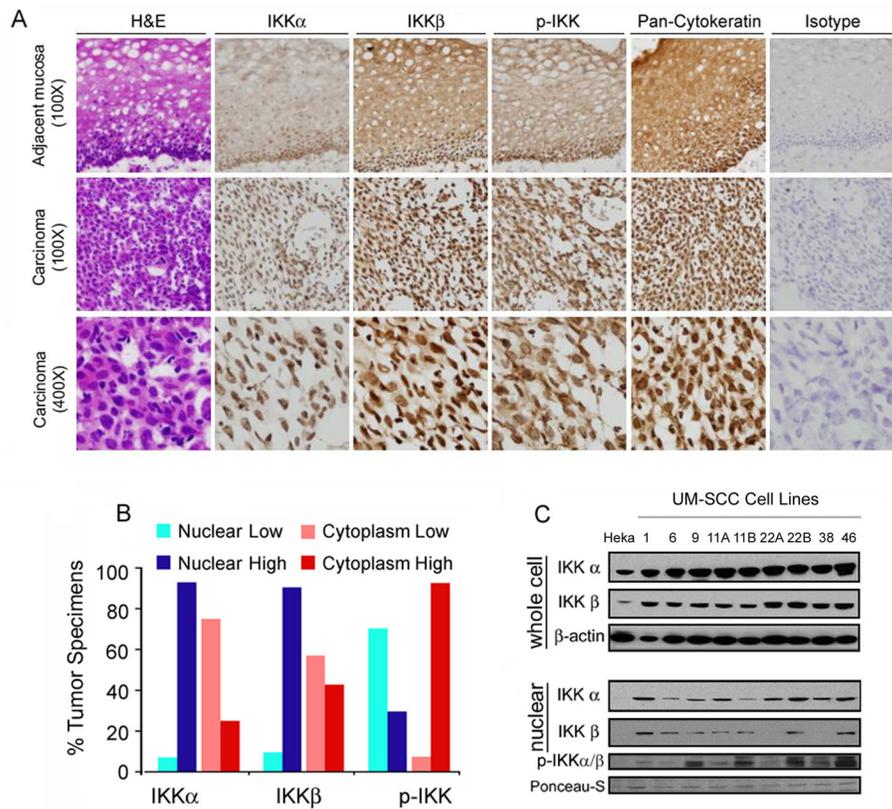
**Figure 1. Aberrant expression of IKKα and IKKβ proteins in HNSCC tumor specimens and cell lines**

A, Immunohistochemical analysis of IKKα, IKKβ, and phospho-IKK in representative tumor specimens and matched mucosa. H&E, pan-cytokeratin and rabbit IgG isotype stains served as controls. Magnification: 100X and 400X. B, Intensities of immunohistochemical staining of nuclear and cytoplasmic IKKs were semi-quantified and presented as histoscores of 18 HNSCC specimens. C, Overexpression of IKKα, IKKβ and phospho-IKK proteins in whole cell and nuclear lysates of 9 UM-SCC lines were compared with normal human primary keratinocytes (HeKa). β-actin and Ponceau-S staining as loading controls.
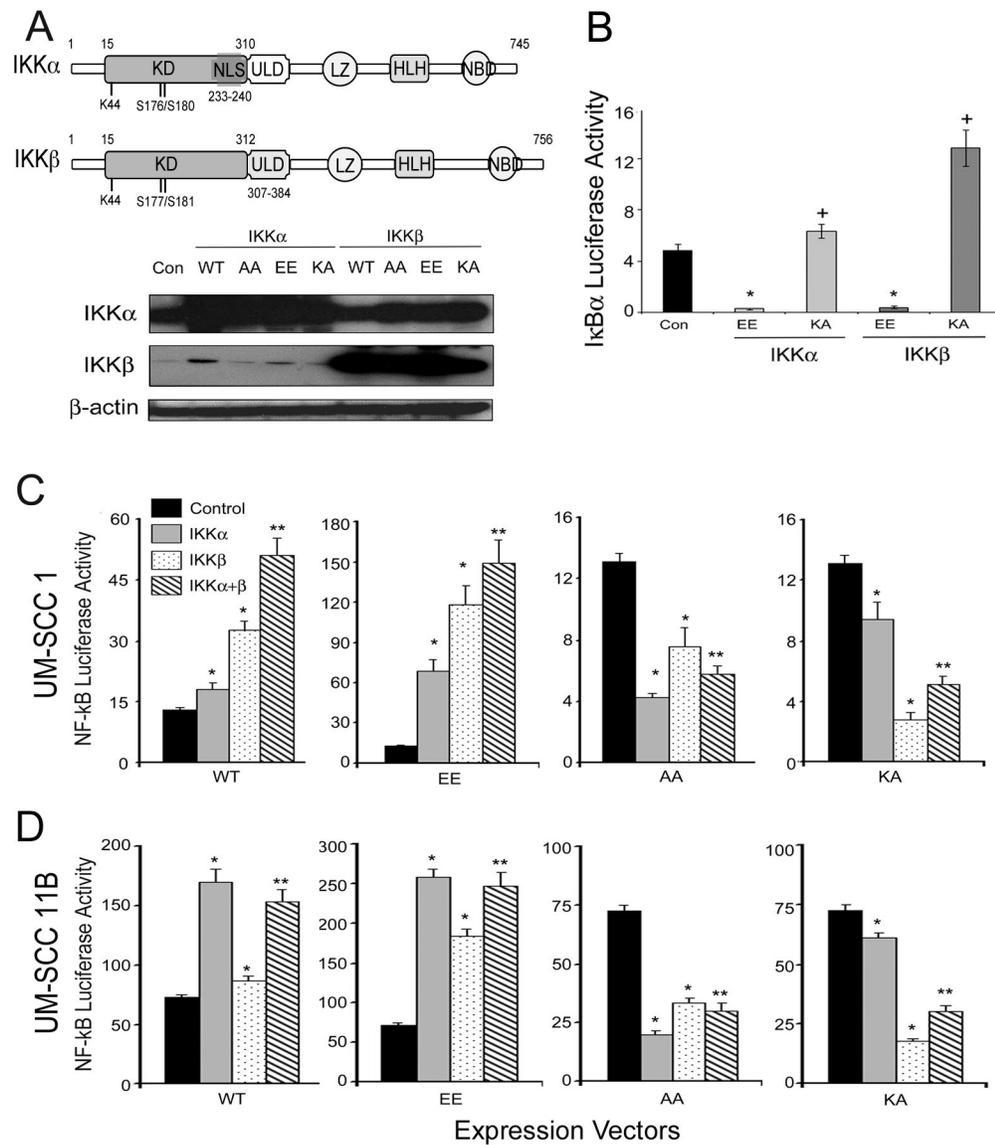
**Figure 2. Overexpression of IKKα or β mutant vectors differentially modulates IκB-α and NF-κB mediated reporter activities**

A, Schematic depiction of functional domains of IKKα and IKKβ (upper). KD, Kinase domain; NSL, Nuclear localization signal; ULD, Ubiquitin-like domain; LZ, Leucine zipper; HLH, Helix-loop-helix; NBD, Nemo binding domain. K44, Lysine44; S176/S180, Serine176 and 180; S177/S181, Serine177 and 181. Western blot analysis of IKKα or IKKβ expression of control (Con), wild type (WT), phospho-resistant (AA), constitutively activated (EE), or kinase inactive (KA) IKK plasmids in whole cell lysates of UM-SCC1 cells 48h post-transfection (lower). β-actin as loading control. B, Relative IκBα-luciferase expression levels with EE or KA mutant of IKKα or IKKβ vector in UM-SCC1 cells 48h post-transfection. Statistical significance (t-test, *p<0.001, +p<0.05) compared to the control. Experiments were performed in triplicate and presented as a mean with standard deviation. Relative NF-κB reporter activity measured 48 hrs after overexpression of mutant
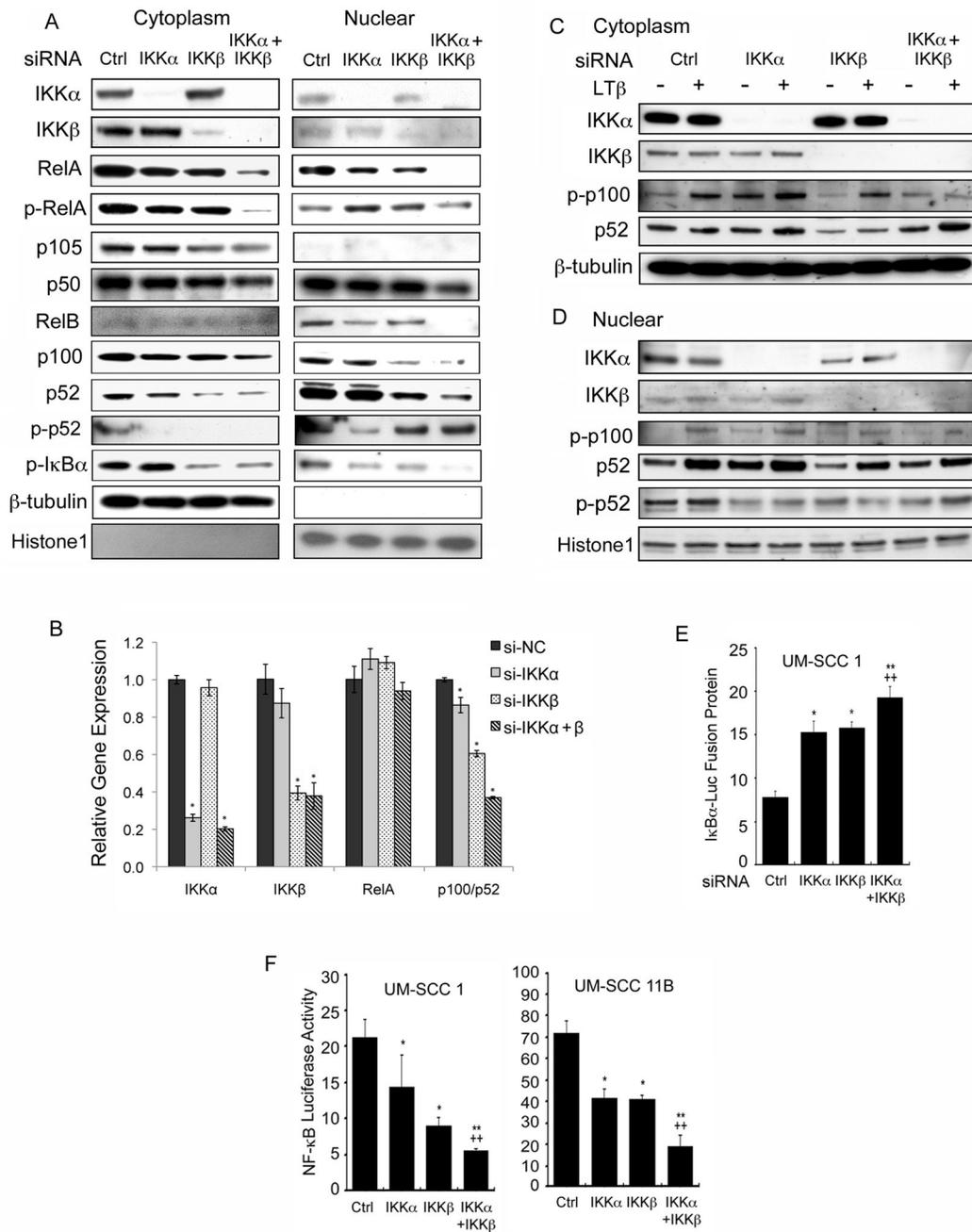
IKK plasmids in UM-SCC1 (C) and UM-SCC11B cells (D). Statistically significant differences between individual IKK constructs (*) or dual IKK overexpression (**) with the control plasmid (t-test, p<0.05).

**Figure 3. A combinatory effect of dual IKKα and IKKβ knockdown by siRNA in blocking NF-κB signaling molecule expression and reporter activity**

A, Knockdown efficiency of IKKα and IKKβ individually or in combination, and effects on downstream NF-κB subunits RelA, p-RelA (ser536), p105, p50, RelB, p100, p52, p-p52 and p-IκBα (ser32/36) after knockdown for 48 hours and treatment with TNF-α for 1 hour. β-tubulin and Histone1 served as loading and fractionation controls. B, Knockdown of IKKs affected mRNA expression of NF-κB p100/p52 subunits. UM-SCC1 cells were transfected with siRNA of IKKs under the same experimental condition as shown above, and RNAs were harvested and relative fold-change in mRNA expression was measured via quantitative

RT-PCR. Starred (*) values indicate a p-value<0.05 by t-test vs. negative control. p-p100 and p52 subunits in cytoplasmic (C) or nuclear (D) fraction were tested after IKK knockdown for 44 hours and treatment with lymphotoxin beta ($\alpha$1/$\beta$2; 100ng/ml) for 4 hours. E, Greater I$\kappa$B$\alpha$ luciferase fusion protein was observed in the double IKK knockdown than individual knockdowns. F, Conversely, the greater combinatory inhibitory effect of NF-$\kappa$B reporter activity was observed in the IKK double knockdown over the single knockdowns in UM-SCC1 (left), UM-SCC11B (right), and UM-SCC6 (data not shown). Statistical significance of t-test, * single knockdowns compared to negative control: p<0.01; ** double knockdowns compared to negative control: p<0.001; ++ dual knockdowns compared to single knockdowns: p<0.001.
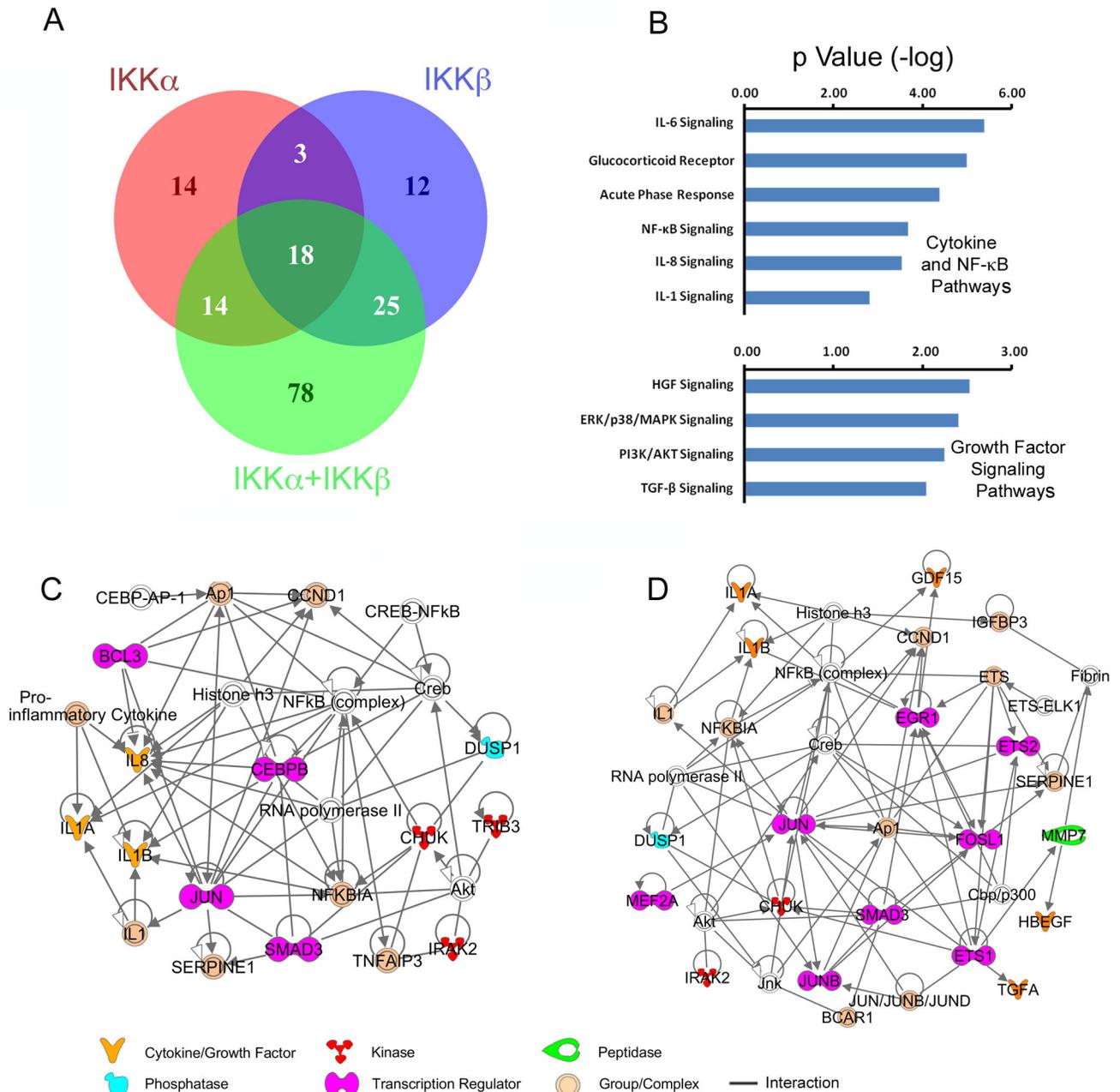
**Figure 4. Array profiling of gene expression after IKK single or dual knockdowns by siRNA in UM-SCC1 cells**

A, Venn-diagram of down-regulated gene expressions and overlaps in IKKα, IKKβ, IKKα & IKKβ knockdown samples compared with control scramble siRNA transfected cells. B, Enriched signaling pathways were annotated among down-regulated genes, and p value (−log) depicted the significance. Ingenuity Pathway Analysis (IPA) constructed networks using the genes involved in the cytokine and NFκB pathways (C), and the growth factor signaling pathways (D).
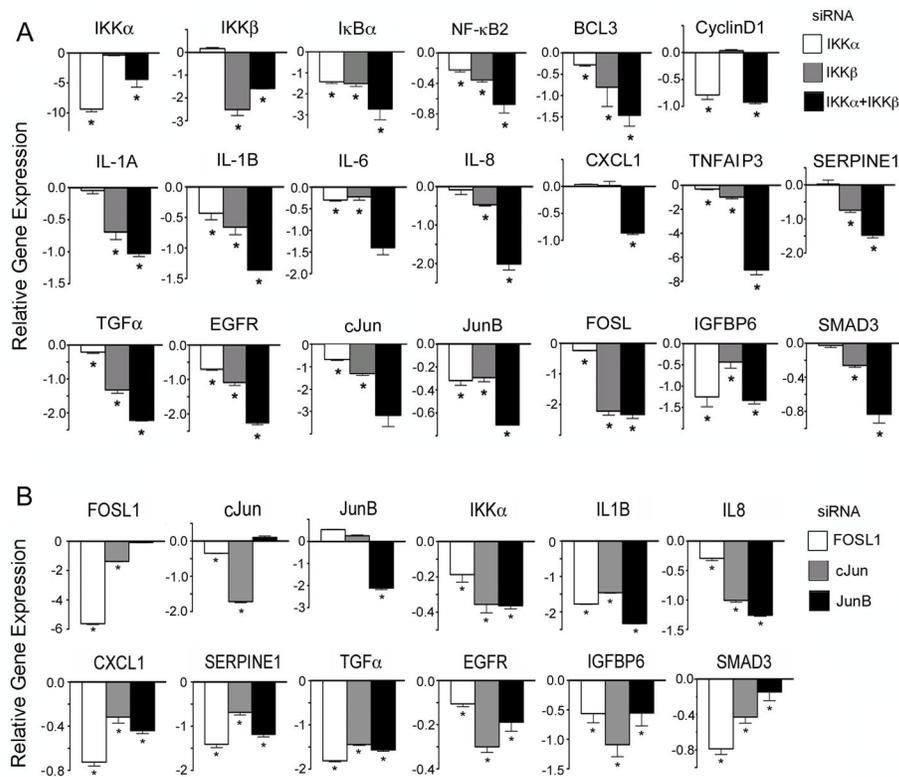
**Figure 5. Knockdowns of IKKs and AP1 subunits down-regulate a partially overlapped gene program involved in NF-κB and EGFR/AP1 signaling pathways**

A, Relative fold-change in mRNA expression after IKK single or double siRNA transfection of UM-SCC1 for 48 hrs were measured by quantitative RT-PCR (white, IKKα; grey, IKKβ; black, IKKα+IKKβ). B, Relative fold-change in mRNA expression after knockdown of AP1 subunits individually by siRNA in UM-SCC1 for 48 hrs were measured by quantitative RT-PCR (white, FOSL1; grey, cJun; black, JunB). $p < 0.05$ by t-test vs. controls (*).
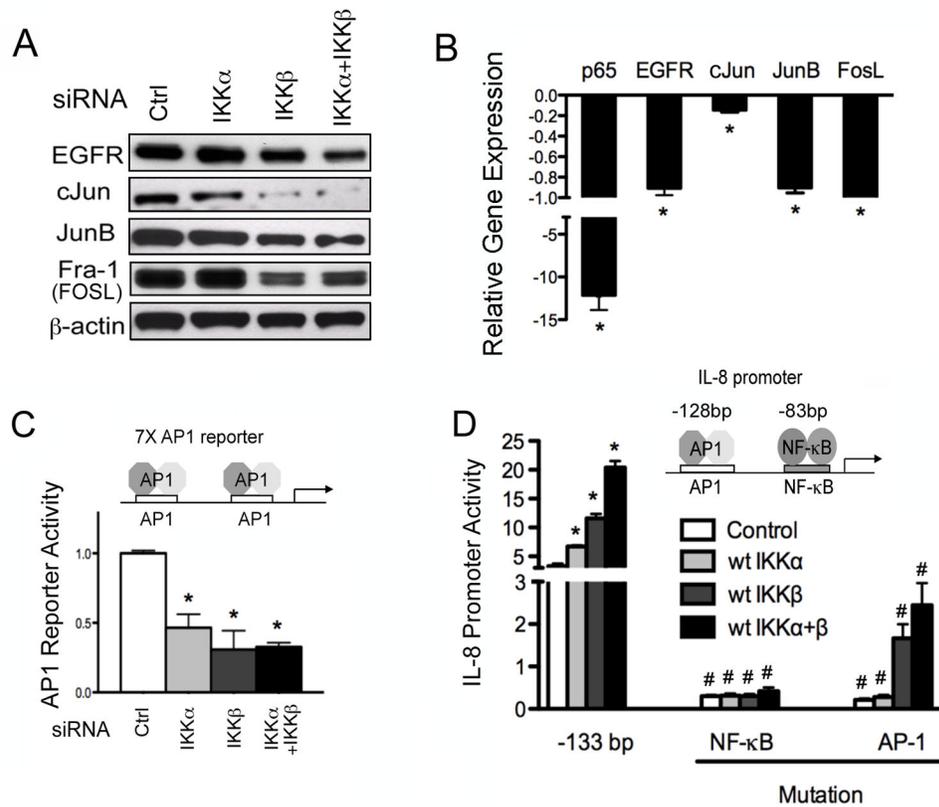
**Figure 6. Knockdowns of IKKs and NF-κB RelA subunits modulated EGFR and AP1 expression and activity**

A, Western blots of UM-SCC1 whole cell lysates showed decreased EGFR and AP1 subunit protein expression after IKK knockdowns. Histone1 as the loading control. B, Relative fold-change in gene expression of EGFR and AP1 subunit molecules after knockdown of NF-κB RelA(p65) in UM-SCC1 for 48 hours. C, Decreased AP1 reporter activity after knockdown of IKKs individually or in combination. D, Overexpressed wild-type IKKs significantly increased IL-8 promoter (−133bp) activity. Point mutation of NF-κB (−83bp) or AP1 (−128bp) binding site abolished or inhibited IL-8 reporter activity. $p < 0.05$ by t-test vs. controls (*), or vs. IL-8 promoter activity when overexpressing IKKs (#).
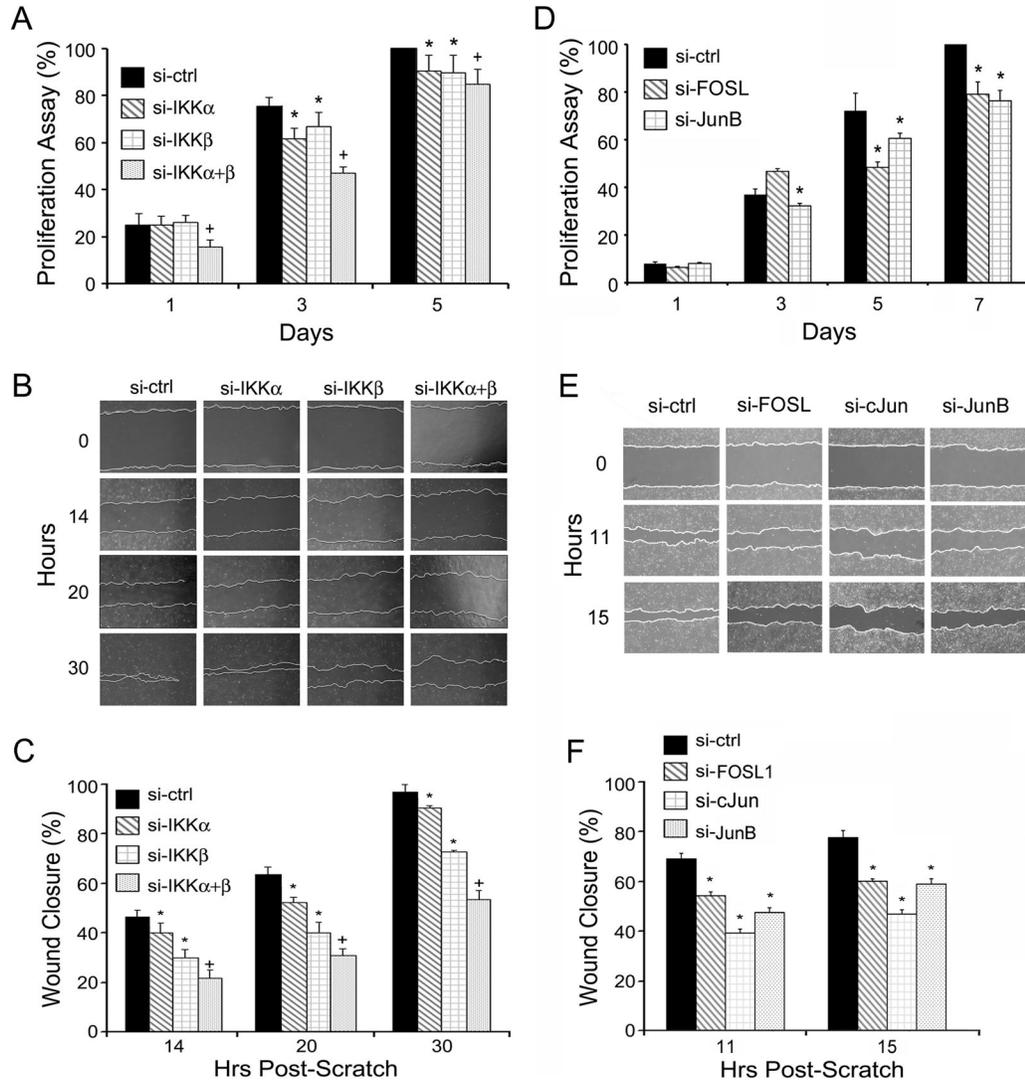
**Figure 7. Knockdown of IKKs or AP1 subunits by siRNAs potently inhibit cell proliferation and migration**

A, IKKα and IKKβ double knockdown by siRNAs potently inhibited cell proliferation over single IKK knockdowns by MTT assay. Statistical significance of t-test, * single knockdown: p<0.05; +double knockdown: P<0.01. B, UM-SCC1 cells were transfected with IKK specific siRNAs for 48 hrs. Scratches were made on cell monolayers and wound closure was monitored at 14, 20, and 30 hrs. C, Quantification of wound healing with statistical significance. The distances between the wounds were measured and significant differences by individual IKK knockdowns were examined by t-test (*, p<0.05), or in dual IKK knockdown (+, p<0.01). D, Knockdown of FOSL and JunB individually by siRNAs inhibited cell proliferation by MTT assay. Statistical significance of t-test, * p<0.05. E, AP1 subunits were individually knocked down for 48 hrs. Scratches were made and wound closure was monitored at 11, and 15 hrs under serum free condition. F, Quantification of wound healing with statistical significance. The distances between the wounds were measured and significant differences were examined by t-test. *, p<0.05.
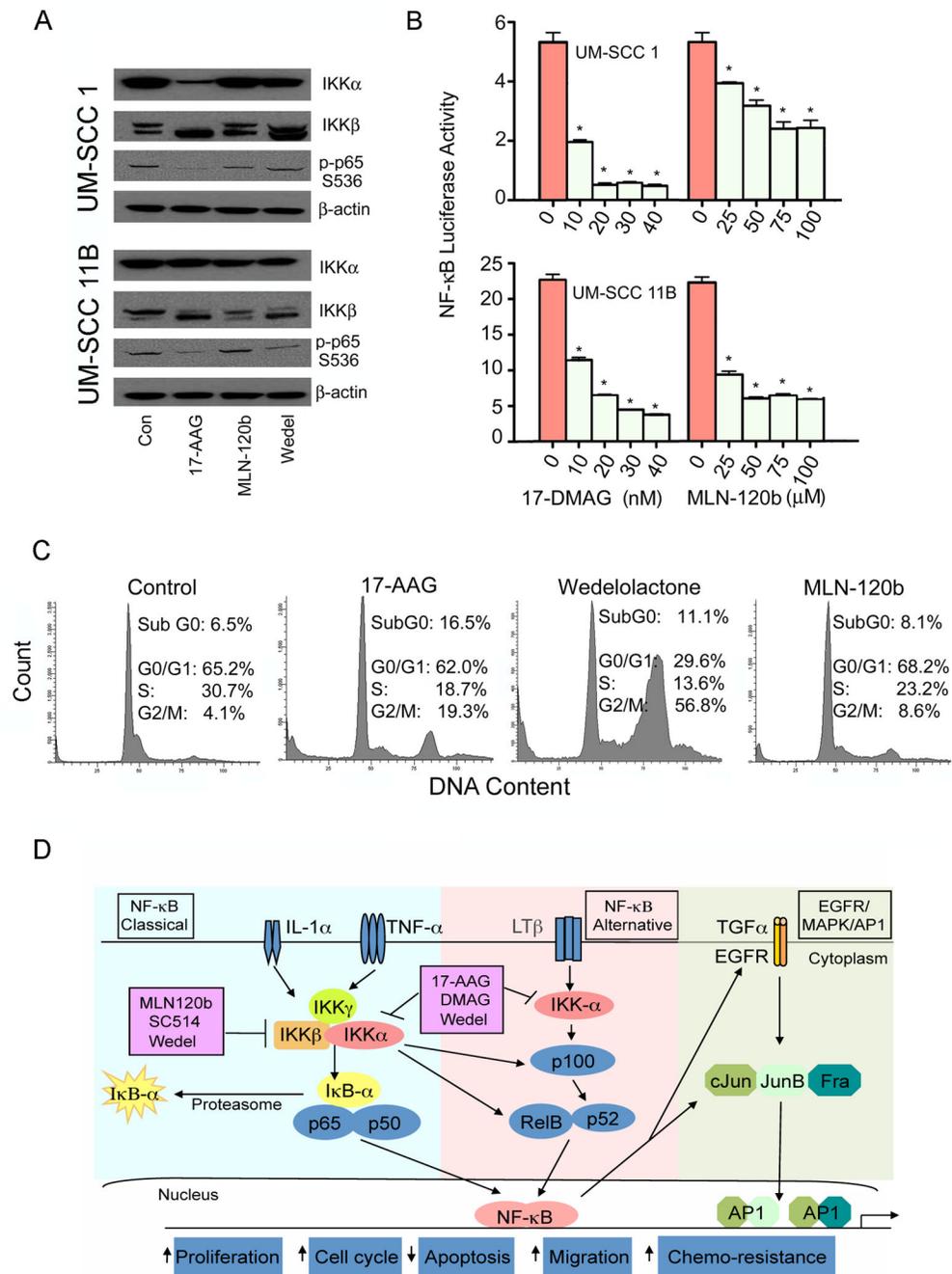
**Figure 8. Drugs targeting IKKα and IKKβ inhibit NF-κB activity, and promote cell cycle arrest and apoptosis of UM-SCC cells**

A, UM-SCC1 and UM-SCC11B cells were treated with 17-AAG (50nM), MLN-120b (40μM), Wedelolactone (20μM) for 24hrs. IKKα, IKKβ, and phospho-p-65 S536 protein were tested in whole cell lysates by Western blots. β-actin as the loading control. B, Inhibition of NF-κB activity in UM-SCC1 or UM-SCC11B cells transfected with constitutively activated IKKα or IKKβ EE expression vector then treated with various doses of 17-DMAG or MLN-120b for 48hrs. Data calculated from triplicates and presented as mean with standard deviation. *Statistical significance compared to controls (t-test, $p<0.05$).

C, Flow cytometry was performed to test cell-cycle and DNA fragmentation in sub-G0/G1 phase (% cell death) by UM-SCC1 and UM-SCC11B (data not shown) 24 hrs post treatment with 17-AAG (50nM), Wedelolactone (20μM), MLN-120b (40μM), and SC-514 (50μM, Supplemental Fig. 3A). D, Schematic of classical and alternative NF-κB pathway depicting the specific activation of IKK/NF-κB subunits as targets by each chemical inhibitors and the cross-induction of EGFR/AP1 expression and signaling.