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## Author Manuscript

*Mol Cancer Ther.* Author manuscript; available in PMC 2009 July 1.

Published in final edited form as:

*Mol Cancer Ther.* 2008 July ; 7(7): 1827–1835. doi:10.1158/1535-7163.MCT-08-0321.

## Active Roles for IKK $\alpha$ and IKK $\beta$ in NF- $\kappa$ B-mediated Chemoresistance to Doxorubicin

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### Abstract

Chemotherapy agents have been shown to induce the transcription factor NF- $\kappa$ B and subsequent chemoresistance in fibrosarcomas and other cancers. The mechanism of NF- $\kappa$ B-mediated chemoresistance remains unclear, with a previous report suggesting that doxorubicin induces this response independent of the inhibitory  $\kappa$ B kinases (IKKs). Other studies have indicated that IKK $\beta$ , but not IKK $\alpha$ , is required. Mouse embryo fibroblasts (MEF) devoid of IKK $\alpha$ , IKK $\beta$ , or both subunits (DKO) were treated with doxorubicin. The absence of either IKK $\alpha$  or IKK $\beta$  or both kinases resulted in impaired induction of NF- $\kappa$ B DNA-binding activity in response to doxorubicin. To provide a valid clinical correlate, HT1080 human fibrosarcoma cells were transfected with small interference RNAs (siRNAs) specific for IKK $\alpha$  or IKK $\beta$  and then subsequently treated with doxorubicin. Knockdown of IKK $\alpha$  severely impaired the ability of doxorubicin to initiate NF- $\kappa$ B DNA-binding activity. However, a decrease in either IKK $\alpha$  or IKK $\beta$  resulted in decreased phosphorylation of p65 in response to doxorubicin. The inhibition of doxorubicin-induced NF- $\kappa$ B activation by the knockdown of either catalytic subunit resulted in increased cleaved caspase 3 and cleaved PARP, and increased apoptosis when compared to doxorubicin alone. The results of this study validate current approaches aimed at NF- $\kappa$ B inhibition to improve clinical therapies. Moreover, we demonstrate that IKK $\alpha$  plays a critical role in NF- $\kappa$ B-mediated chemoresistance in response to doxorubicin and may serve as a potential target in combinational strategies to improve chemotherapeutic response.

### Keywords

IKKalpha; IKKbeta; NF-kappaB; Chemoresistance; Sarcoma

### Introduction

Nuclear factor  $\kappa$ B (NF- $\kappa$ B) is a transcription factor with critical roles in a number of inflammatory responses, as well as oncogenesis, metastasis and chemoresistance. There are five members of the NF- $\kappa$ B family: p50/p105 (NF- $\kappa$ B1), p52/p100 (NF- $\kappa$ B2), c-Rel, RelB, and p65 (RelA), characterized by the presence of the Rel homology domain which is involved in DNA binding, dimerization, and regulation by inhibitory proteins (1-3). NF- $\kappa$ B exists as hetero- and homodimers, with certain dimers (including the classic p50/p65 heterodimer)

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<sup>@</sup>Research was supported from both laboratories (H.J. Kim and A.S. Baldwin) equally

forming a complex with inhibitory  $\kappa$ B proteins (I $\kappa$ B). Stimulation of cells with inflammatory cytokines leads to activation of the canonical pathway, which is mediated by phosphorylation of the I $\kappa$ B proteins by inhibitory  $\kappa$ B kinases (IKKs) (2). The phosphorylated I $\kappa$ B proteins are targeted for ubiquitination and subsequent degradation by the 26S proteasome. Once freed from its binding proteins in the cytoplasm, NF- $\kappa$ B is able to accumulate in the nucleus and interact with regulatory elements and affect gene specific transcription (1,4).

NF- $\kappa$ B activation is known to regulate an array of downstream anti-apoptotic genes, such as cIAP-2 and Bcl-xL (5,6). It is this anti-apoptotic function of NF- $\kappa$ B that has implicated this family of transcription factors in the development, chemoresistance, and potential treatment of many cancers. In the field of oncology, NF- $\kappa$ B has been shown to be constitutively active in numerous neoplasms (7,8). However, NF- $\kappa$ B can also be activated in response to treatment with specific chemotherapy agents or irradiation. Previous work in our laboratory demonstrated that NF- $\kappa$ B is vital to the inducible chemoresistance of colon cancers. More specifically, inhibiting NF- $\kappa$ B activation in response to chemotherapy significantly enhanced the cytotoxic effects of camptothecin (9,10). This relationship has been reported in a variety of cancer cell types, including sarcomas (11,12). Soft tissue sarcomas remain a relatively rare malignancy, affecting approximately 8000 new patients each year (13). Despite recent advances in understanding the biology of many of these soft tissue sarcomas, surgery remains the mainstay of treatment since most sarcomas are largely resistant to chemotherapy (14,15). Surgery and radiotherapy can play important roles in the multimodality treatment of localized sarcomas, but the overall survival rate of less than 50% speaks to the chemoresistance of this malignancy in advanced stages (14,15). The signaling mechanisms involved in NF- $\kappa$ B-mediated chemoresistance have not yet been completely elucidated. In fact, some studies have challenged the notion of chemoresistance, suggesting that NF- $\kappa$ B can play a pro-apoptotic role in response to specific chemotherapy agents, including doxorubicin (16-18).

Overall, these preclinical results have generated increased pharmacologic interest in agents aimed at inhibiting the activation of the NF- $\kappa$ B signaling pathway. Inhibition of the 26S proteasome, which is involved in regulating I $\kappa$ B protein degradation, remains the strategy that has been studied most extensively. The generalized proteasome inhibitors have been tested in several early phase human clinical trials in both hematologic malignancies and solid tumors. In fact, Velcade, a proteasome inhibitor approved by the Federal Drug Administration, has shown efficacy in multiple myelomas, both alone and in combination with doxorubicin (19, 20). Unfortunately, clinical trials in solid tumors using Velcade have been limited by toxicity and a lack of significant improvement in survival (21-23). Additionally, there is increasing evidence that proteasome inhibitors have broad effects, with varied responses both related and non-related to NF- $\kappa$ B activation. As a result, efforts have been redirected toward finding targets for inhibition that have a greater specificity for the NF- $\kappa$ B pathway.

One such potential target in the NF- $\kappa$ B pathway is the IKK complex, which consists of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory protein known as IKK $\gamma$ /NEMO. This complex serves a critical role in the activation pathway of NF- $\kappa$ B by liberating it from its inhibitory molecules. Understanding the roles that these various subunits play in the activation of NF- $\kappa$ B in the setting of cancer and chemotherapy is critical to identifying more effective signaling targets. There are conflicting reports regarding the importance of the different IKK subunits in the NF- $\kappa$ B response to chemotherapy (24,25). Similar to the classical response seen with Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ), some cite an IKK $\beta$  dominance in mouse embryo fibroblasts (MEF) treated with doxorubicin (25). In a more recent report in a similar *in vitro* model, the same group suggests that an IKK-independent, PI3-kinase-dependent pathway is responsible for the late activation of NF- $\kappa$ B by doxorubicin (24). Delineating the role of the IKK subunits in NF- $\kappa$ B-mediated chemoresistance is crucial to the development of selective inhibitory strategies targeting these kinases. As such, examining this question using a clinically

relevant chemotherapy agent in human cancer cells will be important in gaining a better understanding of the signaling steps leading to NF- $\kappa$ B-mediated chemoresistance.

In this study, we used a human fibrosarcoma model to examine the potential involvement of IKK $\alpha$  and IKK $\beta$  in controlling the activation of NF- $\kappa$ B in response to the standard chemotherapy agent, doxorubicin. Additionally, we will further elucidate whether NF- $\kappa$ B activation is pro- or anti-apoptotic in this experimental setting.

## Materials and Methods

### Cell Culture

The human fibrosarcoma cell line HT1080 was obtained from American Type Culture Collection (ATCC; Rockville, MD). The HT1080 cells are grown in Minimum Essential Medium (MEM) Alpha containing L-glutamine supplemented with 10% fetal bovine serum and 100 $\mu$ g/ml penicillin and 100 $\mu$ g/ml streptomycin. Cell cultures were maintained at 37°C with a mixture of 95% air and 5% CO<sub>2</sub>. Mouse embryo fibroblast (MEF) cells devoid of IKK $\alpha$  (IKK $\alpha$ <sup>-/-</sup>), IKK $\beta$  (IKK $\beta$ <sup>-/-</sup>), or both IKK $\alpha$  and IKK $\beta$  (DKO) (generously donated by Dr. I. Verma and Dr. M. Karin) were grown in Dulbecco's Minimal Essential Medium supplemented with 10% fetal bovine serum and antibiotics as above. Cells were treated with doxorubicin (1.5 $\mu$ g/ml) and harvested at the time points indicated (1, 3, 6, 18, 24 hours). The positive control used for these experiments was treatment with TNF $\alpha$  (50ng/ml) for 15 minutes.

### Small Interference RNA (siRNA) Transfection

For transfection of siRNA in HT1080 cells 5 $\times$ 10<sup>4</sup> cells were plated in standard 6-well plates and incubated overnight. The transfections were then carried out according to the manufacturer's protocol. Briefly, the media was removed and replaced with transfection media containing siGENOME SMARTpool siRNA (sequences below) for siControl #1, siIKK $\alpha$ , or siIKK $\beta$  (final concentration 10-20nM) and DharmaFECT4 transfection reagent (Dharmacon, Inc., Lafayette, CO). The cells were maintained in transfection media for 24 hours. The transfection media was then removed and replaced with standard growth media. The cells were then grown for an additional 48 hours to achieve maximum protein knockdown. The cells were then treated with doxorubicin as described above. The knockdown of IKK proteins was confirmed with western blot.

### Western Blot Analysis

The media was removed and the cells were washed with phosphate buffered saline (PBS) and then lifted in the same buffer. Cytoplasmic extracts were prepared using a hypotonic buffer [10mM Hepes (pH 7.6), 60 mM KCL, 1mM EDTA, 1mM DTT, 0.2% NP-40, 1mM PMSF with 1 $\mu$ l each of aprotinin, leupeptin, and pepstatin]. The extracts were isolated by sequential centrifugation at 2500rpm for 4 minutes to separate the nuclei and then at 13,000rpm for 5 minutes to remove any remaining cell fragments. Whole cell extracts were obtained using a lysis buffer [20mM Tris-HCL (pH 7.5), 150mM NaCl, 1mM Na<sub>2</sub>EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM  $\beta$ -glycerophosphate, 1mM Na<sub>3</sub>VO<sub>4</sub>] supplemented with protease and phosphatase inhibitors. The cells were incubated on ice in the whole cell lysis buffer for 5 minutes and the cellular fragments were then separated using centrifugation at 13,000rpm for 10 minutes.

Western blot analysis was then performed following the protocol provided by Invitrogen (Carlsbad, CA) by separating proteins (20-30 $\mu$ g) on NuPAGE 4-12% Bis-Tris gels. Antibodies for I $\kappa$ B $\alpha$ ,  $\beta$ -Tubulin, GAPDH, and p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used at a dilution of 1:1000 in 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in TBS-T. Antibodies for caspase 3, cleaved PARP, PARP (Cell Signaling Technology, Inc.,

Danver, MA) were also used at 1:1000 dilution, while antibodies for IKK $\alpha$ , IKK $\beta$ , phosphorylated p65 at serine 536, and cleaved caspase 3 (Cell Signaling Technology, Inc., Danver, MA) were used at a 1:500 dilution. The blots were incubated with the primary antibody overnight at 4°C. The membranes were then incubated with secondary anti-rabbit antibody diluted 1:10,000 in TBS-T for 1 hour and then developed using Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, England).

### TUNEL Assay

HT1080 cells transfected with siRNA as described above. The cells were then treated with doxorubicin (1.5 $\mu$ g/ml) for 24 hours. The cells were then trypsinized and isolated by centrifugation at 2500 rpm for 5 minutes. The cell pellet was then washed with cold PBS and fixed with 10% formalin. The cells were then embedded in paraffin, sectioned, and mounted on microscope slides. Apoptosis was then measured by labeling DNA strand breaks using the TUNEL method with the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) following the manufacturer's protocol. Results are reported as the percentage of 1000 cells that stained positive. Experiments were conducted in triplicate.

### Electrophoretic Mobility Shift Assay (EMSA)

Cells were harvested after treatment as indicated above, and the EMSAs performed as previously reported (26). In brief, the cytoplasmic extracts were isolated as described above. The nuclei were then suspended in a high-salt buffer (20mM Tris pH 8.0, 420 mM NaCl, 1.5mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1mM PMSF, 25% glycerol, and 1 $\mu$ l each of aprotinin, leupeptin, and pepstatin) for ten minutes. The samples were then placed in a centrifuge at 13,000 rpm for 10 minutes. The supernatant was then separated and protein concentration determined with Bradford assay. Approximately 5 $\mu$ g of nuclear extract was incubated with 1 $\mu$ g/ $\mu$ l polyIdC in binding buffer (50mM Tris pH 7.6, 5mM DTT, 2.5mM EDTA, 50% glycerol) with an oligonucleotide radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP for 15 minutes at room temperature. The probe contains an NF- $\kappa$ B consensus binding site for the H-2 $\kappa$ B promoter (5'-GGGGATTCCC-3'). The samples were then separated on a polyacrylamide gel and developed with autoradiography. For supershifts, the nuclear extract was incubated in binding buffer with 1 $\mu$ l of p65X, p50X (200 $\mu$ g/0.1ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p52, RelB, or cRel (200 $\mu$ g/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 15 minutes and then incubated for an additional 15 minutes with polyIdC and the radiolabeled probe.

### Statistics

All experiments were conducted in triplicate to ensure the accuracy of the results. Statistical comparison was conducted for the TUNEL assay using normal approximation with the assistance of the University of North Carolina Biostatistics Core.

## Results

### Doxorubicin induces NF- $\kappa$ B DNA-binding in MEF cells through both IKK $\alpha$ and IKK $\beta$

In order to examine the roles of the catalytic subunits of the IKK complex in doxorubicin-induced NF- $\kappa$ B activation, wild type (WT) MEF cells and MEF cells devoid of IKK $\alpha$ , IKK $\beta$  or both subunits were stimulated with doxorubicin (1.5 $\mu$ g/ml). Cells were harvested at 3h, 6h, 18h, and 24h time points and nuclear extracts were prepared and evaluated for NF- $\kappa$ B DNA-binding activity using EMSA (Figure 1A). Induction by TNF $\alpha$  served as a positive control and untreated cells served as a negative control. WT MEFs demonstrated basal NF- $\kappa$ B DNA-binding activity, which was significantly increased when exposed to TNF $\alpha$  or doxorubicin (Figure 1A). The time course of induction was similar to that seen in other *in vitro* human cancer models in response to clinically relevant chemotherapeutic agents (9, 12). Supershift

analysis demonstrated that the activated form of NF- $\kappa$ B seen with doxorubicin consists of p65, p50 and p52 subunits as evidenced by the shifting of the complex in the p65 column and the decreased binding of the probe in the p50 and p52 columns (Figure 1B).

In contrast to the WT MEFs, both the IKK $\alpha$ <sup>-/-</sup> MEFs and IKK $\beta$ <sup>-/-</sup> MEFs demonstrated impaired NF- $\kappa$ B DNA-binding activity at baseline and in response to both TNF $\alpha$  and doxorubicin (Figure 1A). More specifically, the lack of IKK $\alpha$  nearly completely abolished the doxorubicin-induced NF- $\kappa$ B activity, whereas the absence of IKK $\beta$  was less effective in deterring NF- $\kappa$ B DNA-binding activity in response to doxorubicin. These results support a significant role for IKK $\alpha$  in the NF- $\kappa$ B response to doxorubicin in MEF cells. It is important to note that the genetic depletion of either catalytic subunit in isolation was not sufficient to completely prevent the activation of NF- $\kappa$ B. Moreover, the fact that double knockout (DKO) MEFs are deficient in NF- $\kappa$ B DNA-binding activity basally and in response to both TNF $\alpha$  and doxorubicin (Figure 1A) indicates that the signaling from doxorubicin to NF- $\kappa$ B does in fact occur through both IKK $\alpha$  and IKK $\beta$  subunits.

### Doxorubicin activates NF- $\kappa$ B and induces I $\kappa$ B $\alpha$ degradation in HT1080 cells

Although sarcomas are largely chemoresistant, doxorubicin remains a standard cytotoxic agent in the treatment of patients with systemic or locally advanced disease. Therefore we analyzed the pattern of NF- $\kappa$ B activation in HT1080 fibrosarcoma cells in response to doxorubicin (1.5 $\mu$ g/ml). Cytoplasmic and nuclear extracts were harvested following 1, 3, 6, 18, and 24 hours of treatment with doxorubicin. TNF $\alpha$  served as a positive control and untreated cells functioned as a negative control. Interestingly, stimulation with doxorubicin resulted in a biphasic pattern of NF- $\kappa$ B DNA-binding activity (Figure 2A). The peak activation was identified at 3-6 hours, similar to the MEF cells. However, rather than a steady decline of activity over time, the HT1080 cells demonstrated a small increase in activity seen again from 18 hours to 24 hours of treatment with doxorubicin. Supershift analysis confirmed that the activated NF- $\kappa$ B complex consisted of both p65 and p50 subunits (Figure 2A).

Next, the effects of doxorubicin treatment on the protein levels of I $\kappa$ B $\alpha$  were examined. In contrast to the pattern seen with NF- $\kappa$ B DNA-binding seen on EMSA, the decrease in I $\kappa$ B $\alpha$  persisted throughout the treatment course (Figure 2B). The initial decrease in I $\kappa$ B $\alpha$  was identified at the 6-hour time point, which coincides with the first peak in NF- $\kappa$ B nuclear activity. However, I $\kappa$ B $\alpha$  protein levels continued to decrease at later time points despite the weaker NF- $\kappa$ B DNA-binding activity. We attribute the initial decrease in I $\kappa$ B $\alpha$  at the 6-hour time point to degradation by the 26S proteasome as it correlates with the initial increase in NF- $\kappa$ B DNA-binding activity. However, the mechanism leading to the decrease in I $\kappa$ B $\alpha$  protein levels with prolonged exposure to doxorubicin remains unclear.

### IKK $\alpha$ is critical to Doxorubicin-induced NF- $\kappa$ B DNA-binding activity in HT 1080 cells

The roles for IKK $\alpha$  and IKK $\beta$  in the induction of NF- $\kappa$ B DNA-binding activity by doxorubicin in HT1080 cells were delineated using siRNA strategies to knock down the expression of IKK subunits. Small interference RNA for IKK $\alpha$  (10nM) or IKK $\beta$  (20nM) was transfected into HT1080 cells to achieve a selective decrease in each catalytic subunit. The cells were then stimulated with doxorubicin (1.5 $\mu$ g/ml) for 6 hours, which was previously identified as the point of maximal NF- $\kappa$ B DNA-binding activity. Nuclear extracts were evaluated by EMSA to assess NF- $\kappa$ B DNA-binding activity and cytoplasmic extracts were evaluated with western blot to determine the protein levels of the IKK subunits. The positive control in this experiment was HT1080 cells treated with doxorubicin alone. In order to control for effects of the transfection alone, a nonspecific control siRNA was employed. We demonstrated a decrease in doxorubicin-induced NF- $\kappa$ B DNA-binding only in the absence of IKK $\alpha$  (Figure 3A). Moreover, the knockdown of IKK $\beta$  did not significantly alter the activation of NF- $\kappa$ B at the

nuclear level by doxorubicin (Figure 3A). Importantly, the siRNA treatments were successful in achieving a selective and substantial decrease in the IKK $\alpha$  and IKK $\beta$  subunits (Figure 3B). These results are similar to those seen in the MEF cells and support an important role for IKK $\alpha$  in the signaling of doxorubicin to NF- $\kappa$ B.

### **Both IKK $\alpha$ and IKK $\beta$ are important to I $\kappa$ B $\alpha$ degradation and p65 phosphorylation**

In order to further evaluate the activation of NF- $\kappa$ B by doxorubicin, the effects of knocking down IKK $\alpha$  and IKK $\beta$  on I $\kappa$ B $\alpha$  degradation and p65 phosphorylation were studied. For I $\kappa$ B $\alpha$  degradation, selective depletion of either IKK $\alpha$  or IKK $\beta$  was achieved using siRNA and the HT1080 cells were stimulated with doxorubicin for 6 hours. Cellular extracts were then prepared and evaluated for I $\kappa$ B $\alpha$  protein levels. Treatment with doxorubicin alone caused a substantial degradation of I $\kappa$ B $\alpha$ . Interestingly, the knockdown of either catalytic subunit resulted in an increase in I $\kappa$ B $\alpha$  at baseline (Figure 3B, lanes 3 and 4) as well as a decrease in the doxorubicin-induced degradation of I $\kappa$ B $\alpha$  (Figure 3B, lanes 7 and 8). As such, the functions of IKK $\alpha$  and IKK $\beta$  appeared to mirror each other and have comparable roles in the signaling of doxorubicin to NF- $\kappa$ B in response to doxorubicin.

Additionally, we analyzed the effects of IKK $\alpha$  and IKK $\beta$  on the phosphorylation state of the p65 subunit as a result of exposure to doxorubicin. The phosphorylation site analyzed in these experiments was the serine residue at position 536. This particular phosphorylation site has been shown to be important in NF- $\kappa$ B signaling in response to various cytokines (27,28). Moreover, both IKK $\alpha$  and IKK $\beta$  have been shown to be capable of phosphorylating this site (28,29). For these experiments, whole cell extracts were prepared following siRNA transfection and doxorubicin treatment for 6 hours. The exposure to doxorubicin generated a high level of phosphorylated p65 that was not affected by the transfection of the control siRNA (Figure 3C). However, the depletion of either IKK $\alpha$  or IKK $\beta$  significantly decreased the amount of phosphorylation of p65. The results clearly show that in HT1080 cells both IKK $\alpha$  and IKK $\beta$  are required for phosphorylating the p65 subunit in response to doxorubicin. These results again support a critical role for both IKK $\alpha$  and IKK $\beta$  in the signaling pathway that enables fibrosarcoma cells to activate NF- $\kappa$ B when exposed to the DNA damage agent, doxorubicin.

### **Deletion of either IKK $\alpha$ or IKK $\beta$ increases the cytotoxicity of doxorubicin in HT1080 cells**

Having identified that IKK $\alpha$  has a dominant role in NF- $\kappa$ B DNA-binding activity, and that both IKK $\alpha$  and IKK $\beta$  subunits are important kinases in the phosphorylation of the p65 subunit in the face of doxorubicin, the question remains whether the deletion of IKK $\alpha$  or IKK $\beta$  would affect the overall survival of HT1080 cells treated with doxorubicin. In order to answer this question, siIKK $\alpha$  or siIKK $\beta$  was transfected into HT1080 cells that were subsequently stimulated with doxorubicin. The outcomes of these combination treatments on the rate of apoptosis in HT1080 cells were evaluated in two ways. First, cells treated with doxorubicin for 6 hours were harvested and the cellular extracts were evaluated for processing of caspase 3 and PARP, both markers of apoptosis. We demonstrate that transfection alone with either siControl, siIKK $\alpha$ , or siIKK $\beta$  was able to induce some cleavage of PARP at baseline. Notably, the knockdown of IKK $\alpha$  generated the largest increase in cleaved PARP and the only detectable levels of cleaved caspase 3 in unstimulated cells (Figure 4A, lanes 1-4). When evaluating the effects of the deletion of IKK $\alpha$  and IKK $\beta$  on the cytotoxicity of doxorubicin, the results demonstrate that the absence of either catalytic subunit results in a significant increase in both cleaved caspase 3 and cleaved PARP when compared to doxorubicin alone (Figure 4A, lanes 5-8). It is important to note that transfection of the control siRNA also increased the levels of these apoptotic markers when combined with doxorubicin suggesting that transfection alone can be mildly toxic when combined with chemotherapy. Nevertheless, the depletion of IKK $\alpha$  or IKK $\beta$  resulted in even greater toxicity than the control siRNA in the doxorubicin

treatment groups confirming that both of these subunits are important to the NF- $\kappa$ B mediated chemoresistance of HT1080 cells to doxorubicin.

Next, in order to confirm the results seen with cellular markers of apoptosis and to more quantitatively measure the rates of apoptosis, a TUNEL assay was conducted. HT1080 cells were transfected with siIKK $\alpha$ , siIKK $\beta$ , or siControl and subsequently treated with doxorubicin for 24 hours. The cells were stained as previously described and the number of positive, or apoptotic, cells per 1000 total cells were counted. The experiment was conducted in triplicate and the results are reported as the overall percentage of apoptotic cells. The combination of IKK $\alpha$  deletion and doxorubicin resulted in a 57% (54-59%, 95% CI) increase in the incidence of apoptosis compared to doxorubicin alone and a 42% (39-44%, 95% CI) increase when compared to siIKK $\alpha$  alone (Figure 4B). When the cells were treated with siIKK $\beta$ , the results were similar with a 59% (56-62%, 95% CI) increase in apoptosis compared to chemotherapy alone and a 43% (40-45%, 95% CI) increase compared to siIKK $\beta$  alone. Moreover, the percentage of apoptosis in combination treatment groups was also greater than the sum of the individual treatments.

Together, these results confirm that the activation of NF- $\kappa$ B in fibrosarcoma cells functions in an anti-apoptotic manner and is directly linked to the resistance of these cells to doxorubicin. Furthermore, both IKK $\alpha$  and IKK $\beta$  subunits serve a critical role in the signaling pathways involved in the NF- $\kappa$ B-mediated chemoresistance of fibrosarcomas.

## Discussion

The transcription factor NF- $\kappa$ B has been implicated as an important mediator in a number of cellular processes from inflammation to cancer. In neoplasms, NF- $\kappa$ B has received specific attention for its role in oncogenesis (2,4). In addition, inducible chemoresistance is attributed to the activation of NF- $\kappa$ B, which in turn stimulates the transcription of anti-apoptotic genes enabling cells to overcome chemotherapy-induced apoptosis, or programmed cell death (5,6). Previous studies from our laboratory and others have supported this concept by demonstrating that inhibition of NF- $\kappa$ B activation sensitizes neoplastic cells to the genotoxic effects of their relevant anticancer therapies (9,10,12). Coincidentally, there has been increasing pharmacologic interest in inhibition strategies aimed at NF- $\kappa$ B in an effort to improve response rates in chemoresistant cancers. Currently, the only NF- $\kappa$ B inhibitors used clinically are generalized proteasome inhibitors, which clearly have broad effects. As a result, these strategies in early phase clinical trials have been limited in solid tumors by lack of efficacy and increased toxicity (21-23). More recent efforts have focused on identifying more selective inhibitory targets in the NF- $\kappa$ B pathway.

As the role of NF- $\kappa$ B in the chemotherapy response is being elucidated, several recent studies have challenged the notion that the induction of NF- $\kappa$ B leads to an anti-apoptotic response; the authors suggest that depending on the cellular context, NF- $\kappa$ B activation can be required for chemotherapy to cause cell death (16,17,30,31). Therefore, delineating the role of NF- $\kappa$ B activation in specific tumors in response to clinically relevant chemotherapies is critical. Then, further analysis of the mechanisms behind the induction of NF- $\kappa$ B could identify more effective targets for NF- $\kappa$ B inhibition strategies with the potential to improve cytotoxic response rates.

In this regard, physiological stimuli, such as TNF $\alpha$  or LPS, have been reported to activate the "canonical pathway" leading to IKK $\beta$ -dependent phosphorylation and degradation of I $\kappa$ B $\alpha$  and translocation of NF- $\kappa$ B to the nucleus (32). On the other hand, IKK $\alpha$  has been implicated in the noncanonical or alternative pathway, leading to the processing of p100 and the generation of free p52/RelB dimers (33,34). In chemotherapy models, initial studies examining NF- $\kappa$ B induction in response to chemotherapy agents, like doxorubicin, published similar results

labeling IKK $\beta$  as the critical catalytic subunit (25). Recently, it has also been shown that doxorubicin can initiate I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B activation independent of either IKK $\alpha$  or IKK $\beta$  using the same *in vitro* models (24). Moreover, some studies indicate that sumoylation of NEMO is important in NF- $\kappa$ B activation in response to DNA damage agents reportedly through a complex interaction involving the p53-inducible death-domain-containing protein (PIDD) (35,36). As these results unfold, the role of the IKK subunits in response to DNA damaging agents remains unclear; whether IKK $\alpha$  and IKK $\beta$  serve independent, overlapping or complementary roles is yet to be determined. Further clarification of these mechanisms will be important in isolating potential therapeutic targets within the IKK complex relative to NF- $\kappa$ B-mediated chemoresistance.

In this study, we sought to further clarify the roles of IKK $\alpha$  and IKK $\beta$  in the activation of NF- $\kappa$ B in response to doxorubicin, a DNA-damage agent known to induce NF- $\kappa$ B activity in a variety of malignancies (30,31,37). By first examining the effects of doxorubicin in MEF cells devoid of either IKK $\alpha$ , IKK $\beta$ , or both subunits, we demonstrated that early NF- $\kappa$ B activation is clearly not IKK-independent as suggested in other published reports (24). In addition, IKK $\alpha$  was found to have a more significant role compared to IKK $\beta$  in the doxorubicin-induced NF- $\kappa$ B DNA-binding activity as measured by EMSA. However, deleting IKK $\alpha$  did not result in complete abolition of NF- $\kappa$ B activity, suggesting that the induction of NF- $\kappa$ B by doxorubicin is not entirely independent of IKK $\beta$ .

In regards to the role these IKKs play in an oncologic setting, we felt it imperative to examine these relationships using a human cancer cell line in which doxorubicin has clinical relevance. Doxorubicin, in combination with ifosfamide, is well recognized to be a treatment of locally advanced and metastatic sarcomas, although the reality remains that clinical response rates are less than 30% and overall survival rates remain dismal for advanced stage disease (14). As such, we elected to use HT1080 human fibrosarcoma cells manipulated with siRNA strategies to deplete either IKK $\alpha$  or IKK $\beta$  in order to further study the significance of both catalytic subunits in the doxorubicin-induced activation of NF- $\kappa$ B.

Interestingly, doxorubicin-induced NF- $\kappa$ B DNA-binding activity was bimodal with its strongest peak at 3-6 hours post-treatment and a reproducible second weaker increase in DNA-binding noted at 18-24 hours. Others have found that NF- $\kappa$ B activity can oscillate over time in response to the TNF $\alpha$  as a function of the levels of inhibitory  $\kappa$ B proteins in the cell, but this work has not been previously reported when cells have been exposed to chemotherapy (38). Our analysis demonstrated that I $\kappa$ B $\alpha$  protein levels did decrease throughout the duration of doxorubicin treatment and did not correlate with the level of nuclear activity of NF- $\kappa$ B as measured by EMSA. As such, it is possible that this delayed decrease in inhibitory  $\kappa$ B proteins in the face of doxorubicin is the source for the second increase in NF- $\kappa$ B nuclear activity. Further studies will be needed in order to determine significance of these oscillations in nuclear activity and their affects on apoptosis and gene expression.

In order to further clarify the roles of IKK $\alpha$  and IKK $\beta$  in the NF- $\kappa$ B response to doxorubicin, we focused on the early maximal activation of NF- $\kappa$ B seen following 6 hours of exposure to doxorubicin. In our *in vitro* model, the depletion of the IKK $\alpha$  subunit resulted in significant inhibition of doxorubicin-induced NF- $\kappa$ B DNA-binding activity, while the knockdown of IKK $\beta$  had no effect. In order to better understand the significance of these two subunits in the signaling pathways that generate NF- $\kappa$ B activation, we examined I $\kappa$ B $\alpha$  degradation and the phosphorylation of p65. Rather than identifying a dominant subunit, as in the case of NF- $\kappa$ B DNA-binding activity, we demonstrated a shared responsibility in both of these processes. More specifically, the knockdown of IKK $\alpha$  and IKK $\beta$  blunted the ability of doxorubicin to initiate the degradation of I $\kappa$ B $\alpha$  as well as decreased the phosphorylation of p65 at the serine536 residue following doxorubicin treatment. This further supports the concept that both IKK $\alpha$  and



IKK $\beta$  possess the ability to phosphorylate the p65 subunit, and stresses that the absence of either catalytic subunit results in a less profound activation of NF- $\kappa$ B by doxorubicin.

Having identified similar, important roles for both the IKK $\alpha$  and IKK $\beta$  subunits in the NF- $\kappa$ B response to doxorubicin, it was critical to evaluate what effect losing one of these subunits would have on the cytotoxicity of the drug. The evaluation of cellular markers of apoptosis (cleaved PARP and cleaved caspase 3) and quantification of the overall percentage of apoptosis using TUNEL staining revealed that knocking down either IKK $\alpha$  or IKK $\beta$  results in a significant increase in the cytotoxic effects of doxorubicin. Although there was some toxicity associated with the transfection process itself, the combination therapies involving deletion of IKK $\alpha$  or IKK $\beta$  together with doxorubicin resulted in greater toxicity than the sum of the individual treatments.

Given the complexity of the signaling pathway, it will be important to continue exploring the role of these catalytic subunits as well as the importance of the regulatory subunit NEMO in the NF- $\kappa$ B response to DNA damage. Additionally, recent reports have suggested that the NF- $\kappa$ B activity is regulated through p53. Since HT1080 cells maintain wild type p53, it will be important to examine our model of chemoresistance in p53 null sarcoma cells (39).

In conclusion, the results of our study support a novel and important role for IKK $\alpha$  in NF- $\kappa$ B-mediated chemoresistance in fibrosarcomas. This further validates the potential for developing biological means of inhibiting the catalytic IKK subunits. These preclinical studies using human cancer cell lines and clinically relevant cytotoxic agents are important to identify mechanisms of chemoresistance and identify potential targets for future therapeutic strategies. In these aggressive malignancies, a large proportion of the patients present with advanced incurable stage of disease, with treatments limited to systemic chemotherapy. Ultimately, pharmacologic agents aimed at selective inhibition of the NF- $\kappa$ B response to current chemotherapy agents will lead to combinational approaches to improve outcomes in these chemoresistant malignancies.

## Acknowledgements

Funding for this research was supported by the following grants: R01-CA73756, R01-CA75080, K08-CA098240, GI SPORE P50-CA10699-01, T32-CA09688

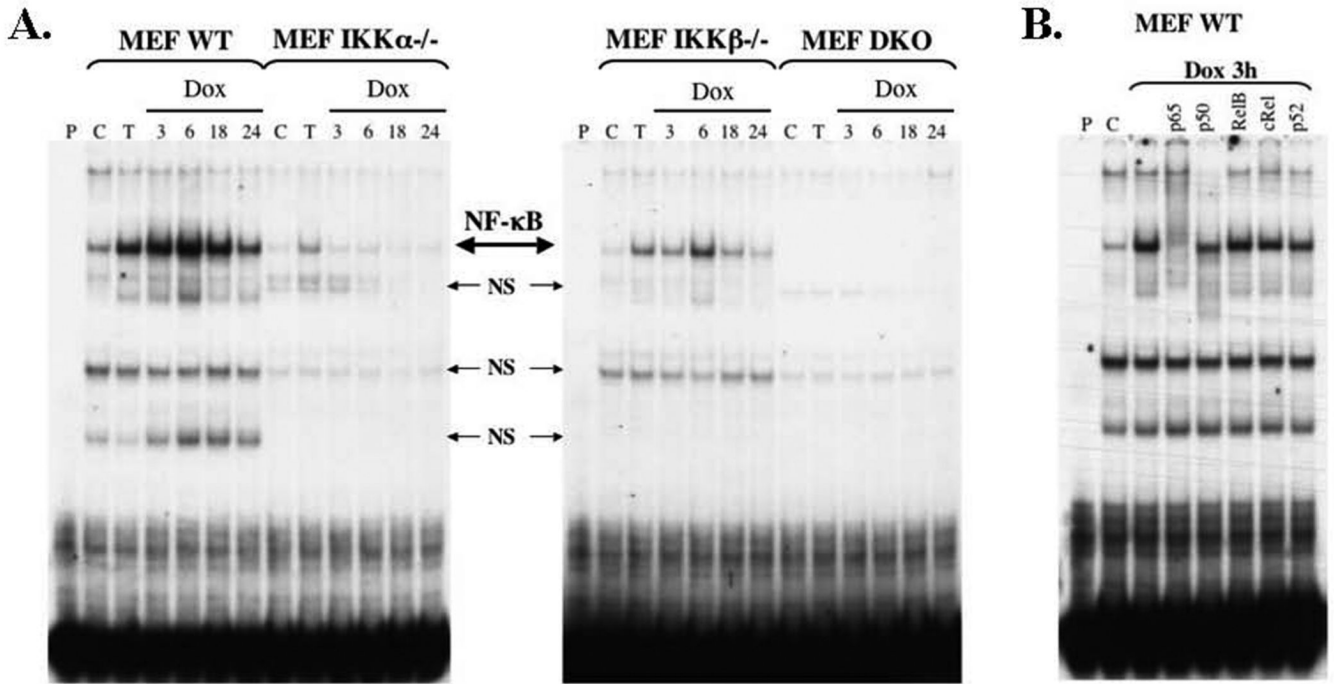
## Abbreviations

IKK, Inhibitory  $\kappa$ B kinase  
siRNA, small interference RNA  
DKO, double knockout  
MEF, mouse embryo fibroblast  
NF- $\kappa$ B, nuclear factor  $\kappa$ B  
I $\kappa$ B, inhibitor  $\kappa$ B  
NEMO, NF- $\kappa$ B essential modulator  
TNF $\alpha$ , tumor necrosis factor alpha  
WT, wild type  
EMSA, electrophoretic mobility shift assay  
TUNEL, terminal transferase dUTP nick end labeling  
PIDD, p53-inducible-death-domain-containing-protein  
LPS, lipopolysaccharide

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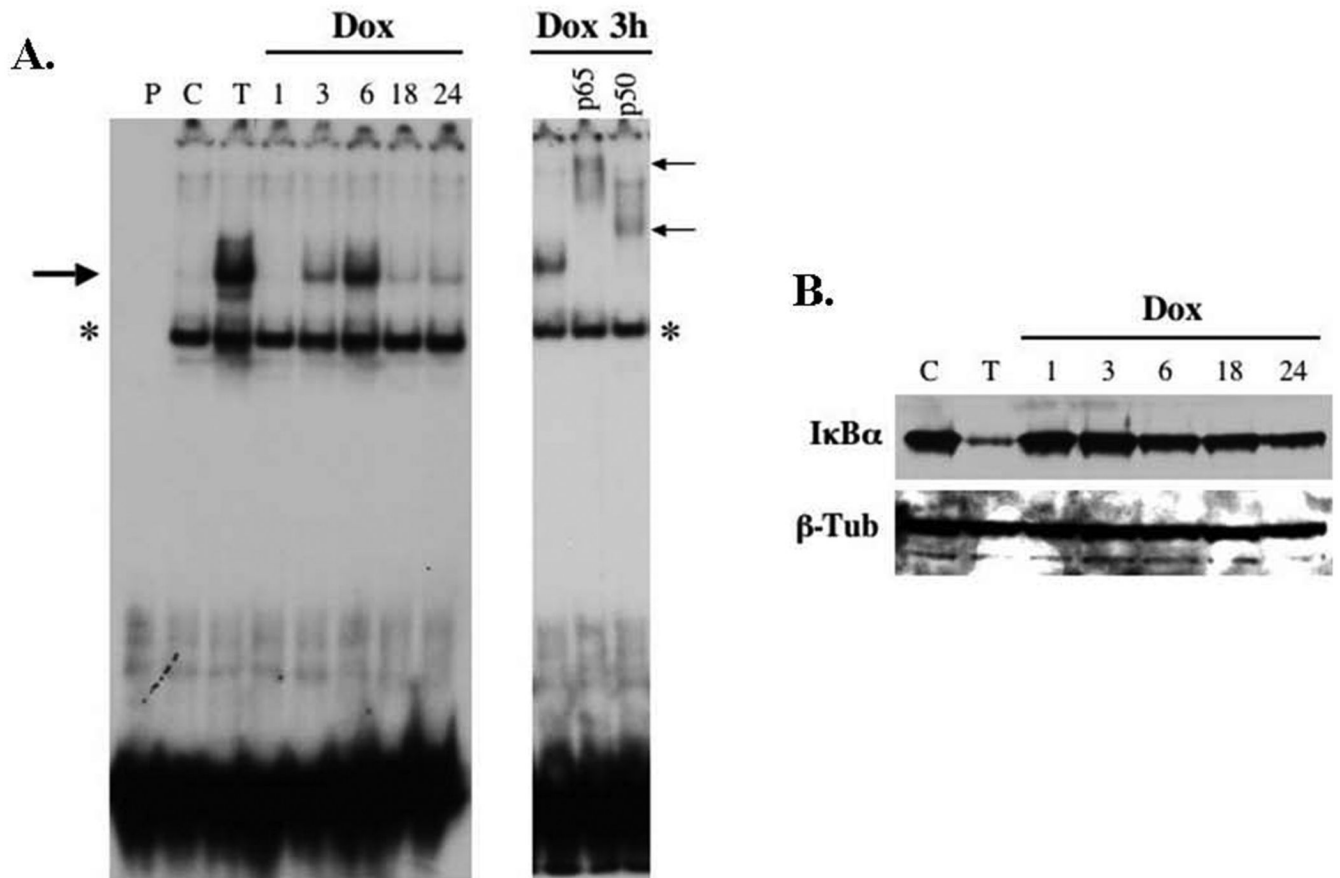
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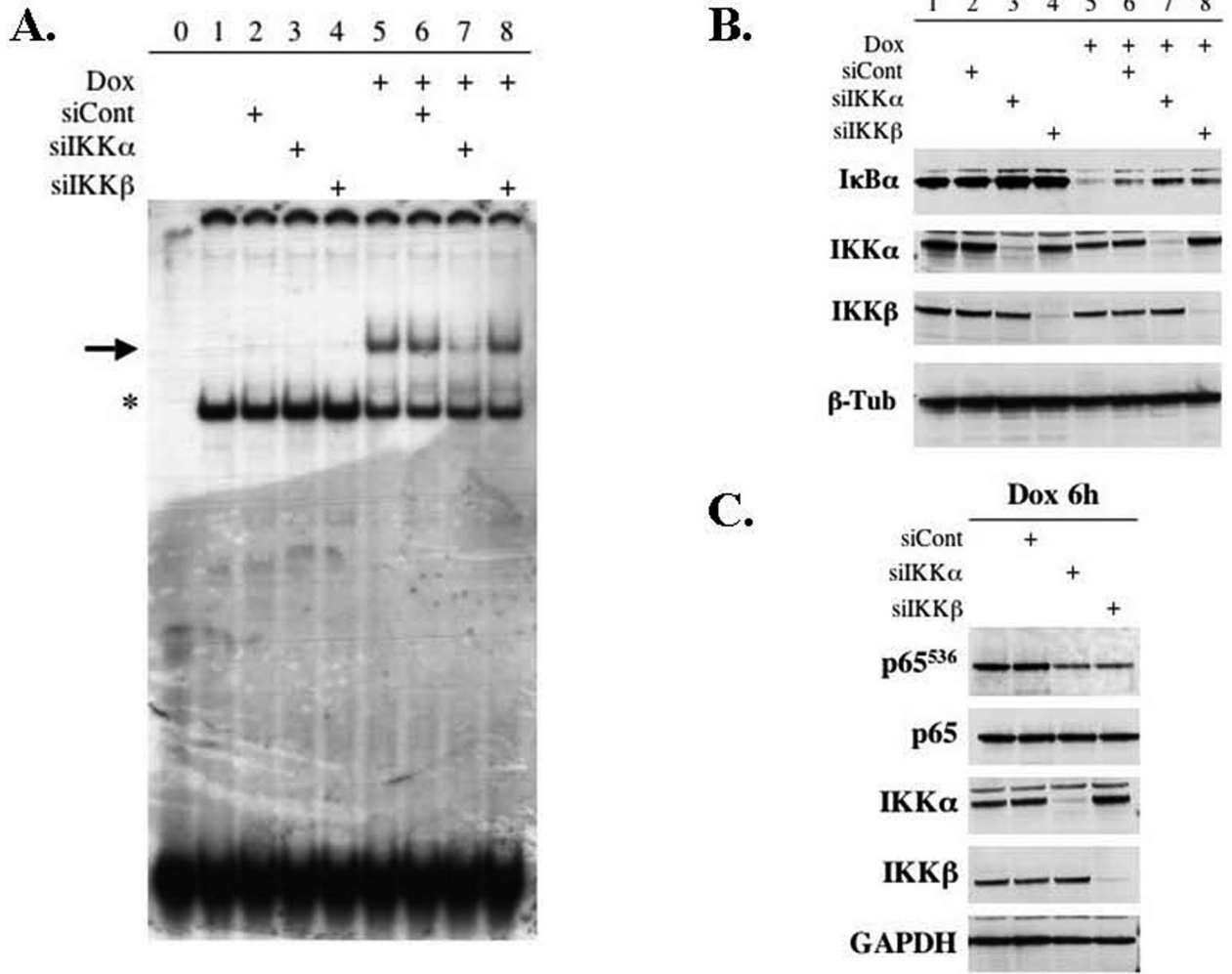
**Figure 1.**

(A) In mouse embryo fibroblasts (MEF), deletion of IKK $\alpha$  impairs nuclear binding of NF- $\kappa$ B (large arrow) in response to doxorubicin (Dox) to a greater extent than deletion of IKK $\beta$ . Wild type (WT), IKK $\alpha$ <sup>-/-</sup>, IKK $\beta$ <sup>-/-</sup> and double knockout (DKO) MEFs were stimulated with TNF $\alpha$  (T) or Dox at a final concentration of 1.5 $\mu$ g/ml for 3, 6, 18, or 24 hours. The results of these treatments were compared to untreated controls (C). The first lane in each gel (P) consists of only the radiolabeled probe, which is collected at the bottom of the gel. The IKK $\alpha$ <sup>-/-</sup> MEFs demonstrated impaired basal NF- $\kappa$ B DNA-binding activity as well as a significant decrease in induced NF- $\kappa$ B DNA-binding activity by both TNF $\alpha$  and dox. The results for IKK $\beta$ <sup>-/-</sup> MEFs were similar in regards to basal and TNF $\alpha$ -induced NF- $\kappa$ B DNA-binding, but the lack of IKK $\beta$  only mildly decreased the ability of doxorubicin to generate NF- $\kappa$ B activity. (B) Supershift analysis performed on the WT MEFs demonstrated that the activated NF- $\kappa$ B complex seen with doxorubicin in MEF cells consists mainly of p65 subunits and to a lesser extent p50 and p52 subunits. Moreover, the use of antibodies to NF- $\kappa$ B subunits clarifies that the additional lighter bands seen are non-specific (small arrows, NS).

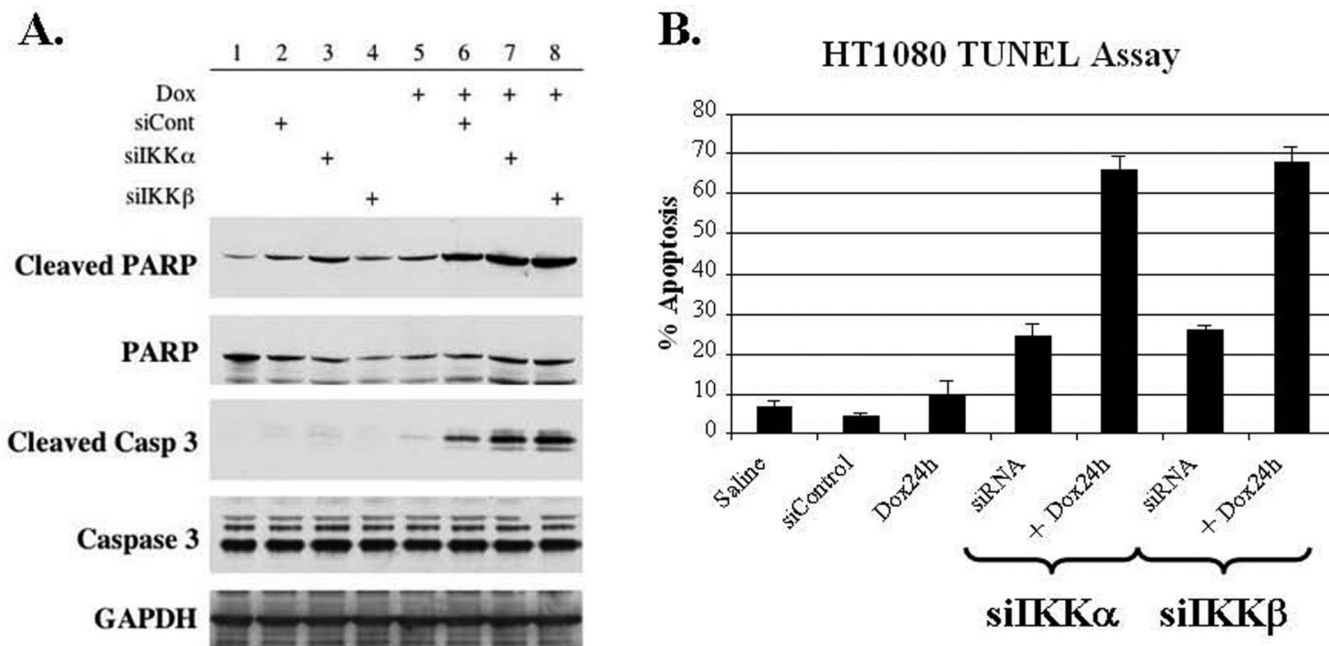


**Figure 2.**

(A) Doxorubicin (Dox) induces NF- $\kappa$ B activity in HT1080 fibrosarcoma cells in a bimodal pattern. HT1080 cells were stimulated with TNF $\alpha$  (50ng/ml) or doxorubicin (1.5 $\mu$ g/ml) and then harvested at 1, 3, 6, 18, and 24-hours. Nuclear extracts were evaluated by EMSA for NF- $\kappa$ B DNA-binding activity (large arrow). Again, the first lane (P) represents radiolabeled probe alone that and the results were compared to untreated cells (C). In the right panel, supershift analysis confirmed that the activated form of NF- $\kappa$ B consists of both p65 and p50 subunits (small arrows) and that the lower band represents a non-specific band (\*). (B) Degradation of I $\kappa$ B $\alpha$  at later time points does not correlate with NF- $\kappa$ B nuclear activity. Cytoplasmic extracts of HT1080 cells were evaluated by western blot for I $\kappa$ B $\alpha$  protein levels and demonstrate a persistent decrease in I $\kappa$ B $\alpha$  over time despite the variable expression of nuclear NF- $\kappa$ B seen by EMSA.



**Figure 3.** (A) Depletion of IKK $\alpha$  causes a significant decrease in NF- $\kappa$ B DNA-binding (large arrow) in HT1080 cells treated with doxorubicin (Dox) for 6 hours. HT1080 fibrosarcoma cells were transfected with siRNA as described. The cells were then stimulated with Dox (1.5 $\mu$ g/ml) for 6 hours. Nuclear extracts were evaluated using EMSA and compared to radiolabeled probe alone (Lane 0) and untreated cells (Lane 1). (B) Cytoplasmic extracts were evaluated by Western blot confirming successful selective depletion of IKK $\alpha$  and IKK $\beta$  using siRNA strategies. Moreover, the lack of either catalytic subunit resulted in a mild increase in I $\kappa$ B $\alpha$  at baseline and resulted in a mild decrease in the ability of Dox to induce the degradation of I $\kappa$ B $\alpha$ . (C) Whole cell extracts were prepared from HT1080 cells following siRNA transfection and exposure to Dox for 6 hours. Evaluation again confirmed specific knockdown of the intended IKK subunit. Moreover, examination of the state of phosphorylated p65 at the serine residue at position 536 (p65<sup>536</sup>) demonstrates that the lack of either IKK $\alpha$  or IKK $\beta$  results in decreased phosphorylation of p65 in response to Dox. Together these results suggest that both IKK $\alpha$  and IKK $\beta$  play a significant independent role in the activation of NF- $\kappa$ B following treatment with chemotherapy.



**Figure 4.**

(A) Following siRNA transfection and exposure to doxorubicin (Dox) for 6 hours, cellular extracts were prepared and evaluated for markers of apoptosis. The ability of Dox to initiate apoptosis is significantly enhanced by preventing the activation of NF- $\kappa$ B through the knockdown of IKK $\alpha$  or IKK $\beta$  resulting in increased cleaved caspase 3 and increased cleaved PARP. (B) Apoptosis following 24 hours of exposure to Dox was quantitated using a TUNEL assay. The knockdown of IKK $\alpha$  combined with doxorubicin (Dox) treatment significantly increased the percentage of apoptosis by 57% (54-59%, 95% CI) when compared to Dox alone and by 42% (39%-44%, 95% CI) when compared to siRNA treatment alone. Similarly, the combination of siIKK $\beta$  and Dox resulted in a significant 59% (56-62%, 95% CI) increase in apoptosis when compared with Dox alone and a 43% (40%-45%, 95% CI) when compared with siRNA treatment alone. The experiments were performed in triplicate.

| Target       | Sense                     | Antisense                     |
|--------------|---------------------------|-------------------------------|
| IKK $\alpha$ | 1. CAAAGAAGCUGACAAUACUUU  | 1. 5'-PAGUAUUGUCAGCUUCUUUGUU  |
|              | 2. CCAGAUACUUUCUUUACUAAU  | 2. 5'-PUAGUAAAAGAAAGUAUCUGGUU |
|              | 3. GAAGUUCGGUUUAGUAGCCUU  | 3. 5'-PGGCUACUAAAACCGAACUUCUU |
|              | 4. AAAUAUGGCAUCUCCUAAAUU  | 4. 5'-PUUAAGGAGAUGCCAUAUUUUU  |
| IKK $\beta$  | 1. GGAAGUACCUGAACCAAGUUUU | 1. 5'-PAACUGGUUCAGGUACUCCUU   |
|              | 2. CCAAUAAUCUUAACAGUGUUU  | 2. 5'-PACACUGUUAAAGAUUAUUGGUU |
|              | 3. GGAUUCAGCUUCUCCUAAAAUU | 3. 5'-PUUUAGGAGAAGCUGAAUCCUU  |
|              | 4. GUGGUGAGCUUAAUGAAUGUU  | 4. 5'-PCAUUCAUUAAGCUCACCACUU  |