INVESTIGATING THE ROLE OF IKKε (EPSILON) IN CANCER-ASSOCIATED NF-κB ACTIVITY

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ABSTRACT

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Investigating the role of IKKε (epsilon) in cancer-associated NF-κB activity

(Under the direction of Prof. Albert S. Baldwin)

Nuclear Factor κB (NF-κB) has been studied extensively as an inducible transcriptional regulator of the immune and inflammatory responses. NF-κB activation downstream of LPS or cytokine stimulation is controlled by the IκB kinase complex which contains IKKα and IKKβ and NEMO. Significantly, the constitutive activity of NF-κB has been implicated as an important aspect of many cancer cells but mechanisms associated with this activity are poorly understood. An inducible kinase, IKK-i / IKKε, related to the catalytic forms of the IκB kinase, has been studied as an anti-viral, innate immune regulator through its ability to control the activity of the transcription factors IRF-3 and IRF-7. Here, we demonstrate that IKK-i / IKKε is expressed in a number of cancer cells and is involved in regulating NF-κB activity through its ability to control basal/constitutive, but not cytokine induced, p65/RelA phosphorylation at ser536, a modification proposed to contribute to the transactivation function of NF-κB. Knockdown of IKK-i / IKKε or expression of a S536A mutant form of p65 suppresses HeLa cell proliferation. The data indicate a role for IKK-i / IKKε in controlling

proliferation of certain cancer cells through regulation of constitutive NF-κB activity. Moreover, microarray analysis of gene expression profiles in cancer cells with RNAi mediated IKKε knock-down demonstrated a number of genes that are highly regulated by IKKε. Among these genes, we were particularly interested in the Bcl-3 oncogene. After the observation that Bcl-3 gene expression is significantly reduced in microarray data following IKKε knock-down, we further confirmed and validated the microarray data by showing that overexpression of IKKε significantly upregulates Bcl-3 gene expression and that IKKε knock down downregulates it. Moreover, our data suggest that Bcl-3 gene expression is defective in IKKε knock-out MEF cells both at the protein level and at the message level. Promoter studies indicate that IKKε regulates Bcl-3 gene expression through NF-κB. We have also observed a significant correlation between IKKε expression and Bcl-3 expression in HCC tumors compared to adjacent tissue. My hypothesis is that IKKε regulation in cancer promotes oncogenic potential partly through the induced regulation of Bcl-3.

DEDICATION

To my wife; Feyza Burak Adli….

for all her support, understanding and sacrifices during this work.

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To the casual observer, a doctoral dissertation may appear to be a solitary work. However, the completion of this work would not have been possible were it not for the support I received from my friends, family and colleagues. I would first like to thank the members of the Baldwin lab, past and present, for their help, discussions and guidance, and for creating an environment conducive to both success and sanity.

I would like to extend my most sincere thanks to my mentor and dear friend Dr. Albert Baldwin for offering me endless opportunities to learn science from him. He had the trust and confidence to allow me to pursue my own ideas and learn from my mistakes. More importantly however, he taught me that a successful scientist can also be a good husband and father, and provides the model for how that can be achieved.

I have been privileged to have the support of a number of scientists throughout my career leading up to and including my graduate work. I would especially like to thank the members of my thesis committee, Jason Lieb, William Marzluff, Aziz Sancar and Brian Strahl, for all of their helpful advice and discussions and for keeping my research focused. I appreciate very deeply all their support, time, patience and counsel for this work.

Finally, I would like to thank my family. I feel especially indebted to my parents, Elmas and Abdulmecit Adli, for their endless, not only financial and but also spiritual, support throughout my life and for sending me their prayers from overseas. I am most grateful to my wife, Feyza Burak Adli, for her tolerance, support and extraordinary understanding. I am thankful to her for putting up with the life of a student and all that entails and having provided all the love, encouragement and support that I ever could have asked for.

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LIST OF ABBREVIATIONS

ARD	ankyrin repeat domain
BAFF	B cell activating factor belonging to the TNF family
Bcl	B-cell lymphoma
B-CLL	B-cell chronic lymphocytic leukemia
BCR	B-cell receptor
bp	Base pair
BRCA	Breast cancer associated
CDK	cyclin dependent kinase
CHK	checkpoint
CBP	CREB binding protein
CDC	cell division cycle
CK2	casein kinase 2
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
IRF	interferon response factor
JNK	c-Jun N-terminal kinase
kD	kilodalton
LT-β	lymphotoxin-beta
ml	milliliter

μl	microliter
LPS	lipopolysaccharide
mM	millimolar
μM	micromolar
NIK	NF-κB-inducing kinase
NF-κB	nuclear factor kappa B
NLS	nuclear localization sequence
RHD	Rel homology domain
RNAi	RNA interference
siRNA	short interfering RNA
TA	transactivation domain
TCR	T-cell receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAF	TNFR associated factor
β-TrCP	beta-transducin repeat-containing protein
ZAP	zeta-chain associated protein

LIST OF SYMBOLS

α	alpha
β	beta
ε	epsilon
γ	gamma
κ	kappa

INTRODUCTION

The Nuclear Factor kappa B (NF-κB)

Since its initial discovery more than 20 years ago, the transcription factor Nuclear Factor-kappaB (NF-κB) is still at the center of intensive research because if its crucial role in inflammation, immunity, controlling cell death and proliferation, which are hallmarks of many challenging human diseases such as cancer [1, 2]. Cells regulate immediate and long-lived cellular responses to environmental stimuli via activation of transcription factors. NF-κB is an evolutionarily conserved inducible transcription factor that responds to a diverse set of environmental changes. Among biological systems, NF-κB plays an essential role in the immune system by regulating the expression of cytokines, growth factors, and effector enzymes in response to ligation of many receptors involved in immunity, including T-cell receptors (TCRs), B-cell receptors (BCRs), TNFR, BAFFR, LTβR and the Toll/IL-1R family [3]. Furthermore, NF-κB also regulates the expression of many genes outside of the immune system such as the development and physiology of the mammary gland, bone, skin and other important biological systems such as the nervous system. Based on strong functional implications in varied essential biological roles, understanding how the transcriptional potential, activity, and selectivity of NF-κB are regulated is therefore the focus of extensive research.

NF-κB family proteins

Mammalian NF-κB is a family of 5 proteins composed of NF-κB1 (p50/p105), NF-κB2 (p52/p100) and 3 Rel family proteins; c-Rel, RelB, RelA (p65). These proteins exist as homo- or heterodimers bound by inhibitory κB (IκB) proteins within the cytoplasm of unstimulated cells [3]. NF-κB proteins share the highly conserved Rel Homology Domain (RHD) located towards the N terminus of the protein that is responsible for dimerization, interaction with IκB proteins and DNA binding. In contrast to the Rel subfamily proteins, which contain C-terminal transactivation domain, the unprocessed p105 and p100 NF-κB subfamily proteins have ankyrin repeats in their long C termini that act as intramolecular inhibitory κB protein (IκB) domains [4]. Upon activation, NF-κB1 and NF-κB2 proteins are processed, partially degraded on their ankyrin repeats in their C-terminal domains to generate p50 or p52 which then dimerize with the Rel proteins to act as transcriptional activators [2, 3]. In unstimulated cells, NF-κB transcription factor is tightly regulated by one of the several inhibitors of NF-κB called inhibitory κB proteins (IκB).

Mammalian Inhibitory kappa B proteins (IκB)

The activity of NF-κB is primarily regulated by interaction with inhibitory IκB proteins. There are several IκB proteins, which have different affinities for individual Rel/NF-κB complexes. They are regulated slightly differently, and are expressed in a tissue-specific manner. The inhibitory kappa B family of proteins include IκBα, IκBβ, IκBε, IκBγ, IκBζ, Bcl-3 and the C terminal precursors of p105 (NF-κB1) and p100 (NF-

κB2) [3, 4]. The interaction with IκB proteins (but Bcl-3) blocks the ability of NF-κB to translocate to the nucleus and bind to DNA and therefore results in the NF-κB complex being primarily in the cytoplasm due to masking of nuclear localization signal on NF-κB dimers and a strong nuclear export signal in IκBα. A large number of intra and extra-cellular stimuli, including ROS, cytokines, PMA, bacterial LPS, viral infection, and T and B cell activation, lead to NF-κB activation which involves phosphorylation and subsequent ubiquitination (generally by a complex called beta-TrCP) and degradation of IκB proteins by the 26S proteosome complex [5, 6]. The released NF-κB transcription factor with unmasked nuclear localization signal then translocates to the nucleus, binds to DNA and regulates the expression of target genes [3]. As described below, IκB phosphorylation by the high molecular weight IκB kinase (IKK) complex (approximately 700 kDa) is one of the critical regulatory steps in the NF-κB activation [7].

IκB kinase (IKK) complex

A large kinase complex responsible for IκB phosphorylation and subsequent degradation was initially partially identified from unstimulated HeLa cells and was later found to be activated in cells treated with TNFα [8, 9]. Subsequently several groups identified two highly related kinases named IKKα (IKK1) and IKKβ (IKK2) as the main kinase components of this kinase complex [10-12]. Both of these kinases have been shown to have specificity for serine residues in the destruction box of IκBα protein and can directly phosphorylate S32 and S36 residues [10]. In addition to IKKα and IKKβ, a non-catalytic, structural component called IKKγ (NEMO) was also identified to be an

essential component of the IKK complex [13-15]. Please refer to [10] for more extensive and detailed review about the IKK complex.

Much of the information about the physiological roles of each of the IKKs comes from gene targeting studies. Extensive research has demonstrated that IKKα, IKKβ and NEMO have distinct roles in terms of regulating differential physiological functions [3, 7]. IKKβ and NEMO knock out mice displayed similar phenotypes of embryonic lethality with severe liver apoptosis. Mouse embryonic fibroblast (MEF) cells that were isolated from IKKβ deficient embryos showed a marked reduction in tumor necrosis factor-alpha (TNFα) and interleukin-1alpha (IL-1α)-induced NF-κB activity and enhanced apoptosis in response to TNFα [16, 17]. In contrast, IKKα deficient mice have a wide range of morphogenesis and developmental defects. Although loss of IKKα did not seem to be critical for IKK activation and IκB phosphorylation, IKKα-deficient mouse embryonic fibroblast (MEF) cells have diminished NF-κB activation induced by TNFα and IL-1 [16, 18]. Therefore, it was claimed that IKKα is essential for NF-κB activation in the limb and skin during embryogenesis, however loss of IKKα does not effect cytokine induced NF-κB activation in fibroblasts and thymocytes [19].

In the light of these genetic studies and additional biochemical studies, it was suggested that IKKα and IKKβ have distinct functional roles [20]. Genetic and mutational analysis, highlighted that IKKβ but not IKKα is the primary component of NF-κB dependent proinflammatory signal transduction [21]. On the other hand, IKKα is believed to be essential in the so-called non-canonical NF-κB activation pathway by

regulating p100 precursor processing and NF-κB dependent developmental processes [7]. Recently some additional distinct nuclear roles of IKKα have been discovered. We and others have demonstrated that IKKα has an important nuclear function by regulating the control of target genes at the level of histone phosphorylation [22, 23]. Although the initial knock-out study for IKKα suggested its role on canonical NF-κB signaling pathway in MEFs [16], this function of IKKα is poorly understood.

NEMO (IKKγ) is the other important structural component of IKK complex. Besides being required for the normal function of the IKK complex, NEMO has been demonstrated to have an essential nuclear function in the activation of IKK signaling in response to DNA damage. Genotoxic stresses, which are caused by irradiation or topoisomerase I- or II-inhibiting drugs and other DNA damaging agents, are well documented to activate cytoplasmic IKK and NF-κB. The signals that are induced upon generation of DNA double-strand breaks by these treatments originate from the nucleus and have to be processed by the cytoplasmic IKK complex [7]. The most important sensor for DNA double-strand breaks is the nuclear kinase ATM, which is rapidly activated by genotoxic stress and activates IKK and NF-κB [24]. Subsequent research has demonstrated that genotoxic stress causes ATM directed phosphorylation of NEMO and subsequent modifications with small ubiquitin-like modifier (SUMO-1), and ubiquitination. It is believed that ubiquitination of NEMO dictates its translocation to cytoplasm where it activates the classical IKK complex and hence NF-κB activation [25].

Other IKK related kinases: IKKε and TBK1

Based on a number of observations, it was assumed that virtually all inducers of NF-κB lead to the activation of a single classical IKKα/β/γ complex. However, recent studies demonstrated the existence of distinct IKK complexes that do not contain IKKα, β, or γ [26]. One of these complexes was described as a PMA-inducible IκB kinase complex, with a critical component being an IKK-related kinase designated IKKε [27], which is identical to a kinase named IKK-i identified via its induction downstream of LPS-induced signaling [19]. IKKε in turn is closely related to another recently discovered IKK-related kinase designated as TBK1 (TANK-Binding Kinase 1) [28] or NAK (NF-κB Activating Kinase) [29]. TBK1, which is highly homologous to IKKε, binds to TANK and TRAF and may form an alternative IKK complex consisting of IKKε and TBK1 [28]. There is significant sequence similarity between IKKε and TBK1 (~66 %) and they share a significant similarity in their kinase domains with IKKα and IKKβ (Figure A).

IKKε and TBK1 are enzymatically distinct from the homologous enzymes IKKα and IKKβ [30] and have been shown to play important roles in the innate immune response. These kinases function as critical components of the interferon regulatory factor 3 (IRF3) and IRF7 signaling pathways involved in responses to viral infection or dsRNA treatment [31, 32]. Recent studies demonstrated that embryonic fibroblasts (MEFs) derived from TBK1-deficient (TBK1-/-) mice show impaired production of NF-κB- [33] as well as IRF3-dependent gene expression [31]. It has also been shown that IFN-β and IFN-inducible gene expression is defective in TBK1 knock-out cells in response to lipopolysaccharide (LPS), poly (I:C) or viral infection [34-36].

The relationships of IKKε and TBK1 with NF-κB activation remain enigmatic.

Although recent studies defined their roles in IRF3 and IRF7 transcriptional activation [31, 32] and suggested their involvement in NF-κB activation [28-30, 33, 37, 38], the exact molecular mechanism of NF-κB activation by these kinases is not clearly understood. One report [39] indicated that IKKε plays a key role in integrating signals induced by pro-inflammatory stimuli by activating C/EBPδ whose expression is regulated by NF-κB. There is a recent report suggesting that IKKε and TBK1 are among the kinases that mediate inducible phosphorylation of p65 at Ser-536 [40], an event proposed to stimulate inherent p65 transactivation function [41]. In this model, TBK1 and IKKε would control NF-κB at a level distinct from the traditional IKK-mediated control of IκB degradation. How IKKε regulates the expression of its target genes is not clearly understood, however our results suggest that p65 phosphorylation at ser 536 position is an important step. In support of our data, IKKε has recently been shown to interact with p52 and promote transactivation via p65 activation [42]. We cannot rule out the possibility that IKKε has other roles in other NF-κB activating pathways as the significant of IKKε involvement is recently being appreciated. One study, for example, demonstrated that TBK1 and IKKε directly phosphorylate the C-terminal domain of cRel in vitro and in vivo and regulate nuclear accumulation of cRel, independently of the classical IkappaB/IKK pathway [32]. While this work was in preparation, Boehm at al., published a paper in the most recent issue of *Cell* that clearly identified IKKε as a Breast Cancer Oncogene by using Integrative Genomic Approaches such as overexpression and siRNA screening as well as comparative genomics analysis [43]. In line with our published studies, they also showed that in many breast cancer cell lines as well as breast cancer carcinomas, IKKε is overexpressed and this overexpression is driving NF-κB

activity which provide prosurvival signal in cancer setting.

In addition to their well established roles in IRF3 and IRF7 activation during innate immunity, recent studies by several groups including ours [43-46] implicated the involvement of IKKε in cancer signaling mechanism. Interestingly TBK1 has also been implicated in tumor cell survival [47]**.** Although research from several groups recently appreciated the implication of IKKε and TBK1 cancer progression and tumor cell survival, the molecular mechanism of IKKε and TBK1 induced cancer signaling mechanism and downstream target genes remain to be identified.

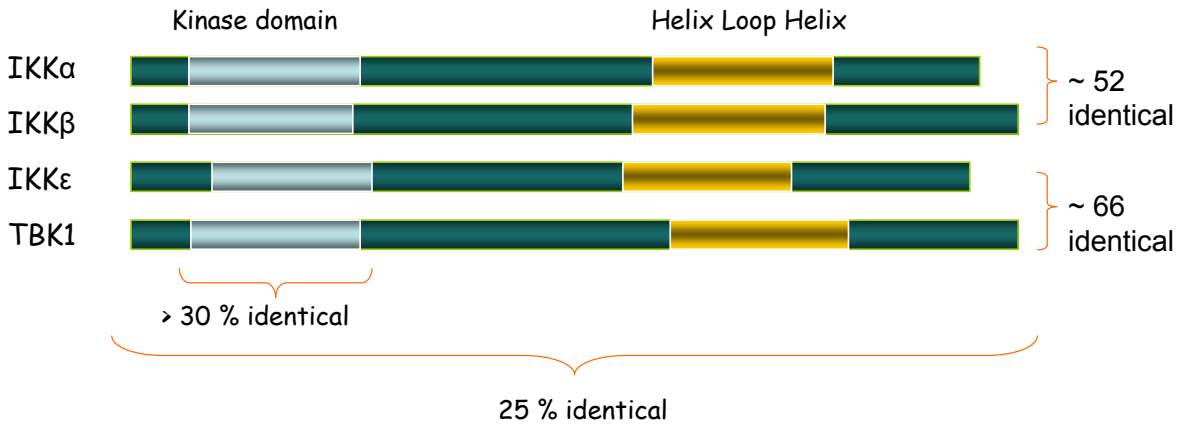

Figure A: Schematic outline of functional domains and sequence similarities in IKKα, IKKβ, IKKε and TBK1. Numbers represent percent sequence similarity between each of kinases.

Overview of the NF-κB activation pathways

NF-κB activation downstream of the classical IKK kinase complex in response to cytokines and other well studied NF-κB inducers is relatively well established. The signaling mechanism of positive and negative regulation of NF-κB upon stimulus induction has been demonstrated to be either canonical (classical) or non-canonical (non-classical) signaling pathway (Figure 1.2). In the canonical NF-κB activation pathway, NF-κB dimers such as p50/RelA are maintained in the cytoplasm by interaction with an independent IκB molecule (often IκBa). In many cases, the binding of a ligand to a cell surface receptor (e.g., tumor necrosis factor-receptor (TNF-R) or a Toll-like receptor) activates the IKK complex (IKKα/β/γ) which leads to phosphorylation and subsequent degradation of the inhibitory subunit (IκB). Released NF-κB dimers then translocate to the nucleus to regulate the expression of target genes [1]. In the non-canonical pathway, certain ligands (e.g., Lymphotoxin β (LTβ), B-cell activating factor (BAFF) and CD40) binding to their cognate cell surface receptors leads to activation of the NF-κB-inducing kinase NIK, which phosphorylates and activates an IKKα complex, which in turn, phosphorylates two serine residues adjacent to the ankyrin repeat C-terminal IκB domain of p100, leading to its partial proteolysis and liberation of the p52/RelB complex [3] (Figure B)

In addition to the well established stimulus-induced positive and negative regulation of NF-κB, there are other NF-κB activating pathways involved especially in constitutive or basal NF-κB activity. Research from our lab and other groups have

suggested IKKε as an important kinase implicated in basal or constitutive activation of NF-κB in cancer-associated signaling mechanisms [44, 45]. Evidence so far suggests that an IKKα- and IKKβ-independent phosphorylation of p65 at the Ser 536 position is critical for this activity. Cancer associated, IKKε-induced basal or constitutive NF-κB activity is critical for survival and proliferation of tested cancer cell lines. Whether this is a general mechanism of NF-κB activation in cancer or only applied to certain cancer cell lines is yet to be understood. Interestingly, IKKε is not involved in cytokine induced NF-κB activation suggesting non-redundant roles for IKKα/β and IKKε [44]. Research from our lab and other groups indicated that Bcl-3, as a NF-κB target gene, is potentially the mechanistic link between constitutive NF-κB activity and cell survival and cell proliferation.

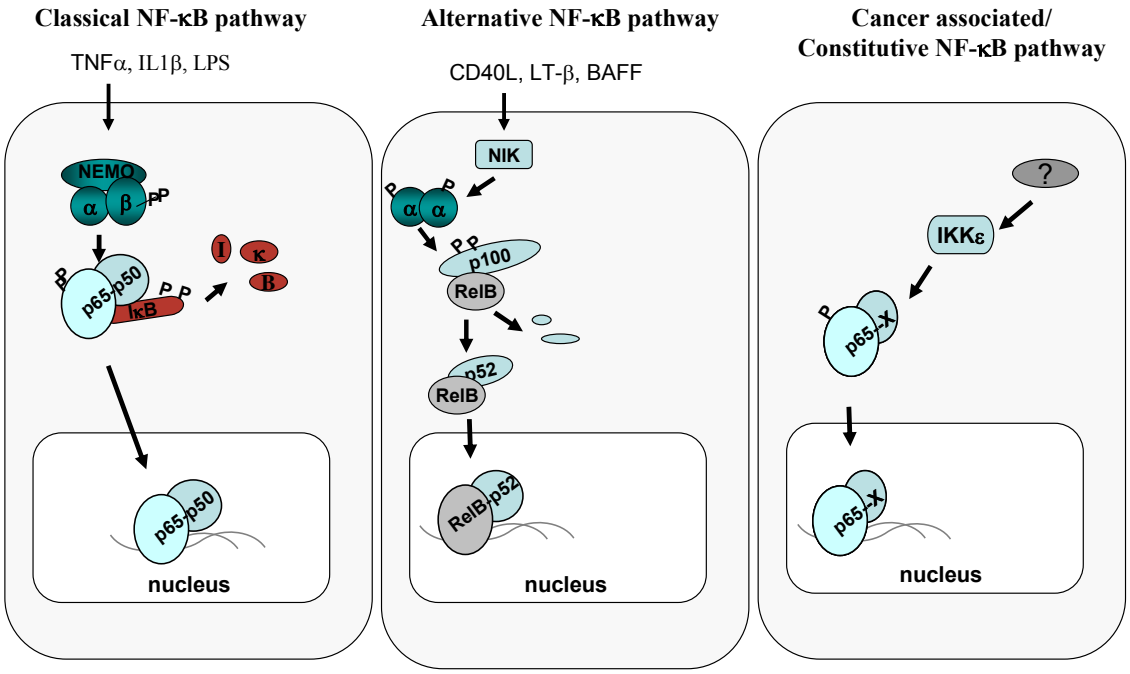

Figure B: Classical and non-classical, alternative pathways of NF-κB activation. A model depicting three different signaling pathways to NF-κB. Classical pathway is mediated by activation of IKKβ by inputs such as TNF, TCR, IL-1β, LPS and many others leading to IKKβ dependent phosphorylation and subsequent degradation of IκB. The alternative pathway involves NIK dependent activation of IKKα which leads to phosphorylation of processing p100, generating p52:RelB heterodimers. Inputs that activate alternative signaling pathway include LTβ, BAFF, and CD40. In constitutive NF−κB activation pathway, without any outside stimuli IKKε and potentially other unknown kinases induces p65 phosphorylation and hence NF-κB activity independent of classical IKK complex.

Bcl-3; not just another inhibitory protein

Bcl-3 is a member of ankyrin-repeat-containing IκB family of NF-κB inhibitors [3]. It was identified 20 years ago by molecular cloning of the breakpoint of the t(14;19) chromosomal translocation from a subset of human B-cell chronic lymphocytic leukemias [48]. Translocation at this point leads to Bcl-3 overexpression that results in dysregulation of mostly unexplored downstream target genes that are involved in differentiation, cell survival and apoptosis [49]. Although Bcl-3 shares significant structural features with IκB proteins [50, 51], Bcl-3 contains two transactivating domains and can function as a transcriptional activator or repressor by forming heterocomplexes with the NF-κB p50 or p52 subunits in a phosphorylation dependent manner [51-55]. Although the molecular mechanism of Bcl-3 dual function is poorly understood, some observations explain how Bcl-3 may act as both transcriptional activator as well as repressor. It was suggested that Bcl-3 can potentially act as a transcriptional activator by removing repressor p50 homodimers from the κB sites [51] and also by acting as a bridging factor between NFκB and other coactivators such as JAB1, Bard1 and Tip60 [56]. Similarly, upon IL-1β stimulation, Bcl-3 recruits Tip60 acetyltransferase containing activator complex to the promoter region of a specific subset of NF-κB dependent genes [57]. Moreover, Bcl-3 was also suggested to function as a coactivator itself by regulating the expression of subset of NF-κB dependent genes in response to TNFα [58]. In contrast to its transactivator potential Bcl-3 has also been suggested to act as a transcriptional repressor. It has been demonstrated that Bcl-3 can physically interact with HDAC-1, -2 and -3 which are involved gene repression [59]. Another study also demonstrated that Bcl-3 inhibits LPS-induced inflammatory responses in macrophages by recruiting

HDAC-1 [60]. Although the opposite dual function of Bcl-3 is poorly understood, one explanation may come from the posttranslational modifications on Bcl-3 or Bcl-3 interacting proteins. Bcl-3 has two residues within its C-terminal domain that are phosphorylated by GSK3 kinase which mediates its interaction with HDAC1 and induce its degradation through the proteosome pathway [59]. Although protein phosphorylation was suggested to be the key mechanism for regulation of Bcl-3 activity [61], other posttranslational modifications such as ubiquitination was recently demonstrated to be critical for Bcl-3 nuclear translocation [62]. For instance, It was clearly demonstrated that TPA or UV light triggers the translocation of tumor suppressor CYLD from the cytoplasm to the perinuclear region, where CYLD binds and deubiquitinates Bcl-3, thereby preventing nuclear accumulation of Bcl-3 and p50/Bcl-3- or p52/Bcl-3-dependent gene expression and subsequent cell proliferation [62].

Bcl-3 has been implicated in many crucial physiological events such as cell proliferation, immune, inflammation and cancer progression. The results from Bcl-3 knock-out mice revealed critical roles for Bcl-3 in antigen-specific priming of T and B cells [63]. Bcl-3 deficient mice are also impaired in germinal center reactions and T cell-dependent antibody responses to influenza virus that may underlie the immunologic defects [64]. It was shown that Bcl-3 associates with NF-κB p50 or p52 subunits and strongly enhances cell proliferation and oncogenesis through activation of the *cyclin D1* promoter [23, 65]. In line with these observations, Eμ–Bcl-3 transgenic mice develop lymphoproliferative disorders such as; Lymphadenopathy, splenomegaly, and altered immunoglobulin production [66]. Furthermore, although the mechanism is poorly

understood, Bcl-3 was shown to directly transform cells [59]. Research has also demonstrated that Bcl-3 exerts anti-apoptotic activity in B and T lymphocytes [67, 68] and controls the death of activated T cells [69]. Despite its well established role in cell proliferation and apoptosis, the signaling pathways and upstream key effecters that activate Bcl-3 gene expression are poorly understood.

CHAPTER I

IKK-i / IKKε (epsilon) controls constitutive, cancer cell-associated NF-kappaB activity via regulation of Ser-536 p65/RelA phosphorylation

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Abstract

Nuclear factor kappaB (NF-kappaB) has been studied extensively as an inducible transcriptional regulator of the immune and inflammatory response. NF-kappaB activation downstream of lipopolysaccharide or cytokine stimulation is controlled by the IkappaB kinase complex, which contains IKKalpha and IKKbeta. Significantly, the constitutive activity of NF-kappaB has been implicated as an important aspect of many cancer cells, but mechanisms associated with this activity are poorly understood. An inducible kinase, IKK-i / IKK epsilon, related to the catalytic forms of the IkappaB kinase, has been studied as an anti-viral, innate immune regulator through its ability to control the activity of the transcription factors IRF-3 and IRF-7. Here, we demonstrate that IKK-i / IKK epsilon is expressed in a number of cancer cells and is involved in regulating NF-kappaB activity through its ability to control basal/constitutive, but not cytokine-induced, p65/RelA phosphorylation at Ser-536, a modification proposed to contribute to the transactivation function of NF-kappaB. Knockdown of IKK-i / IKK epsilon or expression of a S536A mutant form of p65 suppresses HeLa cell proliferation. The data indicate a role for IKK-i / IKK epsilon in controlling proliferation of certain cancer cells through regulation of constitutive NF-kappaB activity.

Introduction

The transcription factor Nuclear Factor-κB (NF-κB) plays a pivotal role in controlling the expression of a diverse set of genes that contribute to a variety of biological functions including cell survival, cell proliferation, and immune and inflammatory responses [3]. The classic form of NF-κB is composed of a heterodimers of the p50 and p65 subunits which is preferentially localized in the cytoplasm as an inactive complex with inhibitor proteins of the IκB family. Following exposure of cells to a variety of stimuli, including inflammatory cytokines and LPS, IκBs are phosphorylated by the IKKα/β complex, polyubiquitinated and subsequently degraded by the 26S proteosome complex [3, 70]. Released NF-κB complexes then accumulate in the nucleus, where they transcriptionally regulate the expression of genes involved in the immune and inflammatory responses [3].

Based on a number of observations, it was assumed that virtually all inducers of NF-κB lead to the activation of a single classical IKKα/β/γ complex. However, recent studies demonstrated the existence of distinct IKK complexes that do not contain IKKα, β, or γ [26]. One of these complexes was described as a PMA-inducible IκB kinase complex, with a critical component being an IKK-related kinase designated IKKε [27], which is identical to a kinase named IKK-i identified via its induction downstream of LPS-induced signaling [19]. IKKε in turn is closely related to another recently discovered IKK-related kinase designated as TBK1 (TANK-Binding Kinase 1) [28] or NAK (NF-κB Activating Kinase) [29]. TBK1, which is highly homologous to IKKε, binds to TANK and TRAF

and may form an alternative IKK complex consisting of IKKε and TBK1 [28].

IKKε and TBK1 are enzymatically distinct from the homologous enzymes IKKα and IKKβ [30] and have been shown to play important roles in the innate immune response. These kinases function as critical components of the interferon regulatory factor 3 (IRF3) and IRF7 signaling pathways involved in responses to viral infection or dsRNA treatment [31, 32]. Recent studies demonstrated that embryonic fibroblasts (MEFs) derived from TBK1-deficient (TBK1-/-) mice show impaired production of NF-κB- [33] as well as IRF3-dependent gene expression [31]. It has also been shown that IFN-β and IFN-inducible gene expression is defective in TBK1 knock-out cells in response to lipopolysaccharide (LPS), poly (I:C) or viral infection [34-36].

The relationships of IKKε and TBK1 with NF-κB activation remain enigmatic. Although recent studies defined their roles in IRF3 and IRF7 transcriptional activation [31, 32] and suggested their involvement in NF-κB activation [28-30, 33, 37, 38], the exact molecular mechanism of NF-κB activation by these kinases is not clearly understood. One report (19) indicated that IKKε plays a key role integrating signals induced by pro-inflammatory stimuli by activating C/EBPδ whose expression is regulated by NF-κB. There is a recent report suggesting that IKKε and TBK1 are among the kinases that mediate inducible phosphorylation of p65 at Ser-536 [40], an event proposed to stimulate inherent p65 transactivation function [41]. In this model, TBK1 and IKKε would control NF-κB at a level distinct from the traditional IKK-mediated control of IκB degradation.

Here, we show that IKKε is expressed in a variety of cancer cell lines. Based on

this, we have investigated a role for IKKε as related to constitutive, cancer-associated NF-κB activity. Our experiments reveal an important role for IKKε in controlling the activation of Ser-536 phosphorylation of the RelA/p65 subunit and functional NF-κB activity in several cancer cell lines and in 293T cells.

Results

IKKε is expressed in a number of cancer cells and in SV40 large T-immortalized 293 cells. In order to address a potential role for IKKε in controlling NF-κB activity, we explored the expression of IKKε in a variety of cell lines. Immunoblotting of extracts of several cancer cell lines revealed constitutive expression of IKKε in breast cancer cell lines MDA MB 468, SK BR3, Sum 226, and MCF7; HeLa cells; PC3 and LNCaP prostate cancer cells; and 293T embryonic kidney cells (Fig. 1). As a marker for endogenous NF-κB activity, we immunoblotted the extracts from these cells with an antibody which recognizes only the RelA/p65 subunit phosphorylated at Ser-536. These data revealed a correlation between IKKε expression and phosphorylated Ser-536 RelA/p65 in most of the cells analyzed. Notably, TBK1 was expressed in these cells but did not consistently correlate with RelA/p65 Ser-536 phosphorylation. The data also reveal that IKKε, while considered an inducible kinase, is found to be constitutively expressed at significant levels in most of the cell lines investigated.

IKKε or TBK1 activates an NF-κB-dependent reporter in a kinase-dependent manner. To investigate a potential role for IKKε and TBK1 in NF-κB regulation, experiments were initiated to analyze their potential involvement in controlling NF-κB-

dependent promoters. Based on the results from Figure 1, we focused these experiments on HEK 293T cells since IKKε is expressed and is potentially active in these cells. Both IKKε and TBK1, but not their kinase mutant forms, activated the 3X-κB and IFN-β luciferase promoter constructs (Figs. 2A and 2B). Unlike the 3X-κB promoter, the IFN-β promoter is considered a complex promoter regulated by coordinate actions of NF-κB and other transcription factors, therefore it is not considered to be regulated exclusively by NF-κB. These results are similar to those of Shimada et al [19]. Interestingly, in all of the assays performed, TBK1 was observed to be a better activator of the reporters (Figs. 2A and 2B). Analysis of the effects of different concentrations of IKKε on activation of the 3X-κB luciferase reporter showed that fold induction of luciferase activity is proportional to the IKKε plasmid concentration, whereas there was no significant induction with the vector control or with the kinase mutant (KM) form of IKKε (Fig. 2C).

IKKε expression induces NF-κB DNA binding activity. We next investigated whether IKKε expression can induce NF-κB binding to a consensus DNA target sequence. Flag-tagged IKKε was transiently expressed in HEK 293T cells for approximately 48 hrs and nuclear extracts were prepared for Electrophoretic Mobility Shift Assays (EMSAs). As shown in Fig. 3A, IKKε effectively induces DNA binding activity of NF-κB. TNF-α is included in this experiment for comparison purposes. It should be noted that there is basal NF-κB DNA binding activity in the VC lane (lane 1), which is better visualized given longer exposure times. TNF-α stimulation of IKKε-overexpressing cells led to more DNA binding activity but this increase appears to be an additive effect of TNF-α and IKKε rather than a synergistic effect. Western blot analysis of cytoplasmic extracts shown in the

lower panel, demonstrates expression levels of IKKε. We next aimed to investigate the nature of the major NF-κB subunits in this bound complex by gel shift assay. For this purpose each of the reactions used in Fig. 3A has been incubated with the indicated antibody and electrophoresed on a separate gel (Fig. 3B). The number on the top of the figure indicates the lane numbers (1-4) from the reactions used in Fig. 3A. In the lanes where an NF-κB complex is detected (2, 3, 4), there is a positive response with the p65 and p50 antibodies. Therefore, we concluded that the complex that bound to this consensus binding site is composed predominantly of p65/p50 heterodimers (Fig. 3B). It should be noted that a single nucleotide change can lead to binding of different NF-κB subunits [58, 71] therefore we can not exclude the possibility that other subunits might also be activated and bind to slightly different NF-κB binding sites. TBK1 expression effects on DNA binding activity of NF-κB yielded very similar results (data not shown).

IKKε and TBK1 expression leads to phosphorylation of endogenous p65 at Ser-536. Recent studies have shown that post-translational modification of NF-κB subunits, such as p65, contribute significantly to NF-κB transactivation potential (reviewed in [3]). Phosphorylation of p65 at Ser-536 is proposed to be a key modification that potentiates p65 transactivation function, hence NF-κB activation [41, 72]. We next tested if IKKε and TBK1 affect p65 phosphorylation. We tested whether ectopically expressed IKKε leads to phosphorylation of endogenous p65 at Ser-536. Expression of GFP-IKKε leads to a significantly higher level of p65 phosphorylation at the Ser-536 position (indicated as P-p65 (Ser-536)) (Fig. 4A). Utilization of GFP-tagged IKKε expression vector allows for distinguishing between ectopically expressed IKKε (GFP-IKKε) and endogenous IKKε.

This experiment reveals that IKKε induces higher levels of endogenous IKKε (Fig. 4A), which has been proposed to be regulated by NF-κB [73]. This observation suggests that ectopically expressed IKKε is able not only to induce phosphorylation of endogenous p65 but also to induce NF-κB dependent gene expression. Next we examined if TBK1, an IKKε homolog, will also induce Ser-536 phosphorylation of p65. As shown in Fig. 4B, WT forms of both IKKε and TBK1 induce p65 Ser-536 phosphorylation however their kinase mutant forms (K38→A) do not lead to phosphorylation of p65. Indeed when analyzed in detail, kinase mutant forms appear to inhibit the basal level of endogenous Ser-536 phosphorylation (compare lane 1 to lane 4 and 6). It is important to note that the phospho-p65 Ser-536 antibody specifically detects only the phosphorylated form of p65, and does not cross-react with unphosphorylated p65 (see Fig. 4B).

Analysis of inducible p65 Ser-536 phosphorylation in IKKε deficient cells. Observing that exogenous IKKε induces p65 phosphorylation, we hypothesized that this post-translational modification on p65 might be defective in IKKε deficient MEFs in response to NF-κB inducers. To test our hypothesis, IKKε deficient and IKKε and TBK1 doubly deficient MEFs (DKO) were stimulated with TNFα, a well known NF-κB inducer, and compared to similarly treated WT MEFs. As seen in Fig. 5A, p65 is phosphorylated at the Ser-536 position in response to TNF-α as early as 5 min. post-stimulation. Interestingly, IKKε deficient cells showed essentially the same pattern of phosphorylation kinetics in response to TNFα stimulation. In addition to p65 phosphorylation, IκBα degradation was also normal in IKKε deficient cells compared to WT MEF cells. Analysis of β-tubulin levels confirmed that loading was essentially equivalent in all lanes

(Fig. 5A). In order to determine if the loss of TBK1 together with IKKε would effect the phosphorylation of p65 and IκBα degradation, WT MEFs and MEFs deficient for both IKKε and TBK1 (double knock-out MEFs (DKO)) have been used under similar experimental conditions. Both p65 phosphorylation and IκBα degradation are normal in DKO MEFs (Fig 5B). This result indicated that NF-κB activation as measured by p65 phosphorylation and IκBα degradation in response to TNF-α is independent of IKKε and TBK1.

We next tested the effect of IL-1β on p65 phosphorylation as well as IκB degradation (Fig. 5C). IL-1β is, like TNF-α, a well known inducer of NF-κB. In as early as 5 min, p65 is phosphorylated maximally, however, maximal IκBα degradation is observed in 10 min. It is interesting again to observe that both p65 phosphorylation at Ser-536 and IκBα degradation are normal in IKKε deficient cells compared to WT cells. In parallel studies, experiments were also performed in cells where both IKKε and TBK1 were deleted (DKO) and we observed no significant difference from WT cells relative to Ser-536 p65 phosphorylation or IκBα degradation after stimulation with TNF-α or IL-1β (Fig 5D). These results suggest a minimal role of IKKε and TBK1 in cytokine induced p65 phosphorylation and IκBα degradation.

The pathway to NF-κB activation in response to LPS has been characterized in molecular detail resulting in the discovery of a novel family of adapter proteins, which serve to regulate and polish up TLR responses. The first identified member of this adapter family was MyD88 [74]. The importance of MyD88 in TLR signaling has been confirmed

by the inability of MyD88-deficient mice to respond properly to a variety of TLR ligands, namely LPS, peptidoglycan and bacterial CpG motifs [19, 34] Interestingly, analysis of MyD88-deficient cells in response to LPS demonstrated the existence of, MyD88-independent, late NF-κB activation and the induction of IRF-3-dependent genes which has recently been verified to be regulated by IKKε and TBK1 kinases [31, 32, 35]. In our experimental system, LPS stimulation of WT MEFs and IKKε deficient cells showed similar levels of inducible p65 phosphorylation (Fig. 5E). As expected, the kinetics of the phosphorylation upon LPS stimulation is not as fast as TNF-α and IL-1β but the pattern of phosphorylation is similar between WT and IKKε deficient cells. In addition to p65 phosphorylation, IκBα degradation was also analyzed and there was no defect in this process. We next tested the effect of PMA stimulation on p65 phosphorylation and did not observe any difference between WT and IKKε deficient MEFs (Fig 5F). Overall our data indicate that inducible Ser-536 phosphorylation is unaffected in IKKε deficient cells, and confirm that IKKε is not significantly involved in mechanisms associated with cytokine-, LPS-, or PMA-induced IκBα degradation.

IKKε controls constitutive p65 Ser-536 phosphorylation. It was surprising to observe that IKKε and TBK1 expression led to the phosphorylation of endogenous p65 but that MEFs deficient for these kinases depict normal phosphorylation patterns. Since we had observed a correlation between IKKε and Ser-536 in certain cancer cells, we therefore hypothesized that these kinases might be involved in basal or constitutive p65 phosphorylation. Since the basal or constitutive level of p65 phosphorylation is quite low in MEF cells, the potential that IKKε could contribute to basal/constitutive levels of Ser-

536 phosphorylation was investigated in HeLa and HEK 293T cells. These cells have higher levels of IKKε expression, constitutive p65 phosphorylation and NF-κB activation compared to MEFs. In order to knock-down IKKε, both plasmid based shRNA and normal siRNA technologies have been utilized against IKKε mRNA. Additionally, an identical control plasmid, which contains a scrambled sequence with no homology to any known human gene product, has been utilized. Extracts from control-treated and siRNA-treated HeLa cells were analyzed for IKKε knock-down as well as for endogenous p65 Ser-536 phosphorylation. Our results indicated that transfection of shRNA against IKKε leads to sustained effective knock down of IKKε. Furthermore, the reduction in IKKε protein level is well correlated with significant reduction in the basal level of Ser-536 phosphorylation in HeLa cells as compared to vector control treated cells (Fig. 6A). Quantitative Real Time-PCR (qRT-PCR) analysis showed more than a 70% reduction in *ikkε* mRNA in HeLa cells when transfected with 2 μg shRNA plasmid (data not shown). To show that this is not a cell line specific observation, similar experiments have been performed in HEK 293T cells. In cells where the shRNA plasmid was transfected, IKKε level were significantly reduced. Again, a significant reduction in Ser-536 phosphorylation of p65 was observed, whereas vector control treated cells exhibited no change in IKKε levels or Ser-536 phosphorylation (Fig. 6B). These results demonstrate that IKKε has a significant role in controlling the basal/constitutive p65 phosphorylation at Ser-536 position in two cell lines. Interestingly, knock-down of TBK1 with siRNA did not show a significant change in p65 phosphorylation at Ser-536 position (Fig. 6C). This result indicates the differential role of IKKε and TBK1 in terms of controlling basal/constitutive p65 phosphorylation, at least in the cell types analyzed.

IKKε knock-down in HeLa cells results in reduced constitutive activity of an NF-κB dependent promoter. In order to better elucidate the role of IKKε in controlling NF-κB activity, and to determine if reduction in p65 phosphorylation upon IKKε knockdown is correlated with reduced constitutive NF-κB activity, we have analyzed NF-κB-dependent luciferase reporter assays in HeLa cells. These cells were transfected with either negative control siRNA and or with IKKε siRNA for 48 hrs followed by transfection with the 3x-κB luciferase promoter construct. Importantly knock-down of IKKε in a dose dependent manner resulted in reduced promoter activity as compared to the control construct (Fig. 7). This result indicates that IKKε controls a significant portion of NF-κB-dependent activity in HeLa cells, presumably through its ability to control Ser-536 p65 phosphorylation.

IKKε and p65 phosphorylation positively mediate HeLa cell proliferation. After observing that IKKε controls basal p65 phosphorylation and NF-κB activity in certain cancer cells, we asked if IKKε provides cell growth/survival functions. For this purpose, MTT cell proliferation assays were performed in HeLa cells transfected with shRNA against IKKε or with a non-phosphorylable form of p65 (S536A) expression construct. For the data presented in both Figures 8A and B, HeLa cells were seeded in 6 well plates and were transfected the next day with the indicated plasmids. 24 hr after transfection, cells were reseeded on 96 well plates and the MTT proliferation assay was performed 24 and 48 hr later. When IKKε is knocked down by shRNA, a significant reduction in cell proliferation is observed at the 48 hr time point as compared to control cells transfected with the scrambled shRNA plasmid (Fig. 8A). In a similar experimental setting, we tested

the effect of IKK□ mediated p65 phosphorylation on cell proliferation. For the purpose, IKKε was transfected with WT or a p65 mutant (S536A) that cannot be phosphorylated at the Ser-536 position. MTT assays read after 24 hr and 48 hr shows that cells with mutant p65 do not proliferate efficiently when compared to cells expressing WT p65. This experiment indicates that p65 phosphorylation at Ser-536 is important for HeLa cell proliferation.

Discussion

The majority of studies analyzing NF-κB activation have focused on induction of this transcription factor downstream of cytokine or LPS-dependent signaling. This response is generally dependent on the classic IKK complex, containing IKKα and IKKβ. Additional evidence has indicated that besides the nuclear translocation of NF-κB, post-translational modifications, like p65 phosphorylation, are required to efficiently activate NF-κB dependent gene transcription [41, 75-80]. It is also well established that a number of cells, particularly those of cancerous origin, exhibit significantly elevated levels of basal or constitutive NF-κB activity. In many cases, the origins of this activity remain unclear.

Here we show that several cancer cell lines, along with the SV40 large T-immortalized 293 cell line, exhibit relatively high levels of expression of IKKε. This is interesting, as IKKε is normally considered a kinase that is induced quantitatively by LPS

or cytokines. We have investigated a potential role for IKKε and TBK1, kinases homologous to the catalytically active IKKα and IKKβ subunits, in controlling NF-κB activity, with the focus being phosphorylation of p65 at the Ser-536 position. Experiments were initiated to study IKKε and TBK1 induced NF-κB-dependent promoter activation.

In agreement with previous results [31, 32], IKKε and TBK1, but not their kinase mutant forms, strongly activate NF-κB regulated reporter constructs. It is important to note that, unlike the 3X-κB promoter, the IFN-β promoter is a complex promoter regulated by coordinate actions of NF-κB and other transcription factors, therefore it is not considered to be regulated exclusively by NF-κB. To confirm our reporter assays, gel shift assays have been performed. As expected, IKKε and TBK1 induced significant NF-κB DNA binding activity. Super shift assays identified p65 and p50 as main subunits of NF-κB complex. Recent studies have shown that post-translational modification of NF-κB subunits, such as p65 phosphorylation, contribute significantly to NF-κB activation. Phosphorylation of p65 at Ser-536 is proposed to be a key modification that potentiates p65 transactivation function, and hence NF-κB activation ability [41, 72, 78].

Recently, it has been reported that overexpression of IKKε or TBK1 together with p65, leads to the phosphorylation of ectopically expressed p65 at Ser-536 [40], however, this group has not analyzed the endogenous phosphorylation at Ser-536 of p65. Our results clearly support the hypothesis that the kinase activity of IKKε and TBK1 may significantly contribute to the constitutive level of S536 phosphorylation of p65. We have also observed that IKKε induces its own expression, which has been shown to be regulated

by NF-κB. This data indicates that ectopically expressed IKKε induces p65 phosphorylation, NF-κB activation and NF-κB dependent gene expression. It is also raises the possibility that IKKε functions in an autoregulatory loop, leading to its own expression.

It was interesting to observe that IKKε deficient cells show a normal pattern of cytokine inducible phosphorylation of p65 and IκBα degradation when compared to WT MEFs. We have tested a series of well known NF-κB inducers (IL-1β, LPS, PMA) that are known to activate NF-κB by utilizing different signaling pathways. Compared to WT MEFs, IKKε null cells (and DKO MEFs for both IKKε and TBK1) allowed inducible RelA/p65 phosphorylation to the same extent. Why is there no defect in inducible Ser-536 phosphorylation of p65 in IKKε deficient cells? The first plausible explanation to this question is that the classical IKK signalsome complex is still intact in IKKε deficient cells. Therefore, this complex likely compensates for the loss of IKKε. Secondly, there are other known and unknown kinases, in addition to IKK complex, that have been claimed to mediate Ser-536 phosphorylation of p65 [41, 80, 81] and in the same way, they may still induce phosphorylation in IKKε deficient cells. The third explanation to this question is that IKKε and TBK1 are not involved in cytokine induced p65 phosphorylation, but rather they are involved in basal/constitutive level of p65 phosphorylation. In one set of experiments, this possibility has been investigated. Since MEF cells have low levels of basal p65 phosphorylation and low levels of IKKε, the potential that IKKε and TBK1 might be involved in constitutive p65 phosphorylation has been investigated in HeLa and HEK 293T cells that demonstrate higher levels of IKKε and constitutive p65

phosphorylation at Ser-536 position. IKKε was knocked down by plasmid based shRNA technology in both HeLa cells and HEK 293T cells. Importantly, shRNA transfection leads to sustained knock down of IKKε and more importantly, reduction in the IKKε level is well correlated with significant reduction in the basal level of Ser-536 phosphorylation (Fig. 6), whereas in vector control transfected cells there is no change in IKKε level and Ser-536 phosphorylation of p65. Interestingly knock-down of TBK1 did not reduce the basal level of p65 phosphorylation. Surprisingly this data suggests that TBK1 and IKKε are not entirely orthologues at least in controlling basal phosphorylation of p65. As suggested earlier [35], IKKε and TBK1 might not be redundant in every signaling pathway that they effect. Thus the data presented here clearly show that IKKε does not mediate cytokine-induced p65 phosphorylation at the Ser-536 position but has a significant role in basal and constitutive phosphorylation of p65 at least in certain cancer cells and in 293T cells. Basal p65 phosphorylation is well correlated with constitutive NF-κB activity, which has been implicated in the pathogenesis of many diseases including cancer. The first evidence to our conclusion that IKKε mediates constitutive NF-κB activity came from a recent report published while this paper was in preparation [45]. In that study, Eddy et al., provided evidences that IKKε contributes to the pathogenesis of breast cancer. Expression of a kinase inactive form of IKKε blocked breast cancer cell colony formation. The results presented in that manuscript are consistent with the findings presented here. We have shown that IKKε contributes to the basal/constitutive p65 phosphorylation and NF-κB activity as measured by EMSA and NF-κB driven luciferase promoter activity. Furthermore, we have also shown that knock-down of IKKε or overexpression of mutated version of p65 (S536A) negatively effects the cell proliferation. These findings indicate an

important role of IKKε and p65 phosphorylation in cancer cell proliferation.

There have been reports suggesting that IKKε and TBK1 may function as IκB kinase kinases [29, 82]. Therefore they might function upstream of the classical IKK complex (IKKα/β/γ). So we questioned if the phosphorylation of p65 at Ser-536 is a direct or indirect effect of IKKε/TBK1. In other words, IKKε and TBK1 might have activated classical IKKs, which then lead to the phosphorylation of p65. In order to test this, we tried to detect activation of IKKβ and IKKα by probing the same blots in Fig. 4 with commercially available, phospho-IKKα/β antibodies, however we could not detect any phosphorylation (data not shown). Although we cannot rule out that IKK□ or TBK1 might function as IκB kinase kinases, our data support a model where these two kinases are direct effectors of p65 activation. There is evidence in the literature supporting this model. First of all, it has been clearly shown that stimulus-coupled IκB degradation and p65 nuclear translocation and DNA binding activity of NF-κB is normal in IKKε and TBK1 deficient cells despite the fact that there is impaired NF-κB-dependent gene transcription [35, 36, 39] This evidence supports the fact that the activity of IKKα and IKKβ is normal in IKKε or TBK1 deficient cells, since there is normal IκB degradation, normal p65 nuclear translocation and normal DNA binding activity. We believe that our data, together with these findings, emphasize the RelA/p65 as the physiological target of IKKε and TBK1 under basal growth situations, at least in certain cells. If IKKε and TBK1 were upstream of classical IKKs, one would expect a deficiency in one of the above processes which are tightly regulated by IKKα and β.

Very recently an article has been published indicating that IKKε mediates inducible phosphorylation of NF-κB p65 at serine 468 but not at serine 536 during T cell costimulation [83]. However, the outcome of the phosphorylation at Ser-468 by IKKε has not been shown, and the phosphorylation at this site by GSK-3β has been claimed by the same group to be associated with negative NF-κB regulation [40].

To summarize, we have provided evidence that IKKε, and not TBK1, controls the constitutive NF-κB activity in certain cancer cells and in 293T cells. This evidence is supported by siRNA experiments and by associated reporter studies. It is presently unclear whether IKKε functions separately from the classic IKK complex, or through distinct regulatory pathways. It is also unclear whether IKKε is the kinase that directly controls Ser-536 p65 phosphorylation. Nevertheless, the data indicate a potentially important role for IKKε in controlling at least part of the constitutive NF-κB activity generated in certain cancer cells, with subsequent downstream effects on cancer cell proliferation.

Figures and Legends

Figure 1.1: IKKε expression is elevated in a number of cancer cells. Total cell extracts from indicated cell lines have been prepared and immuno-blotted with indicated antibodies. IKKε expression is elevated in many cancers cells and the level of the expression is well correlated with p65 phosphorylation at Ser-536. The levels of IKKα, IKKβ and TBK1 are not consistently correlated with p65 phosphorylation level.

Figure 1.1

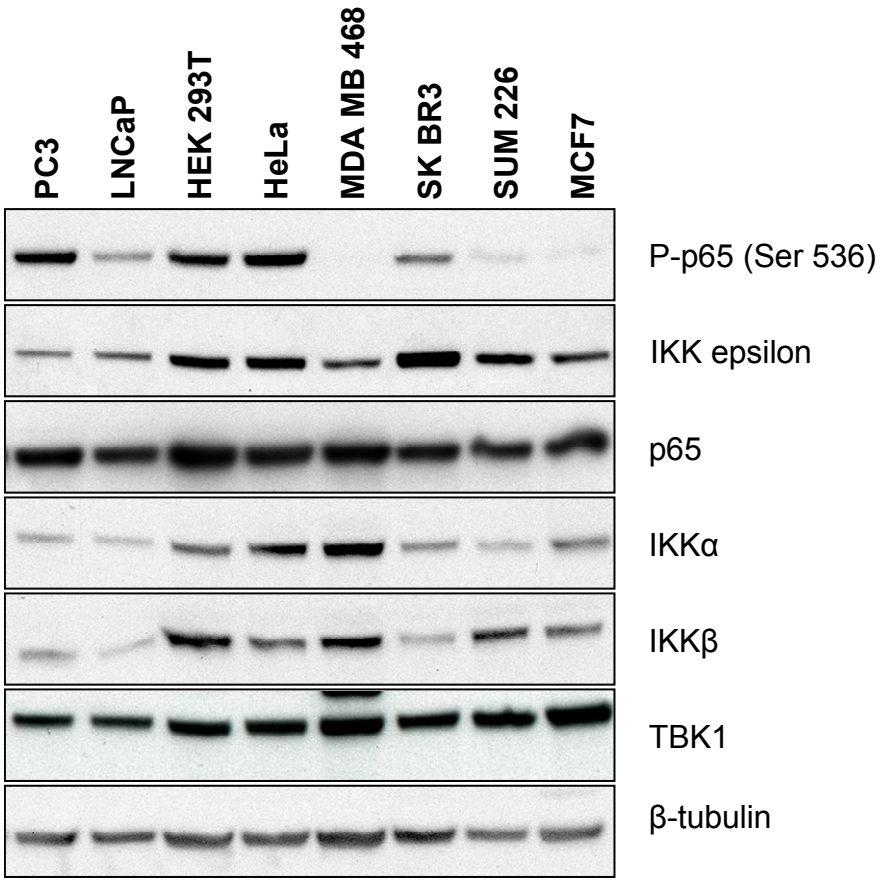
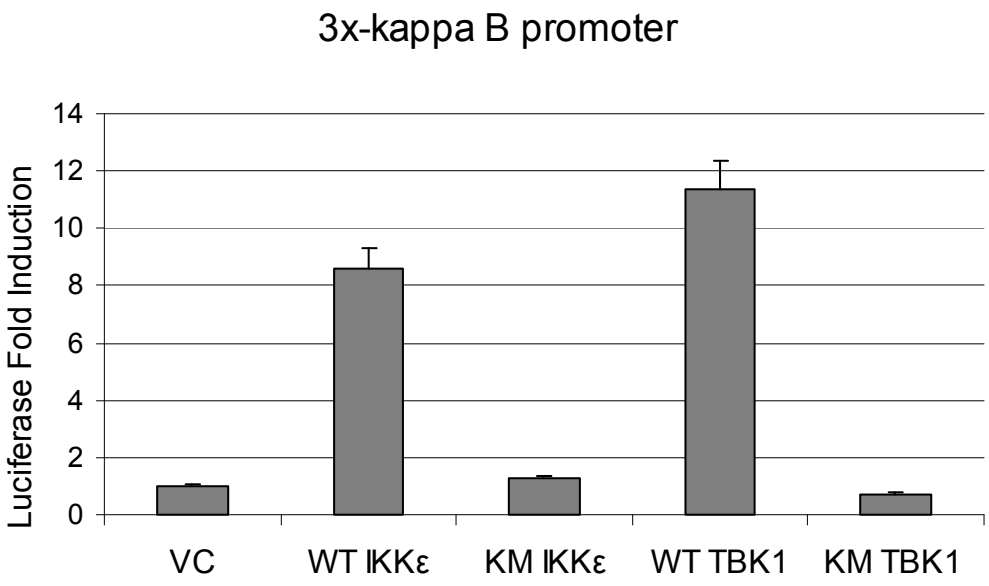

PC3
LNCaP
HEK 293T
HeLa
MDA MB 468
SK BR3
SUM 226
MCF7
P-p65 (Ser 536)
IKK epsilon
p65
IKKα
IKKβ
TBK1
β-tubulin

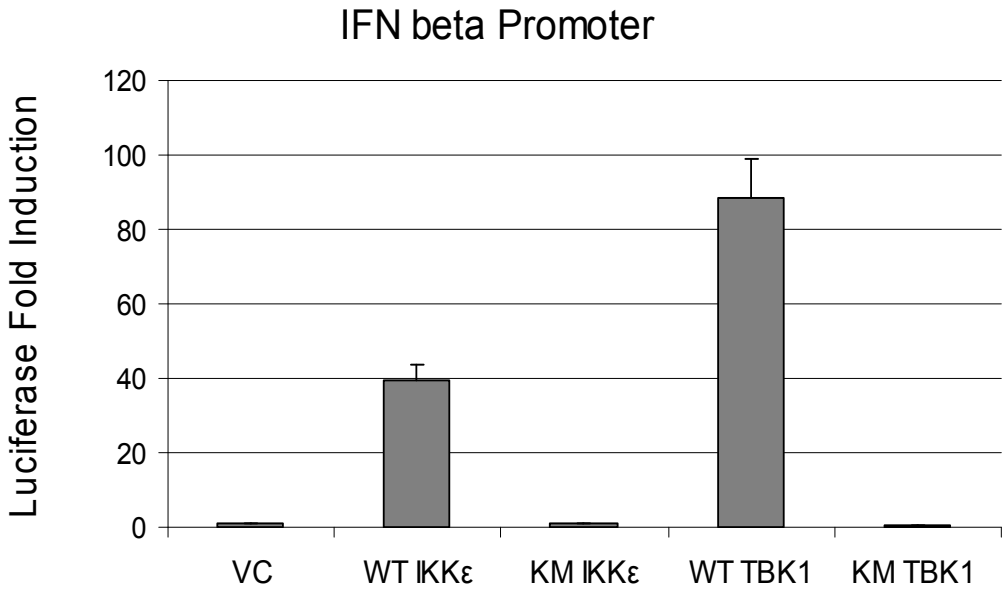
Figure 1.2: IKKε and TBK1 but not their kinase mutant forms activate NF-κB dependent reporter and IFNβ promoter. HEK 293T cells seeded in 24-well plate were transiently co-transfected the next day with an expression vector for IKKε, TBK1 or their kinase mutant forms and vector encoding a reporter gene for 3x-κB reporter (A) and IFNβ promoter (B). Experiments are done at least twice in triplicate and luciferase reporter gene activity was measured 24-48 hr after transfection. (C) IKKε activates NF-κB dependent promoter in a concentration dependent manner. Indicated concentration of IKKε and its kinase mutant form were co-tansfected with 3x-κB reporter gene constructs in 48-well plate.

Figure 1.2

A)


3x-kappa B promoter
Luciferase Fold Induction
14
12
10
8
6
4
2
0
VC
WT IKKε
KM IKKε
WT TBK1
KM TBK1

B)


IFN beta Promoter
Luciferase Fold Induction
120
100
80
60
40
20
0
VC
WT IKKε
KM IKKε
WT TBK1
KM TBK1

C)

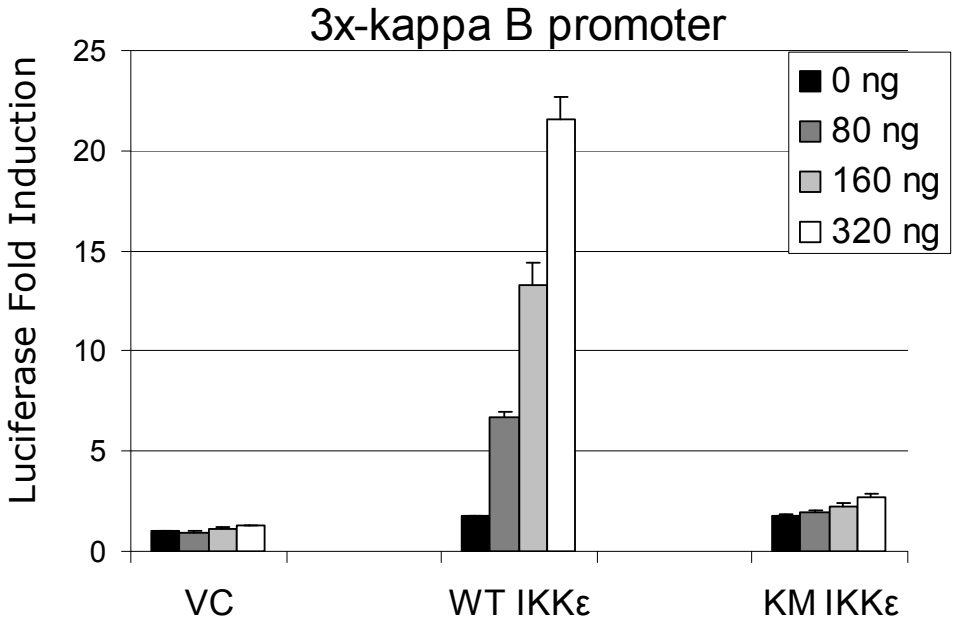

3x-kappa B promoter
Luciferase Fold Induction
25
20
15
10
5
0
0 ng
80 ng
160 ng
320 ng
VC
WT IKKε
KM IKKε

Figure 1.3: IKKε induces significant NF-κB DNA binding activity. (A) HEK 293T cells seeded in 10 cm dishes were transfected with expression vector for IKKε or empty vectors for 48 hr. Indicated cells were treated with 10 ng/ml TNF-α for 30 min. Protein-DNA complex is resolved by EMSA technique as described in materials and methods. TNF-α is included in the experiment for the control purposes. The NF-κB complex and the free probe are indicated by the arrows. Western blot analysis of cytoplasmic extracts is shown in the lower panel for the analysis of IKKε expression (B) NF-κB complex induced by IKKε and TNFα is mainly composed of p65 and p50 heterodimers. Each lane in Fig 3A has been shifted with indicated antibodies. The numbers on the top (1-4) show the lane numbers in Fig. 3A. RelA/p65 or p50 binding activity was assessed by incubation of 5 μg nuclear extracts with either p65 or p50 specific Abs followed by EMSA. In the lanes where an NF-κB complex is detected (2, 3, 4), there is a supershifted band with the p65 and p50 antibodies. ”ns” stands for non-specific binding.

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Figure 1.3

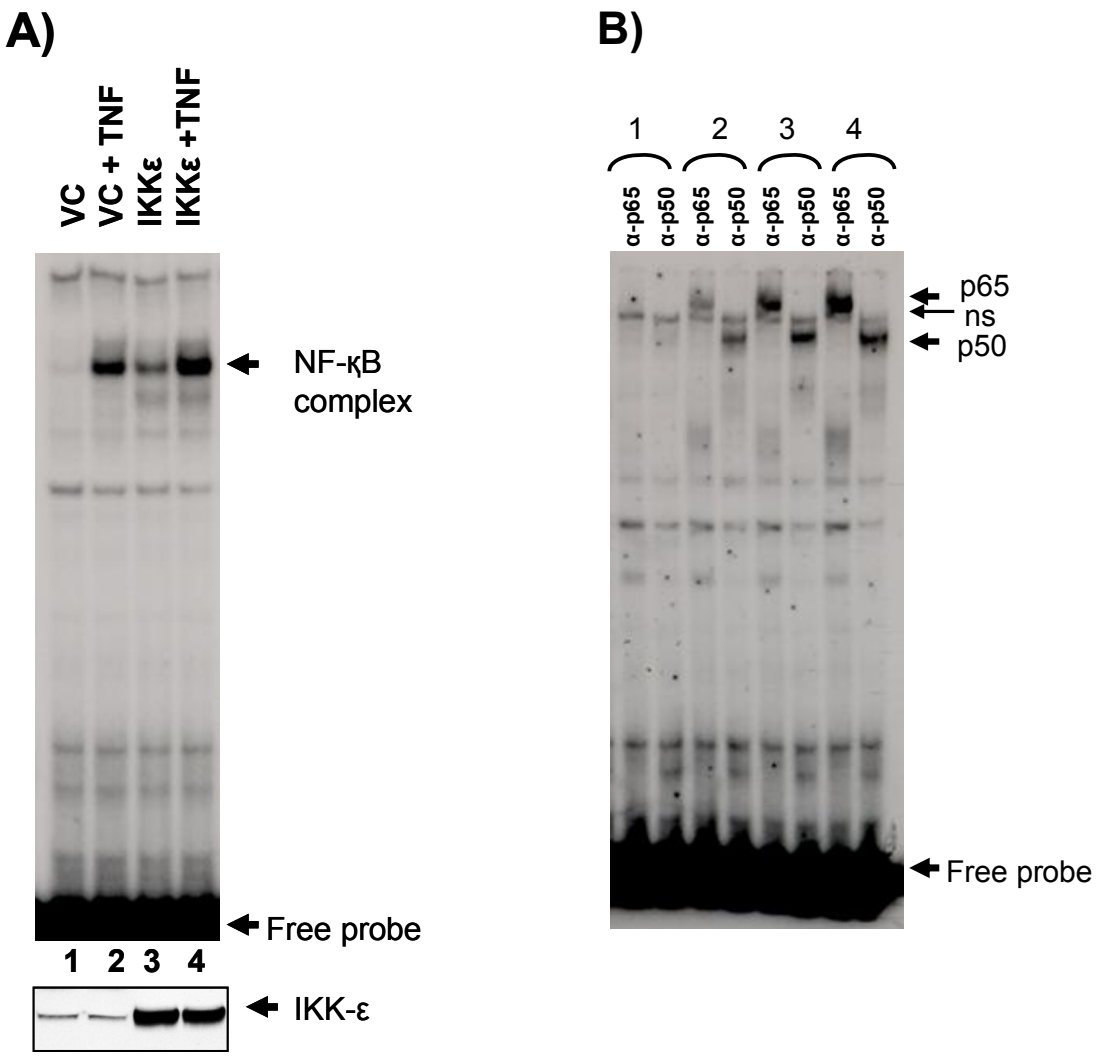
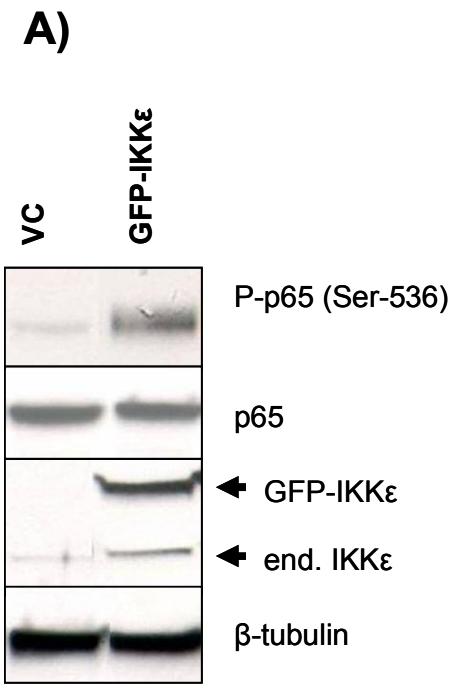

A)
VC
VC + TNF
IKKε
IKKε +TNF
NF-κB complex
Free probe
1 2 3 4
IKK-ε
B)
1 2 3 4
α-p65
α-p50
p65
ns
p50
Free probe

Figure 1.4: IKKε and TBK1 expression leads to phosphorylation of endogenous p65 at Ser-536. (A) IKKε expression induces p65 phosphorylation and its own expression. HEK 293T cells were transiently transfected with 1μg of the GFP-IKKε expression vector. Whole cell lysates were prepared 24 hr after transfection and blotted with p65 phospho specific antibody that detects phosphorylation at Ser-536 position (indicated as P-p65 (Ser-536)). Notably, endogenous IKKε expression is induced by ectopically expressed

Figure 1.4



B)

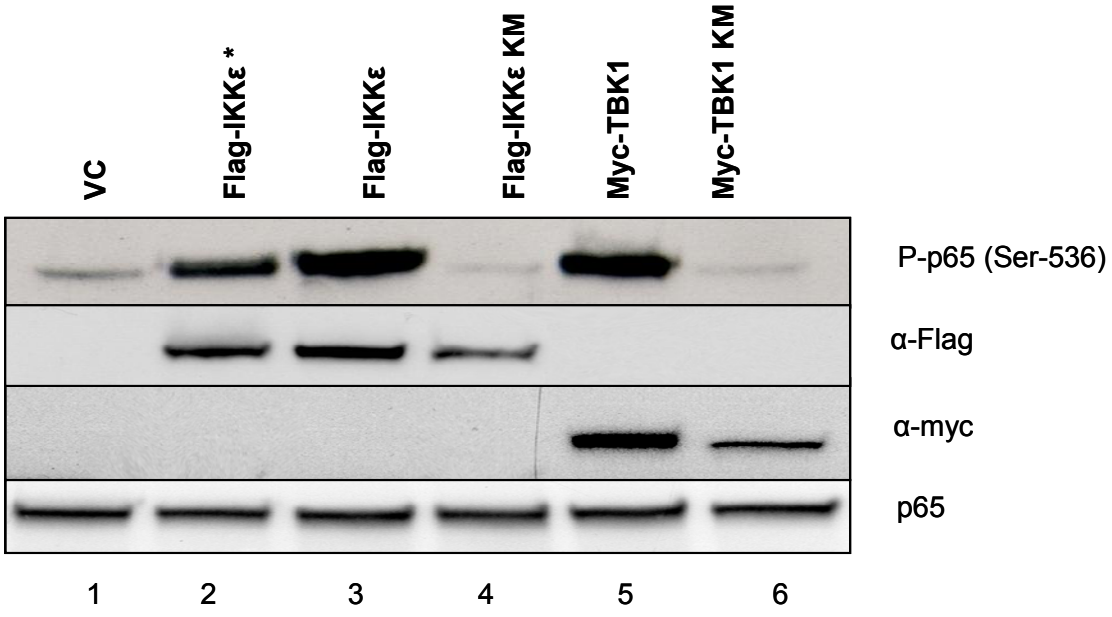
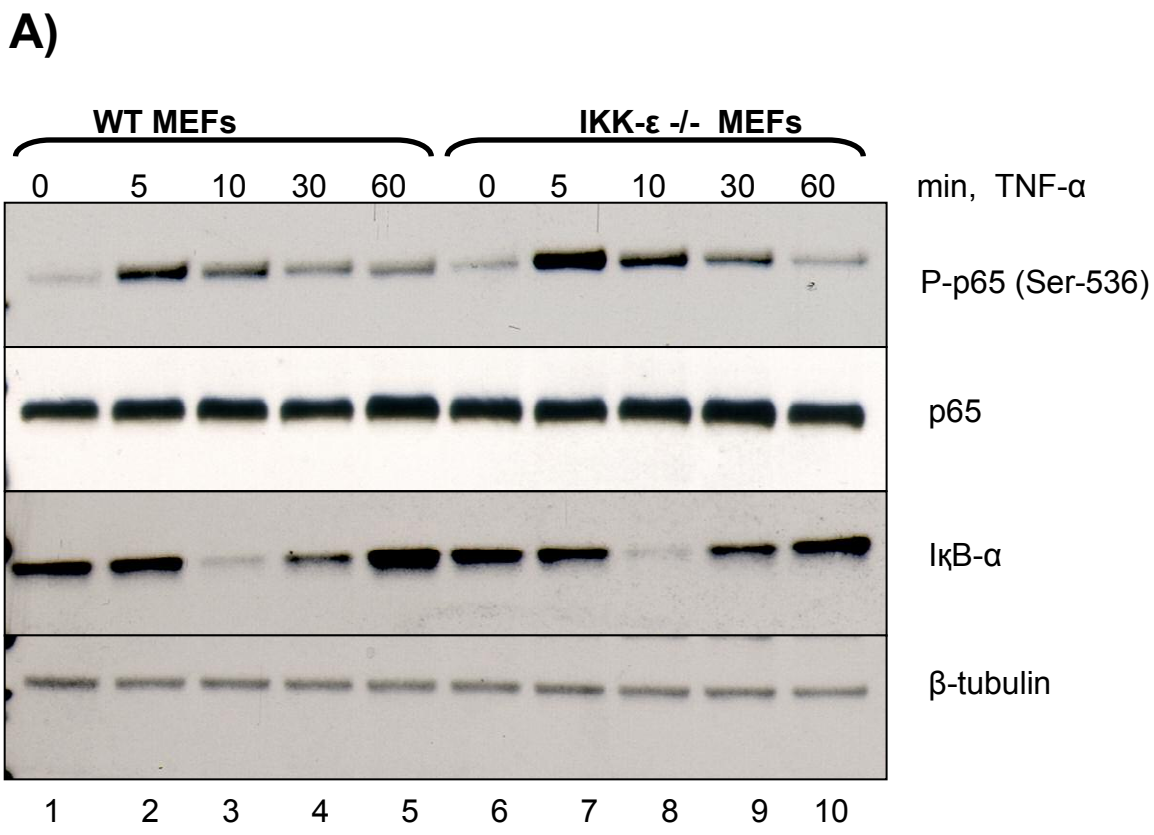
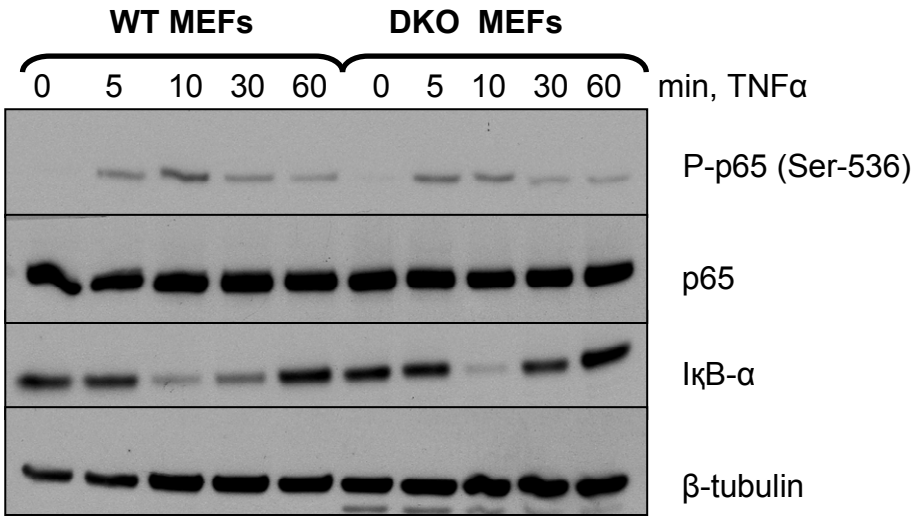

VC
Flag-IKKε *
Flag-IKKε
Flag-IKKε KM
Myc-TBK1
Myc-TBK1 KM
P-p65 (Ser-536)
α-Flag
α-myc
p65
1
2
3
4
5
6

Figure 1.5: p65 phosphorylation at Ser-536 position is normal in IKKε deficient MEFs in response to different stimuli. Cytokine induced p65 phosphorylation in IKKε singly deficient and IKKε and TBK1 doubly deficient MEFs is comparable to WT MEFs. Indicated MEF cells were seeded in 6-well plates and at 70-80 % confluency they were stimulated with 10 ng/ml TNFα (A, B), 10 ng/ml IL-1β (C, D) 1 μg/ml LPS (E) and 100 ng /ml PMA (F) for indicated time points. Whole cell extracts were prepared and blotted with indicated antibodies. Interestingly in all of the stimuli tested, there was no significant difference in terms of either p65 phosphorylation pattern or IκBα degradation kinetic in IKKε deficient or DKO MEFs compared to WT MEF cells. Analysis of β-tubulin levels confirmed that loading was essentially equivalent in all lanes.

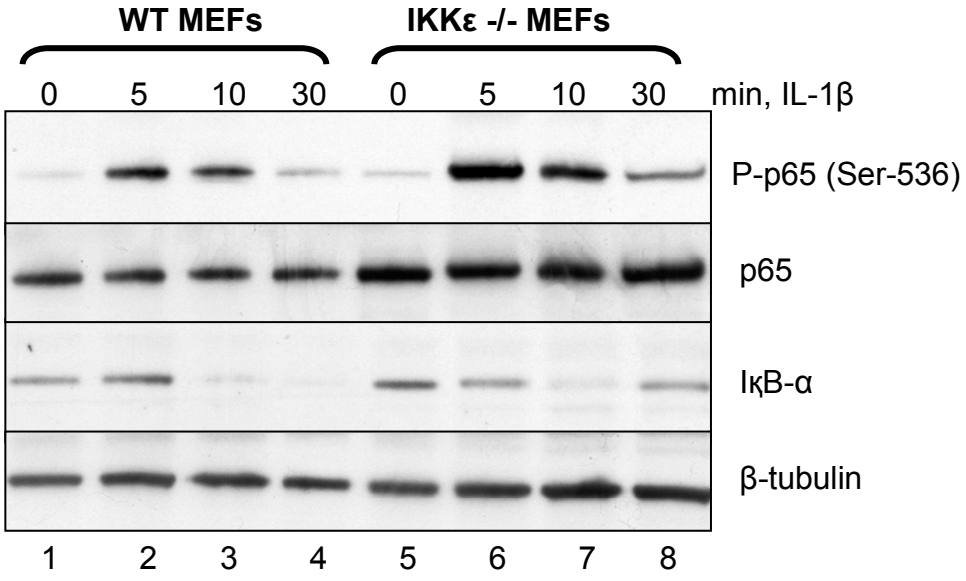
Figure 1.5


A)
WT MEFs
IKK-ε -/- MEFs
0 5 10 30 60 0 5 10 30 60
min, TNF-α
P-p65 (Ser-536)
p65
IκB-α
β-tubulin
1 2 3 4 5 6 7 8 9 10

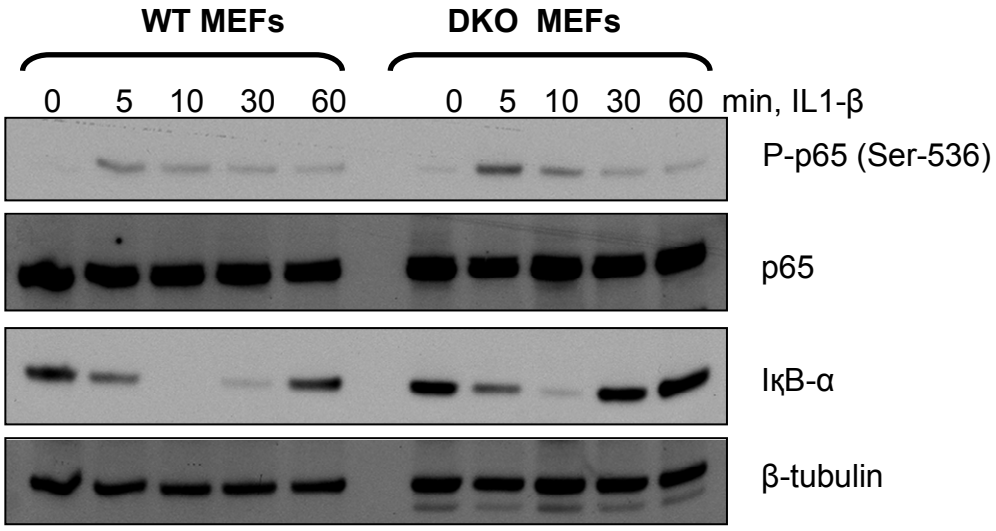
B)


WT MEFs
DKO MEFs
0 5 10 30 60 0 5 10 30 60
min, TNFα
P-p65 (Ser-536)
p65
IκB-α
β-tubulin

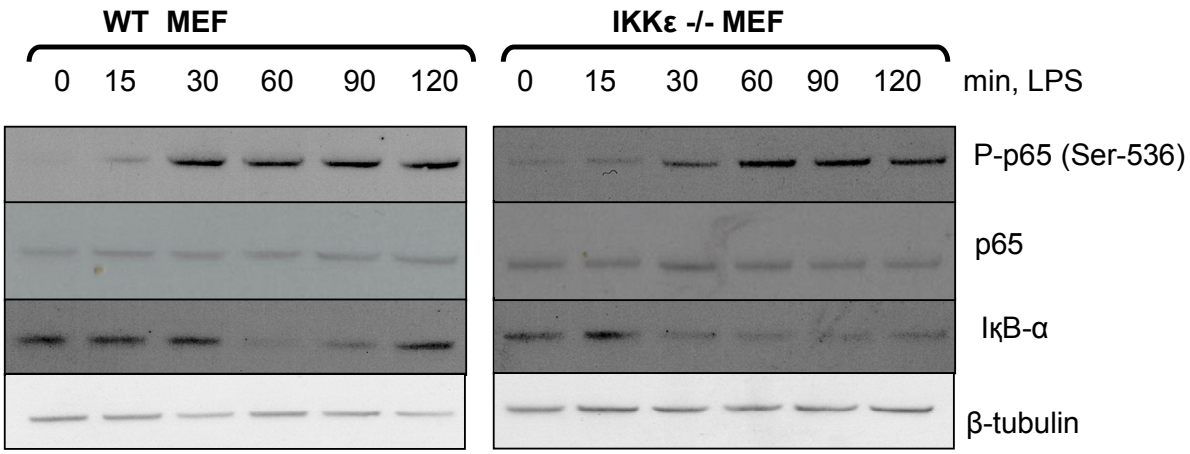
C)


WT MEFs
IKKε -/- MEFs
0 5 10 30 0 5 10 30
min, IL-1β
P-p65 (Ser-536)
p65
IκB-α
β-tubulin
1 2 3 4 5 6 7 8

D)


WT MEFs
DKO MEFs
0 5 10 30 60
0 5 10 30 60 min, IL1-β
P-p65 (Ser-536)
p65
IκB-α
β-tubulin

E)


WT MEF
IKKε -/- MEF
0 15 30 60 90 120
0 15 30 60 90 120 min, LPS
P-p65 (Ser-536)
p65
IκB-α
β-tubulin

F)

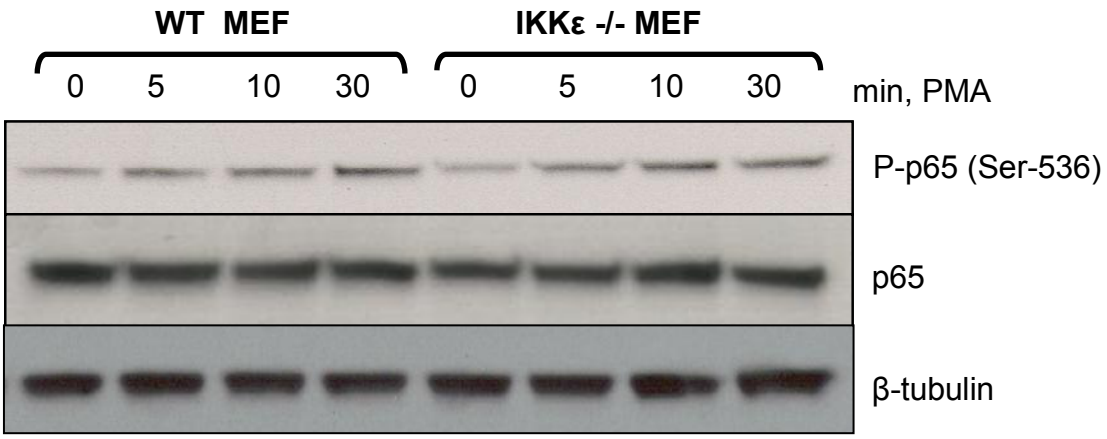
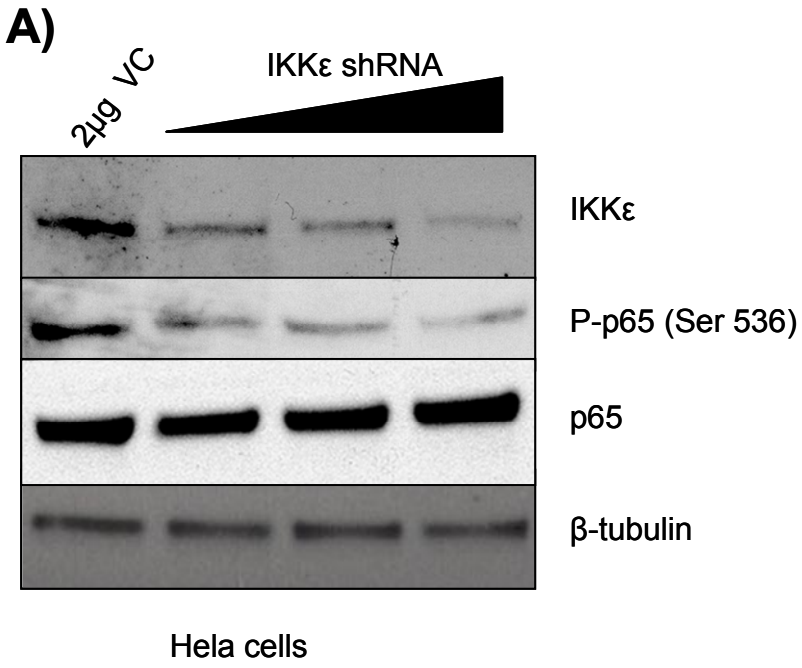
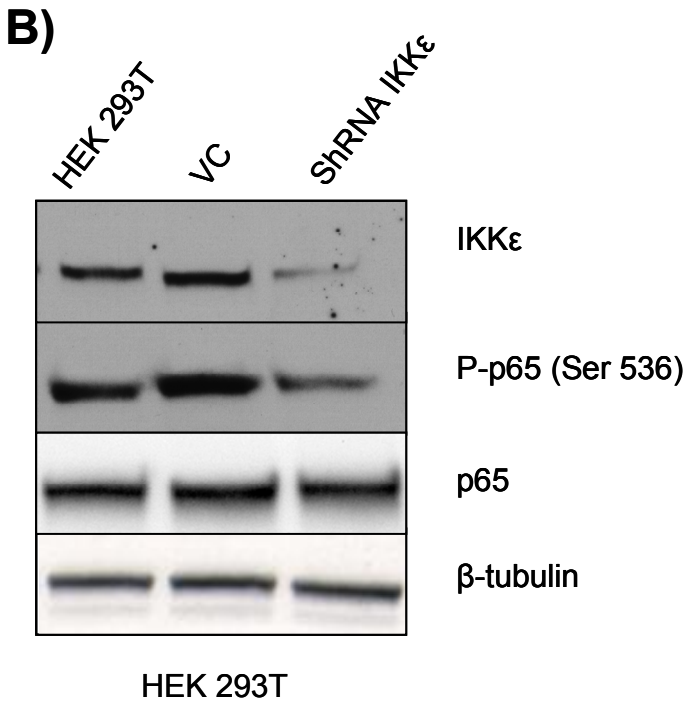

WT MEF
IKKε -/- MEF
0
5
10
30
0
5
10
30
min, PMA
P-p65 (Ser-536)
p65
β-tubulin

Figure 1.6: Sustained knockdown of IKKε leads to impaired Ser-536 phosphorylation of p56. (A, B) Vector encoding shRNA targeting IKKε or vector control (VC) alone was transfected in HeLa cells (A) in 6-well plate with increasing concentration of IKKε shRNA vector (1, 2, 3 μg/well shRNA plasmid) or in HEK 293T cells (B) for 72 hrs. Lysate preparation and western blots are done as described above. A significant knock-down in IKKε level was well correlated with reduction in p65 Ser-536 phosphorylation whereas there is no change in total p65 level. β-tubulin level is shown to demonstrate equal loading in all lanes. (C) siRNA targeting TBK1 was transfected in HeLa cells for 72 hr. As shown, despite the significant reduction in TBK1 level, no significant change in p65 phosphorylation was observed.

Figure 1.6




B)
HEK 293T
VC
ShRNA IKKε
IKKε
P-p65 (Ser 536)
p65
β-tubulin
HEK 293T

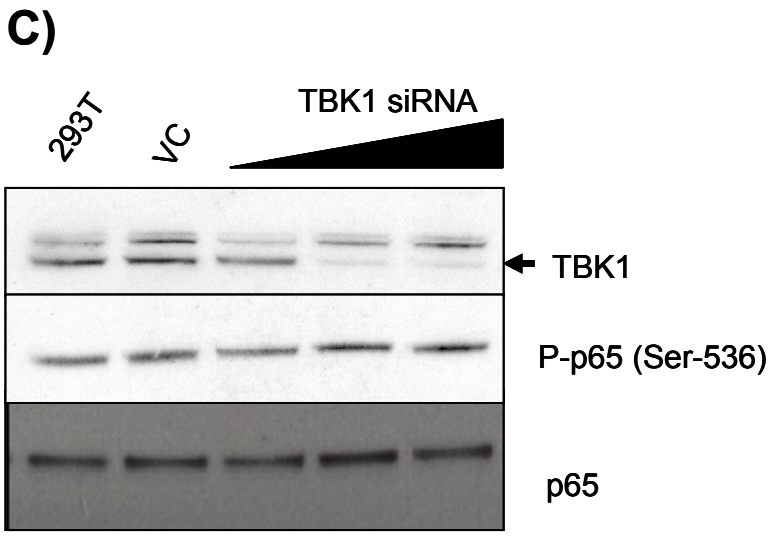

C)
293T
VC
TBK1 siRNA
TBK1
P-p65 (Ser-536)
p65

Figure 1.7: IKKε knock-down in HeLa cells leads reduction in basal activity of NF-κB dependent promoter. HeLa cells seeded in 6 well plates were transfected by siRNA targeting IKKε or negative control (NC) siRNA at indicated concentration for 48 hr. Then cells were split to 24 well plates and were transfected with 3X-κB promoter and *Renilla* Luciferase promoter as an internal control. siRNA was also included in this transfection complex to give a second hit for IKKε knock-down. At indicated concentrations of siRNA (20 and 40 nM), knock-down efficiency of IKKε mRNA was 58% and 79% respectively as measured by QRT-PCR (Data not shown). Reduction in IKKε level is well correlated with the reduction in basal activity of an NF-κB dependent promoter.

Figure 1.7

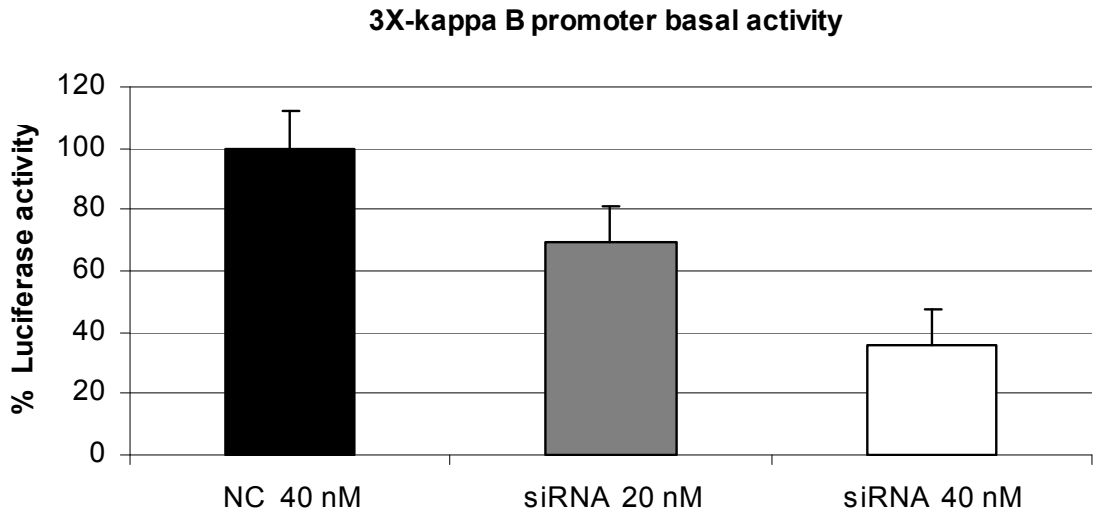
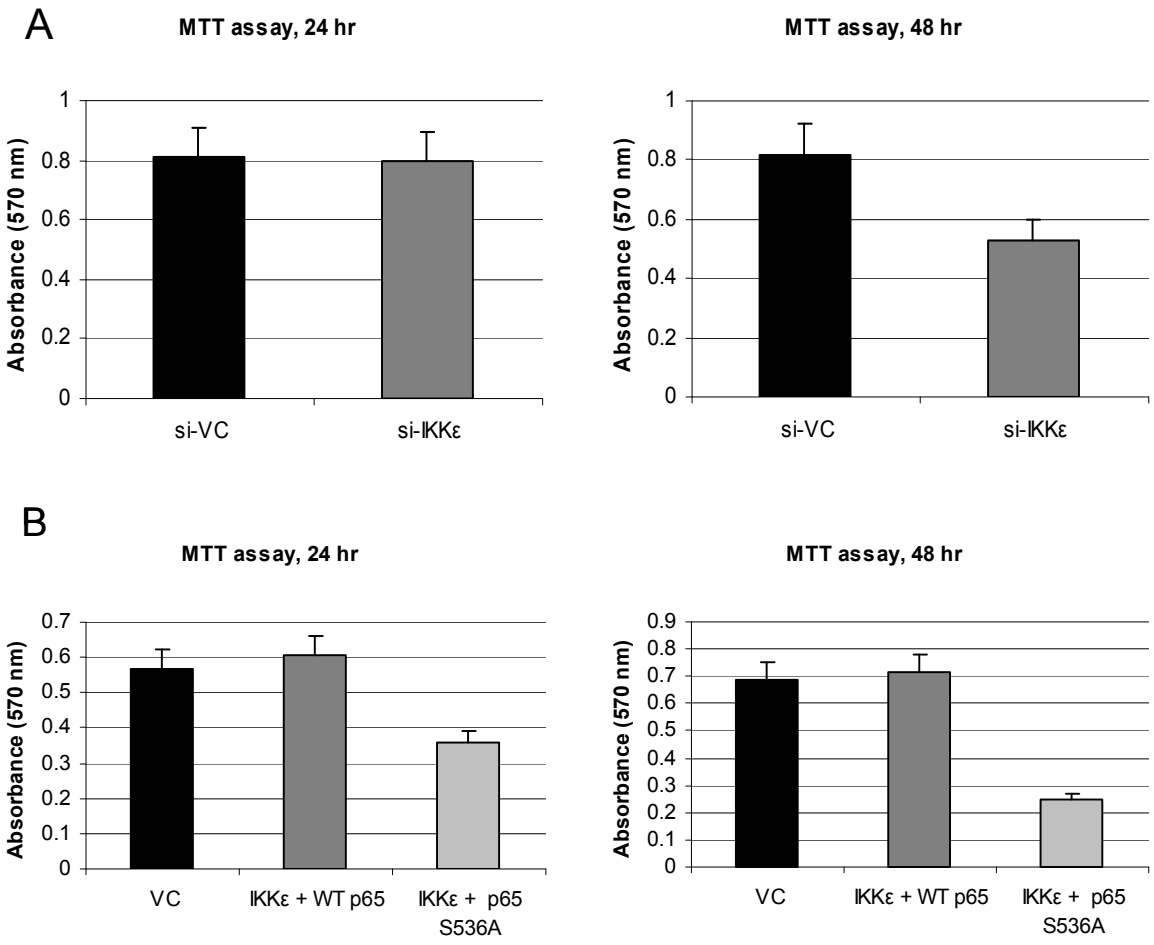

3X-kappa B promoter basal activity
% Luciferase activity
120
100
80
60
40
20
0
NC 40 nM
siRNA 20 nM
siRNA 40 nM

Figure 8: IKKε and p65 phosphorylation mediates cell proliferation. Hela cells seeded in 6 well plates were transfected with indicated plasmids for 24 hr, then cells were trypsinized seeded in 96 well plate in triplicates. After indicated time points, MTT assay has been performed. Results are representative of two independent experiments done in triplicates. When IKKε level has been modified by shRNA, cell proliferation was not as efficient as control shRNA transfected cells. IKKε knock-down with shRNA after 48 hr reduced cell proliferation significantly (A). Si-VC and Si-IKKε stands for vector control shRNA and shRNA against IKKε respectively. IKKe was also overexpressed together with WT and S536A mutant version of p65 to test the significance IKKε mediated of p65 phosphorylation (B). Although IKKε and WT p65 transfected cells proliferate slightly better than vector control transfected cells, when IKKε was overexpressed with S536A mutant version of p65, which can not be phosphorylated at ser 536 position was, there was significantly less cell proliferation. These data suggest that both IKKε level and p65 phosphorylation status is important for proper cell proliferation.

Figure 1.8


A
MTT assay, 24 hr
Absorbance (570 nm)
1
0.8
0.6
0.4
0.2
0
si-VC
si-IKKε
MTT assay, 48 hr
Absorbance (570 nm)
1
0.8
0.6
0.4
0.2
0
si-VC
si-IKKε
B
MTT assay, 24 hr
Absorbance (570 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
VC
IKKε + WT p65
IKKε + p65 S536A
MTT assay, 48 hr
Absorbance (570 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
VC
IKKε + WT p65
IKKε + p65 S536A

Materials and methods

Reagents and materials: All cells were cultured in Dulbecco's modified Eagle's medium (DMEM), complemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin. Generation of Wild type, IKKε single and IKKε- TBK1 double knockout cells were described previously [35] and were the kind gift of S. Akira. A monoclonal antibody against Flag (M2) was obtained from Sigma. An antibody to IKKε and to phospho-specific NF-κB p65 (Ser-536) were obtained from Cell Signaling. Antibodies to β-tubulin and to IκBα were obtained from Santa Cruz. Antibodies to IKKα and IKKβ were obtained from Upstate Biotechnology Inc. LPS (L6529, Sigma) was used at a final concentration of 1 μg/ml. rhTNF-α (Promega) was used at a final concentration of 10 ng/ml. Recombinant mouse Interleukin- 1β (mIL-1β)(Roche Applied Sciences) was used at a final concentration of 10 ng/ml. Effectene transfection reagent obtained from Qiagen was used according to the manufacturer's protocol.

Plasmids and Constructs: 3X-κB luciferase reporter construct contains 3 κB consensus DNA binding sites from the MHC class I promoter fused upstream of firefly luciferase. Wild type and kinase mutant forms of Flag-tagged IKKε (IKKε K38) have been described previously [27]. Wild type and kinase mutant forms of TBK1 (TBK1 K38) have been described previously [32] were the kind gift of J. Hiscott. pLuc-110 IFNβ reporter constructs have been described previously [31] and were the kind gifts of T. Maniatis.

Transfections and Reporter assays: For transient transfections, the indicated cell lines were

seeded in 6-well plates at 30-50 % density and transfected the next day with the indicated expression vectors for 48 hrs using Effectene (Qiagen) transfection reagent according to the manufacturer's instruction. For reporter assays, 2 x 10^5 cells were seeded in 24 well plates and were co-transfected the next day with the indicated luciferase reporter genes and a β-galactosidase reporter gene as an internal control. The total amount of transfected DNA (500 ng DNA) in each well was adjusted by adding empty plasmid vector (pcDNA3.1). Luciferase activity of whole cell lysates was measured by using a luciferase assay kit (Promega). β-galactosidase activity was measured by Liquid β-Gal assay with CPRG substrate. Relative luciferase activity was calculated by normalizing to β-gal expression values. Luciferase fold induction was calculated by normalizing to control treatment which was assumed as 1 fold induction.

Western Blot: After stimulation, cultured cells were lysed on ice for 5 min in RIPA lyses buffer with freshly added protease and phosphatase inhibitor cocktails. Lysates were cleared by centrifugation at 4 °C for 15 min at 13,000 *g*. The amount of total protein was measured and equal amounts (20 μg) were fractionated by NuPAGE Novex 4-12% Bis Tris gels (Invitrogen) and electro-transferred to polyvinylidene difluoride membranes. Membranes were blotted with the indicated antibodies, and proteins were detected using an enhanced chemiluminescence detection system (Amersham Biosciences, Freiburg, Germany). Where indicated, membranes were stripped and re-probed with the indicated antibodies.

Electrophoretic Mobility Shift Assay (EMSA): EMSAs were performed as previously described [23]. Briefly, 4−5 μg of nuclear extracts prepared following cell stimulation were

incubated with a radiolabeled DNA probe containing an NF-κB consensus site. For supershifts, 1 µl of anti-p65 antibody (Rockland) or 2 µl of anti-p50 antibody (Santa Cruz, SC-7178) was added and the binding reaction was allowed to proceed for an additional 15 min. Protein−DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by autoradiography.

siRNA & shRNA transfection: IKKε mRNA was knocked down with the GeneSupressor System (IMGENEX). Plasmids encoding control shRNA and IKKε shRNA were transfected by Effectene transfection reagent (Qiagen) according to manufacturer's instructions for 48-72 hr. Additionally, *Silencer*® predesigned siRNA targeting IKKε and TBK1 have also been utilized and were transfected with *Silencer*™ siRNA Transfection II Kit. Lysate preparation and westerns were performed as described.

Cell proliferation, MTT assay: Cell proliferation assay has been performed as described by TACS MTT assay kit (R&D systems). First, the optimal cell number, which was $1x10^4$ for Hela cells in our system, has been determined. After transfection, equal number of cells has been seeded in 96 well plate for indicated times. 10 µl of MTT reagent was added to each well including the blank wells, and incubated for 4-5 hrs at 37 ^{0}C. Then 100 µl of detergent reagent was added, incubated for at least 12 hr at 37 ^{0}C and absorbances at 570 nm were measured with reference wavelength of 650 nm.

CHAPTER II

Regulation of Bcl-3 proto-oncogene expression by IKK ε (epsilon)

Abstract

We have previously demonstrated that IKK-i / IKKε (epsilon) is expressed in a number of cancer cells and is involved in regulation of constitutive NF-κB activity that mediates cell proliferation [44]. Here we present evidence that Bcl-3 is an important downstream IKKε regulated gene that is potentially the mechanistic link between IKKε and cell proliferation. Research from our lab has recently indicated Bcl-3 as an important suppressor of persistent p53 activity [84]. Bcl-3 was identified by microarray analysis of gene expression profiles in Hela cells with RNAi mediated IKKε knock-down. Here we demonstrate that IKKε knock-down leads to significant reduction in Bcl-3 mRNA and protein level, and exogenous expression of IKKε significantly upregulates Bcl-3 gene expression. Furthermore, basal as well as inducible Bcl-3 gene expression is diminished in IKKε knock-out MEF cells. Data presented here suggests that Bcl-3 gene expression is NF-κB p65 dependent but IKKα/β independent. Interestingly, IKKε is able to phosphorylate p65 in IKKα/β DKO cells, which we think explains how p65 regulates Bcl-3 gene expression independent of IKKα/β. Significant correlation between IKKε and Bcl-3 expression has been observed in HCC tumors compared to adjacent tissue. Moreover, MCF-7 cells stably expressing Bcl-3 proliferate more efficiently than the parental cells suggesting that Bcl-3 is an important mediator of cell proliferation and is potentially the mechanistic link in IKKε induced cell survival and proliferation.

Introduction

Bcl-3 was identified 20 years ago by molecular cloning of the breakpoint of the t(14;19) chromosomal translocation from a subset of human B-cell chronic lymphocytic leukemias [48]. Translocation at this point leads to Bcl-3 overexpression that results in dysregulation of mostly unexplored downstream target genes that are predicted to be involved in differentiation, cell survival and apoptosis [49].

Bcl-3 is a member of the ankyrin-repeat-containing IκB family of NF-κB inhibitors [3]. Although it shares significant structural features with IκB proteins [50, 51], Bcl-3 contains two transactivating domains and can function as a transcriptional activator or repressor by forming heterocomplexes with the NF-κB p50 or p52 subunits in a phosphorylation dependent manner [51-55]. Although the molecular mechanism of Bcl-3 dual function is poorly understood, some observations explain how Bcl-3 may act as both transcriptional activator as well as repressor. It was suggested that Bcl-3 can potentially act as a transcriptional activator by removing repressor p50 homodimers from the κB sites [51] and also by acting as a bridging factor between NFκB and other coactivators such as JAB1, Bard1 and Tip60 [56]. Similarly, upon IL-1β stimulation, Bcl-3 recruits Tip60 acetyltransferase containing activator complex to the promoter region of specific subset of NF-κB dependent genes [57]. Moreover, Bcl-3 was also suggested to function as a coactivator itself by regulating the expression of subset of NF-κB dependent genes in response to TNFα [58]. In contrast to its transactivator potential Bcl-3 has also been suggested to act as a transcriptional repressor. It has been demonstrated that Bcl-3 can

physically interact with HDAC-1, -2 and -3 which are involved gene repression [59]. Another study also demonstrated that Bcl-3 inhibits LPS induced inflammatory responses in macrophages by recruiting HDAC-1 [60]. Although the opposite dual function of Bcl-3 is poorly understood, one explanation may come from the posttranslational modifications on Bcl-3 or Bcl-3 interacting proteins. Bcl-3 has two residues within its C terminal domain that are phosphorylated by GSK3 kinase which mediates its interaction with HDAC1 and induce its degradation through proteosome pathway [59]. Although protein phosphorylation was suggested to be the key mechanism for regulation of Bcl-3 activity [61], other posttranslational modifications such as ubiquitination was recently demonstrated to be critical for Bcl-3 nuclear translocation [62]. For instance, It was clearly demonstrated that TPA or UV light triggers the translocation of CYLD from the cytoplasm to the perinuclear region, where CYLD binds and deubiquitinates Bcl-3, thereby preventing nuclear accumulation of Bcl-3 and p50/Bcl-3- or p52/Bcl-3-dependent gene expression and subsequent cell proliferation [62].

Bcl-3 has been implicated in many crucial physiological events such as cell proliferation, immune, inflammation and cancer progression. The results from Bcl-3 knock-out mice revealed critical roles for Bcl-3 in antigen-specific priming of T and B cells. Bcl-3 deficient mice are also impaired in germinal center reactions and T cell-dependent antibody responses to influenza virus and have partial loss of may underlie the immunologic defects [63, 64]. It was shown that Bcl-3 associates with NF-κB p50 or p52 subunits and strongly enhances cell proliferation and oncogenesis through activation of the *cyclin D1* promoter [23, 65]. In line with these observations, Eμ–Bcl-3 transgenic mice develop

lymphoproliferative disorders such as; Lymphadenopathy, splenomegaly, and altered immunoglobulin production [66]. Furthermore, although the mechanism is poorly understood, Bcl-3 was shown to directly transform cells [59]. Research demonstrated that Bcl-3 exerts antiapoptotic activity in B and T lymphocytes [67, 68] and controls the death of activated T cells [69]. Despite its well established role in cell proliferation and apoptosis, the signaling pathways and upstream key effecters that activate Bcl-3 gene expression are poorly understood.

IKKε was initially identified as PMA inducible kinase [27], which is identical to a kinase named IKK-i identified via its induction downstream of LPS-induced signaling [19]. IKKε in turn is closely related to another IKK-related kinase designated as TBK1 [28] or NAK [29]. TBK1, which is highly homologous to IKKε, binds to TANK and TRAF and may form an alternative IKK complex consisting of IKKε and TBK1 [28]. IKKε and TBK1 have been shown to play important roles in the innate immune response by functioning as critical kinase components of the interferon regulatory factor 3 (IRF3) and IRF7 signaling pathways involved in responses to viral infection or dsRNA treatment [31, 32]. In addition to their well established roles in IRF3 and IRF7 activation during innate immunity, recent studies implicated the involvement of IKKe in cancer mechanism by several groups including us [44-46]. Interestingly TBK1 has also been implicated in tumor cell survival [47].

Although research from several groups recently appreciated the implication of IKKε and TBK1 cancer progression and tumor cell survival, the molecular mechanism of IKKε and TBK1 induced cancer signaling and their downstream target genes remain to be identified.

Here we report Bcl-3 as one of the important target genes downstream of IKKε that is important in cell proliferation and cell survival. Microarray analysis and subsequent biochemical experiments indicated that Bcl-3 is transcriptionally regulated by IKKε in a NF-κB p65 dependent manner. Moreover, Bcl-3 expressing MCF-7B cells proliferate more efficiently than the parental MCF-7 cells. Our data suggest that Bcl-3 is an important mediator of cell proliferation downstream of IKKε and is potentially the mechanistic link in IKKε-induced cell survival and proliferation.

Results

Identification of Bcl-3 as IKKε target gene: We have previously demonstrated that IKKε is an important mediator of NF-κB activity in cancer cell-associated signaling mechanism [44]. Our subsequent research has focused on understanding and identifying IKKε-regulated genes and downstream signaling mechanisms at the genome wide level. HeLa cells have been used since they display significant basal IKKε expression as in many other cancer cell lines. IKKε mRNA levels were knocked down by siRNA technology. RNA from samples where there is at least 75-80 % IKKε knock down was subsequently processed and hybridized to Affymetrix Human Genome Plus U133 Plus 2.0 as described in the materials and methods section. Among a number of potentially interesting genes (Fig. 2.1B) whose expression has changed 2 fold or more at the basal level upon IKKε knock-down, Bcl-3 was especially interesting for us because of our previous research demonstrating the importance of Bcl-3 in cell survival and proliferation. Several publications from our lab have demonstrated that Bcl-3 is over expressed in human tumors, drives cyclin D1 gene expression and negatively regulates p53 signaling [23, 84, 85]. It was interesting to see from the literature and our own

studies that both Bcl-3 and IKKε are overexpressed in number of cancer cell lines and human tumors and that there is a strong positive correlation between IKKε and Bcl-3 mRNA levels from microarray experiments. These observations led us to analyze if IKKε is the key regulator of Bcl-3 gene expression.

IKKε knock-down by Si-RNA reduces, and expression of IKKε induces Bcl3 gene expression:

In order to validate the microarray results IKKε was knocked-down in an independent experiment. IKKε targeted siRNA treatment significantly reduced Bcl-3 both at the mRNA level as well as at protein level in HeLa cells. Total RNA from negative control and IKKε targeted Si-RNA treated samples were prepared and Bcl-3 and IKKε mRNA levels were quantified with qRT-PCR (Figure 2.2A). In addition to mRNA levels, IKKε knock-down significantly reduced Bcl-3 protein levels in HeLa cells. Total cell lysates from different IKKε si-RNA and the negative control (NC) si-RNA treated samples were prepared after 72 hrs of si-RNA treatments. Different si-RNA against IKKε have been used and we could only knock-down IKKε effectively with product si-RNA ID number 919 from Ambion (Figure 2.2B). In addition to gene knock-down experiments, we wanted to test if exogenously expressed IKKε can induce Bcl-3 gene expression. Transiently expressed WT IKKε, but not its kinase mutant form (IKK KM), induced significant endogenous Bcl-3 gene expression (Fig. 2C). Importantly, kinase mutant IKKε (IKK KM) reduces the basal level of Bcl-3 gene expression which suggests that kinase activity of IKKε is important for its function in terms of regulating Bcl-3 gene expression. Moreover, expression of non-degradable form of IκB (IκB super repressor (SR)) inhibits IKKε induced Bcl-3 gene expression however p65

expression significantly up regulates it. In line with overexpression studies, promoter analysis of Bcl-3 also indicated that IKKε is an important regulator of Bcl-3 gene expression (Fig. 2.2D). WT IKKε but not its kinase mutant form induces significant luciferase activity from a construct that has Bcl-3 promoter and an intronic enhancer that has been previously shown to be NF-κB responsive [86]. These results suggest that IKKε control Bcl-3 gene expression in a NF-κB dependent manner. In line with previous observations that Cyclin D1 is one of the Bcl-3 target gene [23, 65] we note that exogenous WT IKKε expression is able to induce cyclin D1 gene expression over 50 %. This increase in cyclin D1 gene expression over the basal level may not seem too much of a fold induction; however, if this effect is true, in a tumor setting, a 50 % increase would give greater advantage to those cells that have higher Cyclin D1 expression.

Basal levels as well cytokine induced levels of Bcl-3 gene expression are defective in IKKε KO MEF cells: Next, IKKε mediated regulation of Bcl-3 gene expression was explored in MEF cells that are deficient for IKKε. For this reason, WT MEF as well as IKKε deficient MEF cell lines have been utilized. First, Bcl-3 gene expression at the basal level was analyzed in both WT as well as IKKε deficient MEF cell line. As shown in Figure 2.3A, IKKε deficient cells have significantly low levels of Bcl-3 mRNA. This further enhances the idea that IKKε is an important mediator of Bcl-3 gene expression. In order to explore inducible regulation of Bcl-3 gene expression, WT MEFs as well as IKKε deficient MEFs were treated with LPS for indicated times, and Bcl-3 mRNA levels are quantified with qRT-PCR. As shown, in Figure 2.3B, cells deficient for IKKε have significantly lower levels of total Bcl-3 message level. Although there is some induction of Bcl-3 in IKKε deficient cells,

compared to WT cells the total induction is significantly less. In addition to the mRNA level, Bcl-3 protein expression is also defective in IKKε deficient cells. As shown in Fig. 2.3C, there is a detectable Bcl-3 gene expression 6 hours after LPS induction, however, this cannot be detected in IKKε deficient cells. It is interesting to observe that Bcl-3 is well induced in WT MEFS, however IKKε deficient cells does not show any detectable Bcl-3 protein expression in response to LPS. Data presented here strongly suggest the transcriptional regulation of Bcl-3 gene expression by IKKε.

IKKα and IKKβ independent. but NF-kB p65 dependent Bcl-3 gene expression: Bcl-3 gene expression has been reported to be regulated by different transcription factors [68, 86]. Since we previously reported that IKKε mediates constitutive NF-κB in cancer, we wondered if the role of NF-κB signaling pathway in regulating Bcl-3 gene expression. For this purpose, MEF cells that are p65 knock-out (KO), and IKKα and IKKβ double knock-out (DKO) have been utilized. Interestingly in p65 deficient MEFs there is significantly less Bcl-3 gene expression at the basal level and there is little or no induction of Bcl-3 in p65 deficient cells upon TNFα treatment for over 7 hr period (Fig. 2.4A). This result clearly indicates that NF-κB p65 is an important mediator of Bcl-3 gene expression as has been suggested before [86]. Since most of the canonical NF-κB signaling comes from IKKα and IKKβ, we wanted to test the role of the classical IKK complex in Bcl-3 gene expression. As shown in Figure 2.4B, there is no significant difference in the level of Bcl-3 gene expression in IKKα/β DKO MEFs and WT MEFs. Both basal and inducible levels seem to be comparable, if not more, in DKO cells. These results suggest that Bcl-3 gene expression is controlled by NF-κB p65 by non-classical

NF-κB kinases. Importantly, as an example of one of these kinases, we and other groups have shown that p65 is phosphorylated by IKKε [40, 44, 83].

IKKα - and IKKβ -independent phosphorylation of p65 by IKKε: The data showing that Bcl-3 gene expression is independent of IKKα/β but p65 dependent was in line with the observation that there are other IKK related p65 kinases like IKKε and TBK1 besides IKKα and IKKβ. Although there is no doubt that IKKε and TBK1 are involved in p65 phosphorylation, there is no direct evidence proving that they are direct kinases and therefore they are suggested to act as IκB kinase (IKK) kinases rather that direct mediators of p65 activation [26]. These observations lead us to investigate if IKKε can function independent of IKKα and β. To test our hypothesis, we have over expressed IKKε in IKKα and IKKβ double knock-out MEFs, and try to see if p65 can still be phosphorylated in the absence of IKKα/β. Interestingly, there is significant p65 phosphorylation in the lane 6, where IKKε and p65 is exogenously expressed in IKKα/β DKO cells (Fig. 2.5). It is important to note that TNF-induced p65 phosphorylation is significantly reduced in IKKα/β DKO cells compared to WT cells (Fig 5, compare lane 2 and lane 4). Inline with previous observations, the data presented here suggest that IKKα and IKKβ are the main p65 kinases upon cytokine induction, however the fact that IKKε can phosphorylate p65 in the absence of IKKα and IKKβ suggests that IKKε functions independent of IKKα/β at least in terms of p65 phosphorylation. It is important to note that p65 can be co-immunoprecipitated with flagged tagged IKKε, which suggest that they interact with each other, and the phosphorylation of p65 by IKKε is a direct effect (data not shown).

Bcl-3 over expressing cells proliferate more efficiently: We previously reported that one of the physiological roles of IKKε is to provide proliferative signaling in cancer cells and that IKKε overexpressing cells proliferate more efficiently [44, 45]. In order understand if Bcl-3 is the main mediator of IKKε induced cell proliferation, we speculate that Bcl-3 overexpressing cells should proliferate more efficiently than the parental cells. In order to test this hypothesis MCF-7B cells where there is stably expressed Bcl-3 gene [84] have been utilized together with parental MCF-7 cells. As shown in Figure 2.6, MCF-7B cells, where there is stable Bcl-3 expression, proliferate more effectively than the control cells as measured my MTT cell proliferation assay over a period of 4 days. The same numbers of cells were seeded in 94 well plate and at indicated time points MTT assay has been performed. Results presented here are perfectly in line with the fact that Bcl-3 is an important mediator of CD1 gene expression, hence provide proliferative and prosurvival signals to cells [23, 67, 68]. These results suggest that Bcl-3 is potentially a mechanistic link between IKKε and cell proliferation probably through activating CD1 gene expression. Experiments are now testing the effects of Bcl-3 knock-down on cell growth control.

Bcl3 and IKKε overexpression in human HCC tumor samples: Significant Bcl-3 overexpression has been observed in human hepatocellular carcinoma (HCC) tumors compared to adjacent tissue sections (Bert O'Neil, Personal communication). In order to understand if there is any correlation between Bcl-3 overexpression with IKKε level in these tumor samples, we have analyzed Bcl-3 mRNA levels as well as IKKε mRNA levels and protein level in different human tumor samples by qRT-PCR and western blot respectively both in adjacent tissue (A) and in tumor sectionsz (T). As shown in Fig 2.7A, there is

significant Bcl-3 overexpression in sample # 2, 3 and 4 specifically in tumor section compared to adjacent tissue. (A= adjacent tissue, T= tumor). In line with this, there is also significant IKKε overexpression in these samples as well as in sample # 1 (Fig 2.7B). We have observed significantly higher IKKε protein expression specifically in tumor section of almost all of the tumor samples (Fig 2.7C). Although there is higher IKKε protein level in sample #5, neither Bcl-3 nor IKKε message level is higher in sample #5 compared to adjacent tissue section. Although we do not know exactly why, we speculate that there might have been miss labeling or RNA degradation during handling process. Over all, there is high correlation between IKKε overexpression and Bcl-3 overexpression in tumor section compared to adjacent tissue; suggesting that, in line with our genetic and biochemical evidences, strong Bcl-3 overexpression in these tumor sections is likely driven by IKKε.

Discussion

IKKε and a related kinase TBK1, are classical I-κB kinase (IKK) related kinases whose importance in cell proliferation, survival and hence cancer mechanism are recently being appreciated. We have previously shown that IKKε is involved in constitutive, cancer cell associated NF-κB activity that provides signaling mechanism for efficient proliferation and survival [44]. In addition, several other groups have also implicated IKKε in cell proliferation, survival and apoptosis [45, 46]. Importantly, IKKε related kinase, TBK1 pathway activation has recently been shown to inhibit apoptosis in tumor cells [47] Currently there is an unmet requirement of dissecting the signaling pathways downstream of IKKε and TBK1 and identifying their target genes that are critical for cell survival, proliferation and

hence tumor formation. Here we describe Bcl-3 as one of those target genes downstream of IKKε.

There is ample evidence in the literature implicating Bcl-3 in cell cycle, immunity and inflammation; however, currently there is an unmet requirement of identifying the upstream signaling mechanism and effectors of regulation Bcl-3 gene expression. Here, we report that that IKKε is important regulator of Bcl-3 gene expression. Identification through microarray analysis of genes that are affected by IKKε knock-down, and subsequent validation biochemical experiments suggested that Bcl-3 gene expression is regulated by IKKε in a NF-κB dependent manner. In addition, our data suggest that regulation of Bcl-3 by IKKε is potentially the mechanistic link and the main mediator of IKKε induced cell survival/proliferation.

Currently we have modest information about the regulation of Bcl-3 gene expression, apart from the fact that chromosomal translocation at t(14;19)(q32.3;q13.2) in B-CLLs leads to Bcl-3 overexpression. We and others have demonstrated that Bcl-3 can be inducibly expressed by DNA damaging agents or some cytokines [16, 22, 84, 87]. Moreover, Bcl-3 expression can be induced by estrogen withdrawal and mediate growth and hormonal independence in breast cancer cells [88]. We have observed selective Bcl-3 overexpression in human breast tumors [85] and reported that Bcl-3 can induce CD1 gene expression [23]. Here we show that IKKε significantly upregulates Bcl-3 gene expression. Importantly, cyclin D1 gene expression was also observed which is a probably consequence of Bcl-3 gene expression.

Upstream effectors and transcription factors regulating Bcl-3 gene expression have not been fully investigated. One study reported that Bcl-3 expression is upregulated

following IL-4 deprivation under the control of AP1 and AP1-like transcription factors [68]. However, Bcl-3 expression was also shown to be regulated by NF-κB transcription factor through an intronic enhancer in T lymphocytes [86]. Whether Bcl-3 is an NF-κB or AP1 dependent genes, or if each transcription factors regulates differentially under different circumstances remains to be identified. Our data presented here suggest that NF-κB p65 is an important mediator of Bcl-3 gene expression. In p65 KO MEFs, there is significant reduction in the basal level of Bcl-3 mRNA and it cannot be induced upon cytokine stimulation. Interestingly, IKKα/β which are the main NF-κB kinases did not seem to be involved in Bcl-3 gene expression. This data suggested the activation of p65 by a third kinase other than IKKα/β. Recently, several groups including us reported that IKKε is an important p65 kinase that phosphorylate at Ser 536 position and contribute to NF-κB regulated gene expression and cell proliferation [40, 44, 45]. Previously, IKKε and TBK1 were suggested to act as upstream kinases of IKK complex [26]. Whether IKKε induces p65 phosphorylation directly or act as an upstream kinase of IKK is currently unknown. We present evidence here that this phosphorylation is a direct effect. First of all, IKKε can induce p65 phosphorylation in IKKα/β double knock-out MEF cells (Fig. 2.5). Furthermore, p65 can be co-immunoprecipitated with IKKε (Data not shown). In addition to this, from the previous research in the literature, we know that stimulus-coupled IκB degradation and p65 nuclear translocation and DNA binding activity of NF-κB is normal in IKKε and TBK1 deficient cells despite the fact that there is impaired NF-κB-dependent gene transcription [35, 36, 39, 44]. This evidence supports the fact that the activity of IKKα and IKKβ is normal in IKKε or TBK1 deficient cells, since there is normal IκB degradation, normal p65 nuclear translocation and normal DNA binding activity. Although we cannot rule out the possibility

IKKε might function as I-κB kinase kinase, our data support a model where IKKε is a direct mediator of p65 activation and is working in parallel with classical IKK complex differentially under different circumstances.

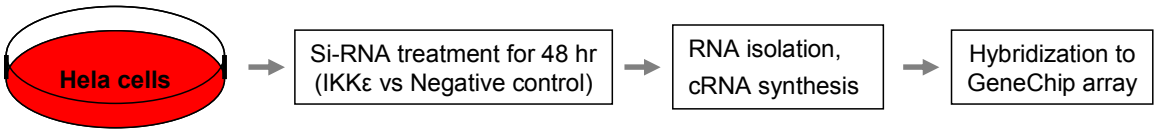
Bcl-3 has been implicated in many crucial physiological events such as cell proliferation, immune, inflammation and cancer progression. We have also observed that cells with a stable Bcl-3 expression priloferate more efficiently than parental cell lines. Bcl-3 induced Cyclin D1 upregulation and inhibition of apoptosis are probably the two main mechanism that accounts for better survival and proliferation in Bcl-3 expressing cells. Bcl-3 upregulation has been observed in many cancer types [85, 89, 90] and hepatocellular carcinomas and pancreatic cancers (Bert O'Neil, unpubl.). Interestingly most of these cancers have elevated basal NF-κB activity and we have reported that IKKε is the major player of cancer cell associated NF-κB activity. We have observed strong correlation between IKKε expression and bcl-3 gene expression specifically in the tumor section of hepatocellular carcinomas samples compared to the adjacent tissue section (Fig. 7), which suggests that IKKε is driving Bcl-3 gene expression in tumor settings. Previous research and the data presented here so far indicate that IKKε is an important mediator of constitutive NF-κB activity in cancer cells and Bcl-3 is one of the important target gene that is upregulated downstream of IKKε induced NF-κB activity.

Figures and Legends

Figure 2.1: Identification of Bcl-3 as IKKε target gene: IKKε mRNA level was knockdown by siRNA technology in HeLa cell lines and the RNA from samples where there is at least 75-80 % IKKε knock down we subsequently processed and hybridized to Affymetrix Human Genome Plus U133 Plus 2.0 as described in materials and methods section. Genes whose expression has changed 2 fold or more at the basal level upon IKKε knock-down were analyzed from two independent experiments. List of genes whose expression has changed more than two fold upon IKKe knock-down by siRNA treatment as identified by microarray technology (Fig. 2.1 B).

Figure 1

A



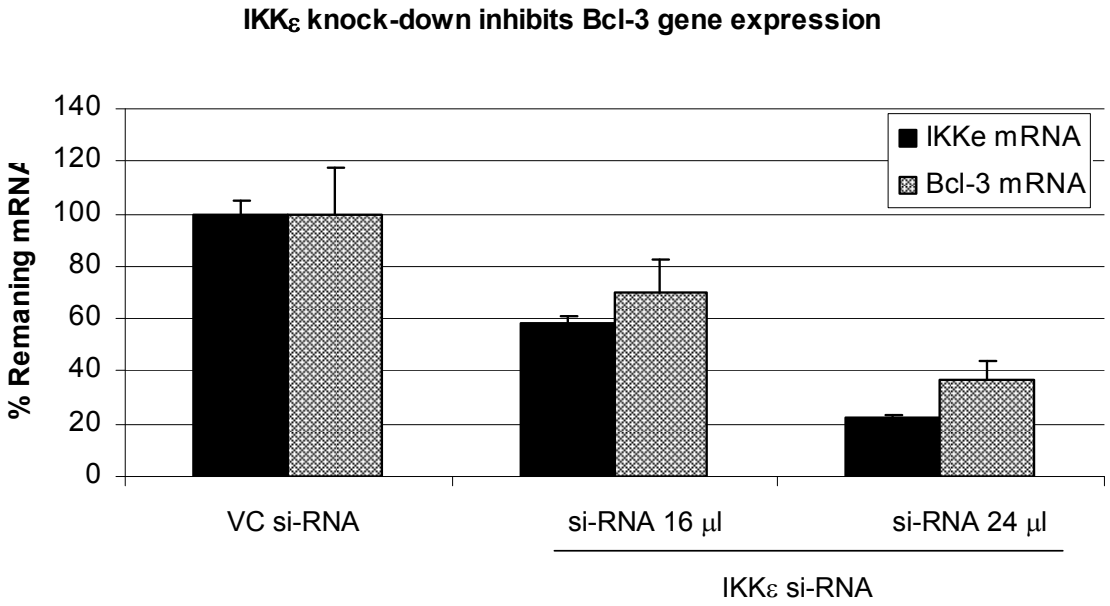
B

Fold Change	Description
2.966	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2
2.897	ADP-ribosylation factor-like 6
2.713	RAB27A, member RAS oncogene family
2.661	interleukin enhancer binding factor 3, 90kDa
2.659	dudulin 2
2.633	talin 1
2.468	tissue factor pathway inhibitor 2
2.188	growth differentiation factor 11
0.488	vesicle-associated membrane protein 3 (cellubrevin)
0.487	Rap guanine nucleotide exchange factor (GEF) 2
0.486	follistatin
0.475	caspase 9, apoptosis-related cysteine protease
0.471	**Bcl-3** (B-cell lymphoma associated protein)
0.471	leucine zipper transcription factor-like 1
0.435	transmembrane protein 2
0.493	**caspase 1**, cysteine protease (interleukin 1, beta, convertase)
0.425	inhibitor of DNA binding 2, dominant negative HLH protein
0.416	glutamine-fructose-6-phosphate transaminase 1
0.392	leucine zipper transcription factor-like 1
0.332	transmembrane 4 superfamily member 3

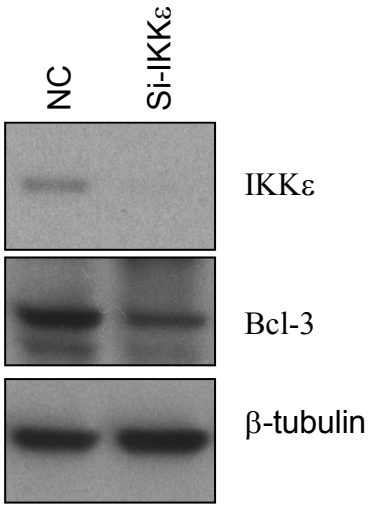
Figure 2.2: IKKε knock-down by Si-RNA reduced Bcl3 gene expression: Microarray result was validated in an independent experiment. IKKε is knocked-down with different amount of siRNA transfections. Reduction Bcl-3 message level is well correlated with the amount of IKKe knock-down as measured by qRT-PCR (Fig. 2.2A). 16 and 24 represents the amount of transfection reagent (in μl) used. In addition to message level, IKKε knock-down significantly reduced Bcl-3 protein level in HeLa cells. Total cell lysates from different IKKε si-RNA and the negative control (NC) si-RNA treated samples were prepared after 72 hrs of si-RNA treatments. Different si-RNA against IKKε has been used and we could only knock-down IKKε effectively with product si-RNA ID number 919 from Ambion (Figure 2.2B). Exogenously expressed WT IKKε but not kinase mutant form (IKKε KM) induce Bcl-3 gene expression. Importantly, expression of IκB super repressor (SR) inhibits IKKε induced Bcl-3 gene expression. p65 expression as well as TNFα treatments induces significant Bcl-3 gene expression Fig. 2.2C). WT IKKε but not its kinase mutant form activate luciferase promoter that has Bcl-3 promoter as well as an NF−κB responsive intronic enhancer (p11 E3+4 luciferase construct). These results suggest that IKKε regulate Bcl-3 gene expression in a NF-κB dependent manner (Fig. 2.2D). WT IKKε can induce cyclin D1 (Fig. 2.2E) gene expression as well. Interestingly kinase mutant IKKε (KM) reduces the basal level of Bcl-3 and cyclin D1 gene expression which suggest that kinase activity of IKKε is important for its function. Vector control, IKKε and IKKε KM, IκB SR and p65 expression vectors were transiently expressed in HEK293T cells

Figure 2.2

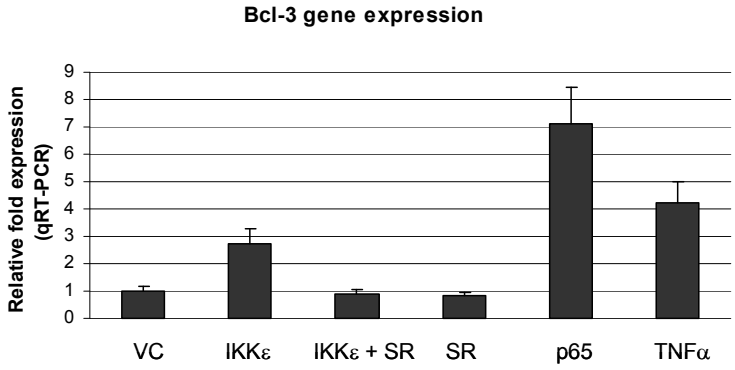
A


IKKε knock-down inhibits Bcl-3 gene expression
% Remaning mRNA
140
120
100
80
60
40
20
0
IKKe mRNA
Bcl-3 mRNA
VC si-RNA
si-RNA 16 μl
si-RNA 24 μl
IKKε si-RNA

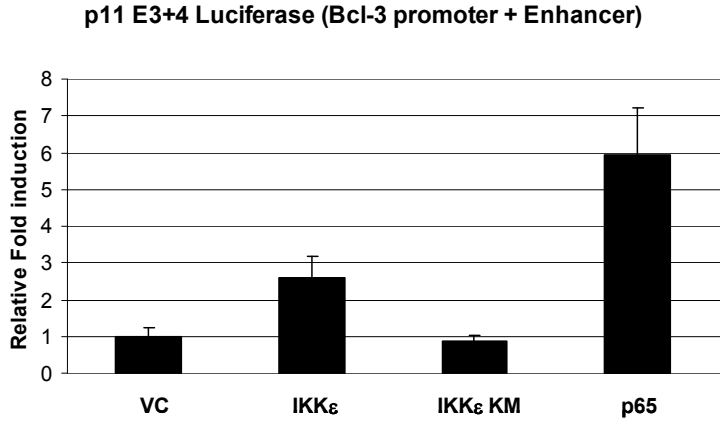
B


NC
Si-IKKε
IKKε
Bcl-3
β-tubulin

C


Bcl-3 gene expression
Relative fold expression (qRT-PCR)
0
1
2
3
4
5
6
7
8
9
VC
IKKε
IKKε + SR
SR
p65
TNFα

D


p11 E3+4 Luciferase (Bcl-3 promoter + Enhancer)
Relative Fold induction
0
1
2
3
4
5
6
7
8
VC
IKKε
IKKε KM
p65

E

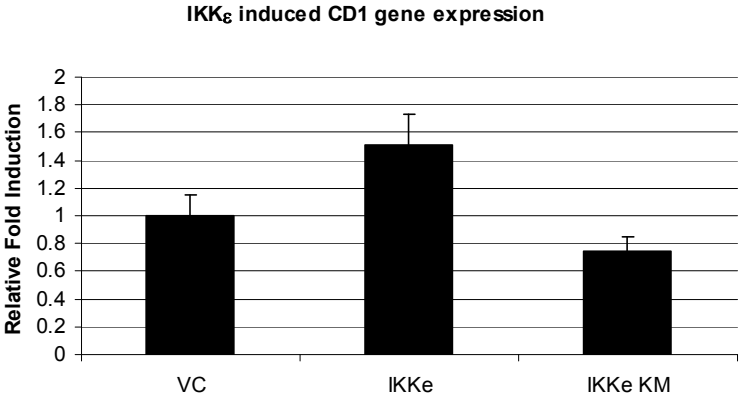
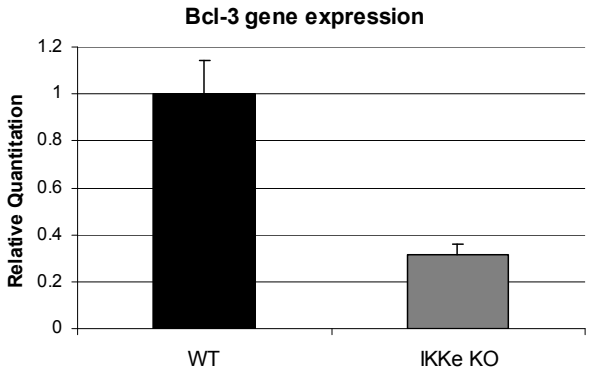

IKKε induced CD1 gene expression
Relative Fold Induction
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
VC
IKKe
IKKe KM

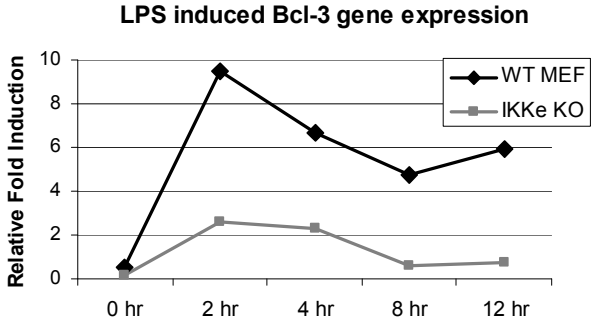
Figure 2.3: Basal level as well cytokine induced level of Bcl-3 gene expression is defective in IKKε KO MEF cells: RNA was isolated from WT MEF as well as IKKε deficient MEF cell under normal growing conditions. Bcl-3 gene expression is significantly low at the basal level in IKKε deficient MEF cell line compared to WT MEF cells as measured by qRT-PCR (Fig. 2.3A). In order to explore inducible regulation of Bcl-3, WT MEFs as well as IKKε deficient MEFs were treated with LPS for indicated times, and Bcl-3 message level was quantified with qRT-PCR. IKKε deficient cells have significantly low level of total Bcl-3 message level upon LPS induction (Fig. 2.3B). Although there is some induction of Bcl-3 in IKKε deficient cells, compared to WT cells this induction is significantly less. Bcl-3 protein level is also defective in IKKε deficient cells. There is a detectable Bcl-3 gene expression 6 hours after LPS induction, however, this can not be detected in IKKε deficient cells (Fig. 3C).

Figure 2.3

A



B



C

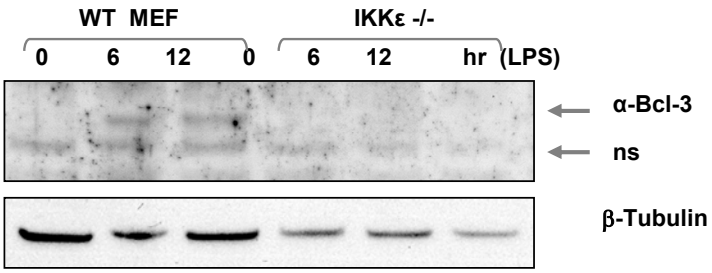
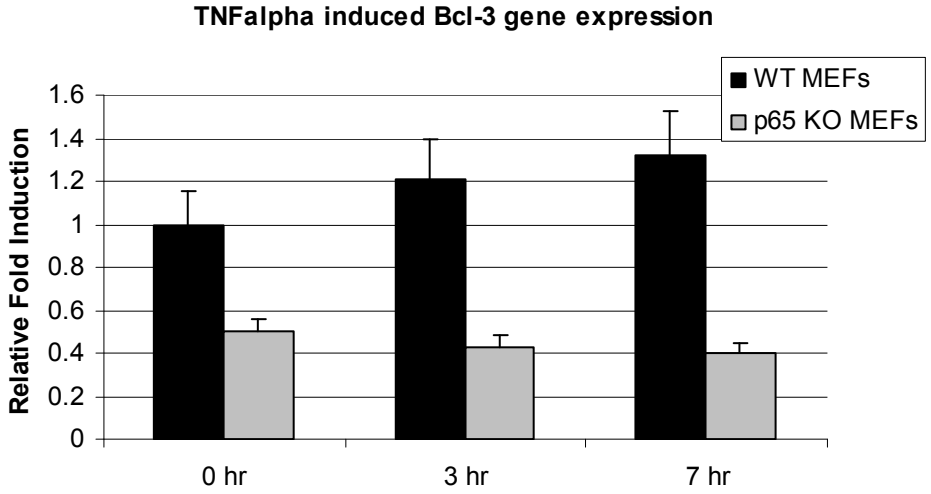


Figure 2.4: IKKα and IKKβ independent, but NF-κB p65 dependent Bcl-3 gene expression:

MEF cells that are p65 knock-out (KO), and IKKα and IKKβ double knock-out (DKO) were grown in 6-well plate and treated 10ng/ml TNFα for indicated time points. p65 deficient MEFs display significantly less Bcl-3 gene expression at the basal level and there seems to be no induction of Bcl-3 in p65 deficient cells upon TNFα treatment for over 7 hr period compared to WT MEF cells as measured by qRT-PCR (Fig. 2.4A). Interestingly, there is no significant difference in the level of Bcl-3 gene expression in IKKα/β DKO MEFs and WT MEFs both basal and inducible levels seem to be comparably if not more in DKO cells (Fig. 2.4B).

Figure 2.4

A


TNFalpha induced Bcl-3 gene expression
WT MEFs
p65 KO MEFs
Relative Fold Induction
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 hr
3 hr
7 hr

B

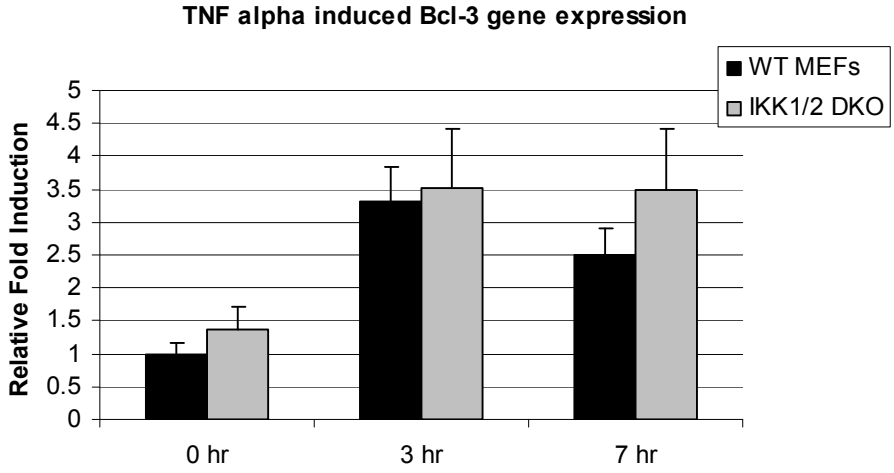

TNF alpha induced Bcl-3 gene expression
WT MEFs
IKK1/2 DKO
Relative Fold Induction
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 hr
3 hr
7 hr

Figure 2.5: IKKα and IKKβ independent phosphorylation of p65 by IKKε: IKKε and p65 was exogenously expressed in IKKα and IKKβ double knock-out MEFs grown under normal conditions in 6-well plate. Significant p65 phosphorylation at Ser 536 position is observed in the lane 6, where IKKε and p65 is together (Fig. 2.5). It is important to note that TNF induced p65 phosphorylation is significantly reduced in IKKα/β DKO cells compared to WT cells (Fig 5, compare lane 2 and lane 4). However, when the film is overexposed we can detect some inducible phosphorylation in DKO cells as well (data not shown). The fact that IKKε can phosphorylate p65 in the absence of IKKα and IKKβ suggest that IKKε function independent of IKKα/β at least in terms of p65 phosphorylation.

Figure 2.5

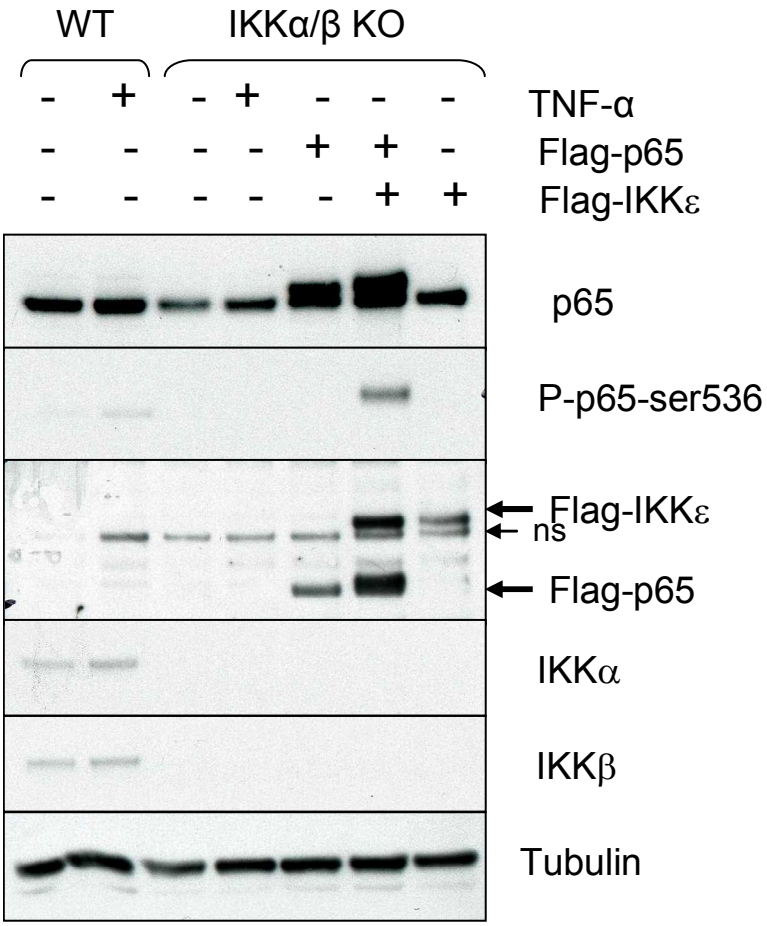

WT
IKKα/β KO
- + - + - - -
- - - - + + -
- - - - - + +
TNF-α
Flag-p65
Flag-IKKε
p65
P-p65-ser536
Flag-IKKε
ns
Flag-p65
IKKα
IKKβ
Tubulin

Figure 2.6: Bcl-3 over expressing cells proliferate more efficiently: MCF-7 cells and the same cell line with stably transfected Bcl-3 gene have been utilized. The same numbers of cells were plated in 4 96-well plates. Every 24 hr, cell proliferation was measured by MTT cell proliferation assay over a period of 4 days. Bcl-3 expressing cells (MCF-7B) proliferate more efficiently than the parental cells (MCF-7) (Fig 2.6). These results suggest that Bcl-3 is potentially a mechanistic link between IKKε and cell proliferation.

Figure 2.6

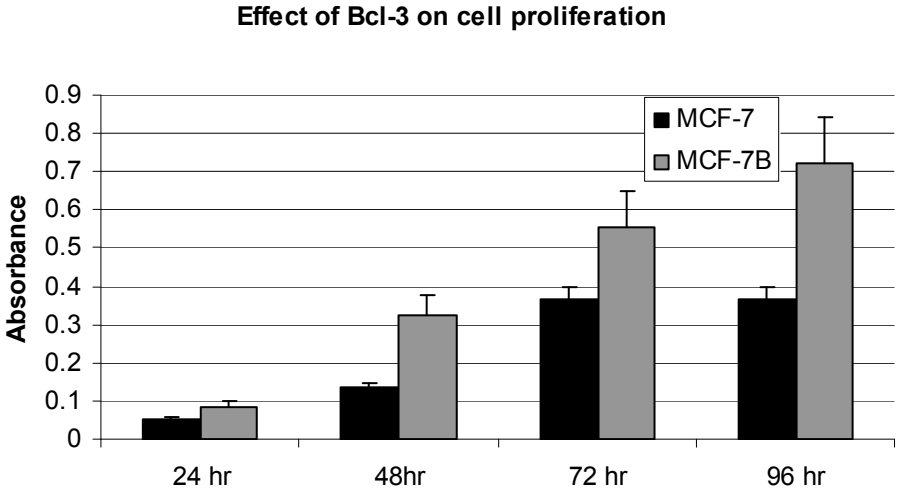
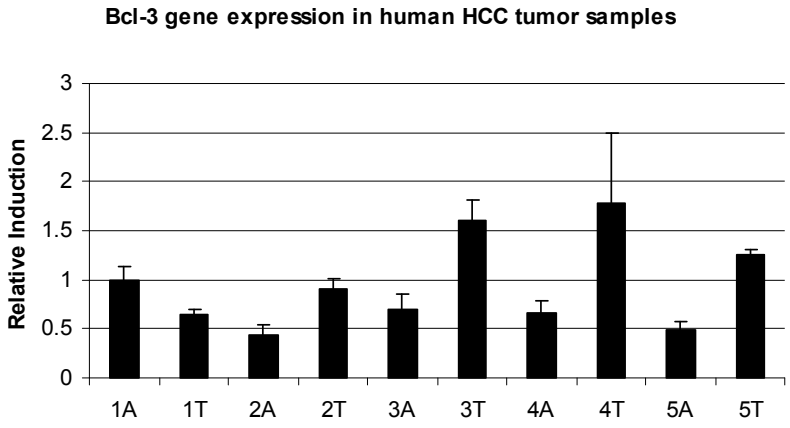

Effect of Bcl-3 on cell proliferation
Absorbance
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MCF-7
MCF-7B
24 hr
48hr
72 hr
96 hr

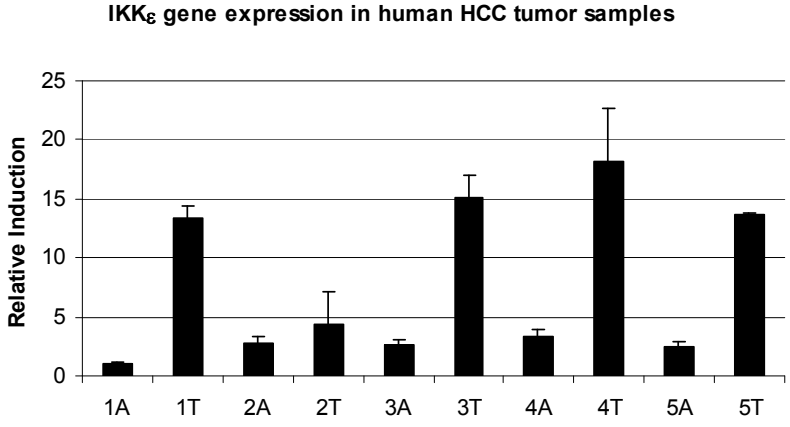
Figure 2.7: Bcl3 and IKKε overexpression in human HCC tumor samples: RNA and proteins from different samples of Human Hepato Cellular Carcinoma (HCC) tumors and adjacent tissue sections were prepared. Bcl-3 message level as well as IKKε message level and protein levels have been quantified in different human tumor samples by qRT-PCR and western blot respectively both in adjacent tissue (A) and in Tumor section (T). There is significant Bcl-3 overexpression in tumor samples 2, 3 and 4 specifically in tumor section compared to adjacent tissue. (A= adjacent tissue, T= tumor) (Fig. 2.7A). In line with this, there is also significant IKKε overexpression in these samples as well as in sample # 1 (Fig. 2.7B). IKKε is overexpressed in almost all of the tumor samples at the protein level (Fig 2.7C). Over all, there is high correlation between IKKε overexpression and Bcl-3 overexpression in specifically tumor section compared to adjacent tissue.

Figure 2.7

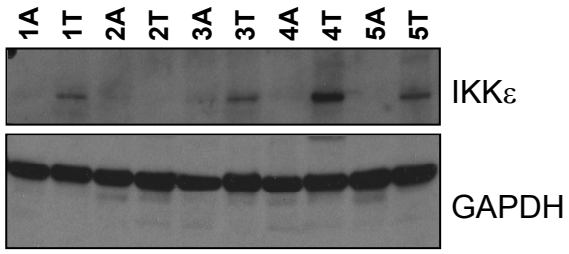
A



B



C



Materials and methods

Reagents: All cells were cultured in Dulbecco's modified Eagle's medium (DMEM), complemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin. An antibody to IKKε, Bcl-3 and to phospho p65 Ser 536 was obtained from Cell Signaling. Antibodies to β-tubulin, GAPDH and to IκBα were obtained from Santa Cruz. LPS (L6529, Sigma) was used at a final concentration of 1 µg/ml. Flag antibody (M2) was purchased from Sigma rhTNF-α (Promega) was used at a final concentration of 10 ng/ml. Effectene transfection reagent obtained from Qiagen was used according to the manufacturer's protocol. Generation of MCF-7B cells stably expressing Bcl-3 gene was previously described [84]. Wild type and kinase mutant forms of Flag-tagged IKKε (IKKε K38A) have been described previously [27]. p11 E3+4 luciferase construct is described in [86] and was a kind gift of Dr. McKeithan.

Transient Transfections: For transient transfections, the indicated cell lines were seeded in 6-well plates at 30-50 % density and transfected the next day with the indicated expression vectors for 48 hrs using Effectene (Qiagen) transfection reagent according to the manufacturer's instruction. The total amount of transfected DNA (500 ng DNA) in each well was adjusted by adding empty plasmid vector (pcDNA3.1).

Western Blot: After stimulation, cultured cells were lysed on ice for 5 min in RIPA lyses buffer with freshly added protease and phosphatase inhibitor cocktails. Lysates were cleared by centrifugation at 4 °C for 15 min at 13,000 *g*. The amount of total protein was measured and equal amounts (20 µg) were fractionated by NuPAGE Novex 4-12% Bis Tris gels

(Invitrogen) and electro-transferred to polyvinylidene difluoride membranes. Membranes were blotted with the indicated antibodies, and proteins were detected using an enhanced chemiluminescence detection system (Amersham Biosciences, Freiburg, Germany).

Si-RNA transfection: IKKε mRNA was knocked down with the IKKe targeted si-RNAs from Ambion (IKKe Si-RNA ID # 918, 919, 9120) and from Dharmacon Company (IKKe targeted ON-TARGET*plus* siRNA and Standard si*GENOME* siRNA). Dharmafect 1 transfection reagent was used for all si-RNA transfections. Lysate preparation and westerns were performed as described.

Real-time PCR: Cells were plated in 100-mm dishes. Treated or untreated cells according to the proposed experiments were washed in PBS and lysed in Trizol Reagent (Invitrogen), and RNA was collected following the manufacturer's protocol. cDNA was generated using the Superscript II Reverse Transcriptase kit (Invitrogen), and quantitative PCR was performed on an ABI Prism 7000 (Applied Biosystems) using gene-specific TAQman primer/probe sets (Applied Biosystems).

Cell proliferation, MTT assay: Cell proliferation assay has been performed as described by TACS MTT assay kit (R&D systems). First, the optimal cell number, which was $1x10^4$ for Hela cells in our system, has been determined. After transfection, equal number of cells has been seeded in 96 well plate for indicated times. 10 µl of MTT reagent was added to each well including the blank wells, and incubated for 4-5 hrs at 37 ^{0}C. Then 100 µl of detergent reagent was added, incubated for at least 12 hr at 37 ^{0}C and absorbances at 570 nm were

measured with reference wavelength of 650 nm.

Microarray Method: 7 μg of total RNA was used to synthesize cDNA. A custom cDNA kit from Life Technologies was used with a T7-$(dT)_{24}$ primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction using the BioArray High Yield RNA Transcript Kit. The cRNA was then fragmented in fragmentation buffer (5X fragmentation buffer: 200mM Tris-acetate, pH8.1, 500mM KOAc, 150mM MgOAc) at 94^{o}C for 35 minutes before the chip hybridization. 15 μg of fragmented cRNA was then added to a hybridization cocktail (0.05 μg/μl fragmented cRNA, 50 pM control oligonucleotide B2, *BioB*, *BioC*, *BioD*, and *cre* hybridization controls, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 100mM MES, 1M [Na^{+}], 20mM EDTA, 0.01% Tween 20). 10 μg of cRNA was used for hybridization. Arrays were hybridized for 16 hours at 45^{o}C in the GeneChip Hybridization Oven 640. The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 400. After this, the arrays were scanned with the Hewlett Packard GeneArray Scanner. Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. Sample quality was assessed by examination of 3' to 5' intensity ratios of certain genes.

SUMMARY AND ADDITIONAL DIRECTIONS

NF-κB is one of the most extensively studied transcription factor since its initial discovery more than 20 years ago. Intensive research has demonstrated that NF-κB is the central mediator of inflammatory responses and immune functions. Moreover, extensive research on NF-κB has also demonstrated that this transcription factor can, in most cases, protects transformed cells from apoptosis and therefore participate in the onset or progression of many human cancers. NF-κB comprises a family of dimeric transcription factors that regulate diverse gene expression programs consisting of hundreds of genes. A family of inhibitor of κB (IκB) proteins controls NF-κB DNA-binding activity and nuclear localization. IκB protein metabolism is intricately regulated through stimulus-induced phosphorylation, degradation and feedback re-synthesis, which allows for dynamic control of NF-κB activity. This network of interactions is called NF-κB signaling module. One of the most important components of this module is kinases that regulate NF-κB activity at several points including IκB phosphorylation and subsequent degradation. A large kinase complex responsible for IκB phosphorylation and subsequent degradation was initially partially identified in unstimulated HeLa cells and was later found to be activated in cells treated with TNFα [8, 9]. Subsequently several groups identified two highly related kinases named IKKα (IKK1) and IKKβ (IKK2) as the main kinase components of this kinase complex [10-12]. Both of these kinases have been shown to have specificity for serine residues in the

destruction box of IκBα protein and can directly phosphorylate S32 and S36 residues [10]. In addition to IKKα and IKKβ, a third non-catalytic, structural component called IKKγ (NEMO) was also identified to be the essential component of the IKK complex [13-15]. Please refer to [10] for more extensive and detailed review about IKK complex.

Based on a number of observations, it was assumed that virtually all inducers of NF-κB lead to the activation of a single classical IKKα/β/γ complex. However, recent studies demonstrated the existence of distinct IKK complexes that do not contain IKKα, β, or γ [26]. One of these complexes was described as a PMA-inducible IκB kinase complex, with a critical component being an IKK-related kinase designated IKKε [27], which is identical to a kinase named IKK-i identified via its induction downstream of LPS-induced signaling [19]. IKKε in turn is closely related to another recently discovered IKK-related kinase designated as TBK1 (TANK-Binding Kinase 1) [28] or NAK (NF-κB Activating Kinase) [29]. TBK1, which is highly homologous to IKKε, binds to TANK and TRAF and may form an alternative IKK complex consisting of IKKε and TBK1 [28]. There is significant sequence similarity between IKKε and TBK1 (~66 %) and they share a significant similarity in their kinase domains with IKKα and IKKβ (Figure 1.1).

Here, we have investigated the role of IKKε in NF-κB activity. Our research has shown that IKKε is expressed in a variety of cancer cell lines. Based on this, we have investigated a role for IKKε as related to constitutive, cancer-associated NF-κB activity. Our experiments reveal an important role for IKKε in controlling the activation of Ser-536 phosphorylation of the RelA/p65 subunit and functional NF-κB activity in several cancer cell

lines and in 293T cells. Additionally, we have investigated IKKε regulated gene expression at genome wide level and have identified Bcl-3 and Caspase 1 as critical IKKε target genes.

Here we show that several cancer cell lines, along with the SV40 large T-immortalized 293 cell line, exhibit relatively high levels of expression of IKKε. This is interesting, as IKKε is normally considered a kinase that is induced quantitatively by LPS or cytokines. We have investigated a potential role for IKKε and TBK1, kinases homologous to the catalytically active IKKα and IKKβ subunits, in controlling NF-κB activity, with the focus being phosphorylation of p65 at the Ser-536 position. Experiments were initiated to study IKKε and TBK1 induced NF-κB-dependent promoter activation.

In agreement with previous results [31, 32], IKKε and TBK1, but not their kinase mutant forms, strongly activate NF-κB regulated reporter constructs. It is important to note that, unlike the 3X-κB promoter, the IFN-β promoter is a complex promoter regulated by coordinate actions of NF-κB and other transcription factors, therefore it is not considered to be regulated exclusively by NF-κB. To confirm our reporter assays, gel shift assays have been performed. As expected, IKKε and TBK1 induced significant NF-κB DNA binding activity. Super shift assays identified p65 and p50 as main subunits of NF-κB complex. Recent studies have shown that post-translational modification of NF-κB subunits, such as p65 phosphorylation; contribute significantly to NF-κB activation. Phosphorylation of p65 at Ser-536 is proposed to be a key modification that potentiates p65 transactivation function, and hence NF-κB activation ability [41, 72, 78].

Recently, it has been reported that overexpression of IKKε or TBK1 together with p65, leads to the phosphorylation of ectopically expressed p65 at Ser-536 [40], however, this group has not analyzed the endogenous phosphorylation at Ser-536 of p65. Our results clearly support the hypothesis that the kinase activity of IKKε and TBK1 may significantly contribute to the constitutive level of S536 phosphorylation of p65. We have also observed that IKKε induces its own expression, which has been shown to be regulated by NF-κB. This data indicates that ectopically expressed IKKε induces p65 phosphorylation, NF-κB activation and NF-κB dependent gene expression. It is also raises the possibility that IKKε functions in an autoregulatory loop, leading to its own expression.

It was interesting to observe that IKKε deficient cells show a normal pattern of cytokine inducible phosphorylation of p65 and IκBα degradation when compared to WT MEFs. We have tested a series of well known NF-κB inducers (IL-1β, LPS, PMA) that are known to activate NF-κB by utilizing different signaling pathways. Compared to WT MEFs, IKKε null cells (and DKO MEFs for both IKKε and TBK1) allowed inducible RelA/p65 phosphorylation to the same extent. Why is there no defect in inducible Ser-536 phosphorylation of p65 in IKKε deficient cells? The first plausible explanation to this question is that the classical IKK signalsome complex is still intact in IKKε deficient cells. Therefore, this complex likely compensates for the loss of IKKε. Secondly, there are other known and unknown kinases, in addition to IKK complex, that have been claimed to mediate Ser-536 phosphorylation of p65 [41, 80, 81] and in the same way, they may still induce phosphorylation in IKKε deficient cells. The third explanation to this question is that IKKε and TBK1 are not involved in cytokine induced p65 phosphorylation, but rather they are

involved in basal/constitutive level of p65 phosphorylation. In one set of experiments, this possibility has been investigated. Since MEF cells have low levels of basal p65 phosphorylation and low levels of IKKε, the potential that IKKε and TBK1 might be involved in constitutive p65 phosphorylation has been investigated in HeLa and HEK 293T cells that demonstrate higher levels of IKKε and constitutive p65 phosphorylation at Ser-536 position. IKKε was knocked down by plasmid based shRNA technology in both HeLa cells and HEK 293T cells. Importantly, shRNA transfection leads to sustained knock down of IKKε and more importantly, reduction in the IKKε level is well correlated with significant reduction in the basal level of Ser-536 phosphorylation (Fig. 6), whereas in vector control transfected cells there is no change in IKKε level and Ser-536 phosphorylation of p65. Interestingly knock-down of TBK1 did not reduce the basal level of p65 phosphorylation. Surprisingly this data suggests that TBK1 and IKKε are not entirely orthologues at least in controlling basal phosphorylation of p65. As suggested earlier [35], IKKε and TBK1 might not be redundant in every signaling pathway that they effect. Thus the data presented here clearly show that IKKε does not mediate cytokine-induced p65 phosphorylation at the Ser-536 position but has a significant role in basal and constitutive phosphorylation of p65 at least in certain cancer cells and in 293T cells. Basal p65 phosphorylation is well correlated with constitutive NF-κB activity, which has been implicated in the pathogenesis of many diseases including cancer. The first evidence to our conclusion that IKKε mediates constitutive NF-κB activity came from a recent report published while this paper was in preparation [45]. In that study, Eddy et al., provided evidences that IKKε contributes to the pathogenesis of breast cancer. Expression of a kinase inactive form of IKKε blocked breast cancer cell colony formation. The results presented in that manuscript are consistent with the

findings presented here. We have shown that IKKε contributes to the basal/constitutive p65 phosphorylation and NF-κB activity as measured by EMSA and NF-κB driven luciferase promoter activity. Furthermore, we have also shown that knock-down of IKKε or overexpression of mutated version of p65 (S536A) negatively effects the cell proliferation. These findings indicate an important role of IKKε and p65 phosphorylation in cancer cell proliferation.

There have been reports suggesting that IKKε and TBK1 may function as IκB kinase kinases [29, 82]. Therefore they might function upstream of the classical IKK complex (IKKα/β/γ). So we questioned if the phosphorylation of p65 at Ser-536 is a direct or indirect effect of IKKε/TBK1. In other words, IKKε and TBK1 might have activated classical IKKs, which then lead to the phosphorylation of p65. In order to test this, we tried to detect activation of IKKβ and IKKα by probing the same blots in Fig. 4 with commercially available, phospho-IKKα/β antibodies, however we could not detect any phosphorylation (data not shown). Although we cannot rule out that IKKε or TBK1 might function as IκB kinase kinases, our data support a model where these two kinases are direct effectors of p65 activation. There is evidence in the literature supporting this model. First of all, it has been clearly shown that stimulus-coupled IκB degradation and p65 nuclear translocation and DNA binding activity of NF-κB is normal in IKKε and TBK1 deficient cells despite the fact that there is impaired NF-κB-dependent gene transcription [35, 36, 39] This evidence supports the fact that the activity of IKKα and IKKβ is normal in IKKε or TBK1 deficient cells, since there is normal IκB degradation, normal p65 nuclear translocation and normal DNA binding activity. We believe that our data, together with these findings, emphasize the RelA/p65 as

the physiological target of IKKε and TBK1 under basal growth situations, at least in certain cells. If IKKε and TBK1 were upstream of classical IKKs, one would expect a deficiency in one of the above processes which are tightly regulated by IKKα and β.

Furthermore, we have focused our research on understanding the downstream target genes whose expression is regulated by IKKε. Interestingly Bcl-3 was identified as one of those genes that is regulated by IKKε and display similar phenotype as IKKε in terms of cell proliferation and survival. There is ample evidence in the literature implicating Bcl-3 in cell cycle, immunity and inflammation; however, currently there is an unmet requirement of identifying the upstream signaling mechanism and effectors of regulation Bcl-3 gene expression. Here, we report that that IKKε is important regulator of Bcl-3 gene expression. Identification through microarray analysis of genes that are affected by IKKε knock-down, and subsequent validation biochemical experiments suggested that Bcl-3 gene expression is regulated by IKKε in a NF-κB dependent manner. In addition, our data suggest that regulation of Bcl-3 by IKKε is potentially the mechanistic link and the main mediator of IKKε induced cell survival/proliferation.

Upstream effectors and transcription factors regulating Bcl-3 gene expression have not been fully investigated. One study reported that Bcl-3 expression is upregulated following IL-4 deprivation under the control of AP1 and AP1-like transcription factors [68]. However, Bcl-3 expression was also shown to be regulated by NF-κB transcription factor through an intronic enhancer in T lymphocytes [86]. Whether Bcl-3 is an NF-κB or AP1 dependent genes, or if each transcription factors regulates differentially under different

circumstances remains to be identified. Our data presented here suggest that NF-κB p65 is an important mediator of Bcl-3 gene expression. In p65 KO MEFs, there is significant reduction in the basal level of Bcl-3 mRNA and it can not be induced upon cytokine stimulation. Interestingly, IKKα/β which are the main NF-κB kinases did not seem to be involved in Bcl-3 gene expression. This data suggested the activation of p65 by a third kinase other than IKKα/β. Recently, several groups including us reported that IKKε is an important p65 kinase that phosphorylate at Ser 536 position and contribute to NF-κB regulated gene expression and cell proliferation [40, 44, 45]. Previously, IKKε and TBK1 were suggested to act as upstream kinases of IKK complex [26]. Whether IKKε induces p65 phosphorylation directly or act as an upstream kinase of IKK is currently unknown. We present evidence here that this phosphorylation is a direct effect. First of all, IKKε can induce p65 phosphorylation in IKKα/β double knock-out MEF cells (Fig. 5). Furthermore, p65 can be co-immunoprecipitated with IKKε (Data not shown). In addition to this, from the previous research in the literature, we know that stimulus-coupled IκB degradation and p65 nuclear translocation and DNA binding activity of NF-κB is normal in IKKε and TBK1 deficient cells despite the fact that there is impaired NF-κB-dependent gene transcription [35, 36, 39, 44] This evidence supports the fact that the activity of IKKα and IKKβ is normal in IKKε or TBK1 deficient cells, since there is normal IκB degradation, normal p65 nuclear translocation and normal DNA binding activity. Although we cannot rule out the possibility IKKε might function as I-κB kinase kinase, our data support a model where IKKε is a direct mediator of p65 activation and is working in parallel with classical IKK complex differentially under different circumstances.

Bcl-3 has been implicated in many crucial physiological events such as cell proliferation, immune, inflammation and cancer progression. We have also observed that cells with a stable Bcl-3 expression priloferate more efficiently than parental cell lines. Bcl-3 induced Cyclin D1 upregulation and inhibition of apoptosis are probably the two main mechanisms that accounts for better survival and proliferation in Bcl-3 expressing cells. Bcl-3 upregulation has been observed in many cancer types [85, 89, 90] and hepatocellular carcinomas and pancreatic cancers (B. O'Neil, unpubl.). Interestingly most of these cancers have elevated basal NF-κB activity and we have reported that IKKε is the major player of cancer cell associated NF-κB activity. We have observed strong correlation between IKKε expression and bcl-3 gene expression specifically in the tumor section of hepatocellular carcinomas samples compared to the adjacent tissue section (Fig. 7), which suggests that IKKe. is driving Bcl-3 gene expression in tumor settings. Previous research and the data presented here so far indicate that IKKε is an important mediator of constitutive NF-κB activity in cancer cells and Bcl-3 is one of the important target gene that is upregulated downstream of IKKε induced NF-κB activity.

In addition to Bcl-3, our data suggest Caspase 1 as another IKKε regulated gene. Caspase 1 is an important component of the inflammasome complex and has been shown to mediate chemo resistance in pancreatic carcinoma cell lines by autocrine production of interleukin 1beta (IL-1β) that confers constitutive nuclear factor kappaB activity [91]. Experiments are further testing the role of Caspase 1 involvement in cell survival and growth downstream of IKKε. How IKKε regulate the expression of its target genes is not clearly understood, however our results suggest that p65 phosphorylation at ser 536 position is an

important step. In support of our data, IKKε has recently been shown to interact with p52 and promote transactivation via p65 activation [42]. We cannot rule out the possibility that IKKε has other roles in other NF-κB activating pathways as the significant of IKKε involvement is recently being appreciated. One study, for example, demonstrated that TBK1 and IKKε directly phosphorylate the C-terminal domain of cRel in vitro and in vivo and regulate nuclear accumulation of cRel, independently of the classical IkappaB/IKK pathway [32]. While this work was in preparation, Boehm at al., published a paper in the most recent issue of *Cell* that clearly identified IKKε as a Breast Cancer Oncogene by using Integrative Genomic Approaches such as overexpression and siRNA screening as well as comparative genomics analysis [43]. In line with out published studies, they also showed that in many breast cancer cell lines as well as breast cancer ca2rcinomas, IKKε is overexpressed and this overexpression is driving NF-κB activity which provide prosurvival signal in cancer setting.

To summarize, we have provided evidence that IKKε, and not TBK1, controls the constitutive NF-κB activity in certain cancer cells and in 293T cells. This evidence is supported by siRNA experiments and by associated reporter studies. It is presently unclear whether IKKε functions separately from the classic IKK complex, or through distinct regulatory pathways. It is also unclear whether IKKε is the kinase that directly controls Ser-536 p65 phosphorylation. Nevertheless, the data indicate a potentially important role for IKKε in controlling at least part of the constitutive NF-κB activity generated in certain cancer cells, with subsequent downstream effects on cancer cell proliferation. Interestingly, IKKε is one of the NF-κB regulated genes therefore there is positive regulatory loop between IKKε expression and NF-κB activity. Based on our current understanding of IKKε and NF-

κB activity, we have developed a model as depicted in Figure A. Furthermore, we have also identified that Bcl-3 is an important IKKε regulated gene downstream of NF-κB activity. This finding is interesting as we know that Bcl-3 is an important mediator of cell cycle and cancer progression and this effect may at least partly come from Bcl-3 regulation of Cyclin D1. Over all these result suggest that Bcl-3 might be the mechanistic link between IKKε induced cell survival and proliferation. Moreover, Caspase1, which has essential role in IL-1B processing and maturation, has also been identified as an IKKε regulated gene and the physiological consequences of this regulation is yet to be understood.

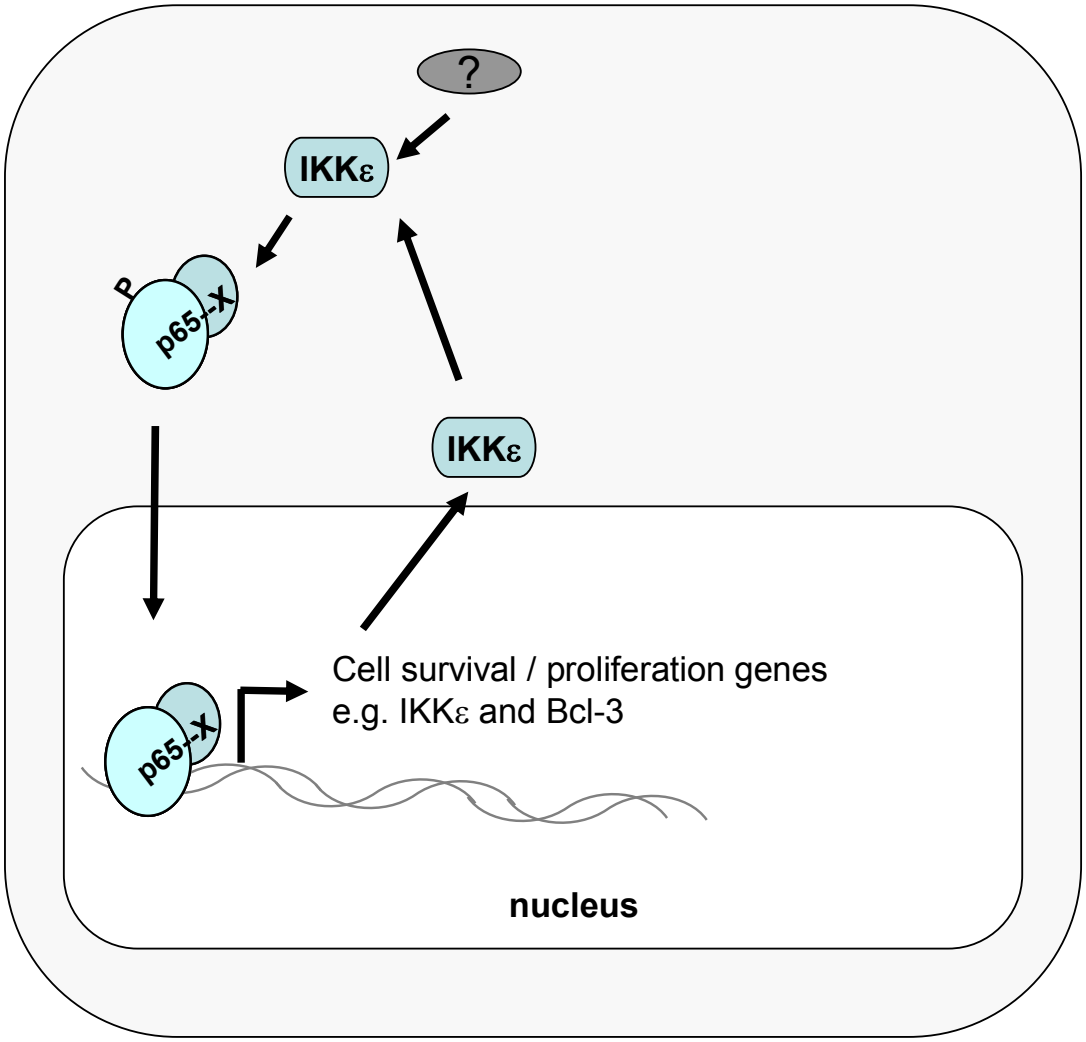


Figure A: IKKε induced cancer cell associated basal NF-κB activity. IKKe leads to constitutive NF−κB activation in cancer associated settings. Without any outside stimuli, or yet to be identified activation signal, IKKε and potentially other unknown kinases induces p65 phosphorylation at ser 536 position and hence activate NF-κB independent of classical IKK complex which then activate genes required for cell survival and proliferation including Bcl-3 and IKKe itself.

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