IKK FAMILY KINASES REGULATE CELL GROWTH, METABOLISM AND AUTOPHAGY BY PHOSPHORYLATION OF KEY SUBSTRATES

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ABSTRACT

Ricardo J. Antonia: IKK family kinases regulate cell growth, metabolism and autophagy by phosphorylation of key substrates
(Under the direction of Albert S. Baldwin)

Inflammation is now widely accepted to be an essential mediator of the balance between cell growth, metabolism, and autophagy. A group of kinases belonging to the IκB kinase (IKK) family has emerged as critical regulators of the interplay between inflammation and energy usage in both healthy and diseased tissues. They accomplish this task by phosphorylation of a variety of protein targets. My dissertation work has focused on two of these phosphorylation targets. In Chapter 2, I characterized the role of IKK in regulating the AMP-activated protein kinase (AMPK), the primary sensor of ATP levels in a cell. I found that IKK directly phosphorylates Thr172 within the AMPK activation loop, both basally in cancer cell lines and in response to cytokine stimulation. Thr172 phosphorylation of AMPK is well-characterized to increase the activity of AMPK and to phosphorylation of downstream AMPK targets. By understanding the mechanism of IKK-mediated AMPK activation, I was able to devise a strategy for exploiting a metabolic weakness of LKB1 deficient lung cancers, which involved combining an IKK inhibitor with the mitochondrial complex I inhibitor phenformin. In Chapter 3, I characterized the mechanism whereby the IKK-related kinase, TANK-binding kinase 1 (TBK1) phosphorylated a critical component of mTORC1, Raptor, which could represent a mechanism whereby TBK1 promotes autophagy.

Overall, the work presented here adds to our understanding of the mechanisms of how inflammation can regulate metabolism. Knowledge of these mechanisms will hopefully shed new light on ways to therapeutically restrict the metabolic changes induced by inflammation for therapeutic benefit.

To my friends and family

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PREFACE

A portion of the Introduction in Chapter 1 and the work in Chapter 2 has been adapted from a manuscript that has been accepted for publication in *Science Signaling*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC acetyl Co-A carboxylase

ADP adenosine diphosphate

AMP adenosine monophosphate

AMPK 5' adenosine monophosphate-activated protein kinase

ATP adenosine triphosphate

BAFF B cell activating factor of the TNF family

CD-40 Cluster of Differentiation 40

GAP GTPase-activating proteins

GTP Guanosine-5'-triphosphate

IκBα I kappa B alpha

IKK I kappa B kinase

IL-1β. interleukin 1 beta

IR Insulin Receptor

IRF Interferon regulatory factor

LKB1 liver kinase B1

MAVS Mitochondrial antiviral-signaling protein

MEF mouse embryonic fibroblast

MEKK3 Mitogen-activated protein kinase kinase kinase 3

NEMO Nuclear factor kappa B essential modulator

NF-κB Nuclear factor kappa B

NIK Nuclear factor kappa B inducing kinase

PFKFB3 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase

PKC protein kinase C

PTEN Phosphatase and tensin homolog

RANK Receptor activator of nuclear factor kappa-B

Raptor Regulatory-associated protein of mTOR

RPS3 40S ribosomal protein S3

SGK Serum and gluccocorticoid serum and glucocorticoid-regulated kinase

STING Stimulator of interferon genes

TAK1 Transforming growth factor activated kinase 1

TBK1 Tank binding kinase 1

TLR Toll like receptor

TNF-α Tumor necrosis factor alpha

TSC Tuberous sclerosis

ULK1 Unc-51 like kinase 1

PMA phorbol 12-myristate 13-acetate

CHAPTER 1: INTRODUCTION

1.1 Introduction to the IKK family of kinases.

In the NF-κB signaling pathway, the canonical IKK complex was initially described as the kinase responsible for the inducible phosphorylation of IκBα[1-4], which in turn leads to its ubiquitination and proteasomal degradation[5]. Upon degradation, IκBα can no longer inhibit p50-p65/RelA, which allows this heterodimer to enter the nucleus and drive transcription of a variety of genes[4]. While the role of IKK in the NF-κB pathway has been extensively studied (for further review of this pathway see Hayden and Ghosh[6]), it is now appreciated that IKK also phosphorylates other substrates that are not involved in NF-κB mediated transcription [7] (and see below).

The canonical IKK complex consists of two catalytic protein kinase subunits, IKKα and IKKβ, as well as a scaffold/substrate specificity factor known as IKKγ or NEMO [1,2]. Like many kinases, IKKα and IKKβ are activated by phosphorylation of critical residues in their activation loops. Activation of IKK can either be accomplished by an upstream kinase, such as TAK1, or by trans-autocatalytic phosphorylation[8]. The canonical IKK complex is activated by various inflammatory cytokines, such as IL-1β and TNF-α, as well as pathogen-associated molecules such as LPS, and downstream of oncogenic factors such as RAS. [6] Roles for IKK in promoting

cancer have been described, controlling both NF-κB-dependent and –independent oncogenic mechanisms.

In addition to the canonical IKK complex, there exists the so-called non-canonical IKK complex that is comprised of IKKα homodimers, lacking both IKKβ and NEMO (see Fig.1.1). This complex is activated by the NF-κB inducing kinase (NIK), which is induced by stimuli such as CD-40 or RANK ligand[9]. There are two other kinases in the IKK family of kinases: IKKε and TANK-Binding Kinase 1 (TBK1). These kinases are most well studied for their ability to stimulate the production of interferon genes by phosphorylation of IRF3 and IRF7, especially in the innate immune system. However, recent studies indicate that these kinases play critical roles in the regulation of cellular growth and metabolism and both are linked with cancer.

1.2 Activation and Regulation of the IKK kinases.

Like other kinases, the IKK kinases are activated by phosphorylation of serine residues within their activation loops[1]. The activation of the canonical IKK complex is most well studied in response to the cytokines TNF-α and IL-1β. Upon engagement of these ligands to their respective receptors, the canonical IKK complex is brought into proximity with the TGF-β Activated Kinase 1 (TAK1), and TAK1 phosphorylates two key activation loop serines 177 and 181 in IKKβ and the analogous residues in IKKα within the canonical IKK complex. IKKα and IKKβ can also be transautophosphorylated in this complex and may also be activated by other upstream kinases such as MEKK3[10].

The non-canonical IKK complex is activated by phosphorylation via another upstream kinase, the NF-κB Inducing Kinase (NIK)[9]. In an unstimulated cell, NIK is continuously synthesized and rapidly degraded by the proteasome. However, stimuli such as lymphotoxin B1

or RANK ligand lead to the stabilization of NIK, which in turn promotes phosphorylation of IKKα homodimers in their activation loops. Interestingly, NIK is phosphorylated by IKK✔ to limit its activity[11].

TBK1 activity is stimulated by phosphorylation of S172 by an autocatalytic activity or by phosphorylation through ULK1, which is downstream of AMPK (and see below)[12]. IKKε is also regulated by phosphorylation of an analogous residue in its kinase domain. However, while TBK1 is thought to be constitutively expressed, expression of IKKε mRNA is increased in response to certain stimuli[13].

While the activation of the IKK kinases in response to cytokines and TLR ligands is relatively well established, much less is known about how IKK activity responds to nutrient availability and growth signals.

For growth signals, such as insulin, Akt was shown phosphorylate IKKα, and this was associated with the induction of IKK activity. Serum and other growth signals can also lead to the induction of IKK activity[14].

In addition to growth signals, the activation of IKK is also observed with changes in nutrient status. Total nutrient deprivation[15], as well as the withdrawal of the individual amino acids leucine[16] and glutamine,[17] have all been associated with the induction of IKK activity. Nutrient stress may be accompanied by an increased in reactive oxygen species, which are known to activate IKK. Other changes in nutrient status are known to inhibit IKK activity. For example, the ATP/AMP sensor AMPK is known to have anti-inflammatory functions in part by reducing IKK activity[18]. IKK can be activated through O-linked β-N-acetylglucosamine modification, which occurred following the loss of p53 function and changes in glucose metabolism[19].

The way that IKK and IKK related kinase recognize substrates and the factors that cause them to phosphorylate one substrate over another is still an open area of research. For the canonical IKK kinases, substrate recognition likely occurs through NEMO, which can act as a substrate specificity factor[20] TBK1 and IKKε are often brought into proximity with their substrates by adaptor molecules such as STING or MAVS[21].

Figure 1.1 Comparison of the Canonical and Non-Canonical IKK Complex

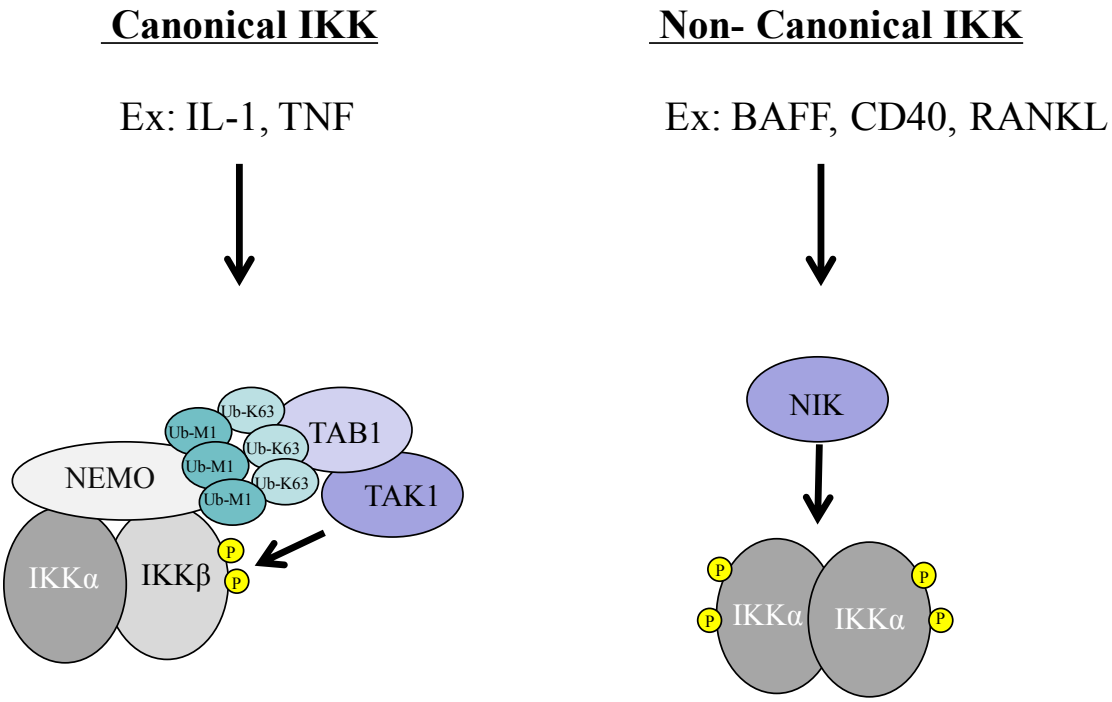


Figure 1.1: This diagram shows that composition and mechanism of activation for the canonical and non-canonical IKK complexes. The canonical IKK complex contains two kinase catalytic subunits, IKKα and IKKβ as well as the scaffold and specificity factor NEMO. This complex is activated when stimuli such as IL-1 or TNF stimulate the formation of K63-linked and linear ubiquitin chains, and these chains bring the IKK activating kinase, TAK1, into proximity with the IKK complex, which in turn allows for phosphorylation of the IKKβ activation loop serines. The non-canonical IKK complex consists exclusively of IKKα homodimers. This complex is activated when stimuli such as BAFF, CD40 and RANK ligand induce the stabilization of NIK, which in turn has a high level of activity towards IKKα homodimers.

1.3 Cell Growth and Metabolism

An essential prerequisite for cell division is an increase in the biomass in the cell. Before committing to increasing biomass, cells must sense whether there is a sufficient amount of energy and nutrients (primarily ATP and amino acids). In multicellular organisms, the decision for individual cells to grow or not to grow must be coordinated between all of the cells in the body. Examples where a coordinated growth pattern is necessary, include development, inflammation and tissue repair. Altered growth signaling pathways are often associated with diseases such as cancer, obesity, diabetes and pathogen infection. Therefore, understanding growth signaling pathways has important consequences for understanding normal human biology as well as the progression of diseases.

Concerning nutrient and energy status, the decision to grow is relatively straightforward: if the cell has sufficient levels of nutrients and energy it can grow; otherwise, no growth can occur. In contrast, many biological processes that are carried out by multicellular organisms have more nuanced growth demands that require more complex signaling processes. For example, during viral pathogen infection, the organism benefits by stimulation of growth and proliferation of immune cells but by the limitation of growth and division of cells infected by the virus. Thus a signal is necessary that stimulates the growth of specific cells and inhibits the growth of others.

Various signaling molecules have evolved to detect these signals and to transduce them to promote the appropriate balance of catabolism and anabolism for a given stimulus. The two major nutrients that a cell requires to grow are ATP and amino acids. A multitude of sensors for the presence of amino acids converge on the Ragulator complex[22] and the sensor for ATP levels is the AMP-activated protein kinase (AMPK)[23,24].

For more complex biological processes, numerous signaling pathways exist that can transduce a wide variety of stimuli into a coordinated physiological response, which results in

either anabolism or catabolism. While growth signaling can be quite complex, several essential signaling nodes integrate many different signals: mTOR, AMPK, and AKT. As mentioned earlier many of the growth signaling cascades converge on the mTOR pathway, which has been extensively reviewed elsewhere[25]. Briefly, in response to insulin and other growth hormones and cytokines, the Phosphoinositde 3-kinase (PI3K) catalyzes the creation of phospholipids at the plasma membrane which leads to the activation of the kinase Akt. Akt inhibits TSC2 by direct phosphorylation, which in turn relieves the inhibition of mTORC1 by the GTP binding protein Rheb. Amino acids and ATP also regulate mTOR. When there are sufficient levels of amino acids present, the Ragulator complex promotes mTOR signaling. AMPK on the other hand, which is potently activated by an increase in the ADP/ATP ratio, inhibits mTOR activity.

Insulin is the hormone that indicates to cells that there is sufficient glucose in the bloodstream. Treatment of cells with insulin potently induces glucose uptake and can promote cellular growth. An important consequence of stimulation of insulin is the induction of the PI3K/Akt pathway[26]. The PI3K/Akt is activated by agonists of the insulin receptor, insulin, and insulin-like growth factor, as well as by other growth factors[27].

These various signals often converge on the mechanistic target of rapamycin (mTOR) signaling pathway. When this pathway is turned on, cells stimulate different growth processes, notably protein synthesis. The kinase mTOR exists in two complexes mTORC1 and mTORC2. mTORC1 phosphorylates 4EBP1 and p70S6K to stimulate protein synthesis by mTOR. In contrast turning off mTORC1 potently induces a process of intracellular lysosomal degradation known as autophagy. Autophagy has the effect of recycling intracellular proteins and organelles. Induction of autophagy allows cells to survive periods when nutrients are scarce[28]. mTORC2 on the other hand phosphorylates Akt, SGK, and PKC.

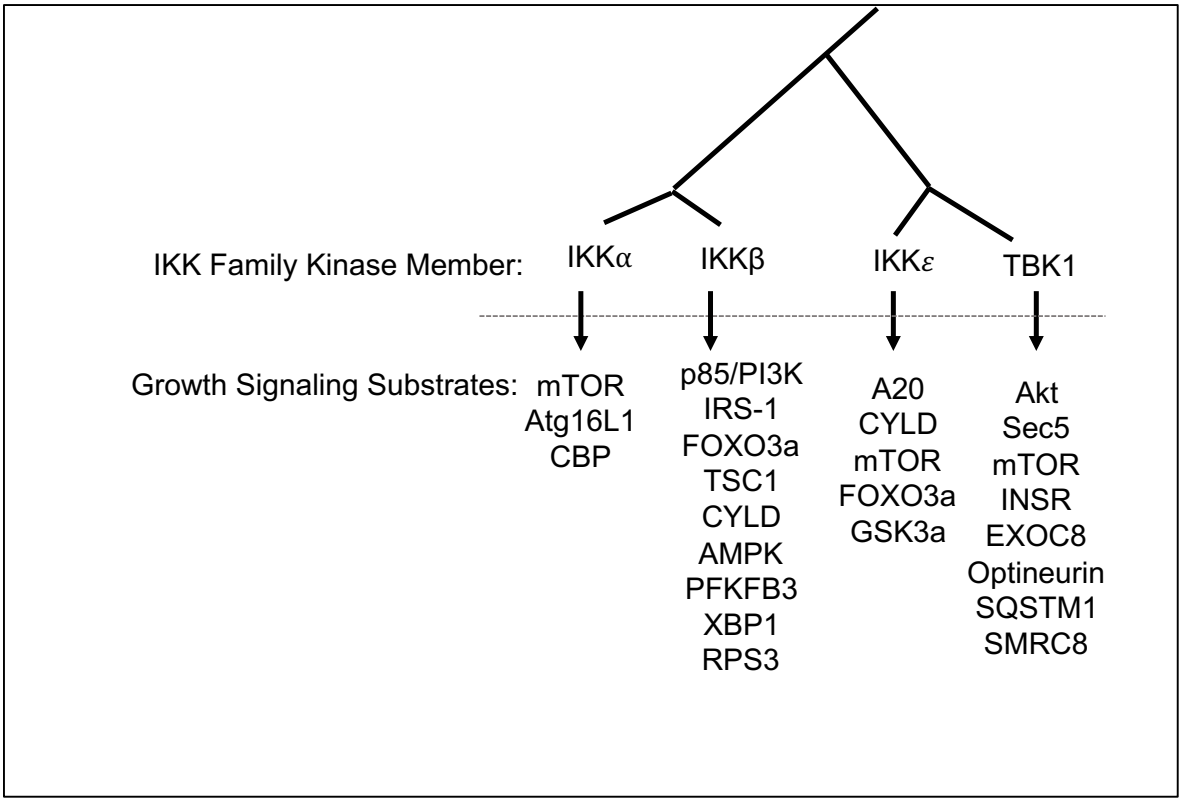


Figure 1.2 Substrates of the IKK Family Kinases involved in cell growth and autophagy

1.4 Beyond Inflammation: IKK and IKK related kinases regulate growth, metabolism, and autophagy.

An emergent regulator of growth signaling pathways is the IKK family, which is well known for their role inflammation. Inflammation is rapidly becoming appreciated as a critical regulator of growth signaling. The IKK family of kinases is now recognized to be a crucial mediator of the cross-talk between inflammation and growth signaling. They regulate cellular growth and metabolism by phosphorylation of various protein substrates. IKK family members are known for promoting pro-survival responses. IKK mediated regulation of cellular growth and metabolism may be a way to ensure that cells have the proper metabolism to survive their specific situation.

While the role of the IKK family members in promoting inflammatory cytokine production via NF-κB and IFR3/7 dependent transcription has been extensively studied, it is now appreciated that these kinases phosphorylate other substrates that are not directly involved in the regulation of NF-κB or IRF 3/7 transcription (see Fig. 1.2).[20] One primary function of these kinases is to mediate the cross-talk between inflammation and cell growth or autophagy.

There is a clear link between inflammatory states and altered metabolism. However, the type of metabolic modification and the consequences of the metabolic changes are highly context dependent. For instance, the inflammatory cytokine TNF-α, a potent inducer of canonical IKK activity was also known as "cachexin" because it induced an extreme form of catabolism that manifested in wasting in cancer patients[29]. Obesity in humans and mice is accompanied by inflammation, manifested by up-regulation of TNF-α and other inflammatory cytokines such as IL-6 and activation of local tissue macrophages, which contributes to insulin resistance [30].

These early findings that TNF-α, a well-known inducer of IKK activity, could play a role in both cancer wasting and obesity likely led researchers to study the effects of IKK on insulin signaling and metabolism. Indeed early studies in mice indicated that IKK induces insulin resistance *in vivo*, providing some of the first evidence that IKK played a vital role for in the cross-talk between the inflammatory and metabolic signaling pathways. [31,32] [33] Many reports have now demonstrated that all of the members of the IKK family can play an essential role in regulating metabolism in various contexts. In certain situations, the IKKs appear to promote growth, and in others, it seems to promote autophagy. For instance, IKKβ is known to inhibit Akt signaling by phosphorylation of the p85 subunit of PI3K, but also to promote mTOR signaling by phosphorylation of TSC1. This seemingly paradoxical role for IKK likely reflects the importance of the context for the IKK activity or could reflect mechanisms to finely tune the amount of anabolism and catabolism that occur in the cell.

Below I will describe the some of the IKKα and IKKβ mediated phosphorylation events that could explain some of the metabolic phenotypes observed above (see Fig. 1.3).

Figure 1.3 Diagram of how IKKα/β promote growth signaling or autophagy

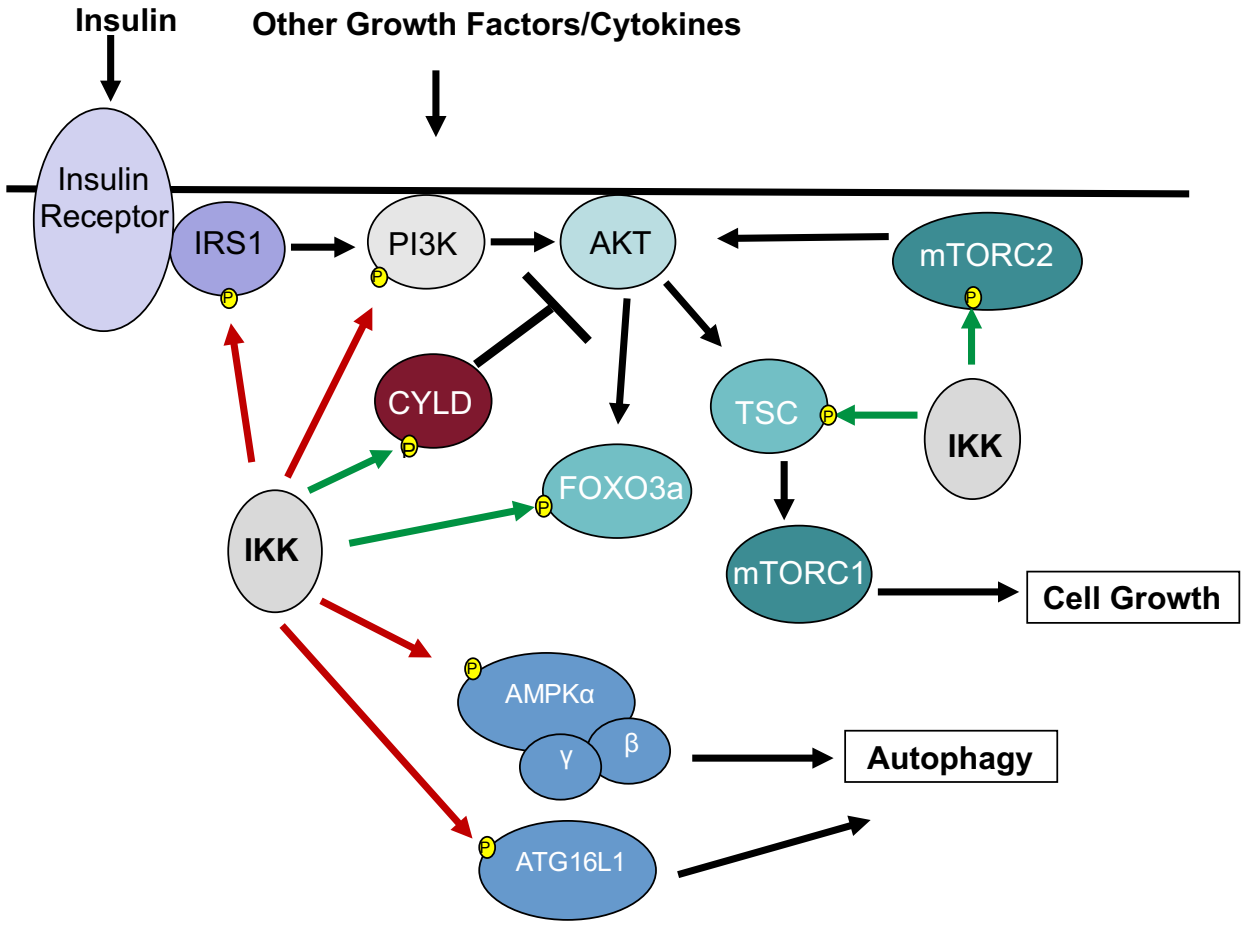


Figure 1.3: Diagram of the major IKKα/β driven phosphorylation events in the growth and autophagy pathways. Green arrows indicate that the phosphorylation event is associated with the promotion of cell growth. Red arrows indicate either inhibition of cell growth or promotion of autophagy.

1.5 Promotion of growth signaling by IKKα and IKKβ through phosphorylation of substrates in the Akt/mTOR pathway

IKKβ promotes mTOR signaling by phosphorylation of TSC1.

The tuberous sclerosis 1 (TSC1) gene encodes a tumor suppressor protein that is part of a critical complex with tuberous sclerosis 2 (TSC2), which is well-known for its role in mTOR regulation[34]. TSC1 is required for stabilization of TSC2 by preventing its degradation by the ubiquitin-proteasome system. [35,36] TSC2 contains a GTPase Activating Protein (GAP) domain that primarily promotes the GTPase activity of the Rheb, which results in inhibition of mTORC1. [37-39] Phosphorylation of the TSC1-TSC2 complex is a way to integrate a diverse set of signals (including growth factors, cytokines, and cellular stress) to the mTOR complex, with many different upstream kinases acting to phosphorylate the complex[34]. IKKβ was shown to inhibit the TSC1-TSC2 complex and thus promote mTOR signaling downstream of TNF-α[40]. IKKβ specifically phosphorylated TSC1 at serines 487 and 511. Phosphorylation of these two serine residues served to stabilize TSC1, as phosphomimetic mutations in serine 487 and 511 significantly decreased the half-life of the TSC1 protein. The authors also show that phosphorylation of these two serine residues contributes to the formation of the TSC1/TSC2 complex. By inactivating a negative regulator of mTOR, IKKβ could efficiently promote mTORC1. Our group, however, has found that this phosphorylation of TSC1 only occurred in certain cell lines[41].

IKKα can also promote growth by phosphorylation of mTOR at serine 1415

Our group found that IKK is required for mTOR activation downstream of AKT in prostate cancer cells with a loss of the tumor suppressor PTEN and downstream of insulin and TNF-α[41,42]. Based these studies, our group concluded that IKKα within the canonical IKK complex plays a more critical role than IKKβ with respect to mTOR signaling, especially in prostate cancer cells that have constitutive Akt activation due to a loss of the tumor suppressor PTEN. Our group also demonstrated that IKKα phosphorylates mTOR at serine 1415 and that this phosphorylation promoted mTOR association with Raptor and mTORC1 mediated phosphorylation of p70-S6K [43]. IKKα promotes Akt activation by increasing mTORC2 activity; however, the mechanisms by which this occurs are still unknown[44]. Importantly, mTOR was required for regulation of NF-κB downstream of AKT[45].

It is interesting that IKKα and IKKβ can both positively regulate mTOR signaling, by direct phosphorylation of two distinct components of the pathway. The canonical IKK complex can both inactivate a negative regulator of mTOR and also directly promote mTOR by phosphorylation of the kinase domain itself. This redundancy may be a way to ensure that mTOR is activated if either IKKα or IKKβ is inhibited.

IKK inhibits CYLD (cylindromatosis)-a negative regulator of Akt activation by growth factors

TRAF6 mediated Akt ubiquitination is required for Akt membrane localization and activation[46]. TRAF6 specifically adds K63-linked polyubiquitin chains to its targets. CYLD is a lysine 63 deubiquitinase that deubiquitinates Akt in serum-starved cells, thus negatively regulating its activity[47]. CYLD also negatively regulates NF-κB signaling through its deubiquitinase activity.[48] The canonical IKK complex phosphorylates CYLD to inhibit its

deubiquitinase activity. [49] It is, therefore, possible that IKK could promote Akt activity by inhibiting CYLD mediated Akt deubiquitination, although this relationship needs to be further explored. NEMO is required for CYLD mediated phosphorylation, but IKKα and IKKβ have similar activities towards CYLD.

IKK down-regulates FOXO3a by phosphorylation of Serine 644.

In addition to directly promoting Akt pathway signaling components, IKK and Akt also share a common effector substrate: FOXO3a. In this instance, IKK can functionally mimic Akt activity in the absence of Akt activity. Akt phosphorylates several of the FOXO transcription factors[26]. Phosphorylation of these sites by Akt creates 14-3-3 binding sites leads to their exclusion from the nucleus, thus preventing them from activating transcription. [50-53] In addition to its exclusion from the nucleus, Akt phosphorylation could lead to proteasomal degradation of FOXO3a[54].

IKK also inhibits FOXO3a by phosphorylating serine 644, which serves to either destabilize FOXO3a or exclude it from the nucleus. In acute myeloid leukemia, IKK could exclude FOXO3a from the nucleus even when Akt was inhibited. [55] This exclusion from the nucleus was dependent on phosphorylation of S644.

However, instead of creating a 14-3-3 binding site as is the case in Akt mediated inhibition of FOXO transcription factors, phosphorylation at serine 644 by IKK leads to ubiquitination and proteasome-mediated degradation[56]. IKK mediated FOXO3a regulation is thought to be independent of Akt, as IKK could still repress FOXO3a transcription on a mutant of FOXO3a that had all of the Akt dependent serine phosphorylation sites mutated to alanine. Interestingly, it was also reported that the IKK-related kinase-IKKε is also capable of phosphorylating FOXO3a S644[57].

IKKβ phosphorylates XBP-1 to promote insulin sensitivity

One report indicated that in a mouse model of chronic liver inflammation driven by liver-specific expression of constitutively active IKKβ, the mice surprisingly showed an improvement in glucose homeostasis. [58] This observation was surprising as many others had implicated IKKβ in insulin resistance and poor glucose homeostasis. Mechanistically, the authors of this report linked this improvement in glucose homeostasis with the endoplasmic reticulum stress response, which is regulated by XBP1. Constitutively active IKKβ increases the stability of XBP1 by phosphorylation of Thr48 and Ser148. IKKβ interacted with XBP in lean mice but not in obese mice and was associated with higher levels of endoplasmic reticulum stress due to the lack of XBP1 protein. These defects could be rescued by overexpression of constitutively active IKKβ in the livers of obese mice. This result would indicate that activation of IKKβ in the livers of obese patients could promote healthy glucose homeostasis.

1.6 IKK can also oppose mTOR/Akt signaling

- IKKβ inhibits insulin receptor signaling by phosphorylation IRS-1 and the p85 subunit of PI3K.

As stated in the introduction, obesity often results in low-level, chronic inflammation and this inflammation leads to insulin resistance, which often precedes diabetes. IKKβ and possibly IKKα can inhibit insulin signaling by phosphorylation of IRS-1 and the p85 subunit of PI3K.

The Insulin Receptor(IR) and IGF-1 Receptor (IGFR) are receptor tyrosine kinases[59]. Upon binding of their respective ligands, IR and IGFR phosphorylate a family of proteins in the Insulin Receptor Substrate (IRS) family, including IRS-1 through IRS-6. Phosphorylation of these proteins creates a binding scaffold for SH2-domain-containing protein that leads to activation of the PI3K/Akt signaling pathway. In contrast to tyrosine phosphorylation, serine/threonine

phosphorylation is thought to downregulate IRS-1 both by preventing tyrosine phosphorylation and by promoting its degradation[60].

Treatment of cells with insulin induces phosphorylation of IRS-1 Serine 312, and this phosphorylation can lead to its degradation, which serves as a negative feedback mechanism for insulin signaling.[61]. Chronic TNF-α stimulation can also lead to serine phosphorylation and degradation of IRS-1, and this lack of IRS-1 protein is associated with insulin resistance, as IRS-1 can no longer transmit signals from the insulin receptor.

To understand how IKK regulates insulin resistance, Gao et al. [62] showed that IKK could directly phosphorylate IRS-1 Ser 312. They demonstrated that induction of IKK activity by TNF-α and calyculin-A was correlated with induction of IRS-1 Ser 312 phosphorylation in HepG2 liver cancer cells. The authors focused on IRS-1 Serine 312 as there is an abundance of functional data indicating the importance of that particular phosphorylation site. However, the authors also suggest that there may be other IKK dependent phosphorylation sites in IRS-1. The functional relevance of these different phosphorylation sites remains to be determined.

One may speculate that this could serve as a mechanism whereby TNF-α treated cells restrict pathogen replication by making cells insensitive to insulin-induced glucose uptake. This would prevent intracellular pathogens from using glucose and thus starving them of the energy that they would need to replicate properly.

IKKα and IKKβ immunoprecipitated separately from HEK293T cells had a similar kinase activity towards recombinant IRS-1. However, since IKKα and IKKβ are frequently found in complexes together, it is possible that these immune complexes contained both IKKα and IKKβ. It is therefore impossible to know which kinase is responsible for direct IRS-1 phosphorylation.

Future studies are necessary to determine whether the canonical or non-canonical IKK complex can phosphorylate IRS-1.

Work from our group previously published that IKK was involved in feedback inhibition of PI3K by phosphorylation of the p85 subunit of PI3K [16]. In this study both IKKα -/- and IKKβ-/- MEFs failed to efficiently shut off Akt S473 phosphorylation after starvation of all nutrients in Hank's Balanced Salt Solution. Treatment of the MEFs with the NEMO Binding Domain Peptide (NBD) that is known to disrupt the canonical IKK complex also prevented starvation induced Akt shut-off. However, both IKKα and IKKβ were able to phosphorylate p85-PI3K at Ser690 efficiently. Phosphorylation of this site blocked p85 phosphotyrosine binding, thus preventing it from interacting with growth factor receptors. All of the data presented above led to the model whereby both IKKα and IKKβ within the canonical IKK complex phosphorylate p85 to downregulate Akt phosphorylation in response to starvation.

IKKβ phosphorylates RPS3

The Ribosomal Protein S3 (RPS3) is a component of the 40S ribosome but contains some non-ribosomal activities, including acting as a specificity factor for NF-κB transcription factor complexes.[63,64] IKKβ phosphorylates RPS3 at S209, which in turn induces its translocation into the nucleus[64]. Nuclear RPS3 was able to function to alter which promoters are bound by NF-κB transcription factors. While there is an apparent effect on nuclear translocation, it will be interesting in the future to determine what effect, if any, RPS3 S209 phosphorylation has on protein translation, which is an important output of mTOR regulation.

IKKβ phosphorylates PFKFB3 Ser269 to promote metabolic adaptation to glutamine deprivation.

In addition to phosphorylation of protein substrates that transduce extracellular signals, IKKβ also phosphorylates PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3) which is a metabolic enzyme to adapt to periods of glutamine deprivation.[17] In this case, IKKβ inhibits aerobic glycolysis, which is the type of metabolism favored by rapidly growing cells.

PFKFB3 is a crucial driver of aerobic glycolysis. In the case of glutamine deprivation, aerobic glycolysis does not promote survival. If glutamine starved cells fail to switch from aerobic glycolysis to oxidative phosphorylation, they die. During glutamine deprivation, IKKβ promoted oxidative phosphorylation by promoting phosphorylation of PFKFB3, which had the effect of inhibiting the activity of this enzyme. This study somewhat contradicts others in that they reported that IKK deficiency leads to an increase in aerobic glycolysis, whereas others have observed that IKK deficiency limits the Warburg effect. For example, in cancer cells, IKKβ was shown to play an essential role in controlling Warburg metabolism and glucose import by regulating NF-κB dependent induction of metabolic genes. [65]

1.7 IKKα and IKKβ can promote autophagy

Turning off mTORC1 potently induces a process of intracellular lysosomal degradation known as autophagy. Autophagy has the effect of recycling intracellular proteins and organelles. Induction of autophagy allows cells to survive in nutrient stressful conditions, such as during low concentrations of amino acids or ATP[28]. IKK family proteins are directly linked with control of autophagy.

In 2010 Criollo et al. [15] demonstrated that IKK was involved in the induction of autophagy in response to several known autophagy inducers including starvation media and rapamycin. This group showed that overexpression of constitutively active IKK was sufficient to promote the induction of autophagy and that this induction required the AMP-activated protein kinase (AMPK). Importantly, overexpression of constitutively active IKK was adequate to promote AMPK activation in HeLa cells, which lack the major AMPK activating kinase LKB1. Our group expanded on this work to demonstrate that IKK could directly phosphorylate AMPK in its activation loop and promote its activity (see below and Chapter 2).

IKKα stabilizes the autophagy protein Atg16L by phosphorylation.

In a mouse model of colitis induced inflammation, IKKα phosphorylates Atg16L1 at serine 278 to stabilize it.[66] Atg16L is an essential autophagy protein [67]. Interestingly, in this study, the author's found that knockout of IKKα but not p100/NF-κB2 in intestinal epithelial cell exacerbated colitis symptoms. This observation indicates that IKKα has some functions in colitis that are independent of activating the non-canonical NF-κB pathway. The authors in this study did not relate their findings to autophagy, however, and their conclusions were more associated with the ER-stress response pathway.

A naturally occurring mutation in Atg16L1, which prevents its Caspase-mediated degradation, is associated with Crohn's disease. The authors of this study thus hypothesized that IKKα might be exacerbating colitis in this model by affecting Atg16L stability. While the authors of this study found that phosphorylation of Atg16L1 by IKKα could promote its stability, the authors did not look at the effects of this phosphorylation on the process of autophagy. One might hypothesize that increased Atg16L stability would increase autophagic

flux and an important future direction will be to determine whether this phosphorylation site can enhance or inhibit autophagy.

Figure 1.4 Diagram of how TBK1/IKKε promote growth signaling or autophagy

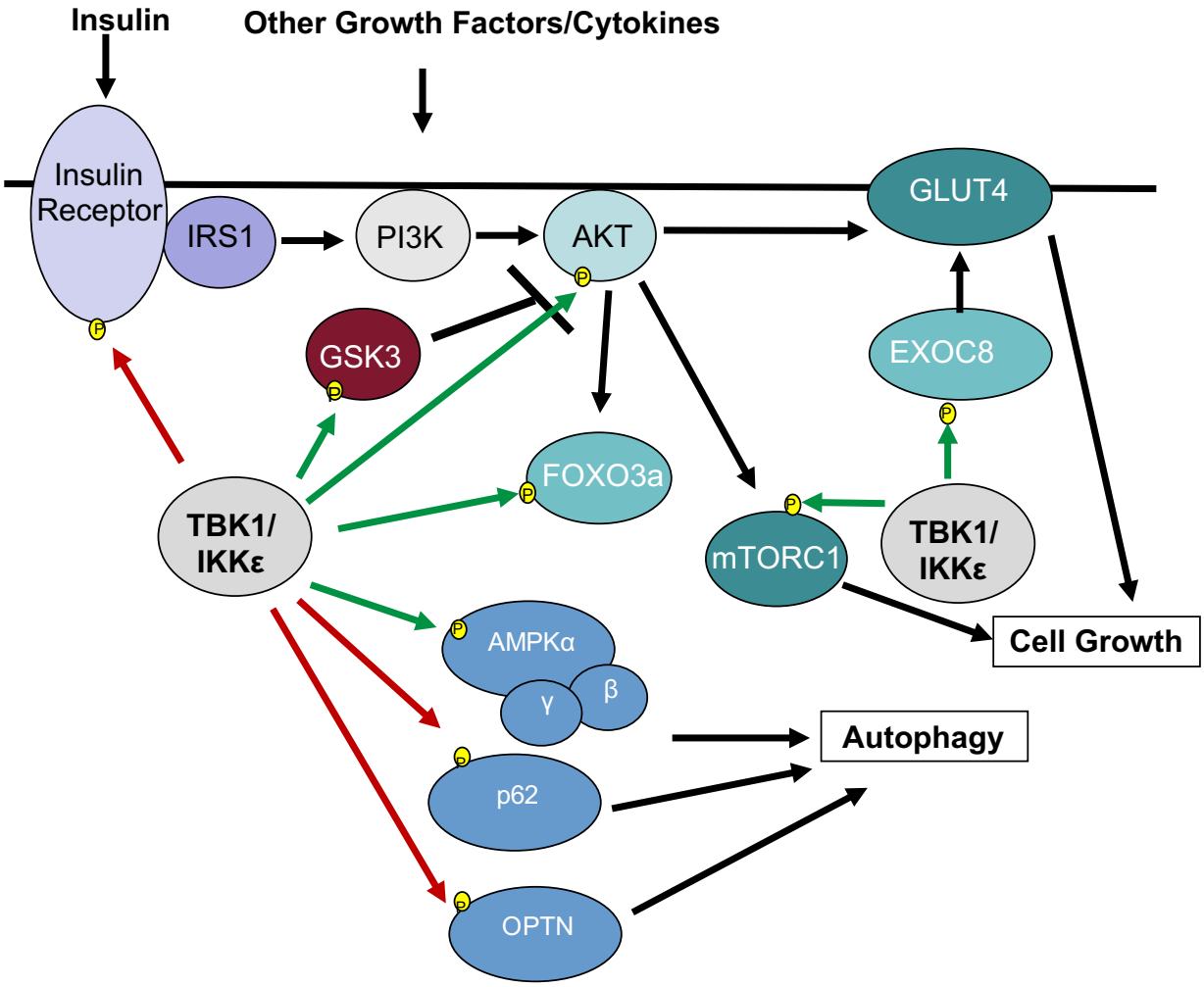


Figure 1.4: Diagram of the major TBK1/IKKε driven phosphorylation events in the growth and autophagy pathways. Green arrows indicate that the phosphorylation event is associated with the promotion of cell growth. Red arrows indicate either inhibition of cell growth or promotion of autophagy.

1.8 Regulation of growth signaling pathways via TBK1/IKKε

The IKK related kinases TBK1 and IKKε are also known to be important regulators of cell growth and autophagy. Similar to IKKα and IKKβ, TBK1 and IKKε can also promote cell growth or induce autophagy depending on the context.

Several lines of work now indicate that TBK1 and IKKε are induced in mouse models of obesity and that they play a significant role in the pathology of this disease. IKKε and TBK1 expression are induced in mouse models of obesity and IKKε regulates energy expenditure in this model[68]. Deletion of IKKε in mice prevents weight gain caused by high-fat diet and increases insulin sensitivity and obesity induced inflammation. Knockout of TBK1 in adipocytes also improved the clinical features of obesity in mice. A small human clinical trial of a TBK1/IKKε inhibitor, Amlexanox, in diabetic patients showed a significant decrease in markers of the disease and was able to restore insulin sensitivity in a select group of the patients on the trial. In addition to diabetes and obesity, IKKε was shown to be involved in glucose reprogramming in pancreatic ductal adenocarcinoma, and TBK1 was shown play a significant role in the regulation of Akt/mTOR signaling in lung cancer cell lines[69]. [70]

Below I will describe the some of the TBK1 and IKKε mediated phosphorylation events that could explain some of the metabolic phenotypes observed above (see Fig. 1.4) .

TBK1/IKKε can promote Akt activation by direct phosphorylation

As mentioned in the introduction, a key effector of PI3K activation is the kinase Akt. Akt kinase activity is activated by phosphorylation of Thr308 and Ser473.[71] Two reports have been published that demonstrate that TBK1 and IKKε can directly phosphorylate and activate Akt. [72,73] One study reported that in MEFs, EGF and LPS, but not insulin induced Akt activation was

TBK1 dependent. TBK1 was able to phosphorylate both Akt Thr308 and Ser476. As for the relevance of TBK1 mediated Akt regulation in cells other than MEFs, the same groups recently published that in cancer cell lines, the role of TBK1 in regulating Akt is dependent on many factors, including whether or not a cell has undergone an epithelial to mesenchymal transition[69]. Another study utilizing TBK1/IKKε double knockout MEFs found that both kinases were required for activation of Akt by a wide range of stimuli, including insulin[73]. This difference between TBK1 -/- and TBK1/IKKε double knockout MEFs for only certain stimuli, indicates that these two kinases may have both redundant and non-redundant roles with respect to Akt, dependening on the stimulus.

IKKε inhibits GSK3α Serine 21 to promote mTOR/AKT.

In addition to direct phosphorylation, IKKε was shown to promote Akt activation by negative regulation of an AKT suppressor, GSK3α. IKKε was shown to phosphorylate GSK3α Ser21, which interestingly is also a substrate for Akt. Gullen et al. noted that IL-1β induced PI3K/Akt independent GSK3α Serine 21 phosphorylation, a phosphorylation site known to inhibit GSK3α kinase activity [74]. The authors found that in the context of IL-1β signaling in Th17 cells, GSK3α suppresses Akt activity by phosphorylation of Akt Thr312, near Akt Thr308, a site well-characterized to promote Akt activity. Akt Thr312 is located within the region of Akt required for substrate recognition. Phosphorylation of Thr312 was predicted to inhibit substrates from entering the Akt active site. IKKε inactivated GSK3 by phosphorylation of serine 21, which relieved inhibition on Akt and had the overall effect of promoting Akt/mTOR activation. This study focused on Th17 cells, so it will be interesting in the future to learn if this mode of regulation can occur in other cell types or contexts.

TBK1/IKKε can promote mTOR activation by phosphorylation of Ser 2159 in the mTOR activation loop.

Phosphorylation of mTOR at serine 2159 in the kinase domain was shown to be sufficient to promote mTOR kinase activity,[75] and knock-in mice with an mTOR Serine 2159 to Alanine mutation showed defective mTOR signaling *in vivo*. Interestingly, Insulin but not EGF was capable of inducing this phosphorylation site (mTOR S2159), in a TBK1/IKKε dependent manner. TBK1 and IKKε were identified in a screen for kinases that could directly phosphorylate this site *in vitro*.[76] Of the wide panel of kinases tested, TBK1 and IKKε showed the most robust levels of activity towards this site, indicating that these are likely to be the most significant kinases for mTOR Ser2159 *in vivo*. Overall, these studies suggest that TBK1/IKKε can promote mTOR activity by phosphorylation of Ser2159, but it should be noted that there is also a significant amount of data to indicate that TBK1/IKKε also inhibit mTOR (see below). It is interesting to note that IKKα can also phosphorylate mTOR to promote mTORC1 signaling and an interesting future direction will be to study the cross-talk between IKKα and TBK1/IKKε with respect to this pathway. One group described a mechanism whereby TBK1 inhibited the upstream IKKα activating kinase NIK by direct phosphorylation[77].

TBK could promote mTOR signaling by inhibition of AMPK activity by Ser459 and Ser 476 phosphorylation

A recent study also indicates that TBK1 can directly inhibit AMPK by promoting phosphorylation of AMPK and Ser459 and Ser476[78]. Phosphorylation of these sites was associated with a decrease in AMPK activity as measured by phosphorylation of AMPK Thr172. In cell-free kinase assays, TBK1 was unable to phosphorylate AMPK, and the authors speculated

that another factor was required in the reaction for TBK1 to promote efficient phosphorylation of AMPK. Future studies are necessary to determine the identity of this factor and to determine whether TBK1 can directly phosphorylate AMPK. Interestingly, AMPK could promote TBK1 phosphorylation by promoting ULK1 mediated phosphorylation of TBK1 Ser172. Therefore, TBK1 mediated AMPK inhibition represents a feedback mechanism on AMPK.

In contrast to TBK1 and IKKα, which both phosphorylate mTOR to promote its activity, IKKβ and TBK1 appear to exert opposite effects on AMPK by direct phosphorylation. Our work in Chapter 2 will describe in detail how IKKβ promotes AMPK Thr172 phosphorylation.

TBK1 phosphorylates the EXOC8 to promote glucose uptake by trafficking GLUT4 to the plasma membrane.

Upon stimulation with insulin, AKT induces glucose uptake by causing a rapid translocation of the glucose transporter GLUT4 to the plasma membrane. The exocyst complex controls trafficking of GLUT4 to the plasma membrane in response to insulin. [79] A recent report indicated that TBK1 phosphorylates a component of the exocyst, EXOC8, to promote Glut4 trafficking to the cell membrane in response to insulin. [80] Insulin-stimulated glucose uptake in 3T3-L1 adipocytes was attenuated by knockdown or inhibition of TBK1 but not by knockdown of either IKKα, IKKβ or IKKε. This difference in phenotype between the different IKK family members indicates that while the IKK and IKK related kinases often share overlapping substrates, they do not have completely redundant functions.

1.9 Inhibition of Akt/mTOR signaling via TBK1/IKKε

TBK1 inhibits the Insulin Receptor by phosphorylation of Serine 994.

In addition to promoting Insulin signaling by activation Akt, TBK1 and IKKε are also known to contribute to insulin resistance[81]. TBK1/IKKε could contribute to insulin resistance by direct inhibition of the Insulin Receptor (IR). In addition to serine phosphorylation of IRS proteins as described earlier, serine phosphorylation of the IR itself is also associated with inactivation of the receptor. Among the serine residues that are found to be phosphorylated is serine 994. Mutation of this serine to alanine increased the tyrosine kinase activity of the insulin receptor when expressed in HEK 293 cells[82]. TBK1 activity was associated with Insulin Receptor 994 phosphorylation *in vivo*. TBK1 displayed activity towards a peptide that contained Serine994, but not a peptide in which Serine994 was mutated to alanine in a cell-free kinase assay. [83] These experiments indicate that TBK1 could inhibit the tyrosine kinase activity of IR by phosphorylation of IR Ser994.

Roles for TBK1 in down-regulating mTOR and promoting Autophagy

Critical signaling pathways in response to nutrients or nutrient deprivation integrate signaling which leads to regulation of mTORC1 and autophagy. These responses play critical roles in promoting growth or promoting autophagy for survival. Crosstalk between mTORC1 and autophagy signaling have been extensively reviewed[84]. Several studies have linked TBK1 with regulation of mTORC1 as well as AMPK, an upstream regulator of the autophagic response (see below).

In cancer, TBK1 mediated suppression of mTOR was linked to prostate cancer dormancy. In this study overexpression of wt but not kinase-dead TBK was sufficient to block mTORC1 activity as measured by p70S6K phosphorylation[85]. In a model of chronic innate immune

activation induced by knockout of the Three-prime repair exonuclease 1 (TRex1), which mimics chronic STING activation, TBK1 was also linked to suppression of mTORC1 activity[86].

The SMRC8 gene product is expressed in neurons and is associated with amyotrophic lateral sclerosis and frontotemporal dementia. Interestingly loss of function TBK1 mutations were found in patients with the same disease. [87,88]SMRC8 was found in a complex with the autophagy initiating kinase Ulk1 and is described to be important for autophagy initiation[89]. It was found that TBK1 phosphorylates this protein and that this phosphorylation is required for autophagy initiation.[90] Recent work also indicates that ULK1 can act as an upstream kinase for TBK1 Ser172[78]. SMRC8 may act as a recruitment factor, bringing ULK1 in close proximity to TBK1 to promote TBK1 Ser172 phosphorylation. Pili et al demonstrated that TBK1 is required for autophagosome maturation in response to several inflammatory stimuli[91]. TBK1 phosphorylated the autophagy cargo adaptor p62 (SQSTM1) and this was required for p62's functions in promoting autophagy. Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria[92].

1.10 Introduction to AMPK (adapted from Antonia and Baldwin, Science Signaling 2018)

AMPK (5' AMP-activated protein kinase) is a highly conserved master energy-sensing kinase that phosphorylates a variety of substrates to promote ATP conservation. [24,93] The most well-characterized effector substrate of AMPK is Acetyl-Co-A Carboxylase (ACC), which promotes fatty acid biosynthesis[94]. AMPK phosphorylation of ACC inhibits its activity and therefore inhibits fatty acid synthesis, a process that consumes large amounts of ATP. AMPK also phosphorylates ULK1 to promote autophagy[95,96], and Raptor to inhibit mTORC1 signaling[97]. Many other substrates have been defined for AMPK, and in general phosphorylation of these substrates serves the function of increasing the levels of ATP in the cell.

AMPK opposes inflammation and NF-κB signaling

In addition to its well-defined role in promoting energy conservation, AMPK is also known to modulate inflammation[98,99]. Evidence primarily comes from that fact that AMPK agonists can reduce inflammation markers in certain inflammatory disease models[100,101]. Some have suggested that AMPK regulates NF-κB indirectly through modulation of PCG-1α[102] or SIRT1.[103,104] However, it remains possible that AMPK regulates NF-κB signaling by direct phosphorylation of components of the NF-κB signaling machinery.

Regulation of AMPK activity

The AMPK holoenzyme is composed of three subunits, AMPKα, AMPKβ, and AMPKγ. The kinase domain lies within the AMPKα subunit. AMPKγ contains nucleotide binding domains that bind either AMP or ATP, which allows for allosteric regulation of the enzyme. The AMPKβ subunit serves scaffolding and structural roles. AMPK kinase activity is regulated by phosphorylation of Thr172 in the kinase domain activation loop of the kinase domain in AMPKα and allosterically by adenosine nucleotide binding [105-107]. Binding of AMP to the gamma subunit of AMPK induces an increase in AMPK activity, and binding of either AMP or ADP promotes Thr172 phosphorylation in the kinase domain by causing a conformational change in the AMPK holoenzyme that prevents phosphatases from accessing Thr172, which leads to an increase in the levels of Thr172 phosphorylation. In cell-free kinase assays, allosteric binding of AMP to the gamma subunit causes a 5-fold increase in AMPK activity while phosphorylation of Thr172 causes a 100-fold increase in AMPK activity, thus Thr172 phosphorylation is the primary point of AMPK regulation both by adenosine nucleotides and by upstream kinases[105,108,109].

Three kinases have been shown to phosphorylate AMPK at Thr172: LKB1 (liver kinase B1), CAMKK2 (calcium/calmodulin-dependent protein kinase kinase 2) and TAK1 (transforming growth factor-activated kinase-beta-1). The kinase upstream of AMPK is dependent on the signaling cascade or specific stressor. When the AMP/ATP ratio is increased in cells, a conformational shift occurs that makes Thr172 more amenable to phosphorylation by LKB, a robust and well-characterized AMPK kinase. [110-112] However, LKB1 is a tumor suppressor that is lost in many cancers, and these cancers still require AMPK for growth and survival. [113]

Stimuli such as ionomycin that lead to an increased concentration of intracellular calcium cause CAMKK2 to phosphorylate Thr172 [114-116]. Additionally, cytokines such as IL-1, TNF, and TRAIL lead to AMPK Thr172 phosphorylation in a TAK1-dependent manner [117,118], although much less is known concerning the mechanism of how TAK1 promotes AMPK Thr172 phosphorylation.

Role of AMPK in Cancer and Disease

Many diseases are associated with altered metabolism and AMPK as a master energy sensor plays an important role in diseases such as cancer, diabetes, heart disease and pathogen infection. Each of the three AMPK subunits has redundant coding regions. There is an AMPKα1 and an AMPKα2, AMPKβ1 and AMPKβ2, AMPKγ1, AMPKγ2, and AMPKγ3. Mutations in AMPKγ2 leads to a genetic heart disorder known as Wolf-Parkinson-White disease[119].

AMPK loss of function mutations or deletions are rarely observed in human cancers. Moreover, since each AMPK subunit contains redundant coding regions, a complete loss of AMPK activity is almost never observed in human cancers. AMPK appears to play a dual role in cancer depending on the context. AMPK can promote NADPH balance and mitophagy (autophagic degradation of mitochondria) to favor tumor survival under metabolic stress, as is

frequently observed in tumors. However, AMPK can inhibit growth signaling and protein synthesis pathways (notably mTOR signaling) to limit tumor growth. [113] The major upstream AMPK activating kinase, LKB1, is a tumor suppressor that is lost in many cancers, and is thought to exert its tumor suppressive actions by promoting AMPK activity.[120]

Since the role of AMPK in cancer is so context dependent it is critical to understand the role of AMPK in cancer and how it is regulated.

CHAPTER 2: IKK PROMOTES CYTOKINE-INDUCED AND CANCER-ASSOCIATED AMPK ACTIVITY AND BUFFERS AGAINST PHENFORMIN-INDUCED CELL DEATH IN LKB1-DEFICIENT CELLS[1]

2.1 Overview

The 5' AMP-activated protein kinase (AMPK) is a master energy sensing kinase that is regulated by phosphorylation of Thr172 in its activation loop. Three kinases have been described to phosphorylate AMPK at Thr172: the tumor suppressor LKB1, CAMKK2 and TAK1. While LKB1- and CAMKK2-mediated AMPK Thr172 phosphorylation have been well-characterized, much less is known about TAK1-dependent AMPK phosphorylation. An important target of TAK1 is IκB kinase (IKK) which controls NF-κB transcription factor activation. Here, we tested the hypothesis that IKK acts downstream of TAK1 to activate AMPK by phosphorylating Thr172. IKK is required for AMPK Thr172 phosphorylation induced by IL-1β and TNF-α treatment and by TAK1 overexpression. Additionally, IKK regulates basal AMPK Thr172 phosphorylation in several cancer cells independent of TAK1, indicating that other modes of IKK activation can lead to AMPK activation. Mechanistically, we found that IKK directly phosphorylates AMPK at Thr172 and that IKK promotes AMPK Thr172 phosphorylation independent of LKB1 expression or energy stress. This indicates that while

[1] This chapter has been adapted from a manuscript that has been accepted for publication in *Science Signaling* The original citation is as follows: "Antonia RJ. and Baldwin AS. IKK Promotes Cytokine-Induced And Cancer-Associated AMPK Activity And Buffers Against Phenformin-Induced Cell Death In LKB1-Deficient Cells. Science Signaling 2018.

LKB1 activates AMPK as a sensor of energetic stress, IKK activates AMPK in response to extracellular inflammatory signals and through distinct pathways that activate IKK. Accordingly, in LKB1-deficient cells IKK inhibition caused a reduction in AMPK Thr172 phosphorylation in response to the mitochondrial inhibitor phenformin. This response led to enhanced apoptosis and suggests that IKK inhibition in combination with phenformin could be used clinically to treat patients with LKB1-deficient cancers.

2.2 Introduction

AMPK (5' AMP-activated protein kinase) is a master energy sensing kinase that phosphorylates a variety of substrates to promote ATP conservation [24,93]. AMPK appears to play a dual role in cancer depending on the context. AMPK can promote NADPH balance and mitophagy to favor tumor survival under metabolic stress, as is frequently observed in tumors. However, AMPK can inhibit growth signaling and protein synthesis pathways (notably mTOR signaling) to limit tumor growth [113]. AMPK activity is regulated by phosphorylation of Thr172 in the kinase domain activation loop and allosterically by adenosine nucleotide binding [105-107]. Binding of AMP to the gamma subunit of AMPK induces an increase in AMPK activity, and binding of either AMP or ADP promotes Thr172 phosphorylation in the kinase domain by causing a conformational change in the AMPK holoenzyme that prevents phosphatases from accessing Thr172 [106,107]. In cell-free kinase assays, allosteric binding of AMP to the gamma subunit causes a 5-fold increase in AMPK activity while phosphorylation of Thr172 causes a 100-fold increase in AMPK activity, thus Thr172 phosphorylation is the major point of AMPK regulation[105,108,109,121]. Three kinases have been shown to phosphorylate AMPK at Thr172: LKB1 (liver kinase B1), CAMKK2 (calcium/calmodulin dependent protein kinase kinase 2) and TAK1 (transforming growth factor activated kinase-beta-1)[93]. The kinase upstream of AMPK is

dependent on the signaling cascade or specific stressor. When the AMP/ATP ratio is increased in cells, a conformational shift occurs that makes Thr172 more amenable to phosphorylation by LKB1, a robust and well-characterized AMPK kinase [110,112,122]. However, LKB1 is a tumor suppressor that is lost in many cancers and these cancers still require AMPK for growth and survival [113]. This implies that other sources of AMPK activation are important in cancer.

Stimuli such as ionomycin that lead to an increased concentration of intracellular calcium cause CAMKK2 to phosphorylate Thr172 [114-116]. Additionally, cytokines such as IL-1, TNF and TRAIL lead to AMPK Thr172 phosphorylation in a TAK1-dependent manner [117,118], although much less is known concerning the mechanism of how TAK1 promotes AMPK Thr172 phosphorylation.

TAK1 is activated by inflammatory signals such as IL-1 and TNF, and in turn activates several downstream pathways including the NF-κB transcription factor pathway, the c-Jun N-terminal kinase (JNK) pathway, p38 pathway and AMPK [123]. To activate p38 and JNK pathways, TAK1 phosphorylates mitogen activated protein kinases (MAPKs). To activate the NF-κB pathway, TAK1 directly phosphorylates IκB Kinase (IKK) to promote its activity. TAK1 promotes NF-κB dependent transcription by directly phosphorylating IKK at serines 177 and 181 which lie within the IKKβ activation loop [8]. The canonical IKK complex consists of two highly related kinases IKKα and IKKβ [1,2], and a scaffolding protein IKKγ (also known as NEMO) [20,124]. In addition to activation by TAK1, IKK can also be activated by trans-autophosphorylation especially in the presence of linear ubiquitin molecules [125]. IKK was originally described as the kinase responsible for the inducible phosphorylation of IκBα [6]. Phosphorylation of IκBα by IKK leads to its rapid proteasome-mediated degradation, which in turn allows NF-κB transcription factors to enter the nucleus and promote transcription. Although IKK is best known

for its role in promoting the NF-κB transcription activation pathway, NF-κB independent roles for IKK have been described [126]. The exact mechanisms that lead IKK to phosphorylate one substrate over another are incompletely understood, however this likely involves NEMO, which can act as a substrate specificity factor [20]. Among the NF-κB-independent roles for IKK is a role in promoting AMPK-dependent autophagy [15,20,127]. Previous work demonstrated that expression in HeLa cells (LBK1-deficient) of a mutant IKKβ that mimics 177 and 181 phosphorylation is sufficient to induce AMPK Thr172 phosphorylation. This mutant IKK mimics constitutive phosphorylation by TAK1, thus these results suggested that IKK could play a role downstream of TAK1 in activating AMPK, independent of LKB1[15].

In this study we explored a potential relationship between IKK and AMPK, and demonstrate that IKK is required for basal and cytokine-induced AMPK Thr172 phosphorylation in a variety of cell lines, regardless of LKB1 status. In response to the inflammatory cytokines IL-1β and TNF-α, IKKβ promoted AMPK Thr172 phosphorylation downstream of TAK1. However, we also identified several cancer cell lines where IKK regulates AMPK independent of TAK1. Since there are situations where IKK regulates AMPK independent of TAK1, we assessed whether IKK could directly phosphorylate AMPK. Importantly, we found that IKK phosphorylates Thr172 in cell free kinase assays, promoting AMPK activity.

The mitochondrial complex I inhibitor phenformin causes energetic stress by rapidly depleting ATP [128]. This leads to activation of AMPK in an LKB1-dependent manner, which in turn leads to energy conservation and growth arrest. Cells that lack LKB1 undergo apoptosis in response to phenformin treatment, which led to the concept that phenformin could be used therapeutically to treat LKB1- deficient cancers [128,129]. Since IKK is shown to be important for AMPK regulation in LKB1-deficient cells, we asked whether IKK inhibition would further sensitize these cells to

phenformin. IKK inhibition led to a decrease in AMPK Thr172 phosphorylation in LKB1-deficient cells when treated with phenformin, which resulted in enhanced apoptosis. These results suggest that a combination of IKK inhibition and phenformin treatment could be a useful therapeutic strategy for LKB1-deficient cancers.

2.3 Results

IKK is required for AMPK Thr172 phosphorylation induced by IL-1β

Previously it was demonstrated that two known inducers of IKK activity, IL-1 and TNF, are capable of increasing AMPK Thr172 phosphorylation in serum-starved HeLa cells, which lack the major upstream AMPK Thr172 kinase LKB1 [118]. Since IL-1 is a potent inducer of IKK activity, we tested whether IKK is required for AMPK Thr172 phosphorylation in response to IL-1β. Pre-treatment with the IKK specific inhibitor Compound A (Bay 65-1942) [130] prevented IL-1β from inducing AMPK Thr172 phosphorylation (Fig. 2.1A). Phosphorylation of AMPK at Thr172 mirrored the changes in phosphorylation of IKK and amount of IκBα protein, which served as markers of IKK activity (Fig 2.1A). [1]. Compound A effectively inhibited IL-1β-induced AMPK Thr 172 phosphorylation at 1μM (Fig. 2.6A).

To validate the findings with Compound A, we expressed either wild type (wt) or dominant negative/kinase dead (kd, K44A) IKKβ in HeLa cells and then treated with IL-1β for 5 minutes. IL-1β induced AMPK Thr172 phosphorylation in cells expressing wtIKKβ but not in cells expressing kdIKKβ (Fig. 2.1B). Knockdown of the essential IKKβ scaffold protein NEMO by siRNA was also sufficient to block IL-1β induced AMPK Thr 172 phosphorylation in the LKB1-deficient A549 cells (Fig 2.1C). This indicated that the canonical IKK complex (which is defined by the presence of NEMO) was required for IL-1β induced AMPK Thr 172 phosphorylation. Since the previous data were all conducted in LKB1-deficient cells, we next

tested whether the loss of LKB1 activity was required for IL-1β to induce IKK-dependent AMPK Thr172 phosphorylation. Wild type mouse embryonic fibroblasts (wtMEFs) also showed an increase in AMPK Thr172 in response to IL-1β that was blocked by knockout of IKKβ (Fig. 2.1D) or Compound A (Fig. 2.6B), indicating that LKB1 loss is not required for IL-1β to induce IKK-dependent AMPK Thr172 phosphorylation.

While it has been reported that serum starvation induces AMPK Thr172 phosphorylation[131], our data indicate that IL-1β induces AMPK Thr172 phosphorylation in the presence of full serum and that cells grown in full media (DMEM with 10% serum) exhibited a level of IL-1β induction of AMPK Thr172 phosphorylation that is similar to stimulated cell starved of all nutrients with Earle's Balanced Salt Solution (EBSS) for 2 hours (Fig. 2.6C). This indicated that IL-1β can induce AMPK Thr172 in LKB1-deficient cells experiencing energy stress.

Previously, others demonstrated that IL-1β promotes AMPK Thr172 and ACC Ser79 phosphorylation [117,118]. To further validate that IL-1β could induce the phosphorylation of downstream AMPK targets we treated wtMEFs or AMPKα1 -/-, AMPKα2 -/- knockout (AMPK -/-) MEFs [132] with IL-1β. Treatment of wtMEFs with IL-1β but not AMPK -/- MEFs with IL-1β for 20 minutes increased the amount of phosphorylation of the two known AMPK substrates ACC S79 and ULK S555 (Fig. 2.1E). IL-1β also induced ACC Ser79 phosphorylation in A549 cells after a 30 min treatment, and this was blocked by Cmpd A (Fig. 2.1F).

One important consequence of AMPK activation is the induction of autophagy [95,96]. Previously it was shown that IKK is involved in the induction of autophagy in response to known autophagy inducers, including rapamycin and starvation media [15]. Moreover, IKK-mediated autophagy induction required AMPK, as knockdown of AMPK prevented constitutively active IKKβ from inducing autophagy[15]. Other reports have indicated that IL-1β could induce

autophagy in macrophage and epithelial cell lines [91,117,133]. Given that IL-1β induces AMPK activity in LKB1-deficient A549 cells, we determined whether IL-1β could induce IKK-dependent autophagosome formation in these cells. IL-1β increased the abundance of the autophagosome marker LC3-II (faster migrating LC3 band) within 20 minutes of treatment in A549 cells (Fig. 2.1G). Pre-treatment with Cmpd A (Fig. 2.1H) or the AMPK inhibitor Dorsomorphin [134,135] (Fig. 2.61D) prevented LC3-II induction upon treatment with IL-1β, indicating that IL-1β induced autophagosome formation required IKK and AMPK activity. The changes in LC3 mobility strongly correlated with ACC Ser79 phosphorylation, which served as a marker of AMPK activity (Fig. 2.1F). IL-1β also increased the amount of staining by an acidified autophagosome specific dye (Fig. 2.1I). The increased autophagosome staining was blocked by Compound A, indicating that IKK and AMPK activity are required for IL-1β to induce autophagosome formation. Bafilomycin-A1, an established inhibitor of autophagosome acidification[133], also prevented IL-1β induced staining (Fig. 2.1I). This indicated that the increased signal from the dye was specific to increased autophagosome formation. These results were not due to direct off-target inhibition of AMPK by Compound A, since Compound A was unable to inhibit recombinant AMPK kinase activity in a cell-free peptide based kinase assay (Fig. 2.61E and see Fig. 2.7B). As a positive control, Compound A inhibited recombinant IKKβ activity in a peptide based kinase assay (Fig. 2.61E).

The increase in phosphorylation of downstream AMPK targets and the increase in autophagosome formation in response to IL-1β indicate that the increased AMPK Thr172 phosphorylation in response to IL-1β correlated with an increase in AMPK activity and promoted downstream signaling events.

TAK1 promotes IKK-mediated AMPK phosphorylation in response to cytokines, but TAK1 only partially regulates basal IKK-mediated AMPK regulation

The signaling to AMPK from IL-1β and TNF-α occurs through TAK1-dependent canonical IL-1 and TNF receptor signaling pathways respectively, as pre-treatment with the TAK1 inhibitor 5z-7-oxozeanol [136] prevented IL-1 or TNF induction of AMPK Thr172 phosphorylation to a similar extent as Compound A (Fig. 2.2A). Since IKK was required for cytokine-induced AMPK Thr172 phosphorylation and IKK is generally thought to be downstream of TAK1 in cytokine signaling cascades, we next explored whether IKK functions downstream of TAK1 with respect to AMPK Thr172 phosphorylation. Overexpression of TAK1 and the adaptor protein TAB1 in HeLa cells is known to be sufficient to induce AMPK Thr172 phosphorylation [118]. To determine if IKK is required for AMPK Thr172 induction upon TAK1 and TAB1 overexpression, HeLa cells were transfected with TAK1 and TAB1 for 48 hours and then treated with either DMSO or Compound A (5μM) for 1 hour. Treatment with Compound A reduced both basal and TAK1/TAB1-induced AMPK Thr172 phosphorylation (Fig. 2.2B). To further address the relationship between TAK1, IKKβ and AMPK, IKKβ was inhibited in mouse embryonic fibroblasts (MEFs) that lack TAK1 kinase activity (TAK1 -/-) [137]. Knockdown of IKKβ or treatment with Compound A was sufficient to decrease AMPK Thr172 phosphorylation in TAK1 -/- MEFs (Fig. 2.2C) indicating that IKKβ can regulate AMPK Thr172 phosphorylation in the absence of TAK1. TAK1 -/- MEFs had lower amounts of basal AMPK Thr172 phosphorylation than their wild type counterparts and the residual AMPK Thr172 phosphorylation in TAK1 -/- MEFs was almost completely ablated by Compound A treatment. Similar to Compound A treatment, knockdown of IKKβ decreased AMPK Thr172 phosphorylation in TAK1 -/- MEFS (Fig. 2.2D). The data above indicate that TAK1 is upstream of IKK with respect to AMPK. However, in the absence of TAK1, residual IKK activity is

important for regulating the remaining AMPK Thr172 phosphorylation. In this regard, there are other potential sources of IKK activation including trans-autocatalytic phosphorylation (induced by forced proximity) [125] or activation by an alternative upstream IKK kinase such as NIK or MEKK3 [10]. In contrast to MEFs where a large portion of the basal AMPK Thr172 phosphorylation was dependent on TAK1 (Fig. 2.2B), basal AMPK Thr172 phosphorylation in the LKB1-deficient cancer cell line A549 was independent of TAK1. Treatment of A549 cells with TAK1 inhibitors, NG-25 [138]or 5z-7-oxozeanol, completely abolished phosphorylation of p38 [139] (a marker of TAK1 pathway activation), but had no effect on either AMPK Thr172 phosphorylation or p65 Ser536 phosphorylation (a marker of IKK activity) (Fig. 2.2E). This indicated that the majority of the basal IKKβ and AMPK activity in these cells is independent of TAK1. To determine whether IKK regulates basal TAK1 activity, A549 cells were treated with Compound A and the amount of phospho-p38 were determined. Compound A treatment abolished phospho-p65, but had no effect on the ratio of phospho-p38 to total p38 (Fig. 2.2E). This indicated that TAK1 does not regulate basal AMPK or IKK activity in A549 cells, and that IKK does not regulate basal TAK1 activity.

Canonical IKK regulates AMPK Thr172 phosphorylation in cancer cell lines

The canonical IKK complex is a high molecular weight complex containing the kinases IKKα and IKKβ bound to the scaffold protein, NEMO. IKKα is also found as a homodimer in the non-canonical IKK complex [6,140]. In order to determine the relative contributions of the canonical and non-canonical IKK complex in regulation of AMPK Thr172 phosphorylation, individual components of IKK were knocked down or inhibited. Knockdown of NEMO, the essential scaffold protein of the canonical IKK complex [124], decreased AMPK Thr172

phosphorylation and downstream ACC Ser79 phosphorylation in A549 and MDA-MB-231 cells (Fig. 2.3A). Two distinct siRNAs targeting NEMO also inhibited AMPK Th172 phosphorylation in A549 cells, indicating that the effects of NEMO knockdown were not due to an off-target effect of the siRNA (Fig. 2.7A). Knockdown of either IKKα or IKKβ in A549 cells also led to a decrease in AMPK Thr172 phosphorylation (Fig. 2.3B). However, IKKβ knockdown decreased AMPK Thr172 phosphorylation and ACC Ser79 phosphorylation to a greater extent than IKKα (only knockdown of IKKβ caused a statistically significant decrease in AMPK Thr172 phosphorylation). These results suggest that the canonical IKK complex plays a more important role in regulating AMPK Thr172 phosphorylation than the non-canonical IKK complex in the A549 cells. IKKα-/- and IKKβ -/- MEFs mirrored the knockdown experiments in A549 cells, where IKKβ -/- MEFs displayed a greater decrease in AMPK Thr172 phosphorylation compared to IKKα -/- (Fig. 2.3C). To corroborate the observations using genetic knockdowns or knockouts, A549 (LKB1-deficient) and MDA-MB-231 (normal) were treated with increasing concentrations of Compound A for 1 hour. Compound A treatment led to a dose-dependent decrease in AMPK Thr172 phosphorylation. Importantly, Compound A also dose-dependently decreased the ACC Ser79 phosphorylation in both cell lines (Fig. 2.3D). The effect of Compound A on AMPK Thr172 phosphorylation was not due to off-target inhibition of other kinases as Compound A had no effect on AMPK Thr172 phosphorylation in IKKα -/-, IKKβ -/- double knockout MEFs (DKO, Fig. 2.7B.). Another IKK inhibitor, the NEMO Binding Domain Peptide (NBD peptide) which works via a distinct mechanism from Compound A [141], also decreased AMPK Thr 172 phosphorylation in A549 cells (Fig. 2.7C). In addition, an inactive enantiomeric mixture of Compound A, labeled Compound B, did not have any effect on

AMPK Thr172 phosphorylation in A549 cells (Fig. 2.7D), further supporting the specificity of Compound A towards AMPK Thr172 phosphorylation.

Time course experiments demonstrated that a decrease in AMPK Thr172 phosphorylation could be observed as early as 15 minutes after Compound A treatment while maximal inhibition occurred from 30 to 60 minutes (Fig. 2.3E). The rapid kinetics of Compound A mediated inhibition of AMPK Thr172 phosphorylation suggested the possibility that IKK directly regulates this phosphorylation site.

IKK regulates AMPK Thr172 phosphorylation independent of changes in the energy status of the cell

AMPK activity is tightly regulated by changes in the ratio of ADP/ATP and AMP/ATP[106,107] [108]. An increase in the ratio of AMP/ATP or APD/ATP is indicative of energetic stress and is generally associated with an increase in AMPK activity. IKK and NF-κB are also known to regulate cellular metabolism in certain contexts [17,65], thus we sought to determine if IKK inhibition affects ADP/ATP and AMP/ATP ratios. A 30 minute treatment with Compound A (5μM) increased both the ratios of AMP/ATP and ADP/ATP (Fig. 2.8A), which indicates energetic stress and would be expected to increase AMPK Thr172 phosphorylation in cells with LBK1 activity. However, at this time and concentration, Compound A decreases AMPK Thr 172 phosphorylation in A549 cells (Fig. 2.3E). In addition, Compound A decreased Thr172 phosphorylation on an AMPK kinase domain mutant that cannot bind to AMP [142] (Fig. 2.8B). These data indicate that the effects of Compound A on AMPK activity are independent of changes in ADP/ATP or AMP/ATP ratios.

IKK phosphorylates AMPK Thr172 in cell free kinase assays and induces its activity

To determine whether IKK regulates AMPK via direct phosphorylation, inactive recombinant AMPK trimers, that contain AMPKα1, AMPKβ1 and AMPKγ1 [143] (purified from bacterial cells), were incubated with GST-IKKβ (1-786) (purified from Sf9 insect cells), in the presence of ^{32}P-γ-ATP. AMPKα1 incorporated ^{32}P when incubated with IKKβ (Fig. 2.9A). To map the IKK dependent phosphorylation sites on AMPK, a parallel reaction was performed with cold ATP and subjected to mass spectrometry. Among the phosphopeptides identified was a peptide that corresponded to phosphorylated Thr172 (Fig. 2.9A). Mutation of Thr172 to strongly reduced incorporation of ^{32}P into AMPKα1 in the presence of active IKKβ (Fig. 2.9B). These findings were confirmed by western blot, where increasing amounts of IKKβ led to increased Thr172 phosphorylation on AMPK trimers (Fig. 2.4A).

Since phosphorylation of AMPK at Thr172 is sufficient to increase the kinase activity of AMPK, we predicted that IKKβ would increase AMPK activity. AMPK activity, as measured by the SAMS peptide kinase assay (see Materials and Methods), was indeed strongly increased after incubation with IKKβ (Fig. 2.4B). IKK also efficiently phosphorylated a kinase-dead mutant of AMPK (Fig. 2.4C) indicating that the IKKβ effect on AMPK Thr172 is independent of AMPK autocatalytic activity. To determine if IKKα and IKKβ have differential efficacy relative to AMPK Thr172 phosphorylation, cell-free kinase assays were performed with either IKKα or IKKβ. IKKβ phosphorylated approximately 5-fold more AMPK Thr172 than IKKα (Fig. 2.4D). Moreover, IKKβ is able to bind more effectively to the AMPK kinase domain than did IKKα, as evidenced by co-precipitation experiments in which a GST-tagged AMPK kinase domain was precipitated from HEK293T cells (Fig. 2.4E). This suggested that IKKβ is the dominant AMPK Thr172 kinase within the canonical IKK complex. Compound A effectively inhibited IKKβ-mediated AMPK Thr172 phosphorylation, but did not inhibit either TAK1 or CAMKK2 (two

other known upstream AMPK kinases) from phosphorylating AMPK Thr172 (Fig. 2.4F). Thus the effect of Compound A on AMPK Thr172 phosphorylation in cells is independent of inhibition of either TAK1 or CAMKK2.

IKK inhibition leads to a reduction in AMPK Thr172 phosphorylation in phenformin-treated LKB1-deficient cells and promotes apoptosis

Phenformin is an inhibitor of complex I of the mitochondria [144-146] and leads to a depletion of ATP [128]. To counteract the action of phenformin, AMPK is activated to conserve ATP [129]. Phenformin- induced AMPK activity is largely dependent on the upstream AMPK activating kinase LKB1, and LKB1-deficient cancer cell lines are particularly sensitive to phenformin induced apoptosis (as measured by Caspase 3/7 activation) [128,129]. Since IKK regulates AMPK in LKB1-deficient cells and since IKK-mediated AMPK regulation is independent of energy status (Fig. 2.6C), we asked whether IKK inhibition would further sensitize these cells to phenformin-induced apoptosis. To inhibit IKK, A549 (LKB1-deficient) and MDA-MB-231 cells (LKB1-positive) were transfected with siRNA targeting NEMO. These cells were then treated with phenformin for 18 hours (Fig. 2.5A). Both A549 cells and MDA-MB-231 cells had lower basal AMPK Thr172 phosphorylation when NEMO was knocked down (Fig 2.3A and Fig 2.5A). Phenformin treatment induces AMPK Thr172 phosphorylation only in MDA-MB-231 cells and this effect did not change when NEMO was knocked down (Fig. 2.5A). Accordingly, NEMO knockdown led to phenformin-induced apoptosis as measured by Caspase 3/7 activity in A549 cells. On the other hand, NEMO knockdown had no effect on Caspase 3/7 induction by phenformin in MDA-MB-231 cells (Fig. 2.5B). These results were corroborated with Compound A, where lower levels of AMPK Thr172 phosphorylation are observed upon Compound A treatment in combination with phenformin in A549 cells and Compound A does not effect

AMPK Thr172 phosphorylation in response to phenformin in MDA-MB-231 (Fig. 2.5C). This is consistent with previous work indicating that LKB1 is required for phenformin to induce AMPK Thr172 phosphorylation. This data also suggested that IKK is not required for phenformin induced AMPK activation, consistent with our previous findings that IKK regulates AMPK independent of cellular metabolic changes.

We next tested the effects of Compound A, phenformin or the combination of the two in a panel of LKB1-deficient (HeLa, A549, NCI-H23, and NCI-H460) or LKB1 wt cell lines (IMR90, NCI-H441, MDA-MB-231 and MiaPaca-2). 1mM phenformin was used as this concentration since it is sufficient to induce AMPK Thr172 in LKB1 expressing cells, but does not induce measureable amounts of apoptosis in LKB1-deficient cells 18 hours after treatment as measured by Caspase 3/7 activity. On average the combination of phenformin and Compound A induced more Caspase 3/7 activity in LKB1-deficient cells than in LKB1-expressing cells (Fig. 2.5D). Taken together these results indicate that IKK inhibition sensitizes LKB1-deficient cells to phenformin-induced apoptosis by decreasing AMPK Thr172 phosphorylation.

2.4 Discussion

Three kinases have been previously described to phosphorylate AMPK Thr172: LKB1, CAMKK2 and TAK1 [93]. While the molecular mechanisms and the biological context of LKB1 and CAMKK2 regulation of AMPK Thr172 phosphorylation have been well described, much less is known about the mechanism of TAK1 regulation of AMPK Thr172. In this study, we show that IKK plays an important role downstream of TAK1 in regulating AMPK Thr172.

Previous studies demonstrated that overexpression of TAK1 along with the adaptor protein TAB1 is sufficient to promote AMPK phosphorylation. Moreover, TAK1/TAB1 fusion proteins can directly phosphorylate AMPK Thr172 in cell-free kinase assays [117,118,147]. It is interesting to

note, however, that TAK1 is not active in vitro unless the adaptor protein TAB1 is also present, and thus cannot phosphorylate AMPK in the absence of recombinant TAB1[147]. Stimuli such as IL-1β, TNF-α and TRAIL that activate TAK1 lead to the induction of AMPK activity [117,118,147]. Here we provide evidence (Fig. 2.2A) that inhibition of TAK1 prevents these stimuli from inducing AMPK Thr172 phosphorylation. All of this evidence led to the view that cytokine-induced TAK1 activity leads to direct phosphorylation of AMPK Thr172 by TAK1.

However, others published that expression of a constitutively active IKKβ mutant in HeLa cells (LKB1-deficient), that mimics TAK1 phosphorylation, leads to an increase in AMPK Thr172 phosphorylation [15]. This result suggested a potential role for IKKβ downstream of TAK1 in regulating AMPK. Here we demonstrate that IKK activates AMPK downstream of TAK1 in response to IL-1β or TNF-α.

The canonical IKK complex, which is defined by the presence of NEMO [6], promoted AMPK activity in response to IL-1β and TNF-α (Fig. 2.1, Fig. 2.2A). In addition to the scaffold NEMO, the canonical IKK complex consists of two related protein kinases IKKα and IKKβ. While IKKα and IKKβ share homology, their activity towards different substrates in the NF-κB pathway varies. For example, IKKβ has higher activity towards IκBα than IKKα, but IKKα has a higher activity towards p100 than IKKβ [148]. With respect to AMPK, IKKβ shows a 5-fold preference towards Thr172 phosphorylation as compared with IKKα (Fig. 2.4D). It is possible that IKKβ has more kinase activity towards AMPK Thr172 or it could be that IKKβ is directed to and binds AMPK more efficiently than IKKα, as was observed with GST-pull downs in HEK293T cells (Fig. 2.4E). This indicated that it was IKKβ within the canonical complex that directly phosphorylated AMPK Thr172. However, knockdown of IKKα in A549 cells or in MEFs leads to a decrease in AMPK Thr172. This can be explained in a variety of ways. For

instance, the non-canonical IKK complex could be regulating the canonical IKK complex. Alternatively, IKKα could be regulating IKKβ activity within the canonical IKK complex, either through phosphorylation of IKKβ activation loop serines or by stabilizing the canonical complex. Future studies are necessary to determine whether stimuli (such as RANK ligand or CD40 ligand) that specifically activate the non-canonical IKKα complex promote activation of AMPK. Although, IKKα had less activity towards AMPK Thr172 than IKKβ, it is still possible that a stimulus that specifically over activates IKKα could promote AMPK activity.

The IKK/NF-κB pathway is known to promote its own feedback in order to limit the response to IL-1β and TNF-α, notably by inducing the transcription of IκBα [149]. Activation of AMPK may be another way to limit NF-κB signaling in response to these cytokines, as several studies have indicated that AMPK activity opposes NF-κB [150,151]. One report indicated that TNF-α-induced apoptosis was opposed by AMPK [152]. This is in line with the role of IKK in promoting survival over apoptosis in response to TNF-α. Consistent with the idea that IL-1β induces AMPK in an IKK-dependent fashion, IL-1β promotes autophagosome formation in an IKK-dependent manner in A549 cells. Previous work had demonstrated that IL-1 could promote autophagy in breast epithelial cells [117] and in macrophages [91], which both express LKB1. Here we show that IL-1β induces autophagosome formation in an LKB1-deficient cell line, A549, indicating that IL-1β induced autophagy does not require LKB1 but instead required IKK. In macrophages, Il-1β was critical for clearance of certain pathogens [91]. Several studies have demonstrated that IKK is involved in autophagy induction, however the mechanism whereby IKK promotes autophagy has been poorly understood [20]. It is known that IKK promotes transcription of autophagic genes [127], and is involved in induction of autophagosomes in response

to several known autophagy inducing stimuli. Importantly, knockdown of AMPK prevented the induction of autophagosomes by overexpression of constitutively active IKK, indicating that AMPK is downstream of IKK with respect to autophagy [15]. Data presented here suggest a mechanism where IKK could be promoting autophagy by direct phosphorylation of AMPK Thr172.

We also demonstrated that IKK regulates basal AMPK activity independent of TAK1, as inhibition of IKK in TAK1 -/- MEFs led to a decrease in AMPK Thr172 phosphorylation. In addition to TAK1 phosphorylation, IKKβ can also be activated by trans-auto-phosphorylation, which is induced by forced proximity in the presence of linear ubiquitin molecules [125], or another upstream signal, such as from MEKK3 or NIK [10]. Regardless of the source of the residual IKK activity, it is clear that inhibiting IKK lowers the basal AMPK Thr172 phosphorylation in the absence of TAK1 activity (Fig. 2.2C-D). This is similar to what occurs in A549 cells, where basal IKK activity was independent of TAK1 activity. Treatment of A549 cells with TAK1 inhibitors had no effect on either IKK activity (as measured by phosphorylated p65) or AMPK activity (as measured by Thr172 phosphorylation). However, treatment of A549 cells with an IKK inhibitor rapidly depleted both phosphorylated AMPK and phosphorylated p65 (Fig. 2.3E).

The loss of LKB1 in cancer promotes tumor growth, but also leaves the cells vulnerable to metabolic stress, such as with treatment with the mitochondrial complex I inhibitor phenformin [128,129]. Despite knowledge of this vulnerability, little progress has been made in the clinic towards exploiting this vulnerability. An explanation could be that LKB1-deficient cancer cells promote basal AMPK phosphorylation via IKK to buffer against energetic stress. In this study we demonstrated that IKK inhibition in LKB1-deficient cells leads to a decrease in AMPK Thr172 phosphorylation, even when the cells are treated with phenformin (Fig. 2.5A and B). This

decrease in AMPK Thr172 was associated with apoptosis, as measured by Caspase 3/7 activity (Fig. 2.5C and D). In cells expressing LKB1, IKK had no effect on the ability of phenformin to induce AMPK Thr172 phosphorylation. Phenformin alone or in combination with IKK inhibition did not induce apoptosis cells that express LKB1. A future therapeutic strategy might be to combine IKK inhibition with phenformin treatment in LKB1-deficient tumors.

2.5 Materials and Methods

Cell Culture

All cell lines were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Facility and cultured in DMEM (Gibco) or RPMI (Gico), depending on ATCC recommendations, supplemented with 10% fetal bovine serum (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), and 1 mM glutamine. Immortalized wild type mouse embryonic fibroblasts (MEFs) of the indicated genotypes were as described previously [127]. All cell lines were maintained in a humidified incubator at 37°C and 5% CO_2.

Antibodies and Compounds

All antibodies were purchased from Cell Signaling Technology (Danvers, Massachusetts). Compound A (Bayer, Bay65-1942) and Compound B were dissolved in DMSO. The TAK1 inhibitor 5z-7-oxozeanol and the AMPK inhibitor Dorsomorphin were purchased from Tocris Biosciences and NG-25 was purchased from MedChem Express (Monmouth Junction, NJ). Phenformin Hydrochloride was purchased from Sigma Aldrich. Recombinant IL-1β was purchased from Peprotech (Rocky Hill, NJ) and recombinant TNF-α was purchased from Promega (Madison, WI). Phenformin was purchased from Cayman Chemical Company (Ann Arbor, MI). The NBD peptide was synthesized by American Peptide.

siRNA Transfections

ON TARGETplus SMART pool siRNA or individual siRNAs (all siRNAS were pooled unless otherwise indicated), as well as negative control siRNA were purchased from Dharmacon (Thermo Scientific Pierce, Rockford, IL). Each pool was reconstituted in 1X siRNA buffer (Dharmacon) and diluted in DEPC-treated water to a final concentration of 20 μM. Briefly, $1.5X10^5$ cells were plated onto 10cm dishes and cultured as described above. The following day Dharmacon Transfection reagent #1 (Thermo Scientific) and 200 pmol of siRNA mixture were incubated for 20 minutes at room temperature and added to the cells in serum-free media. Cells were harvested 48-72 hours post-transfection for protein extraction preparation.

Plasmid Transfections

Plasmids were transfected using the XtremeGene Transfection Reagent (Roche Life Science, Indianapolis, IN) according to the manufacturer's protocol. Mutations were confirmed by Sanger Sequencing at the UNC Genome Analysis Core. The AMPK kinase domain pEBG-AMPKα1(1-312) (Addgene plasmid # 27632). Kinase dead AMPK 1-312 was created by mutating D157 to A in pEBG-AMPKα1(1-312) using a quick-change mutagenesis protocol. AMPK T172A was created by cloning pDONR223-AMPKα1 (Addgene plasmind # 82274) into the pDEST-27 to add a GST-tag using Gateway Cloning (Invitrogen). Thr172 was then mutated to alanine using a quick-change mutagenesis protocol. The wild type and kinase dead mutant (K44A) IKKβ, as well as the TAK1 and TAB1 plasmids were kind gifts from Dr. Lewis Cantley's lab.

Kinase Assays

Kinase assays were performed as described previously[16]. For the kinase assays using recombinant proteins, AMPK trimers purified from *E. coli* were used as previously described [143]

(a kind gift from Dr. J. Brenman, UNC Chapel Hill). Purified GST- tagged IKKα and β from Sf9 insect cells were obtained from SignalChem. The kinase buffer contained 1mM beta-glycerolphosphate, 20mM Tris pH 7.4, 12mM MgCl2 and 100μM cold ATP. γ^{32}P-ATP was added where indicated. The reactions were then subjected to SDS-PAGE and either transferred to a nitrocellulose membrane for western blotting (see below) or were Coomassie stained for mass spectrometry. The mass spectrometry experiments were performed with the UNC Proteomics Core Facility. The band corresponding to the AMPKα1 subunit was excised from the gel and digested with trypsin. Phospho-peptides were enriched using titanium dioxide, then subjected to LC-MS using an LTQ Orbitrap Velos (Thermo Scientific) ion trap mass spectrometer. Peptides were identified using Mascot Software. For the SAMS peptides assays [94], the resulting kinase reactions were mixed with SAMS peptide for 15 minutes then spotted on squares of p81 phosphocellulose paper. Each square was washed three times in 1% phosphoric acid, then dried and the remaining radioactivity was measured using a scintillation counter. To determine the effect of Compound A on AMPKα1 or IKKβ activity, peptide based kinase assays were performed as described previously, using 100μM ATP and 5μM Compound A [153]. Active AMPKα1/AMPKβ1/AMPKγ1, the SAMS peptide and the IκBα peptide were obtained from MRC PPU Reagents and Services facility (MRC PPU, College of Life Sciences, University of Dundee, Scotland, mrcppureagents.dundee.ac.uk).

Western Blotting

Whole-cell protein extraction was performed by scraping the cells in cold 1% NP-40 buffer (20mM Tris pH 7.6, 1mM EDTA, 1mM EGTA, 150mM NaCl, 1% Igepal). Protein concentrations were determined using the Bradford Assay (Bio-Rad). Protein lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE),

transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Pittsburg, PA), blocked for 1 hour in 1X TBST containing 5% nonfat milk, and incubated overnight in corresponding primary antibody at 4°C. Blots were then incubated with horseradish peroxidase-labeled secondary antibody and developed using developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburg, PA), or the Biorad chemi-doc system. Densitometry of the relevant bands was performed using ImageJ and the values were normalized to either total protein or Actin as indicated in the figures. The data from independent biological replicates was then plotted using GraphPad Prism. For statistical analysis a two-way ANOVA followed by a Bonferroni post test was performed to determine statistical significance.

GST-Pull downs

Whole cell extracts were prepared in 1% NP-40 buffer and 5% of the extract was saved for use as input. The rest was allowed to rotate overnight at 4°C with Glutathione conjugated beads (Amersham, GE Healthcare Life Sciences). The following day, the beads were centrifuged and washed 3X in 1% NP-40 buffer. Proteins were eluted from the beads by boiling in Laemmeli buffer and then subjected to SDS-PAGE and Western Blotting.

Measurement of ADP/ATP and AMP/ATP Ratios

A549 cells were treated with either DMSO or Compound A (5μM) for 30 minutes. The ratio of ADP/ATP and AMP/ATP was then measured at the UNC Biomarker Mass Spectrometry Core Facility using a protocol similar to that described in Johnsen [154]. Known amounts of stable isotopes of ATP, ADP and AMP (purchased from Millipore Sigma) were added to each sample, the samples were subjected to HPLC using a Surveyor HPLC system and then analyzed on a Thermo Fisher TSQ-Quantum Ultra triple-quadrupole mass spectrometer.

Autophagosome Stain

The autophagosome specific stain was purchased from Sigma-Aldrich (catalog # MAK-138). Cells were seeded at a density of 1000 cells/well of a 96 well dish. The following day the cells were left untreated, treated with IL-1β, or treated with IL-1β in combination with Compound A or bafilomycin. Cells were then washed four times in the wash buffer provided with the detection kit, then the mean fluorescence intensity was measured using a plate reader.

Caspase Assays

The Caspase-Glo reagent was purchased from Promega and used according the manufacturer's instructions. Cells were seeded at a density of 1000 cells per cell of a white-walled 96 well plate. The Caspase-Glo reagent was then directly added to the wells, placed on a shaker at room temperature for 30 minutes then the luminescence read using a plate reader.

Supplementary Material:

Fig. S1. Supplemental data associated with Figure 1. (Fig. 2.6)

Fig. S2. Validation of Cmpd A and siRNA specificity. (Fig. 2.7)

Fig. S3. IKK regulates AMPK independent of changes in energy status/ATP. (Fig. 2.8)

Fig. S4. Identification of AMPK Thr 172 by mass spectrometry. (Fig. 2.9)

2.6 Figures for Chapter 2

Figure 2.1 AMPK Thr172 phosphorylation induced by inflammatory signals requires IKK

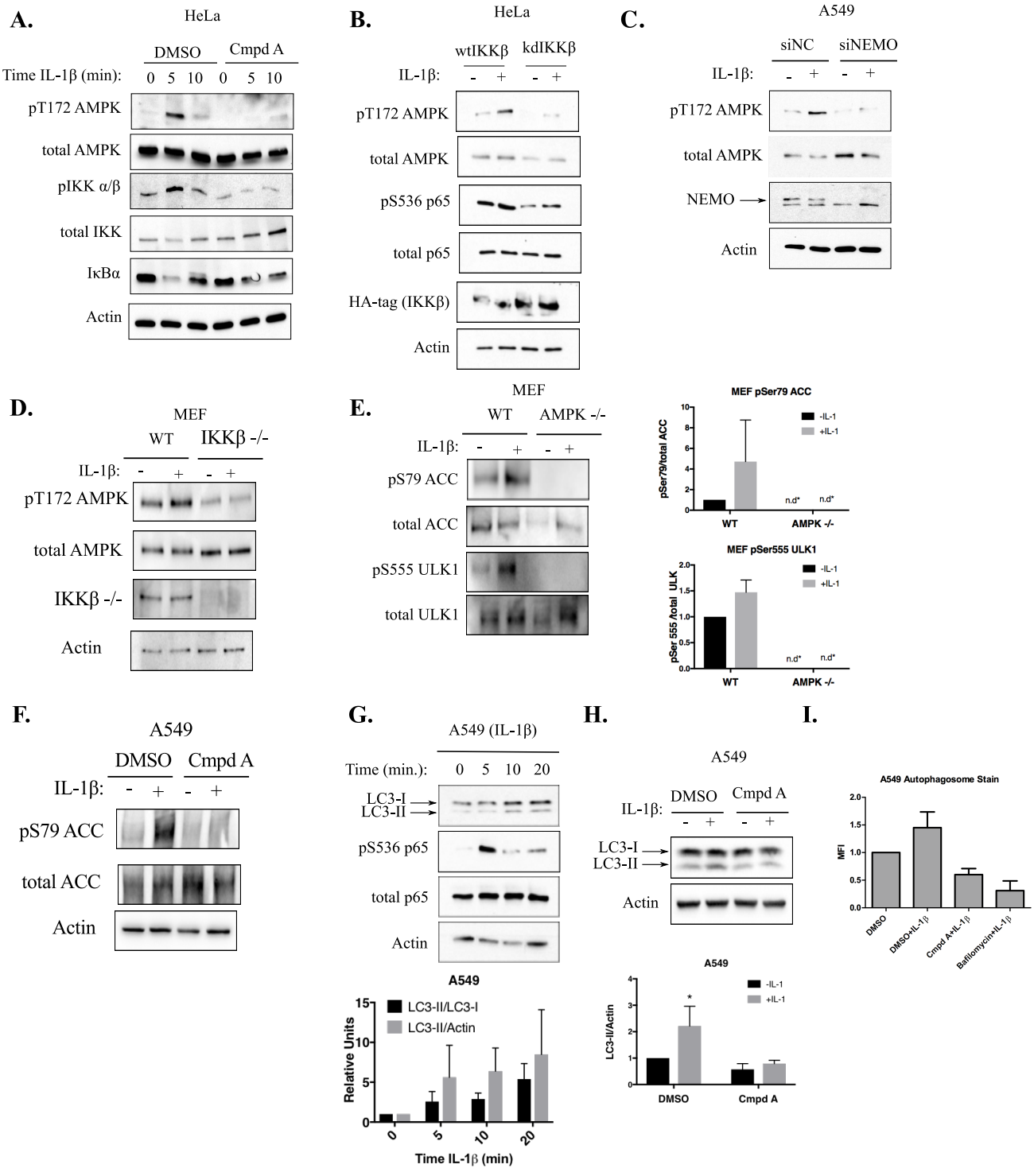


Figure 2.1: AMPK Thr172 phosphorylation induced by inflammatory signals requires IKK

(A.) HeLa cells were pre-treated with either DMSO or 5μM Compound A (abbreviated CmpdA), then treated with IL-1β (15ng/ml) for the indicated time periods. Whole cell lysates were then subjected to western blot analysis using the indicated antibodies (n=3).

(B.) HeLa cells were transfected with either wtIKKβ or kdIKKβ and then treated with IL-1β (15ng/ml) for 5 minutes. Whole cell lysates were then subjected to western blot analysis using the indicated antibodies (n=2).

(C.) A549 cells were transfected with either non-coding siRNA (siNC) or siRNA targeting NEMO, then stimulated with IL-1β (15ng/ml) for 5 min and probed with the indicated antibodies (n=3).

(D.) WT or IKKβ -/- MEFs were treated with IL-1β for 5 minutes followed by western blot analysis of whole cell lysates by western blotting with the indicated antibodies (n=3).

(E.) WT or AMPK -/- MEFs were treated with IL-1β for 30 minutes and then probed with antibodies of known AMPK substrates (n=3, the error bars represent the standard error of the mean,S.E.M, *n.d = not detected).

(F.) A549 cells were treated with IL-1β for 30 minutes and then probed with antibodies against the known AMPK substrate ACC phospho-Ser79.

(G.) A549 cells were treated with 15ng/ml IL-1β for the indicated time periods and probed for the autophagy marker LC3. The ratio of LC3-II/LC3-I and the normalized ratio of LC3-II to actin are displayed in the graph below (n=3, independent biological replicates). To test if there was a positive time trend, we used repeated measures linear mixed-effect model. This analysis revealed that there was a statistically trend of increasing LC3-II (p-value = 0.027).

(H.) A549 cells were pre-treated with either with DMSO or 5μM Cmpd A for 15 minutes then with IL-1β for 20 minutes. Whole cell extracts were then prepared and analyzed by western blot for the indicated proteins. (n=3, the error bars represent the S.E.M, a two-way ANOVA analysis was used to determine which groups were statically different, * = $p<0.05$).

(I.) A549 cells were pre-treated with DMSO, Compound A(5μM) or Bafilomycin A1 (10nM) for 15 minutes then treated with IL-1β for 2 hours. Cells were then stained with a dye that is selective for acidified autophagosomes. Cells were washed 3 times and then the fluorescence intensity was measured (n=3, error bars represent the S.E.M.).

Figure 2.2 IKK is downstream of TAK1 with respect to AMPK

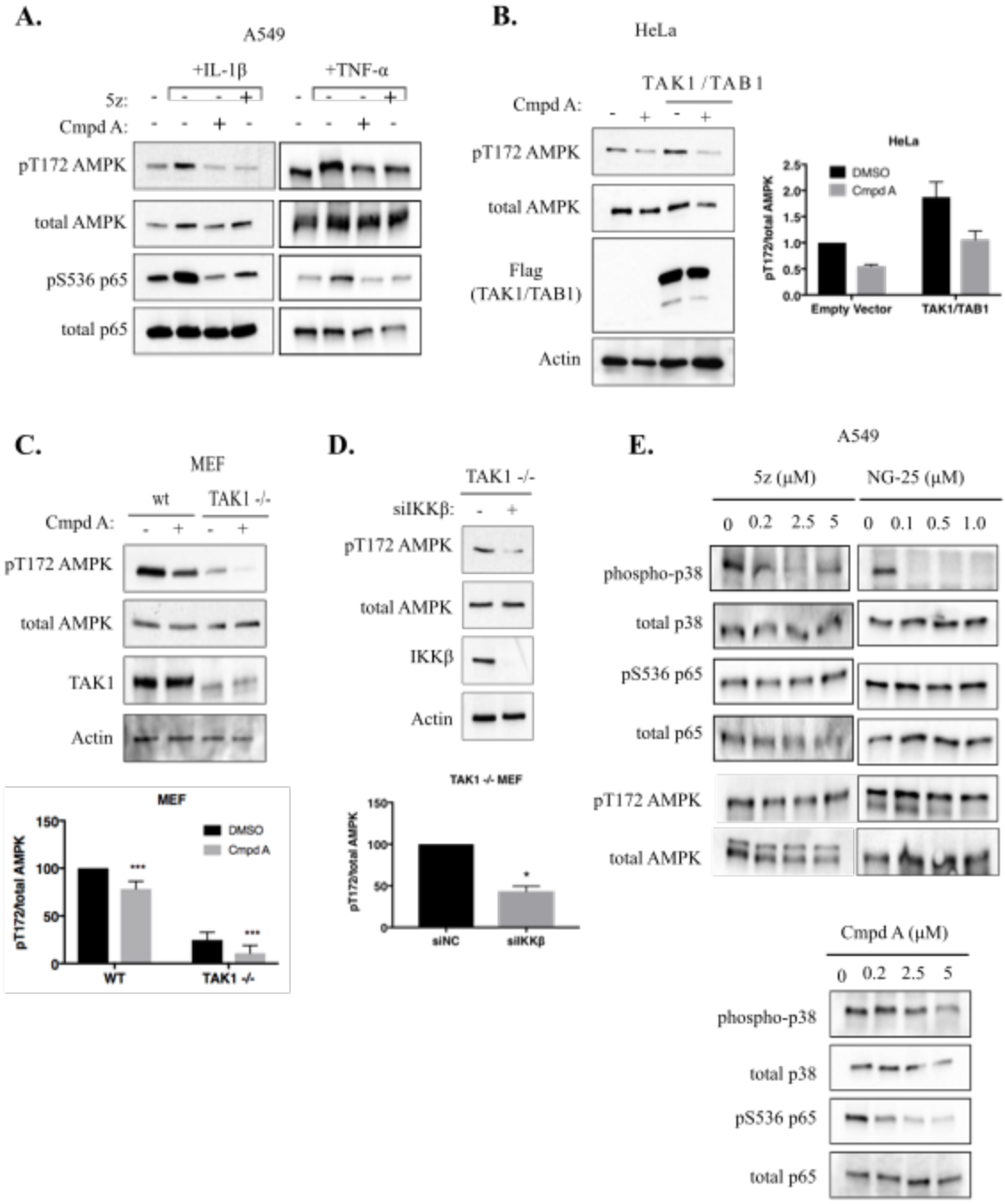


Figure 2.2: IKK is downstream of TAK1 with respect to AMPK

(A.) A549 cells were pre-treated with either the IKK inhibitor Cmpd A (5μM) or the TAK1 inhibitor 5-z-oxozeanol (5μM) for 15 minutes then stimulated with IL-1β (15ng/ml) or TNF-α (15/ng/ml) for 5 min (n=3).

(B.) HeLa cells were transfected with either an empty vector plasmid or plasmids encoding Flag-TAK1 and Flag-TAB1. Cells were then either treated with DMSO or Cmpd A (5μM) for 30 minutes. Whole cell lysates were then subjected to western blot analysis with the indicated antibodies (n=3). A two-way ANOVA analysis was used to determine if the Compound A treatment was different from DMSO treatment in the TAK1/TAB1 overexpressing cells vs empty vector. The difference between the two groups was not statistically significant ($p=0.82$), indicating that Compound A inhibited AMPK Thr172 phosphorylation driven by TAK1/TAB1.

(C.) Wt or TAK1 -/- MEFs were treated with Cmpd A (5μM) for 1 hour, then whole cell lysates were analyzed by western blot for AMPK Thr172 phosphorylation and total AMPK (n=3, the error bars represent the S.E.M., a two-way ANOVA analysis was used to determine which groups were statically different, **= $p<0.01$, *** = $p<0.001$)

(D.) TAK1 -/- MEFs were transfected with siRNA targeting murine IKKβ and blotted for the indicated proteins (n=3, error bars represent S.E.M, a ratio paired t-test was performed to determine if the control and siIKKβ groups were significantly different, *= $p<0.05$).

(E.) A549 cells were treated with either of the TAK1 inhibitors 5z-7-oxozeanol or NG-25 for 30 mins. Whole cell extracts were then analyzed by western blot with the indicated antibodies. (n.d. = non detected). A549 cells were treated with Compound A at the indicated concentrations for 30 minutes and blotted for phospho-p38 (marker of TAK1 activity) or phospho-p65 (marker of IKKβ activity) (n=3).

Figure 2.3 IKK regulates basal AMPK T172 phosphorylation in cell lines

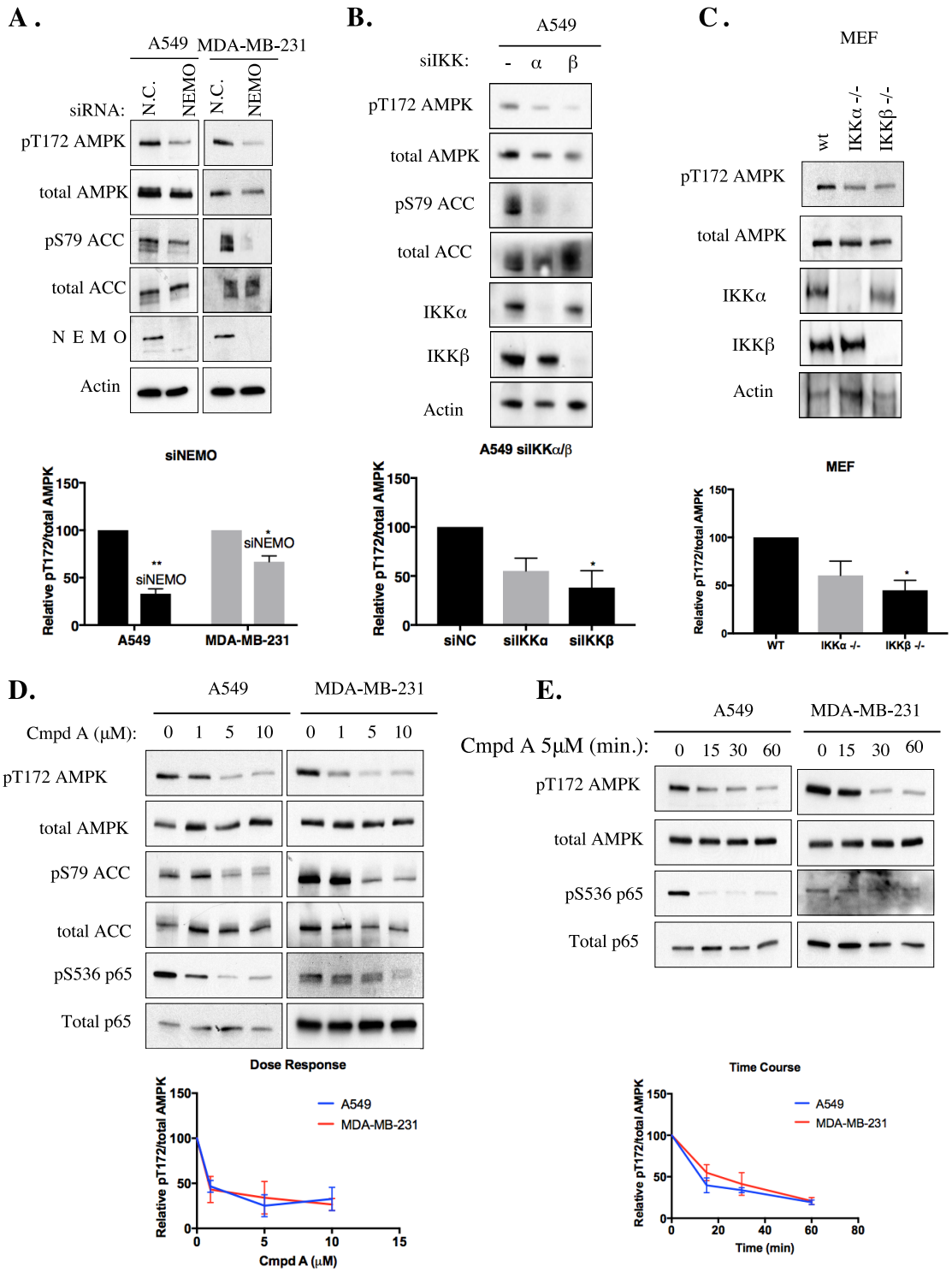


Figure 2.3. IKK regulates basal AMPK T172 phosphorylation in cell lines.
(A.) A549 (LKB1-deficient) and MDA-MB-231 (normal LKB1) were transfected with siRNA targeting NEMO for 48 hours then whole cell extracts were analyzed by Western blot with the indicated antibodies(n=3, error bars represent the S.E.M, a two-way ANOVA analysis was used to determine which groups were statically different, **= p<.01, *p<.05).

(B.) A549 cells were treated with siRNA targeting either IKKα or IKKβ for 48 hours then whole cell extracts were analyzed by western blot (n=3, error bars represent the S.E.M, a two-way ANOVA analysis was used to determine which groups were statically different, * =p<0.05).

(C.) Whole cell extracts from Wt, IKKα -/- and IKKβ -/- mouse embryonic fibroblasts (MEFs) were analyzed by western blot with antibodies for either AMPK pT172 or total AMPK(n=3, error bars represent the S.E.M, a two-way ANOVA analysis was used to determine which groups were statically different, *= p<0.05).

(D.) A549 (LKB1-deficient) and MDA-MB-231 (normal LKB1) cells were grown in full media (DMEM, 10% FBS) and treated with the indicated concentrations of Cmpd A for 1 hour. Whole cell lysates were then evaluated by western blot with the indicated antibodies (n=3, error bars represent the S.E.M)

(E.) A549 and MDA-MB-231 cells were treated with Cmpd A (5μM) for the indicated time periods then analyzed by western blot for AMPK Thr172 phosphorylation(n=3, error bars represent the S.E.M).

Figure 2.4 IKK directly phosphorylates AMPK T172 in cell-free kinase assays

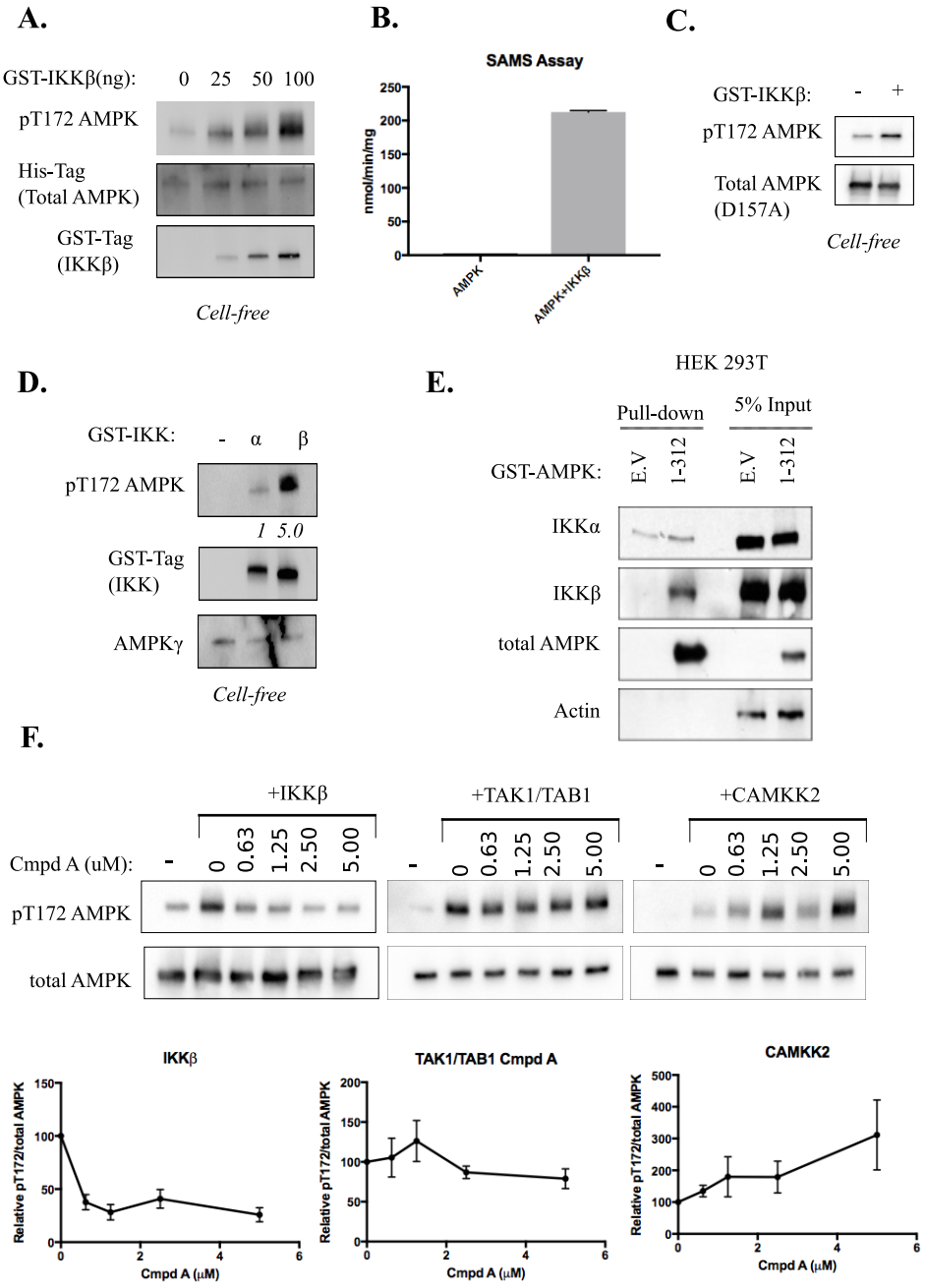


Figure 2.4. IKK phosphorylates AMPK Thr172 in cell free kinase assays and induces its activity.

(A.) Increasing amounts of GST-IKKβ were incubated with recombinant AMPKα1/β1/γ1 trimers in a cell free kinase assay. The reactions were then subjected to SDS-PAGE and western blot analysis with the indicated antibodies.

(B.) A cell free kinase assay was performed as in (A). The SAMS peptide and ^{32}P-ATP were incubated with the reactions then spotted on p81 phosphocellulose paper. The activity of AMPK was calculated after subtraction of the blank reaction and the IKKβ alone reaction.

(C.) A GST-tagged kinase dead mutant of the AMPKα1 kinase domain (AMPK D157A) was purified from HEK293T cells and then used as a substrate for a cell-free kinase assay with active IKKβ.

(D.) Recombinant IKKα and IKKβ were incubated with AMPKα1/β1/γ1 trimers as in (A.) The amount of AMPK pT172 phosphorylation by IKKβ is normalized to the amount of AMPK pT172 phosphorylation by IKKα and is displayed below the western blot.

(E.) A GST-tagged construct of the AMPK kinase domain which is comprised of amino acids 1-312, (the kinase domain), was transfected into 293T cells. The amount of co-precipitation with either IKKα or IKKβ were then measured.

(F.) Effect of Compound A on the phosphorylation of AMPK Thr172 by recombinant IKKβ, TAK1/TAB1 or CAMKK2. (n=5 for each kinase, the ratio of phosphorylated to total AMPK is plotted in the graphs below and the error bars represent the standard error of the mean, S.E.M).

Figure 2.5 IKK/AMPK provide resistance to phenformin in LKB1 -/- cells

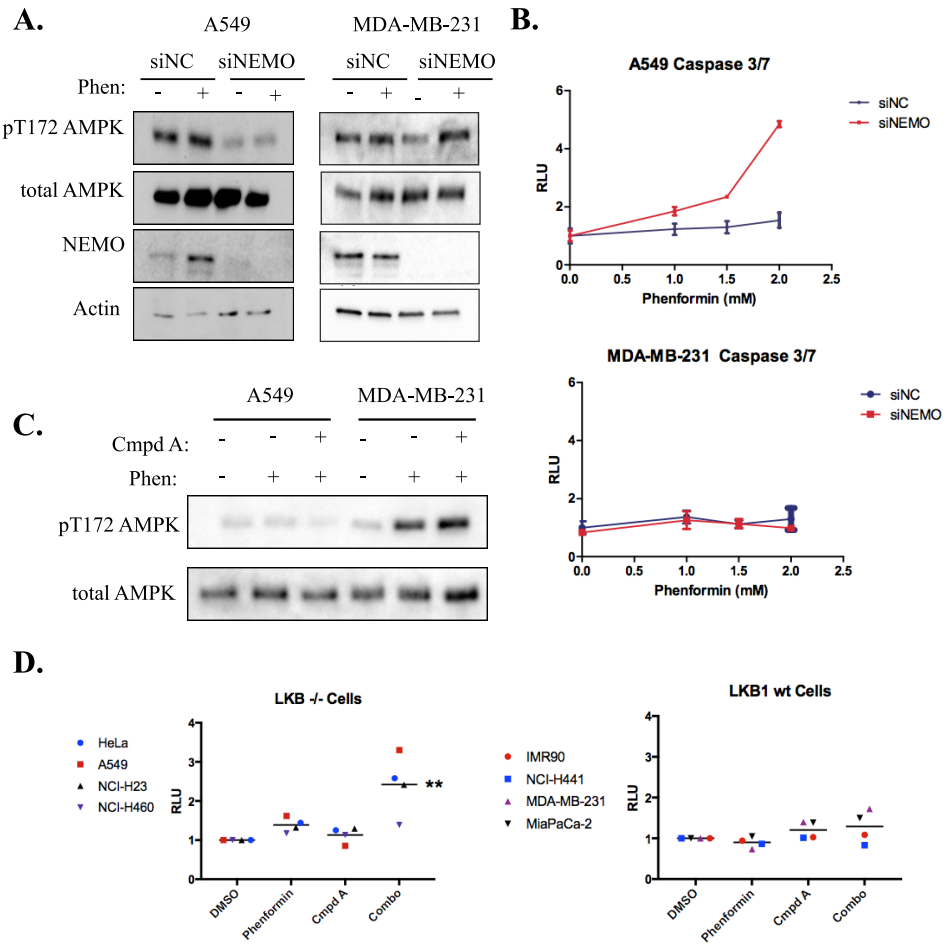


Figure 2. 5: IKK/AMPK provide resistance to phenformin in LKB1 -/- cells

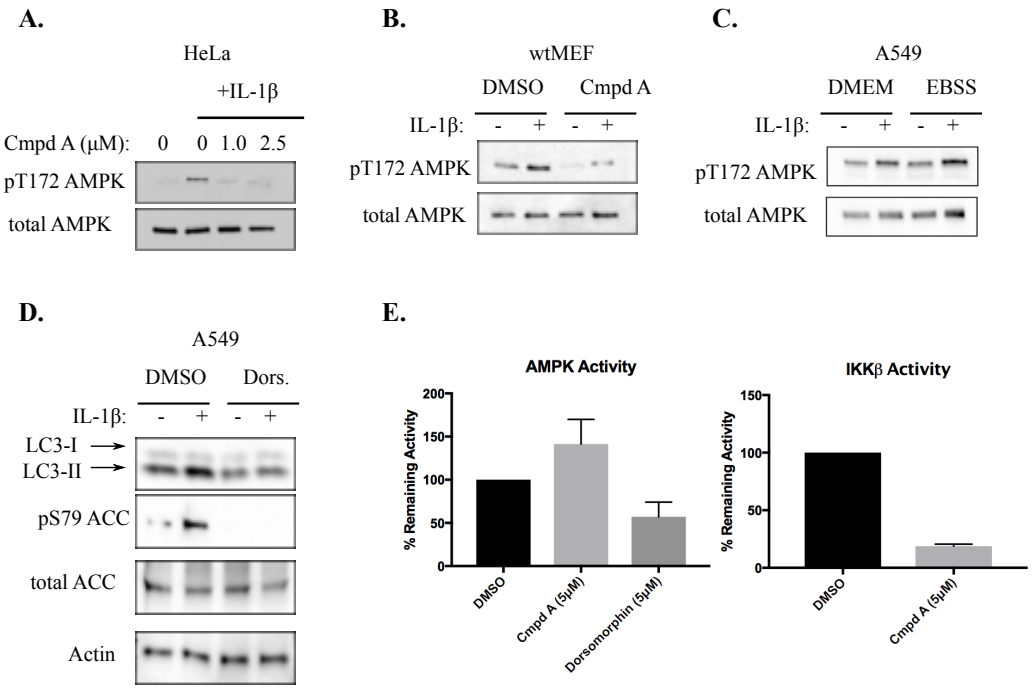
(A.) A549 and MDA-MB-231 cells were transfected with either non-coding siRNA (siNC) or siRNA targeting NEMO (siNEMO). 48 hours after transfection the cells were treated with phenformin (1mM) for 18 hours. Cells were then lysed for analysis by western blot (n=3)

(B.) A549 or MDA-MB-231 cells were pre-treated with Cmpd A (5μM) or DMSO for 15 min, then with phenformin (1mM) for 45 minutes. Cells were then lysed for analysis by western blot (n=3).

(C.) A549 and MDA-MB-231 cells were transfected with either non-coding siRNA (siNC) or siRNA targeting NEMO (siNEMO). 48 hours after transfection the cells were treated with increasing concentrations of phenformin for 18 hours then Caspase 3/7 activity was measured (n=3, the error bars represent the S.E.M of biological replicates).

(D.) A panel of LKB1-deficient (A549, HeLa, NCI-H460 and NCI-H23) and LKB1 wt cell lines (IMR90, NCI-H441, MDA-MB-231 and MiaPaca-2)were treated with either DMSO, phenformin (1mM), Cmpd A (5μM) or both (Combo) for 18 hours, then the Caspase 3/7 activity of each cell line was measured. Each cell line was assayed in 3 independent experiments and the average of the three was plotted on the graph (a two-way ANOVA was performed to determine which groups were statically different ** $p =< .01$).

Figure 2.6 Supplemental data associated with Figure 2.1.



Supplemental Figure 2.6.

(A.) HeLa cells were pre-treated with either DMSO or Cmpd A at the indicated concentrations for 15 minutes then treated with IL-1β for 5 minutes. Whole cell lysates were then subjected to western blot analysis with the indicated antibodies (n=2).

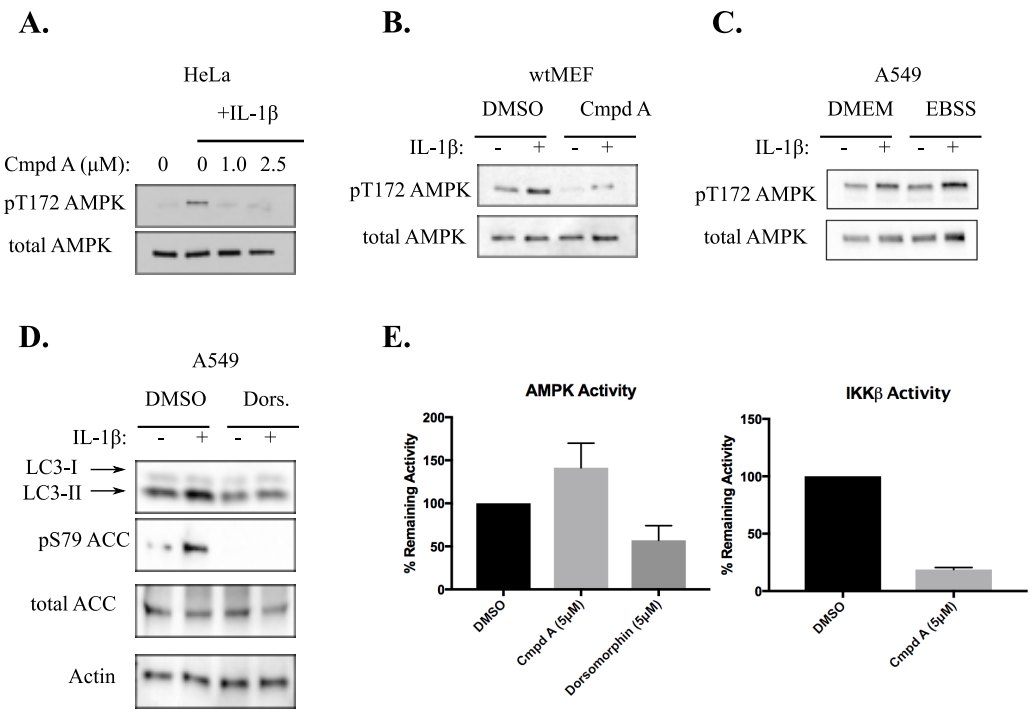
(B.) wtMEFs were pre-treated with either DMSO or Cmpd A for 15 minutes the treated with IL-1β for 5 minutes. Whole cell lysates were then subjected to western blot analysis with the indicated antibodies (n=2).

(C.) A549 cells were either grown in DMEM + 10% FBS or starved for 2 hours using Earle's Balanced Salt Solution (EBSS) then were treated with IL-1β for 5 minutes and then AMPK Thr172 phosphorylation was analyzed by western blot.

(D.) A549 cells were pre-treated with either with DMSO or 5μM Dorsomorphin (n=2) for 15 minutes, then treated with IL-1β for 20 minutes. Whole cell extracts were then prepared and analyzed by western blot for the indicated proteins.

(E.) The activity of recombinant AMPK or IKKβ was measured in the presence of the indicated inhibitors using either the SAMS peptide or a peptide corresponding to IκBα respectively. The graphs represent the S.E.M of three independent experiments.

Figure 2.7 Validation of Cmpd A/siRNA specificity



Supplemental Figure 2.7

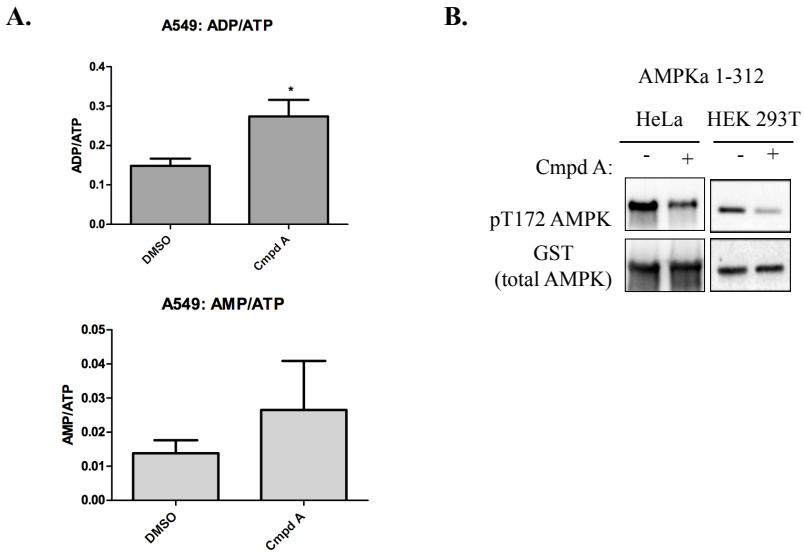
(A.) A549 cells were transfected with either one of two unique siRNA's targeting NEMO for 48hrs. Whole cell lysates were then subjected to western blot analysis with the indicated antibodies (n=3).

(B.) IKKα -/-, IKKβ -/- double knockout MEFs (DKO) were treated with 5 μM Compound A. Whole cell lysates were then analyzed by western blot analysis. The ratio of pT172 to total AMPK was quantified using ImageJ and plotted on the graph below (n=3, the error bars represent S.E.M).

(C.) A549 cells were treated with the indicated concentrations of NBD peptide, whole cell lysates were prepared and blotted for the indicated proteins. The ratio of pT172 to total AMPK was quantified using ImageJ and plotted on the graph below. The error bars represent the S.E.M of three independent experiments.

(D.) A549 cells were treated with 5μM of either Compound A or an inactive enantiomeric mixture of Compound A, known as Compound B .

Figure 2.8 IKK regulates AMPK independent of changes in energy status/ATP.

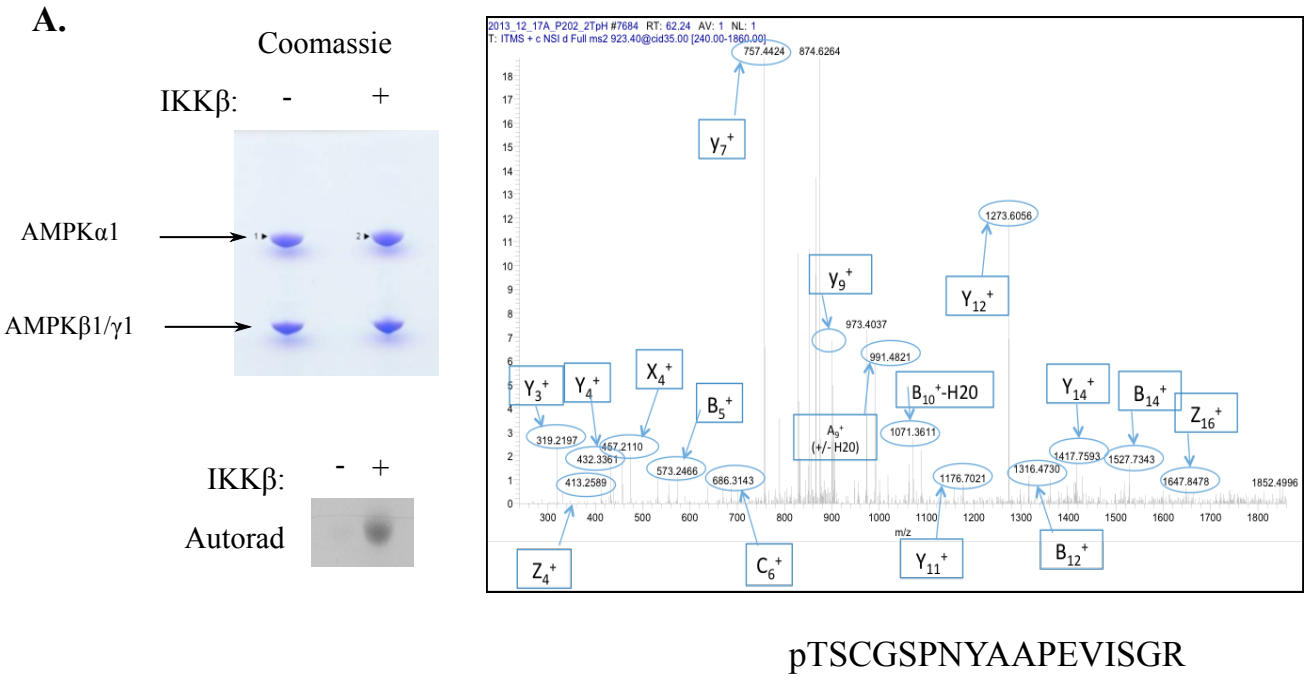


Supplemental Fig 2.8. IKK regulates AMPK independent of changes in energy status/ATP.

(A.) A549 cells were treated with Compound A (5μM) for 30 minutes and the ratio of ADP:ATP and AMP:ATP was measured using LC-MS (N=3, error bars represent the S.E.M).

(B.) A plasmid encoding a GST-tagged kinase domain of AMPKα (1-312), which cannot bind AMPKγ and thus its activity is not affected by the binding of either AMP or ADP, was transfected into HeLa or HEK293T cells. The cells were then treated with either DMSO or Cmpd A (5μM) for 1 hour. GST-AMPK was then isolated using GSH-sepharose beads and western blots were performed for either GST (total AMPK) or AMPK pT172.

Figure 2.9 Identification of AMPK Thr172 phosphorylation by mass spectrometry



Supplemental Fig 2.9. Identification of AMPK Thr172 phosphorylation by mass spectrometry

(A.) Recombinant active IKKβ was incubated with recombinant inactive AMPKα1/β1/γ1 trimers. The resulting reaction was separated by SDS-PAGE. The band corresponding to the alpha subunit was digested with trypsin and subjected to mass spectrometry. A peptide corresponding to phosphorylated Thr172 was identified only in the reaction that contained IKK.

(B.) Recombinant IKKβ was incubated with either wt or T172A GST-AMPKα1 purified from HEK293T cells in the presence of radiolabeled ATP. The resulting reaction was analyzed by SDS-PAGE.

CHAPTER 3: RAPTOR SER877 IS PHOSPHORYLATED BY TBK1 AND IS CORRELATED WITH SUPPRESSION OF MTORC1.

3.1 Overview

The role of Tank Binding Kinase 1 (TBK1) in the regulation of mTORC1 activity has been controversial. Some reports indicate that TBK1 activity represses mTORC1 while others suggest that TBK1 activates mTORC1. We report here that TBK1 is required for the inhibition of mTORC1 in response to serum starvation. Using an unbiased proteomics approach, we found that TBK1 phosphorylates a vital component of the mTORC1 complex, Raptor, at Ser877 both in cell-free kinase assays an in cell culture. Raptor Ser877 phosphorylation was induced by the pathogen-associated molecules double stranded DNA and by LPS in a TBK1 dependent manner. In addition, we find that Herpes Simplex Virus-1 induced Raptor Ser877 phosphorylation is attenuated in TBK1 -/- MEFs. The increase in Raptor Ser877 phosphorylation was correlated with a decrease in mTORC1 signaling as measured by phospho-p70S6K. Thus Raptor Ser877 phosphorylation may be a marker of TBK1 mediated mTORC1 repression. Future studies are required to determine if this phosphorylation site is required for TBK1 mediated mTORC1 repression. Although TBK1 is well known to promote selective autophagy by phosphorylation of autophagy adaptors, this could represent a mechanism whereby TBK1 promotes bulk autophagy though mTOR inhibition.

3.2 Chapter 3 Introduction

The TANK-binding kinase 1 (TBK1) is an IKK related kinase that is known for its role in the innate immune system[21]. In response to pathogens and pathogen-associated molecules, TBK1is recruited to the Interferon Regulatory Factor 3 (IRF3) by adaptor molecules. It then phosphorylates IRF3 at Ser396, which promotes its DNA binding and transcription activation potential. Importantly, IRF3 promotes transcription of interferons which in turn promotes pathogen clearance[21].

In addition to its role in innate immune activation, TBK1 can regulate the mechanistic Target of Rapamycin (mTOR), a kinase known as a master regulator of cell growth [25,155]. When mTOR is active, it promotes cell growth primarily by stimulating protein synthesis and inhibiting autophagy. mTOR is found in two distinct complexes, mTORC1 and mTORC2. In addition to mTOR, mTORC1 contains regulatory proteins Raptor, MLST8, PRAS40, and DEPTOR. The regulatory associated protein of mTOR, Raptor, recognizes substrates by binding to TOS-motif containing proteins and directing them towards the active site of the mTOR kinase domain[156]. The substrates for mTORC1 include several proteins that regulate protein translation (ex: pS70 S6K and 4EBP-1) and autophagy (ex: Ulk1). mTORC2 on the other hand primarily phosphorylates Akt, PKC and SGK1 [157].

While mTORC1 signaling is most well-characterized in response to changes in growth factors and nutrients, it is now appreciated that many different signaling pathways, including TBK1 signaling, converge on mTOR. However, the mechanism and consequences of TBK1 mediated mTOR regulation remain incompletely understood. Several publications indicate that TBK1 represses mTOR activity. Overexpression of wild-type but not kinase-dead TBK1 was sufficient to block mTORC1 activity as measured by p70S6K phosphorylation in prostate cancer

cells and TBK1 mediated suppression of mTOR was linked to prostate cancer dormancy[85]. In a model of chronic innate immune activation induced by knockout of the Three-prime repair exonuclease 1, TBK1 was also linked to suppression of mTORC1 activity [86]. TBK1 is also known to promote autophagy, a process that is strongly opposed by mTORC1[91].

In contrast, several publications indicated that TBK1 promotes the activation of an upstream activator, Akt,[72] and one recent report demonstrated that TBK1 increases mTORC1 activity through direct phosphorylation of mTOR itself[76]. TBK1 could physically interact with multiple components of the mTOR pathway when expressed in 293T cells[69]. In that study, the authors found a wide range of sensitivity of lung cancer cell lines to TBK1 inhibitors. Interestingly, in cell lines that were sensitive to TBK1 inhibition, TBK1 inhibitors decreased markers of mTOR activity, whereas the opposite occurred in TBK1 inhibitor resistant cell lines. This indicates that TBK1 activity has the opposite effect on mTOR activity in different cell lines. This contradiction, where some studies suggest that TBK1 activates mTORC1 while and others suggest that TBK1 inhibits mTORC1, indicates that the role of TBK1 in mTORC1 regulation is likely complex and context dependent.

To further understand the relationship between TBK1 and mTOR signaling we decided to test whether TBK1 phosphorylates a critical component of the mTORC1 complex, Raptor. Raptor is heavily regulated by phosphorylation by a diverse set of signaling cascades and kinases, and this allows for fine-tuning of the mTORC1 signaling. Using a proteomics approach, we found that TBK1 can directly phosphorylate a Raptor at Serine 877, both in cell-free kinase assays and in intact cells. While phosphorylation of Raptor Serine 877 has been well-documented (see Phosphosite.org), the function of this phosphorylation site, as well as upstream kinases that regulate this site, remains much less characterized. One study found that NEMO-

Like kinase (NLK) could phosphorylate Raptor Ser877, but that the primary phosphorylation site for NLK was the nearby Raptor Ser863[158]. Another study indicated that Raptor Ser877 phosphorylation was decreased in response to the mTOR inhibitors Torin and Rapamycin leading to the view that mTOR itself phosphorylates Ser877 [159]. However, another study indicated that Raptor Ser877 phosphorylation was insensitive to Rapamycin or mTOR activity induced by insulin[160].

The Unc-51 like kinase 1 (ULK1) is a highly conserved kinase that is required for the initiation of autophagy. Overexpression of ULK1 was sufficient to induce Raptor Ser877 phosphoryalation[161]. Recent work has also shown that ULK1 can directly activate TBK1 by phosphorylation of TBK1 S172[12]. Thus, the induction of Raptor Ser877 phosphorylation by Ulk1 could be a direct consequence of TBK1 activation.

Serine 877 is located within a cluster of phosphorylation sites on Raptor known as "Cluster 2."[160] The most well-characterized phosphorylation site in this cluster is Ser863, which can promote mTOR activity or inhibit mTOR activity depending on the context[158,160]. Only one study has been published in which Ser877 itself was mutated and a phenotype assessed[158]. In that particular study, expression of a phosphomimetic mutant of Raptor Ser877 did not affect the ability of Raptor to bind the Ragulator complex proteins. However, phosphomimetic mutations can only approximate serine phosphorylation, as they only have half of the negative charge as a true phosphorylation site. It is thus still possible that phosphorylation of Raptor Ser877 disrupts binding with the Ragulator proteins.

Over-expression of Rheb, a GTP-binding protein that is known to promote mTORC1, was sufficient to increase the amount of this phosphorylation of Raptor Ser877[160]. Since Raptor Ser877 is followed by a proline residue, several groups have hypothesized that Ser877 is

phosphorylated by ERK/MAP kinases. However, Raptor Ser877 phosphorylation was not affected by PMA or an ERK inhibitor[162]. Similarly, we found that Raptor Ser877 phosphorylation was only weakly affected by PMA in cells. Nocodazole treatment increased Raptor Ser877 phosphorylation; however, this was also not dependent on ERK activity [163]. One study looked at the cellular distribution of Raptor Ser877 phosphorylation and found that the majority of the Raptor phosphorylated at Ser877 was localized to the centrosome in mitotic cells [164].

In this study, we have identified Raptor Ser877 as a direct target for TBK1, both in cells and in cell-free kinase assays. We found that TBK1 -/- MEFs have greater levels of mTOR activity than their wild-type counterparts when serum starved. Importantly, this phosphorylation site was correlated with a decrease in mTORC1 activity. Thus TBK1 mediated phosphorylation of Raptor Ser877 could be a marker of TBK1 mediated mTOR repression. However, future studies are necessary to determine whether Raptor Ser877 phosphorylation required for TBK1 mediated mTORC1 repression.

3.3 Results

TBK1 is required mTORC1 inhibition in response to serum starvation.

A recent report indicated that TBK1 is required for full mTORC1 activation in response to certain stimuli after overnight serum starvation[76]. However, another recent report indicated that the kinase ULK1, whose activity is known to be induced by serum starvation, was recently demonstrated to activate TBK1 by phosphorylating Ser172 in the TBK1 activation loop[12]. Accordingly, we found that serum starvation potently induced TBK1 Ser172 phosphorylation (Fig. 3.1A). Since TBK1 activity was induced by serum starvation, we next tested whether TBK played a role in regulating mTOR signaling upon serum withdrawal, as mTOR is known to be

rapidly inhibited by serum starvation. In wtMEFs, serum starvation led to a decrease in the phosphorylation of the known mTOR substrate, p70-S6K, and marker of its activity, phosphorylated S6 (Fig. 3.1B). In contrast TBK1 -/- MEFs failed to fully suppress the levels of phospho-p70S6K in response to serum starvation, indicating that TBK1 is required for repressing mTORC1 in response to serum starvation.

TBK1 phosphorylates Raptor Ser877 in cell-free assays and in cells

We hypothesized that TBK1 suppresses mTOR by direct phosphorylation of a component of the mTORC1 complex. We immuno-precipitated myc-mTOR from HEK293T cells and combined the resulting precipitation with recombinant TBK1 in the presence of radiolabeled ATP. The addition of TBK1 to the reaction did not increase the levels of ^{32}P-γ-ATP incorporated into the mTOR itself(Fig. 3.2A). However, ^{32}P-γ-ATP was incorporated into a protein with an apparent molecular weight of around 150 kDa. Since the mTOR used in this reaction was precipitated from HEK293T cells, we speculated that TBK1 phosphorylated a protein that is typically found in a complex with mTOR (Fig. 3.2A). Two proteins, Raptor (149 kDa) and Rictor (192 kDa) were candidate proteins for that particular band as both proteins are known to be tightly associated with mTOR. We immuno-precipitated tagged Raptor or Rictor from HEK293Ts and found that recombinant TBK1 only showed a significant amount of activity towards Raptor (Fig. 3.2B).

To map the exact TBK1 dependent phosphorylation sites on Raptor we utilized mass spectrometry in a cell-based model and in cell-free kinase assays. To determine which sites on Raptor were phosphorylated in cell-free kinase assays, we performed a reaction as in Fig. 3.2B, except that we did not include radiolabeled ATP in the reaction. Three reactions were performed:

(1) HA-Raptor (2) HA-Raptor+ATP or (3) HA-Raptor +ATP and +TBK1. The peptides identified from the second reaction are presumed to be from another kinase that could be co-purified with HA-Raptor, such as mTOR. In this way, we could tell which sites were specifically due to TBK1 activity and not a contaminating kinase that might be co-purified with HA-Raptor. The peptides enriched in the third reaction were presumed to be due to TBK1 activity. Each reaction was separated using SDS-PAGE and then stained with Coomassie. The band corresponding to Raptor was then excised and subjected to mass spectrometry. In total, we identified nine phospho-peptides that were enriched in the samples incubated with TBK1. The phosphorylation sites corresponded to S44, S101, S704, S877, S982, S1027, and S1171 (Table 1).

We next determined which of these candidate phosphorylation sites were dependent on TBK1 activity in intact cells. HA-Raptor was immunoprecipitated from HEK 293T cells either unstimulated, stimulated with a known inducer of TBK1, phorbol myristoyl acetate (PMA) [165] , or a combination of PMA in conjunction with the TBK1 inhibitor AZ-5E. [166]. As in the other approach, we excised the band that corresponded to Raptor and performed mass spectrometry. Only Serine 877 was identified as TBK1 dependent in cell-free kinase assays and in cells (Fig. 3.2C and Table 1). Several other TBK1 dependent phosphorylation sites on Raptor were also identified in HEK293T cells, including S696 and S863. While it appears that TBK1 can regulate these sites in cells, it likely does so indirectly as these sites were not TBK1 dependent in cell-free kinase assays (Table 1).

To validate the mass spectrometry findings, we used a commercially available antibody that specifically recognizes Raptor when phosphorylated at Ser877. Overexpression of wt- but not kinase-dead (kdTBK1, K38A), TBK1 in HEK293T cells induced Raptor Ser877 phosphorylation

(Fig. 3.2D). The signal from the antibody was specific to Raptor Ser877 phosphorylation as mutation of Ser877 to alanine completely erased the signal.

To determine whether endogenous TBK1 could regulate endogenous Raptor Ser877 phosphorylation, we treated of the lung cancer cell line A549 with the dual TBK1/IKKε inhibitor, which led to a decrease in the relative levels of Raptor Ser877 phosphorylation (Fig. 3.2E). In addition, we treated MEFs with an ATP-competitive TBK1 selective inhibitor, Compound 1, that does not inhibit IKKε[167]. Compound 1 decreases the levels of Raptor S877 phosphorylation in wild-type mouse embryonic fibroblasts (MEFs) in a dose-dependent manner (Fig. 3.2F), indicating that inhibition of TBK1 activity alone is sufficient to decrease the levels of Raptor Ser877 phosphorylation [167].

TBK1 agonists LPS and dsDNA induce Raptor S877 phosphorylation in wt but not TBK1 -/- MEFs

We next treated either wt or TBK1 knockout MEFs (TBK1 -/-) with two known TBK1 agonists, double-stranded DNA or LPS for 2 hours. Both agonists stimulated the Raptor Ser877 phosphorylation in wild-type MEFs but not in TBK1 -/- MEFs. This induction of Raptor Ser877 phosphorylation was associated with an increase in phospho-p70S6K relative to the total levels.

HSV induces TBK1 dependent Raptor Ser877 phosphorylation independent of ICP 34.5

Since double-stranded DNA could induce TBK1 dependent phosphorylation of Raptor Ser877, we next wondered whether Herpes Simplex Virus 1 (HSV-1) could also induce Raptor Ser877 phosphorylation, as it is known to induce the cytosolic double-stranded DNA response [168]

and STING knockout mice, which lack the capacity to sense foreign cytosolic DNA, are more susceptible to HSV-1 infection than their wild-type counterparts[169].

We choose to compare two strains of HSV, one in which the viral gene ICP34.5 was deleted (D) and one that had this gene put back (R). We decided to use this system since ICP34.5 is known to bind and modulate TBK1 [170]. This gene product can interact with TBK1[171]. However, disruption of the binding of ICP34.5 to TBK1 does not impact the ability of TBK1 to phosphorylate IRF3[172].

Interestingly, HSV was able to robustly induce the phosphorylation of Raptor Ser877 regardless of whether or not ICP 34.5 was present (Fig. 3.4A). It could be that ICP 34.5 inhibits TBK1 with respect to certain substrates but not towards other substrates, such as Raptor Ser877. HSV-1-1ICP35.5-D induction of Raptor Ser877 phosphorylation was significantly attenuated in TBK1 -/- MEFs. We choose to use HSV-ΔICP 34.5 as the HSV gene product ICP 34.5 is known to modulate TBK1 activity[173]. An interesting future direction would to perform an infection with an HSV1 strain that lacks ICP27 as this gene product was shown to disrupt the STING-TBK1 complex and would not be capable of appropriately responding to cytosolic dsDNA[168].

3.4 Discussion

TBK1 is known to both repress and to activate mTOR, depending on the context and cell line. While a mechanism for TBK1 mediated activation of mTOR has been described[76], a mechanism whereby TBK1 inhibits mTOR has not. In this study, we find that TBK1 knockout MEFs have higher levels of mTORC1 activity as measured by the phosphorylation of the mTORC1 target p70-S6K during acute serum starvation. Thus studying MEFs that have been serum starved for short periods of time is a good model to study TBK1 mediated mTOR

inhibition. Other growth conditions, such as overnight serum starvation, may be better for studying TBK1 mediated promotion of mTOR signaling.

Here we describe a possible mechanism whereby TBK1 inhibits mTORC1 signaling, through direct phosphorylation of Raptor, a critical component of the mTORC1 complex. We have found that TBK1 can directly phosphorylate Raptor Ser877 and this phosphorylation is associated with an increase in mTOR activity. However, in this study Raptor Ser877 phosphorylation is merely correlated with repression of mTOR and future studies are required to determine whether Raptor Ser877 phosphorylation is truly required for TBK1 to inhibit mTORC1.

Others have published that TBK1 and IKKε can phosphorylate the mTOR kinase directly to promote its activity [174]. This work used recombinant GST-mTOR that was immobilized, and a panel of kinases was screened against the mTOR *in vitro*. The ratio of Raptor to mTOR was likely very different in those reactions as compared to our studies, and it is possible that the reactions in our study contained higher levels of Raptor (see Fig 3.2B). The increased levels of Raptor in our reactions may explain why we did not find that recombinant TBK1 displayed much activity towards mTOR precipitated from HEK293T cells, as it may have served as a competitive substrate for mTOR.

In the study mentioned above, TBK1 knockout MEFs have less mTOR activity than their wild-type counterparts[76], which is the opposite of what is found in our study. This difference could be attributed to either a different genetic background of the MEFs or different growth conditions. We serum-starved the MEFs for short time periods before our experiments, whereas Bodur et al starved the cells overnight. These differences in growth conditions could explain why we observed that TBK1 inhibits mTORC1 whereas others have observed that it inhibits mTORC1.

Overnight serum starvation can induce many changes in a cells grown in culture. For instance, serum starvation is known to induce degradation of the components of the Hippo pathway YAP/TAZ[175] and these genes are also known to modulate TBK1/IKKε meditated activation of IRF3 in response to cytosolic DNA[176]. While these genes inhibited the ability of TBK1 to promote IRF3 phosphorylation, they could favor TBK1 phosphorylation of other substrates such as Raptor. Moreover, at least one study has indicated that YAP/TAZ can regulate mTOR by promoting the transcription of SLC38A1[177]. Another important consequence of serum starvation is cell-cycle arrest. Both TBK1 activity and Raptor Ser877 phosphorylation are known to be regulated by the cell cycle [163,178,179].

Many studies using both targeted and unbiased proteomics have shown that Raptor is phosphorylated at Ser877 (see Phosphosite.org). However, the upstream kinase of this phosphorylation site has remained elusive. It has been reported that mTOR inhibitors reduce the levels of that Raptor S877, which has led to the conclusion that Raptor S877 is a direct target of mTOR kinase activity. Another possibility in light of the new data presented here, is that mTOR regulates TBK1 activity and that mTOR inhibitors decrease Raptor Ser877 phosphorylation by inhibition of TBK1 activity. In our hands, TBK1 knockout cells have lower levels of Raptor Ser877 phosphorylation than wild-type MEFs even though other markers (phospho-p70S6K) of mTORC1 are higher in the TBK1 -/- MEFs than wtMEFs. This means that in at least some instances TBK1 mediated phosphorylation of Raptor Ser877 is not directly linked to mTOR activity.

The sequence around Ser877 would have led one to predict that ERK or a MAPK would be a likely candidate for the upstream kinase of this site, since the serine is followed by a proline. In contrast the consensus sequence around TBK1 phosphorylation sites (based on peptide library

screens) indicates that TBK1 prefers leucine or isoleucine at the +1 position. However, proline is also tolerated at this position for TBK1 substrates, and several bona fide TBK1 substrates have proline at the +1 position[21]. While other kinases have a very strong preference for the amino acids immediately surrounding the phosphorylation site, TBK1 mediated substrate recognition is likely to be by other means such as forced proximity.

We should note that is is possible that TBK1 phosphorylates other serines or threonines on Raptor in different situations. For practical reasons, the only cell-based model we used for mass spectrometry for phosphorylation site mapping was HEK293T cells stimulated with PMA. It would be interesting to map the phosphorylation sites on Raptor from cells that have been stimulated with other known TBK1 agonists and determine whether the sites identified *in vitro* are also regulated by TBK1 *in vivo*. Several of the phosphorylation sites that we observed in cell-free assays have never been observed before according to Phosphosite.org, meaning that these phosphorylation sites may be artifacts from the in vitro kinase assays conditions or may be novel TBK1 dependent phosphorylation sites with unknown function.

TBK1 and IKKε are very homologous and can often phosphorylate the same substrates. An important future direction is to test whether IKKε also regulates Raptor Ser877. During HSV infection, Raptor Ser 877 phosphorylation is not induced until a much later time point, which could be consistent with a role for IKKε in regulating this site. While TBK1 is constitutively expressed, IKKε's expression is induced at the level of transcription. One possibility is that TBK1 can react rapidly to shut off or limit mTOR by phosphorylation of Raptor Ser877 and IKKε promotes mTORC1 by phosphorylation of the mTOR kinase domain, as described by Bodur et al. at later time points. Future studies are required to determine the relative contribution of TBK1 and IKKε to Raptor Ser877 phosphorylation *in vitro* and *in vivo*.

Future studies are also necessary to determine whether Raptor Ser877 phosphorylation is merely correlated with inhibition of mTORC1 or if it truly plays a role in mTORC1 inhibition. By expressing a mutant of Raptor in which Ser877 is mutated to alanine and can therefore not be phosphorylated, one could determine if the phosphorylation site is required for TBK1 mediated mTORC1 inhibition. Using such a mutant, one could study whether Ser877 phosphorylation is a key determinant in whether TBK1 promotes or inhibits mTOR signaling. This could yield important information about how TBK1 regulates such processes as autophagy and pathogen clearance, as well as provide insight into the role of TBK1 in diseases such as cancer and obesity.

3.5 Materials and Methods

Cell lines, plasmids, virus strains

All cells were maintained in DMEM (4.5g/L glucose) supplemented with 10% FBS and PenStrep. For serum starvation, cells were grown in serum-free media for 1 hour before the experiment. HeLa and HEK293T cells were from the UNC Tissue culture core facility. The wt and TBK -/- MEFs were as described previously[166]. pRK5-HA-Raptor was obtained from Addgene (Plasmid #8513). For immunoprecipitation experiments, HA-tag antibody-conjugated agarose beads were purchased from Cell Signaling Technology. The phospho Raptor Ser877 antibody was obtained from Millipore, and all of the other antibodies were obtained from Cell Signaling Technology. The TBK1 inhibitor, Compound 1, was generously provided by Dr. Qing Zhang (UNC-Chapel Hill). The Herpes Simplex Virus 1 strains are as described previously[170].

Kinase Assay

HEK23T cells were transfected with HA-Raptor. Two days after transfection, cells were lysed and incubated with the HA-antibody conjugated agarose beads for 1 hour. The beads were

then washed three times in lysis buffer and then in kinase assay buffer. The beads were then incubated with the TBK1, ATP or the appropriate negative control at 30 degrees for 30 minutes. The reaction was stopped using Laemmli buffer.

Mass Spectrometry (From Dr. Laura Herring)

The mass spectrometry experiments were carried out at the UNC Proteomics Core Facility. Bands corresponding to Raptor were in-gel digested with trypsin overnight. Extracted peptides were enriched for phosphopeptides using TiO2. The TiO2 elution for each sample was analyzed by LC/MS/MS on an Easy nLC 1000-Qexactive HF. Samples were eluted over a 45 min gradient from 5-35 %B, where mobile phase A = 0.1% formic acid and mobile phase B = acetonitrile with 0.1% formic acid. The top 15 most intense ions were chosen for HCD fragmentation. Data were searched against a reviewed Human UniProt database using Mascot. The parameters used were: 10 ppm precursor ion mass tolerance, 0.02 Da product ion mass tolerance, up to two missed trypsin cleavage sites, carbamidomethylation of Cys was set as a fixed modification and oxidation of M, deamidation of N, Q, and phospho of S, T, Y were set as variable modifications. A peptide false discovery rate of 5% was used to filter all results.

3.6 Chapter 3 Figures

Figure 3.1 TBK1 is required for the mTORC1 repression in response to serum starvation

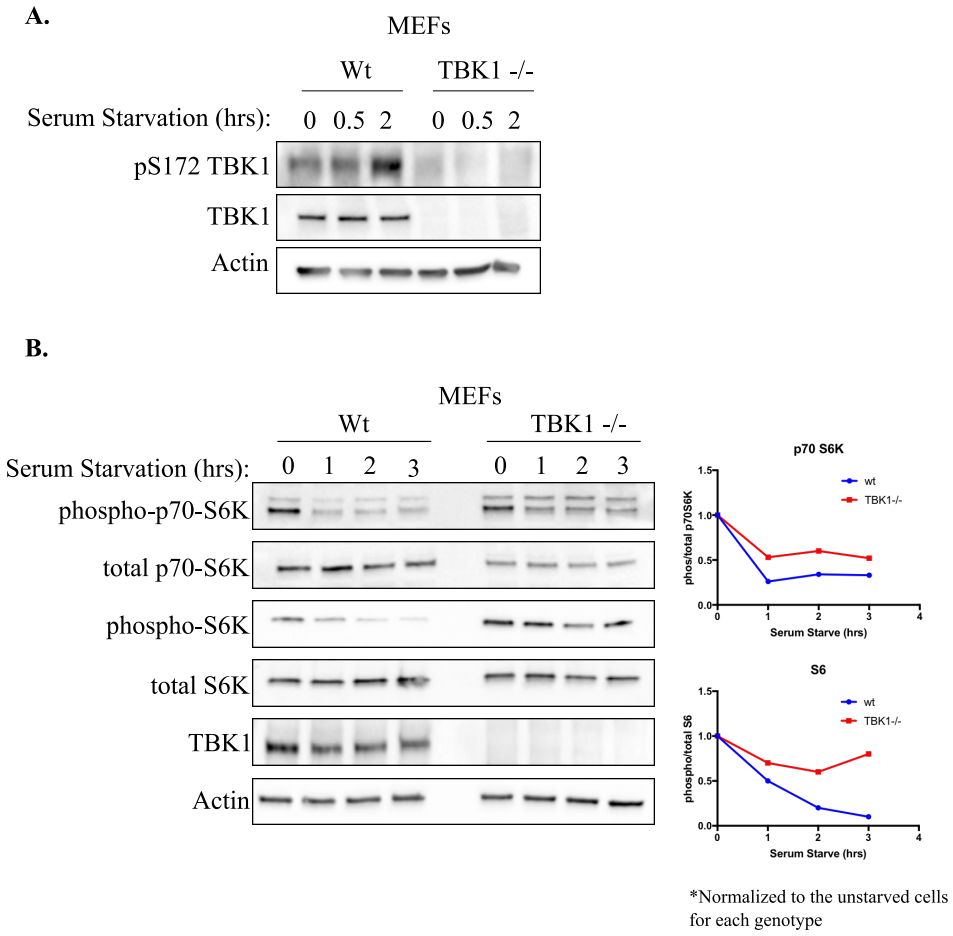


Figure 3.1 : TBK1 is required for mTORC1 repression induced by serum starvation

(A.) Wt or TBK1 -/- MEFs were serum starved for the indicated time periods and then whole cell lysates were prepared and analyzed by western blot.

(B.) As in A. The relative ratio of phosphorylated to total p70-S6K and S6 was determined using ImageJ. Each sample is normalize to the un-starved control for each genotype

Figure 3.2 Identification of Raptor Ser877 as a direct target for TBK1 phosphorylation.

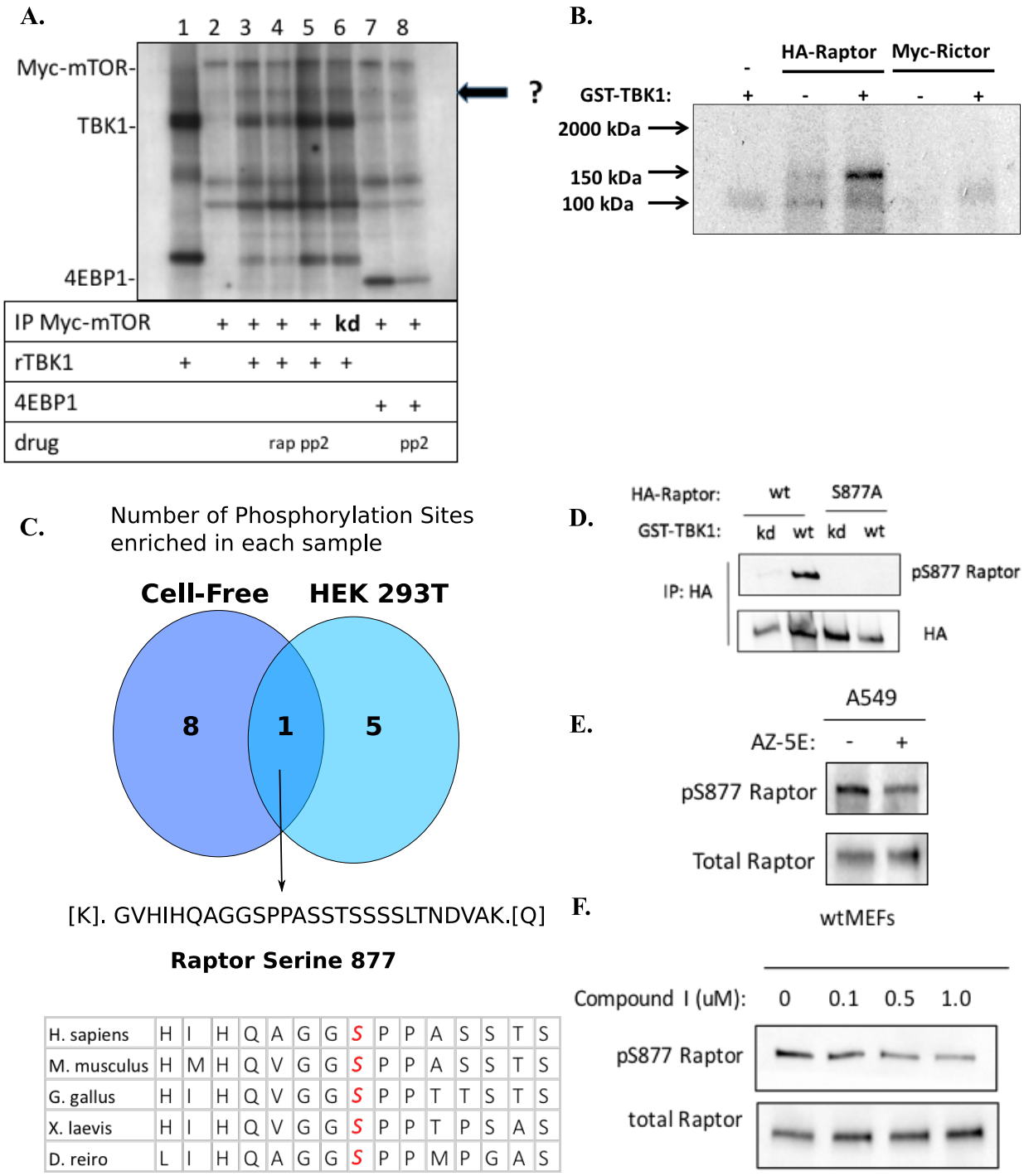


Figure 3.2: Identification of Raptor Ser877 as a direct target for TBK1 phosphorylation.

(A.) A kinase assay was performed with the indicated proteins as described in the materials and methods.

(B.) As in A except that either HA-Raptor or Myc-Rictor were used in the kinase assay instead of Myc-mTOR.

(C.) A Venn Diagram showing the phosphorylation sites identified as TBK1 dependent by mass spectrometry either in cell-free assays or when Raptor was precipitated directly from HEK293T cells. The sequence of the phospho-peptide corresponding to Raptor Ser877 is shown below as well as the sequence conservation of the surrounding amino acids.

(D.) Either GST-wt or GST-kd TBK1 was overexpressed in HEK293T cells along with wither HA-wt-Raptor or HA-S877A-Raptor. HA-Raptor was then immuno-precipitated and analyzed by western blot using either the phospho-Raptor Ser877 antibody or an antibody that recognizes the HA-tag.

(E.) A549 cells serum starved for 1 hour and then were treated with the AZ-5E for 1 hour. Whole cell lysates were prepared and analyzed by western blotting with the indicated antibodies.

(F.) Wt MEFs were treated with the indicated concentrations of Compound 1 and blotted with the Raptor phospho Ser877 antibody.

Table 1. Phosphorylation Sites Identified Using Mass Spectrometry

Phospho Site(s)		Peptide Sequence	Cell-Free			HEK293T		
			Blank	(+ATP)	(+ATP +TBK1)	Blank	PMA	PMA+AZ-5E
44		[K].IEGSKSLAQSWR.[M]	0.3	1.2	1.5			
101		[K].ALETIGANLQK.[Q]	0.0	0.0	1.0			
122		[R].YKQSLDPTVDEVK.[K]	0.0	0.0	1.0			
366		[R].SYNCTPVSSPR.[L]	0.6	2.0	0.4	0.8	0.0	1.2
696	704	[K].NYALPSPATTEGGSLTPVR.[D]	1.2	1.3	0.5	0.0	1.8	0.3
696		[K].NYALPSPATTEGGSLTPVR.[D]				0.3	2.5	0.3
696		[K].NYALPSPATTEGGSLTPVR.[D]				0.0	1.7	0.3
704		[K].NYALPSPATTEGGSLTPVR.[D]	0.4	1.1	1.4			
711		[K].NYALPSPATTEGGSLTPVRDSPCTPR.[L]	0.7	1.6	0.7			
719		[R].LRSVSSYGNIR.[A]	0.3	1.7	0.0			
722		[R].SVSSYGNIR.[A]	0.3	2.1	0.6	1.5	0.0	0.5
836		[K].VLNSIAYK.[A]	0.0	0.0	1.0			
854	855	[R].VLDTSSLTQSAPASPTNKGVHIHQAGGSPPASSTSSSSLTNDVAK.[Q]	0.8	1.2	0.0			
857	863	[R].VLDTSSLTQSAPASPTNK.[G]				1.1	1.6	0.3
859	863	[R].VLDTSSLTQSAPASPTNK.[G]				0.0	1.0	0.0
863		[R].VLDTSSLTQSAPASPTNK.[G]	0.9	1.6	0.5	1.1	1.4	0.5
863		[R].VLDTSSLTQSAPASPTNK.[G]				1.0	1.5	0.5
863		[R].VLDTSSLTQSAPASPTNK.[G]				1.0	1.5	0.5
863	869	[R].VLDTSSLTQSAPASPTNK.[G]	0.9	1.3	0.9			
877		[K].GVHIHQAGGSPPASSTSSSSLTNDVAK.[Q]	0.7	1.8	0.4	0.5	1.5	0.0
877		[K].GVHIHQAGGSPPASSTSSSSLTNDVAK.[Q]	1.1	1.2	0.6	1.4	1.4	0.2
877	884	[K].GVHIHQAGGSPPASSTSSSSLTNDVAK.[Q]	0.0	0.5	2.4			
877	889	[K].GVHIHQAGGSPPASSTSSSSLTNDVAK.[Q]	0.0	0.0	1.0			
Y916		[R].DLPSGRPGTTGPAGAQYTPHSHQFPR.[T]	1.0	1.8	0.1			
916		[R].PGTTGPAGAQYTPHSHQFPR.[T]	0.0	1.8	0.2			
937		[K].MFDKGPEQTADDADDAAGHK.[S]	0.0	1.0	0.0			
937		[K].GPEQTADDADDAAGHKSFISATVQTGFCDWSAR.[Y]	1.8	0.2	0.0			
982		[K].IPEEHDLESQIR.[K]	0.0	0.0	1.0			
1027		[R].NPGVPSVVK.[F]	0.0	0.0	1.0			
1171		[K].VQDIPTGADSCVTSLSCDSHR.[S]	0.0	0.0	1.0			
1200		[R].MALSECR.[V]	0.8	1.9	0.3			

Table 1:

This table displays the phosphorylation sites identified using mass spectrometry as described in the materials and methods. The values in the table represent the normalized area of the peak for the isotopic cluster for the peptide. Cells highlighted in light blue were the phospho-peptides that were called as TBK1 dependent. Cells highlighted in orange indicate phosphorylation sites that were not were previously identified on Phosphosite.org at the time of this writing.

Figure 3.3: LPS and dsDNA induce Raptor pS877 phosphorylation in a TBK1 dependent manner.

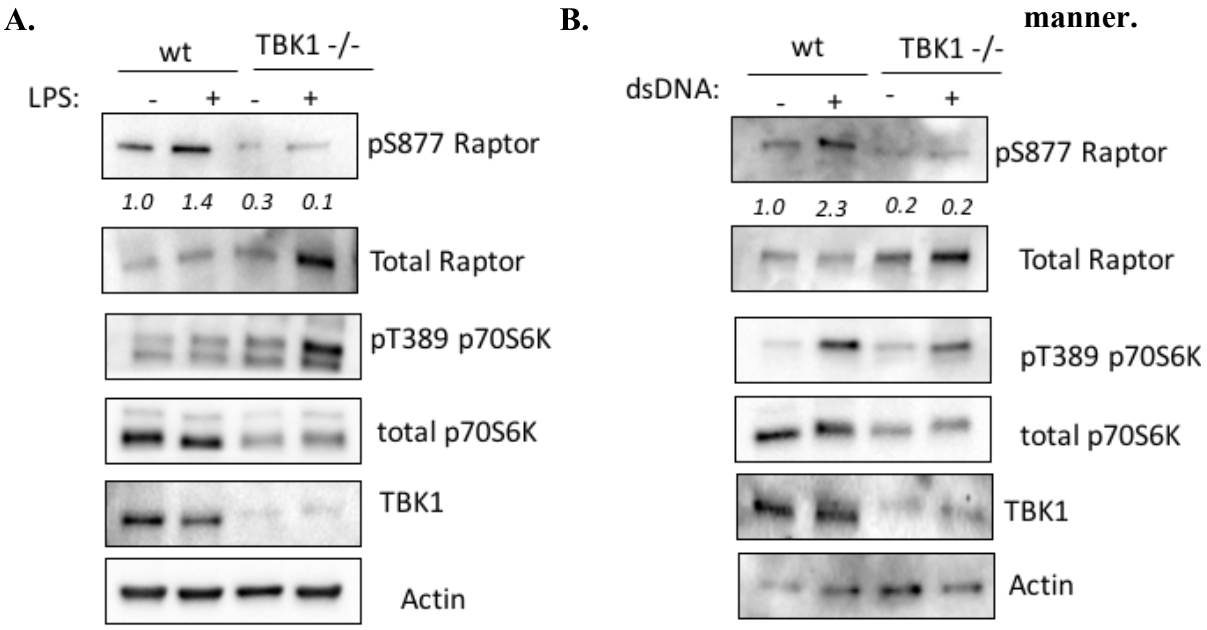


Figure 3.3: LPS and dsDNA induce Raptor pS877 phosphorylation in a TBK1 dependent manner.

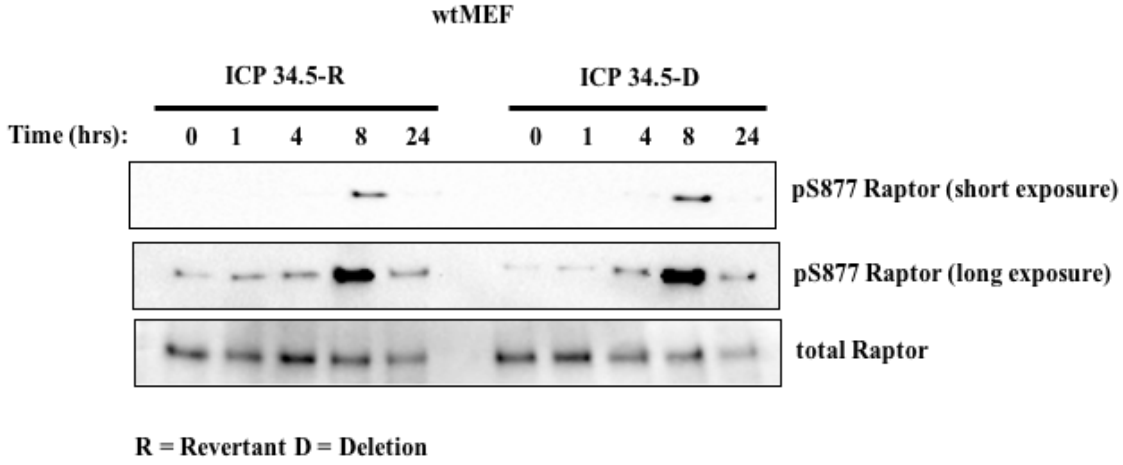
(A.) Wt or TBK1 -/- MEFs were treated with 10ug/ml LPS and then whole cell lysates were prepared and analyzed by western blot for the indicated antibodies.

(B.) As in (A.) except cells were transfected with dsDNA (2ug/ml) for 2 hours.

The relative intensity of the phospho-S877 to total Raptor was assessed using ImageJ. The normalized ratios are displayed underneath the phospho-S877 Raptor western blots.

Figure 3.4 HSV infection induces Raptor Ser877 phosphorylation.

A.



B.

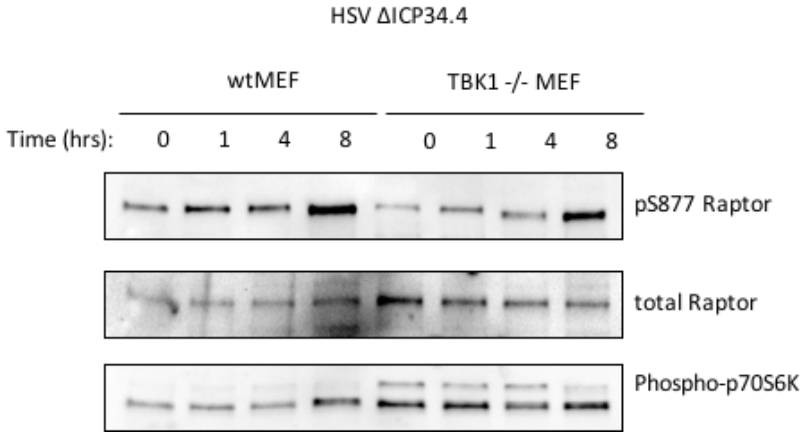


Figure 3.4.

(A.) Wt MEFs were infected with either HSV ICP34.5 deletion (D) or revertant (R) strains and blotted for either phospho Ser877 or total Raptor.

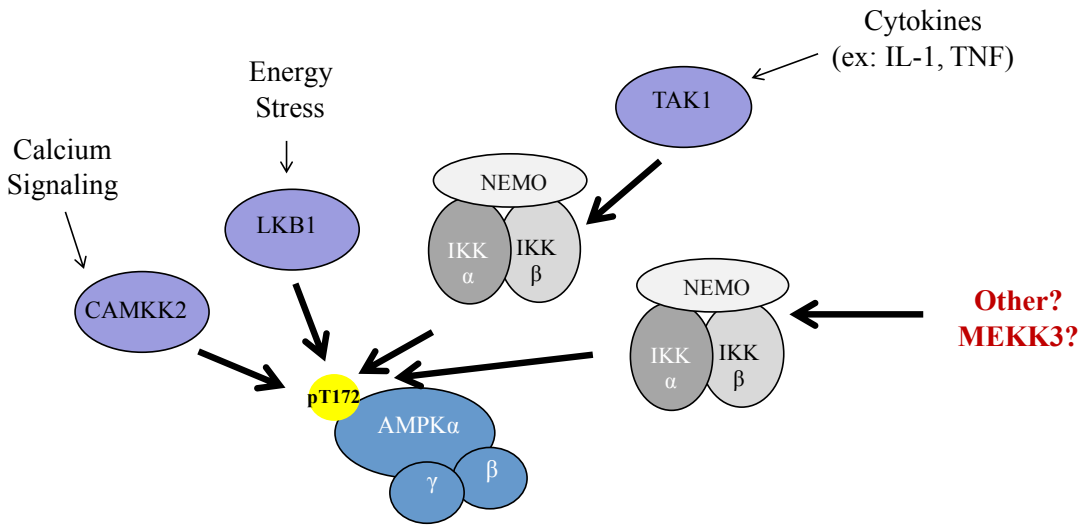
(B.) wt or TBK1 -/- MEFs were infected with HSV ΔICP34.5 for the indicated time periods and then analyzed by western blot analysis with the indicated antibodies.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

This chapter will discuss the ways that the work presented above adds to the body of work described in the introduction concerning the mechanisms by which IKK family kinases regulate cell growth, metabolism and autophagy. I will also present potential future scientific studies and ideas for therapeutic interventions that have emerged as a result of this work.

Figure 4.1 Model of AMPK regulation by Thr172 phosphorylation



4.2 Section 4.2 Future Directions related to Chapter 2: "IKK phosphorylates AMPK Thr172"

In Chapter 2 I describe the mechanism whereby canonical IKK regulates AMPK by direct phosphorylation of Thr172 in the kinase activation loop of the AMPK kinase domain. I established that IKKβ phosphorylates AMPK Thr 172 in response to the cytokines IL-1β and TNF-α. IKK activity was also required for the basal levels of AMPK activity in several cancer cell lines, and this was independent of another upstream AMPK/IKK kinase, TAK1. IKK-mediated activation of AMPK buffered against energetic stress in cancer cells that lack the primary upstream AMPK activating kinase, the tumor suppressor LKB1.

What is the source of basal IKK activity in cancer cells?

Interestingly, inhibition of a known upstream IKK activating kinase TAK1 did not affect the basal IKK activity in specific cancer cell lines. Consequently, TAK1 inhibition did not affect the levels of AMPK activity observed in these same cells. While this demonstrated that IKK could promote AMPK Thr172 phosphorylation in the absence of TAK1 activity, it raises the question of how IKK is activated, if not through TAK1 dependent phosphorylation. TAK1 is well established to phosphorylate IKK activation loop serines in a ubiquitin-dependent manner in response to IL-1, TNF- and other stimuli [8]. However, as demonstrated in Chapter 2, two distinct TAK1 inhibitors failed to inhibit IKK activity as measured by p65 Ser536 phosphorylation in the absence of stimulation. This observation implied to us that another source of IKK activation must be present in these cells.

One potential source of IKK activation could be IKK trans-autophosphorylation. Canonical IKK complexes are known to autophosphorylate, especially in the presence of linear-ubiquitin

molecules [125]. However, these linear ubiquitin chains are thought to be formed primarily upon stimulation, such as with IL-1. NEMO binds tightly to these linear ubiquitin chains which increases the local concentration of canonical IKK complexes. While these linear ubiquitin chains can lead IKK to autophosphorylate due to forced proximity, they also bring IKK into proximity with TAK1 which allows TAK1 to phosphorylate and activate IKK. It therefore possible that in these cells, there exists a mechanism for increasing the local IKK concentration without bringing IKK in proximity to TAK1.

Another potential inducer of IKK activity is MEKK3 [10]. Like TAK1, MEKK3 is a member of the MAP3K kinase family. Knockout of MEKK3 in mouse embryonic fibroblasts blocked TNF from inducing IKK activity and cell-free kinase assays revealed the MEKK3 could directly phosphorylate IKK. However, the exact site of MEKK3 phosphorylation on IKK was not mapped, and it is not known whether MEKK3 has activity towards the activation loop serines of IKK or if it phosphorylates another site on IKK. Other work has demonstrated that overexpression of the adaptor protein p62 was sufficient to activate NF-κB through MEKK3 [180], indicating that MEKK3 could activate IKK through a mechanism distinct from TAK1/TAB1 mediated IKK activation. An inhibitor of MEKK2/3 was recently developed [181] that, importantly, displayed no activity against TAK1 in cell-free kinase assays. An interesting experiment would be to compare the effects of a TAK1 inhibitor with the MEKK2/3 inhibitor on basal IKK activity in cancer cells. Based on our work, one might predict that the MEKK3 inhibitor would have a greater effect on basal IKK activity than a TAK1 inhibitor.

The protein p62 is known to be selectively degraded during autophagy and its degradation is frequently used as a marker of autophagy [182]. p62 is also known to promote IKK activity and this is the mechanism whereby KRas drives tumorigenesis [183,184]. This mode of regulation may

represent a feedback mechanism whereby p62 promotes IKK activity and IKK, in turn, supports the autophagic degradation of p62 by increasing AMPK Thr172 phosphorylation.

How does IKK recognize AMPK for phosphorylation of Thr172?

Another interesting observation is that IKKα/β phosphorylation in the respective activation loop serines is hardly detectable in the absence of stimulation. However, inhibition of IKK can decrease the relative levels of phosphorylation of known IKK substrates even when only low levels of IKK phosphorylation are detectable. Phosphorylation of these kinases is well established to increase the activity of these kinases. This discrepancy implies that other mechanisms of substrate phosphorylation may occur that do not necessarily require an increase in IKK activity, such as forced proximity of IKK to its substrates through a specificity factor(s). For example, the scaffold protein NEMO, which is known to behave like a specificity factor, could bring IKK into proximity with AMPK and allow IKK to phosphorylate AMPK even when IKK activity is relatively low [20].

AMPK Thr172 would not have been predicted to be a substrate for IKK based on previous information that was available about IKK substrate recognition. In screening of peptide libraries, IKK appears to prefer phosphorylation of substrates with a leucine or isoleucine at the +1 position, and has minor preferences for amino acids in other positions that surround the phosphorylation site target [185]. However, many bona fide IKK substrates do not follow this phosphorylation site motif. Several early studies have also suggested that IKK could only phosphorylate serines and could not phosphorylate threonine residues [186]. The fact that AMPK Thr172 does not fit the canonical IKK phosphorylation motif implies that IKK mediated AMPK

Thr172 phosphorylation occurs through a novel substrate recognition mechanism that has yet to be determined.

Do other stimuli lead IKK to phosphorylate AMPK Thr172?

I focused my studies on AMPK activity induced by IL-1β and TNF-α, as these are strong and well-established inducers of IKK activity, particularly of the canonical IKK complex. However, IKK activity is also known to be regulated by diverse other stimuli, such as DNA damage, LPS, and cytosolic double stranded DNA. An important future direction will be to determine whether IKK can lead to AMPK activation in response to pathogen exposure.

In addition, the tumor microenvironment often favors the activation of IKK [187]. An important future direction will be to determine whether the milieu of cytokines present in the tumor microenvironment can induce IKK to phosphorylate AMPK Thr172 and what the consequences of this activation are for tumor growth. IL-1β and TNF-α are potent inducers of the canonical IKK complex, which consists of the kinases IKKα and IKKβ, in addition to the scaffold and specificity factor NEMO. However, another IKK complex exists which includes exclusively IKKα homodimers [9]. This complex is activated by other stimuli, such as RANK ligand [9]. In my studies using a cell-free kinase assay, I found that IKKβ had approximately five times as much activity towards AMPK Thr172 as did IKKα. This observation led me to conclude that, in response to stimuli of the canonical IKK complex, it was IKKβ that was primarily responsible for the induction of AMPK Thr172 phosphorylation. However, IKKα still had a measurable level of activity towards AMPK Thr172. It is, therefore, possible that other stimuli that promote the activity of IKKα, such as BAFF ligand, could cause IKKα to phosphorylate AMPK Thr172.

What's the biological significance of IKK mediated AMPK Thr172 in non-cancerous cells that express wild type LKB1?

For our studies, we primarily used cells that did not express functional LKB1. It is well established that LBK1 is the most robust AMPK Thr172 kinase, and the decision to focus on LKB1 deficient cells was to prevent confounding issues and because a substantial number of patients harbor tumors that lack LKB1 expression. An exciting future direction would be to explore the role of the IKK mediated AMPK Thr172 phosphorylation in normal physiology, when LKB1 is expressed. I did find that IKK could regulate both basal and cytokine-induced AMPK Thr172 phosphorylation in cell lines that express LKB1 (MDA-MB-231, HEK293T and wtMEFs).

One might expect that IKK-mediated AMPK Thr172 phosphorylation is less critical during energetic stress when LKB1 is present since LKB1 is such a potent inducer of AMPK Thr172 phosphorylation. I would hypothesize that IKK-mediated AMPK regulation is more important in response to cytokines or in response to specific developmental cues that are known to be IKK dependent (for example during hematopoiesis).

Another upstream AMPK Thr172 kinase is CAMKK2, which promotes AMPK Thr172 phosphorylation in response to stimuli that increase the concentration of intracellular calcium [115,116,188]. Some speculate that the reason CAMKK evolved to induce AMPK Thr172 phosphorylation is that signals that produce an increase in intracellular calcium often precede an increased energy demand and thus prompt the cell to alter its metabolism to compensate for this increase [189]. A similar situation may be the case for IKK, where cellular stimulation by a cytokine can often lead to substantial shifts in cellular metabolism.

For example, pathogen infection often leads to substantial metabolic changes. Activating AMPK may be a way for the cell to shut down the translation machinery preemptively so that the pathogen can not usurp it for its own replication. Also, AMPK activation leads to the induction of autophagy, which is often used by cells to degrade intracellular pathogens directly. Fighting a pathogen infection can be an energy-intensive process, requiring the mobilization of immune cells and the production of proteins and other chemicals that instantly kill the pathogen [190]. For example, activated B-cells must produce large quantities of antibodies very rapidly in response to pathogens, thereby taxing cellular energy and metabolites [191]. IKK-mediated AMPK Thr172 phosphorylation may be a way to induce the metabolic shift that are necessary for the situations described above.

AMPK mediates epigenetic changes by regulating EZH2 - Is there an involvement of IKK?

A recent report indicates that AMPK phosphorylates the histone methyltransferase EZH2 to drive it away from the polycomb repressive complex, resulting in decreased levels of H3K27me3 [192]. Another paper demonstrated that increasing the levels of H3K27me3 by inhibition of the demethylase prevented TNF-α induction upon LPS stimulation, indicating that H3K27me3 could be repressing inflammation [193]. By driving AMPK Thr172 phosphorylation, IKK could also be driving inhibition of the EZH2-mediated increase in H3K27me3, which could make the promoters of certain pro-inflammatory genes more accessible to transcription factors. Alternatively, IKK could be driving EZH2 away from the canonical repressive complex towards complexes with NF-κB. EZH2 is known to either promote or inhibit NF-κB target genes in breast cancer, depending on the context, by binding to NF-κB transcription factors [194]. Our group also recently published that EZH2 could positively regulate the expression of another NF-κB family transcription factor, RelB, in triple-negative breast cancer[195]. An interesting future

direction will be to determine whether AMPK mediates the effect of IKK on gene expression by modulating epigenetic changes.

What is the role of AMPK in LKB1 deficient cancer cells?

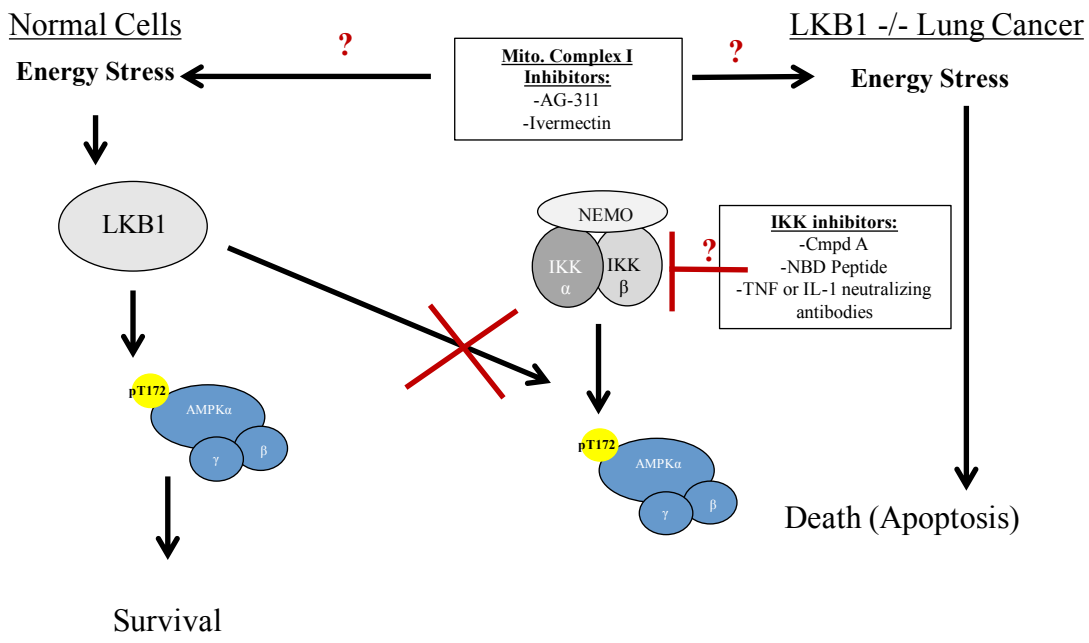
Loss of LKB1 is found in a significant number of human cancers, but loss of AMPK is almost never observed [196]. While in the literature LKB1 loss is often considered synonymous with loss of AMPK activity, there is a detectable level of AMPK activity that remains in LKB1-deficient cells. An important future direction will be to characterize the effect of this remaining AMPK activity on tumorigenesis. While AMPK is known to play a vital role in tumor cell survival during periods of energetic stress, AMPK is also known to have tumor suppressive capabilities [196].

Current thinking in the fields concludes that using AMPK agonists to treat various types of cancer, since AMPK over-activation is known to promote growth arrest. However, this strategy is unlikely to work if the cells do not express functional LKB1, as most of the agonists described to date require LKB1 in order to promote AMPK activity. Therefore, alternative ways to activate AMPK in these cells will be necessary. One possible way to activate AMPK in LKB1 null cells may be to induce IKK mediated AMPK Thr172 phosphorylation. In Chapter 2, I demonstrated that treatment with the inflammatory cytokines IL-1β or TNF-α induce AMPK activity in cells that lack LKB1, and at least one study indicates that IL-1β can cause a cell cycle arrest in specific tumor cell lines [197]. IKK may be required for IL-1β to induce a cell cycle arrest via AMPK, and IL-1β secretion by immune cells may be a mechanism for anti-tumor immunity. One study demonstrated that over activation of IKKβ in cancer associated fibroblasts, which leads to the production of cytokines that could activate IKK in cancer cells, can suppress tumor growth

[198]. Overactive IKKβ in cancer associated fibroblasts inhibit tumor growth by inducing cytokine activated AMPK activity and subsequent growth arrest. Therefore a strategy to inhibit LKB1 deficient cancer growth might be to induce expression of AMPK-inducing cytokines in cancer associated fibroblasts. The observation that IL-1β can promote growth arrest also implies that one may need to use caution when inhibiting inflammatory cytokines, such as with therapeutic monoclonal antibodies, as their inhibition may turn off a vital growth suppressive mechanism.

On the other hand, IKK inhibition may be a way to prevent cancer cells from appropriately responding to energetic stress and thus causing tumor cell death. Cytokine stimulation may be a signal for tumor cells that energetic stress is occurring. The capacity of IKK to regulate AMPK Thr172 phosphorylation was not affected by changes in the ADP/ATP ratio, indicating that IKK mediated AMPK regulation was not affected by energetic stress. In addition, I demonstrated that IL-1 could induce AMPK Thr172 phosphorylation in LKB1 deficient cells that were starved of all nutrients, indicating that in theory inflammatory cytokines may be able to compensate for LBK1 loss in situations of energetic stress with respect to AMPK. Given that IKK mediated AMPK regulation is not dependent on the energy status of the cell, cytokines may be a way to for cells to communicate with each other about nutrient availability. This extracellular communication about nutrient status may be necessary for LKB1-deficient cancers. These solid tumors often experience energetic stress and hypoxia and therefore may need to activate AMPK to survive, but lack a cell intrinsic nutrient sensing mechanism to appropriately do so.

Figure 4.2 Exploiting the vulnerability of LKB1 deficient cells to metabolic stress in the clinic.



In theory LKB1-deficient cancer cells have an "Achilles Heel" that should make them vulnerable to energetic stress, such as during mitochondrial inhibition by phenformin. In pre-clinical studies using an animal model of lung cancer that was driven in part by the loss of LKB1, phenformin produced the most robust levels of apoptosis compared to other pharmacological agents that are known to cause metabolic stress. However, the concentrations of phenformin that were necessary to induce apoptosis in these models would be difficult to achieve in humans without producing significant harmful side-effects. The work described in Chapter 2 indicated that inhibition of IKK could reduce the concentration of phenformin that was necessary to induce apoptosis in LKB1 deficient cells. In order to translate this finding into a clinical setting, it will be necessary to improve the way that both IKK and Complex I of the mitochondria are inhibited *in vivo*.

As of this writing, there are no FDA approved agents that directly target IKK activity. Several natural compounds, such as salicylate and its derivative aspirin can inhibit IKK *in vitro*

[199]. However, the concentrations necessary to inhibit IKK would be difficult to achieve in a human patient.

Most kinase inhibitors are designed to directly compete with ATP in the kinase domain, thus preventing the kinase from transferring a phosphate to its substrates. However, design of a selective ATP-competitive IKK inhibitor has been a challenge since IKK has such a high affinity for ATP [200]. An alternative approach has been to take advantage of the fact that IKKβ binding to NEMO is required for many IKKβ functions. In Chapter 2, I demonstrated that the NEMO Binding Domain Peptide (NBD) peptide, which is known to disrupt the interaction between IKK and NEMO, could lead to a decrease in AMPK Thr172 phosphorylation in LKB1 deficient cancer cells. The challenge with this peptide for clinical use is that it shows poor solubility properties and does not cross the cell membranes very efficiently. An interesting future direction might be to design molecules that functionally mimic the NBD peptide, but have more drug-like properties. There are also several FDA approved drugs that target known activators of IKK such TNF-α and IL-1β. However, it will first be necessary to determine if inhibiting only one of these cytokines is sufficient to inhibit AMPK Thr172 phosphorylation in an *in vivo* tumor model.

Another potential issue with IKK inhibitors could be side effects. IKKβ knockout mice are embryonic lethal, due to excessive liver apoptosis, and show increased sensitivity to TNF-α [201]. In addition, several reports have indicated that in certain circumstances such as in cancer associated fibroblasts, IKK can have tumor suppressive functions [198]. In order to avoid these inhibiting the tumor suppressive characteristics of CAFs, it may be necessary to target IKK specifically in the cancer cells rather than in the entire body.

As far as energetic stressing agents are concerned, we chose to use phenformin as there is pre-clinical data using this drug in various tumor models. An early study compared several

energetic stress inducing agents, and found that phenformin was able to induce the most robust levels of apoptosis in LKB1 deficient cells[129]. While IKK inhibition decreased the concentration of phenformin that was necessary to induce apoptosis in LKB1 deficient cells, very high concentrations were still required to see a maximal apoptotic response. The equivalent concentration of phenformin *in vivo* is likely to be associated with very serious side effects, such as lactic acidosis [202]. To improve upon phenformin, several novel agents. such as AG-311, have been described that can inhibit Complex I of the mitochondria at much lower concentrations than phenformin, [203]. However, the safety profiles of these compounds for use in humans has still not been fully determined. Alternatively, one could use other compounds widely used by humans that can also inhibit mitochondrial Complex I. For example, the anti-helminth drug Ivermectin, which has been used by millions of people for decades with very minor side effects, was shown to inhibit mitochondrial Complex I in glioma cell lines[204].

I demonstrated that Ivermectin can indeed reduce the levels of ATP (as measured by Cell Titer Glo) and induce the activity of Caspase 3/7 (as measured by Caspase Glo) in two LKB1 deficient cell lines (A549 and HeLa). These effects could be observed in the low micromolar range, indicating that it may be reasonable to achieve the concentrations necessary to inhibit Complex I of the mitochondria in patients. In contrast, two non-transformed cell lines (wtMEFs and IMR90) were not sensitive to Ivermectin, indicating that there may be a large therapeutic window for treating lung cancer patients with Ivermectin (see Fig. 4.3).

Figure 4.3 Effects of Ivermectin on cell viability in A549, HeLa, wtMEF and IMR90 cells

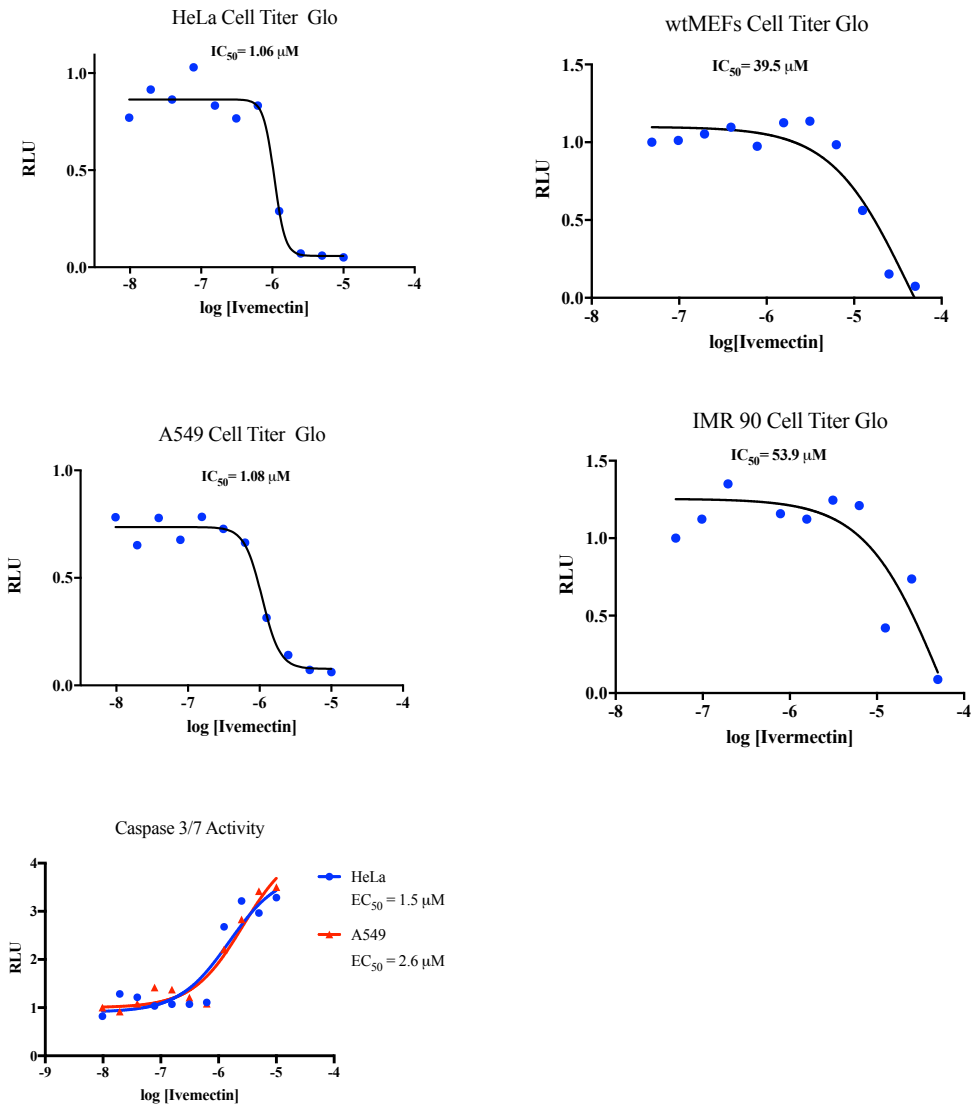
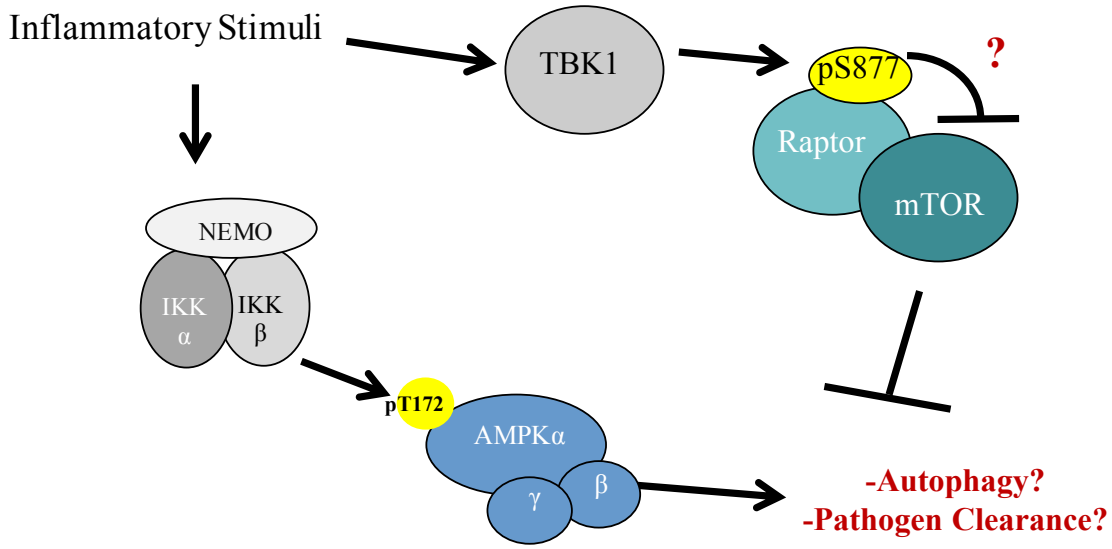


Figure 3. HeLa, A549, IMR90 or wtMEFs cells were plated in white walled 96 well plates and allowed to attach for 5 hours. After attachment, the cells were treated with increasing concentrations of Ivermectin for 14 hrs, at which point either Cell-Titer Glo or Caspase Glo Reagent was added to the wells and analyzed according to the manufacturer's instructions (Promega). The IC_{50} and EC_{50} values were determined using Graphpad Prism software.

4.3 Future Directions related to Chapter3 "TBK1 phosphorylates Raptor S877 and is associated with repression of mTORC1"

Figure 4.4 : Diagram of the cross talk between TBK1 and IKKβ with respect to mTOR and AMPK signaling.



The TANK binding kinase 1 (TBK1) is an IKK-related kinase known for its ability to regulate interferon genes in response to pathogen infection. There have been contradictory reports with respect to TBK1 and mTORC1 signaling. Some reports indicate that TBK1 can inhibit mTORC1 [205], while others report that TBK1 can promote mTORC1 signaling. This likely means that whether TBK1 promotes or inhibits mTORC1 is likely to depend on both the cell type and growth conditions. While a mechanism for TBK1-mediated mTORC1 promotion has been described [76], a mechanism for TBK1 mediated mTORC1 repression has not been described. In Chapter 3, we used a proteomics approach to map a TBK1 dependent phosphorylation site on a critical component of the mTORC1 complex, Raptor.

How does TBK1 recognize Raptor to phosphorylate it?

One important function of Raptor is to bind to substrates containing a TOS motif and direct them towards mTOR for phosphorylation [156]. Interestingly, TBK1 contains a TOS motif and deletion of this motif prevents TBK1 from inhibiting mTOR [85]. This could be an interesting mechanism whereby a regulatory kinase recognizes Raptor for phosphorylation rather than Raptor recognizing a substrate for phosphorylation.

What is the consequence of TBK1 mediated phosphorylation of Raptor Ser877?

The most critical future direction is to determine the functional consequences of Raptor Ser877 phosphorylation. Raptor Ser877 phosphorylation was correlated with suppression of markers of mTORC1 activity, but this correlation does not prove causality. Future experiments will need to compare the levels of mTORC1 activity in cells expressing wild type and mutant Raptor that cannot be phosphorylated at Ser877 (i.e., Raptor serine 887 to alanine). Using this system, one can determine whether Ser877 phosphorylation can regulate mTORC1 complex formation or downstream signaling. In addition, one could determine what the effect of this phosphorylation event is on other outcomes. Below I will describe future experiments that involve assigning a function to TBK1 mediated Raptor Ser877 phosphorylation.

Could TBK1 mediated inhibition of mTORC1 promote Akt activity by promoting mTORC2 activity?

Several reports have indicated that TBK1 and the closely related IKKε could activate Akt by direct phosphorylation [72,73]. However, these reports are controversial in the field and may not reflect the full complexity of the signaling that is occurring. One possibility is that by inhibition

of mTORC1 complex formation or signaling, TBK1 actually favors mTORC2 activity. Greater levels of mTORC2 activation could explain the higher levels of Akt activity observed by others.

Coordination of autophagy between canonical IKK and TBK1 downstream of IL-1

IL-1β was found to promote autophagy in macrophages and this enhanced the clearance of certain pathogens that are known to be suppressed by autophagy [91]. Interestingly, in that particular study the authors found that TBK1 was not required for the initiation of autophagy in response to IL-1, but was required for autophagosome maturation [91]. However, this study did not address the mechanism whereby TBK1 might promote autophagosome maturation, and one possibility is that it does so by inhibition of mTOR through Raptor Ser877. My work in Chapter 2, on the other hand, demonstrated that IKKβ was required for the initiation of autophagy by IL-1β. This could be an interesting mechanism whereby IKK coordinates with TBK1 to promote autophagy. IKKβ induces autophagy rapidly in response to IL-1β and TBK1 is required for autophagosome maturation at later time points after IL-1β stimulation. TBK1 is known to play an essential role in the selective autophagy-mediated degradation of certain types of bacteria as well as mitochondria [206,207]. Future studies are required to determine if Raptor Ser877 phosphorylation is required for cells to clear pathogens or dysfunctional mitochondria by autophagy.

Is TBK1 required for autophagy in KRas driven cancers?

TBK1 was identified in an early shRNA screen for synthetic lethality with mutant KRas [208]. It is possible that TBK1 is required for certain KRas driven cancers by promoting autophagy through limiting mTORC1 signaling. It has now become apparent that autophagy plays an

important role in certain types of cancer [209]. TBK1 could inhibit mTORC1 in these cells by phosphorylation of Raptor Ser877, which would in turn stimulate autophagy.

The role of autophagy in KRas driven cancer is likely to be context dependent, where the role of autophagy might be dependent on p53 mutation status. An important future direction will be to explore whether KRas mutant cancers that rely on autophagy have high levels of Raptor Ser877 phosphorylation and whether this is associated with sensitivity to autophagy inhibitors, such as chloroquine. This could be used as a biomarker to determine whether a patient presenting with a KRas mutant tumor should be treated with autophagy inhibitors.

At the same time, others have demonstrated that whether a cell line is sensitive to TBK1 inhibition is highly context dependent, and in some cases can be associated with the epithelial to mesenchymal transition [69]. If TBK1 inhibitors are ever to be used clinically, it will be important to have a marker of which patients will respond to the inhibitor. As described for autophagy inhibitors, Raptor Ser877 phosphorylation could be a marker of sensitivity to TBK1 inhibitors.

4.4 Concluding Remarks

The IκB kinase (IKK) was initially described as the kinase responsible for phosphorylating IκBα, which leads to its proteasome-mediated degradation and promotion of NF-κB dependent transcription [1,186]. [4,5]. The canonical IKK complex consists of IKKα and IKKβ. Besides the canonical IKKs, there are two other IKK related kinases, TBK1 and IKKε, that are key regulators of interferon production. While the role of the canonical IKK complex and the IKK related kinases in controlling NF-κB and interferon signaling is well established, many other roles for this kinase family have recently emerged [126]. It has now become apparent that IKK and the IKK related kinases are essential regulators of cell growth, metabolism, and autophagy. As

described in Chapter 1, the IKK family of kinases regulates these processes by directly phosphorylating a variety of the proteins involved. In this dissertation, I have characterized two additional substrates for IKKβ and TBK1 respectively, both of which are well known to be involved in cell growth and autophagy. In Chapter 2, I demonstrated that IKKβ phosphorylates AMPK at Thr172 to promote its activity and that this can buffer against phenformin induced energetic stress. In Chapter 3, I demonstrated that the IKK related kinase TBK1 phosphorylates a critical component of the mTORC1 complex, Raptor, at Ser877. This phosphorylation event was associated with repression of mTORC1. The work presented here lays the foundations for future studies concerning the interplay between inflammation and growth signaling, and for exploitation of these pathways for therapeutic benefit.

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