IKK promotes cytokine-induced and cancer-associated AMPK activity and attenuates phenformin-induced cell death in LKB1-deficient cells

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The 5′ AMP-activated protein kinase (AMPK) is an energy sensor that is activated upon phosphorylation of Thr^{172} in its activation loop by the kinase LKB1, CAMKK2, or TAK1. TAK1-dependent AMPK phosphorylation of Thr^{172} is less well characterized than phosphorylation of this site by LKB1 or CAMKK2. An important target of TAK1 is IκB kinase (IKK), which controls the activation of the transcription factor NF-κB. We tested the hypothesis that IKK acted downstream of TAK1 to activate AMPK by phosphorylating Thr^{172}. IKK was required for the phosphorylation of Thr^{172} in AMPK in response to treatment with the inflammatory cytokine IL-1β or TNF-α or upon TAK1 overexpression. In addition, IKK regulated basal AMPK Thr^{172} phosphorylation in several cancer cell types independently of TAK1, indicating that other modes of IKK activation could stimulate AMPK. We found that IKK directly phosphorylated AMPK at Thr^{172} independently of the tumor suppressor LKB1 or energy stress. Accordingly, in LKB1-deficient cells, IKK inhibition reduced AMPK Thr^{172} 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.

INTRODUCTION

AMPK [5′ adenosine monophosphate (AMP)–activated protein kinase] is a master energy-sensing kinase that phosphorylates various substrates to promote adenosine triphospate (ATP) conservation (*1*, *2*). AMPK appears to play a dual role in cancer depending on the context. AMPK can promote redox 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 mechanistic target of rapamycin (mTOR) signaling] to limit tumor growth (*3*). AMPK activity is regulated by phosphorylation of Thr^{172} in the kinase domain activation loop and allosterically by adenosine nucleotide binding (*4*–*6*). Binding of AMP to the AMPKγ subunit induces an increase in AMPK activity, and binding of either AMP or adenosine diphosphate (ADP) promotes Thr^{172} phosphorylation in the kinase domain by causing a conformational change in the AMPK holoenzyme that prevents phosphatases from accessing Thr^{172} (*5*, *6*). In cell-free kinase assays, allosteric binding of AMP to the AMPKγ subunit causes a 5-fold increase in AMPK activity, whereas phosphorylation of Thr^{172} causes a 100-fold increase in AMPK activity; thus, Thr^{172} phosphorylation is the major point of AMPK regulation (*4*, *7*–*9*). Three kinases phosphorylate AMPK at Thr^{172}: LKB1 (liver kinase B1), CAMKK2 (calcium/calmodulin-dependent protein kinase kinase 2), and TAK1 (transforming growth factor β–activated kinase 1) (*2*). The kinase upstream of AMPK depends on the signaling cascade or specific stressor. When the AMP/ATP ratio is increased in cells, a conformational shift occurs that makes Thr^{172} more amenable to phosphorylation by LKB1 (*10*–*12*). However, LKB1 is a tumor suppressor that is lost in many cancers, and these cancers still require AMPK for growth and survival (*3*), implying 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 Thr^{172} (*13*–*15*). In addition, cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) lead to AMPK Thr^{172} phosphorylation in a TAK1-dependent manner (*16*, *17*), although less is known about the mechanism by which TAK1 promotes AMPK Thr^{172} phosphorylation.

TAK1 is activated by inflammatory signals such as IL-1 and TNF and in turn activates several downstream pathways including the nuclear factor κB (NF-κB) transcription factor pathway, the c-Jun N-terminal kinase (JNK) pathway, the p38 pathway, and AMPK (*18*). 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 Ser^{177} and Ser^{181}, which lie within the IKKβ activation loop (*19*). The canonical IKK complex consists of two highly related kinases (IKKα and IKKβ) (*20*, *21*) and a scaffolding protein [IKKγ; also known as NF-κB essential modulator (NEMO)] (*22*, *23*). In addition to activation by TAK1, IKK can also be activated by trans-autophosphorylation especially in the presence of linear ubiquitin molecules (*24*). IKK was originally described as the kinase responsible for the inducible phosphorylation of IκBα (*25*). 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 (*26*). The exact mechanisms that lead IKK to phosphorylate one substrate over another are incompletely understood, but it likely involves NEMO, which can act as a substrate specificity factor (*22*). IKK promotes AMPK-dependent autophagy in an NF-κB–independent manner (*22*, *27*, *28*). Expression in HeLa cells (which are LBK1-deficient) of a form of IKKβ with phosphomimetic mutations at Ser^{177} and Ser^{181} is sufficient to induce AMPK Thr^{172} phosphorylation. This mutant IKK mimics constitutive phosphorylation by TAK1, suggesting that IKK could play a role downstream of TAK1 in activating AMPK, independently of LKB1 (*27*).

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Here, we explored a potential relationship between IKK and AMPK and demonstrated that IKK was required for basal and cytokine-induced AMPK Thr^{172} phosphorylation in various cell lines, regardless of LKB1 status. In response to the inflammatory cytokines IL-1β and TNF-α, IKKβ promoted AMPK Thr^{172} phosphorylation downstream of TAK1. However, we also identified several cancer cell lines in which IKK regulated AMPK independently of TAK1. Because IKK can regulate AMPK independently of TAK1, we assessed whether IKK directly phosphorylated AMPK. We found that IKK phosphorylates Thr^{172} in cell-free kinase assays and increased AMPK activity.

The mitochondrial complex I inhibitor phenformin causes energetic stress by rapidly depleting ATP (*29*), leading to activation of AMPK in an LKB1-dependent manner and, consequently, energy conservation and growth arrest. Cells that lack LKB1 undergo apoptosis in response to phenformin treatment, which led to the notion that phenformin could be used therapeutically to treat LKB1-deficient cancers (*29*, *30*). Because IKK was 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 Thr^{172} phosphorylation in LKB1-deficient cells upon treatment 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.

RESULTS

IKK is required for AMPK Thr^{172} phosphorylation induced by IL-1β

Two inducers of IKK activity, IL-1 and TNF, can increase AMPK Thr^{172} phosphorylation in serum-starved HeLa cells, which lack LKB1, the major kinase that phosphorylates AMPK Thr^{172} (*17*). Because IL-1 is a potent inducer of IKK activity, we tested whether IKK is required for AMPK Thr^{172} phosphorylation in response to IL-1β. Pretreatment with the IKK-specific inhibitor Compound A (Bay 65-1942) (*31*) prevented IL-1β from inducing AMPK Thr^{172} phosphorylation (Fig. 1A). Phosphorylation of AMPK at Thr^{172} mirrored the changes in the phosphorylation of IKK and amount of IκBα protein, which served as markers of IKK activity (Fig. 1A). (*20*). Compound A effectively inhibited IL-1β–induced AMPK Thr^{172} phosphorylation at 1 μM (fig. S1A).

To validate the findings with Compound A, we expressed either wild-type (WT) or dominant-negative, kinase dead (KD; K44A) IKKβ in HeLa cells. IL-1β induced AMPK Thr^{172} phosphorylation in cells expressing WT IKKβ but not in cells expressing KD IKKβ (Fig. 1B). Knockdown of the essential IKKβ scaffold protein NEMO by small interfering RNA (siRNA) also blocked IL-1β–induced AMPK Thr^{172} phosphorylation in the LKB1-deficient A549 cells (Fig. 1C). This finding indicated that the canonical IKK complex (which is defined by the presence of NEMO) was required for IL-1β–induced AMPK Thr^{172} phosphorylation. Because 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 Thr^{172} phosphorylation. WT mouse embryonic fibroblasts (WT MEFs) also showed an increase in AMPK Thr^{172} in response to IL-1β that was blocked by deficiency in IKKβ (Fig. 1D) or Compound A (fig. S1B), indicating that LKB1 loss is not required for IL-1β to induce IKK-dependent AMPK Thr^{172} phosphorylation. Although serum starvation has been reported to induce AMPK Thr^{172} phosphorylation (*32*), our data indicate that IL-1β induced AMPK Thr^{172} phosphorylation regardless of whether the cells were grown in complete media or starvation media (fig. S1C). This finding indicated that IL-1β can induce AMPK Thr^{172} in LKB1-deficient cells experiencing energy stress.

IL-1β promotes AMPK Thr^{172} and acetyl–coenzyme A carboxylase (ACC) Ser^{79} phosphorylation (*16*, *17*). To further validate that IL-1β induced the phosphorylation of downstream AMPK targets, we treated WT MEFs or *AMPKα1*$^{-/-}$, *AMPKα2*$^{-/-}$ knockout (*AMPK*$^{-/-}$) MEFs (*33*) with IL-1β. Treatment of WT MEFs with IL-1β for 20 min increased the amount of phosphorylation of the AMPK substrates ACC Ser^{79} and Unc-51–like autophagy activating kinase (ULK1) Ser^{555}, an effect not seen in *AMPK*$^{-/-}$ MEFs (Fig. 1E). IL-1β treatment for 30 min also induced ACC Ser^{79} phosphorylation in A549 cells, an effect that was blocked by Compound A (Fig. 1F).

An important consequence of AMPK activation is the induction of autophagy (*34*, *35*). IKK is involved in the induction of autophagy in response to rapamycin and nutrient deprivation (*27*), and IKK-mediated autophagy induction required AMPK, because knockdown of AMPK prevented constitutively active IKKβ from inducing autophagy (*27*). IL-1β can induce autophagy in macrophage and epithelial cell lines (*16*, *36*, *37*). IL-1β induces AMPK activity in LKB1-deficient A549 cells, and IL-1β increased the abundance of the autophagosome marker LC3-II (the more quickly migrating LC3 band) within 20 min of treatment in A549 cells (Fig. 1G). Pretreatment with Compound A (Fig. 1H) or the AMPK inhibitor dorsomorphin (fig. S1D) (*38*, *39*) 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 correlated with ACC Ser^{79} phosphorylation, a marker of AMPK activity (Fig. 1F). IL-1β also increased staining by a dye specific for acidified autophagosomes (Fig. 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 inhibitor of autophagosome acidification (*36*), also prevented IL-1β–induced staining (Fig. 1I). These findings indicated that the increased dye signal was due to increased autophagosome formation. These results were not due to direct off-target inhibition of AMPK by Compound A, because cell-free peptide-based assays showed that Compound A inhibited the kinase activity of recombinant IKKβ but not that of recombinant AMPK (figs. S1E and S2B). Together, the increase in phosphorylation of downstream AMPK targets and the increase in autophagosome formation in response to IL-1β indicate that the increased AMPK Thr^{172} 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

Pretreatment with the TAK1 inhibitor 5z-7-oxozeanol (*40*) prevented IL-1– or TNF-mediated induction of AMPK Thr^{172} phosphorylation to a similar extent as Compound A (Fig. 2A). These results indicated that AMPK activation in response to IL-1β and TNF-α occurred through TAK1-dependent canonical IL-1 and TNF receptor signaling pathways, respectively. Because IKK was required for cytokine-induced AMPK Thr^{172} phosphorylation and IKK is generally thought to be downstream of TAK1 in cytokine signaling cascades, we next explored whether IKK functioned downstream of TAK1 with respect

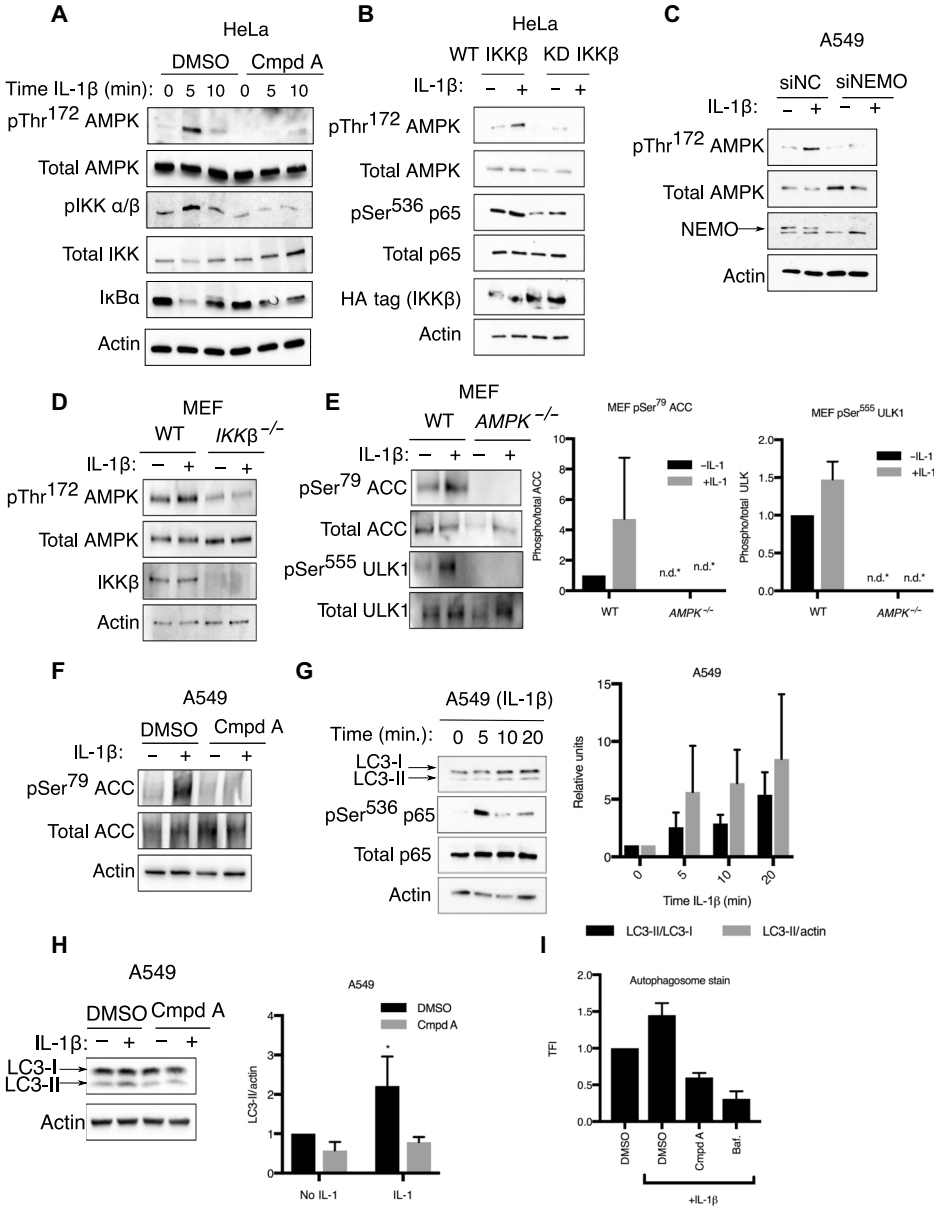

Fig. 1. AMPK Thr^{172} phosphorylation induced by inflammatory signals requires IKK. (**A**) HeLa cells were pretreated with either dimethyl sulfoxide (DMSO) or 5 μM Compound A (Cmpd A) and then treated with IL-1β (15 ng/ml) for the indicated time periods. Whole-cell lysates were then subjected to Western blot analysis using the indicated antibodies. Blots are representative of three independent experiments. (**B**) HeLa cells were transfected with either WT IKKβ or KD IKKβ and then treated with IL-1β (15 ng/ml) for 5 min. Whole-cell lysates were then subjected to Western blot analysis using the indicated antibodies. Blots are representative of two independent experiments. HA, hemagglutinin. (**C**) A549 cells were transfected with either noncoding siRNA (siNC) or siRNA targeting NEMO (siNEMO) and then stimulated with IL-1β (15 ng/ml) for 5 min and probed with the indicated antibodies. Blots are representative of three independent experiments. (**D**) WT or *IKKβ*$^{-/-}$ MEFs were treated with IL-1β for 5 min and immunoblotted with the indicated antibodies. Blots are representative of two independent experiments. (**E**) WT or *AMPK*$^{-/-}$ MEFs were treated with IL-1β for 30 min and then probed with antibodies recognizing ACC and ULK1 phosphorylated at their respective AMPK sites. Blots are representative of three independent biological experiments; the error bars represent the SEM of the normalized densitometry value measured for each experiment (n.d., not detected). (**F**) A549 cells were pretreated with either DMSO or Compound A for 15 min, then treated with IL-1β for 30 min, and then probed with antibodies recognizing ACC phosphorylated at the AMPK site. Blots are representative of three independent experiments. (**G**) A549 cells were treated with IL-1β (15 ng/ml) 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). A repeated measures linear mixed-effect model showed that there was a statistically significant trend of increasing LC3-II with time ($P = 0.027$). (**H**) A549 cells were pretreated with either with DMSO or 5 μM Compound A for 15 min and then with IL-1β for 20 min. Whole-cell extracts were then prepared and analyzed by Western blot for the indicated proteins. Blots are representative of three independent experiments. The error bars represent the SEM of the densitometry values obtained in each experiment. A two-way analysis of variance (ANOVA) was used to determine which groups were statistically different. *$P < 0.05$. (**I**) A549 cells were pretreated with DMSO, Compound A (5 μM), or Bafilomycin A1 (10 nM) for 15 min and then treated with IL-1β for 2 hours. The abundance of autophagosomes was assayed using a commercially available kit. Cells were then stained with a dye that is selective for acidified autophagosomes, and total fluorescence intensity (TFI) was measured. Error bars represent the SEM of the average total fluorescence measured in three independent experiments.

to AMPK Thr^{172} phosphorylation. Overexpression of TAK1 and the adaptor protein TAK1 binding protein 1 (TAB1) in HeLa cells induces AMPK Thr^{172} phosphorylation (*17*). Accordingly, in HeLa cells transfected with TAK1 and TAB1, treatment with Compound A reduced both basal and TAK1/TAB1-induced AMPK Thr^{172} phosphorylation (Fig. 2B). To further address the relationship among TAK1, IKKβ, and AMPK, IKKβ was inhibited in *TAK1*$^{-/-}$ MEFs (*41*). Knockdown of IKKβ or treatment with Compound A was sufficient to decrease AMPK Thr^{172} phosphorylation in *TAK1*$^{-/-}$ MEFs (Fig. 2C), indicating that IKKβ regulated AMPK Thr^{172} phosphorylation in the absence of TAK1. *TAK1*$^{-/-}$ MEFs had lower amounts of basal AMPK Thr^{172} phosphorylation than their WT counterparts, and the residual AMPK Thr^{172} phosphorylation in *TAK1*$^{-/-}$ MEFs was almost completely ablated by Compound A treatment. Similar to the effect of Compound A treatment, knockdown of IKKβ decreased AMPK Thr^{172} phosphorylation in *TAK1*$^{-/-}$ MEFs (Fig. 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 Thr^{172} phosphorylation. In this regard, there are other potential sources of IKK activation including trans-autocatalytic phosphorylation (induced by forced proximity) (*24*) or activation by an alternative upstream IKK kinase such as NF-κB–inducing kinase

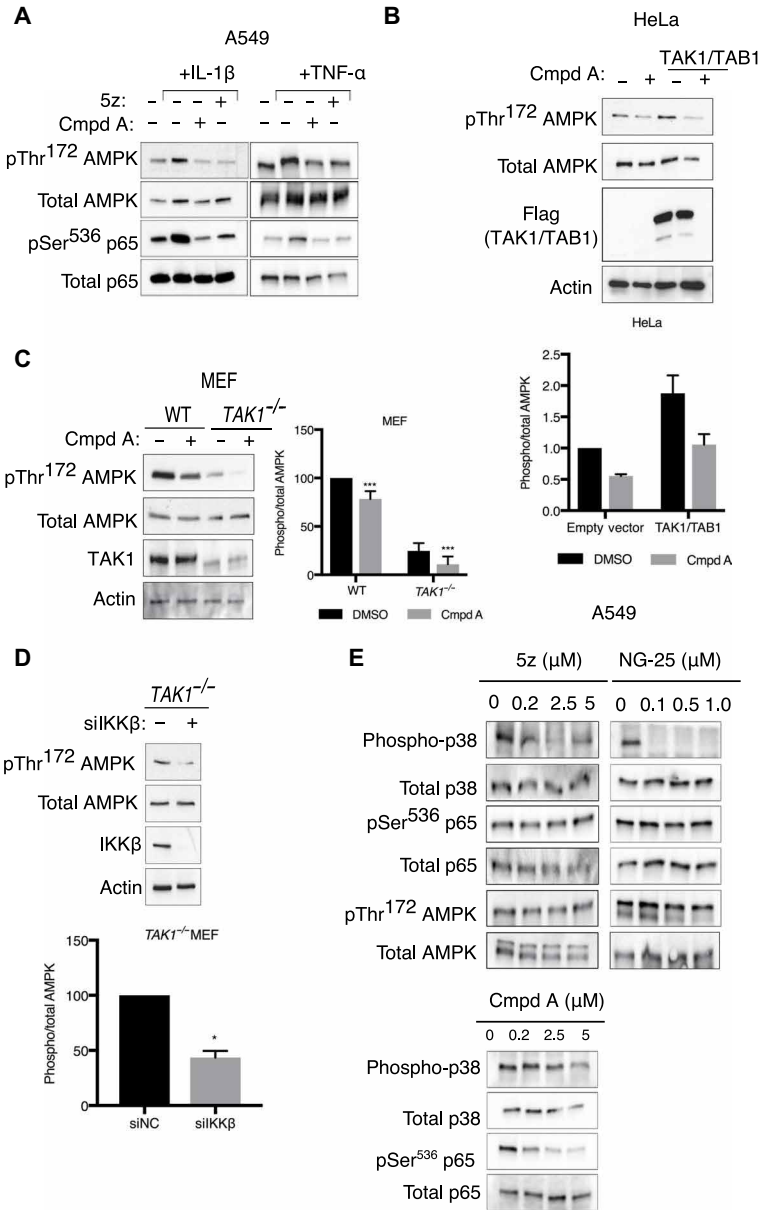

Fig. 2. IKK is downstream of TAK1 with respect to AMPK. (**A**) A549 cells were pretreated with either the IKK inhibitor Compound A (5 μM) or the TAK1 inhibitor 5z-7-oxozeanol (5z) (5 μM) for 15 min and then stimulated with IL-1β (15 ng/ml) or TNF-α (15 ng/ml) for 5 min. Blots are representative of three independent experiments. (**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 Compound A (5 μM) for 30 min. Whole-cell lysates were then subjected to Western blot analysis with the indicated antibodies. Blots are representative of three independent experiments. A two-way ANOVA showed no difference between Compound A or DMSO treatment in TAK1/TAB1 overexpressing cells compared to cells transfected with empty vector ($P = 0.82$). (**C**) WT or *TAK1*$^{-/-}$ MEFs were treated with Compound A (5 μM) for 1 hour, and then, whole-cell lysates were analyzed by Western blot for AMPK Thr^{172} phosphorylation and total AMPK. Blots are representative of three independent experiments. The error bars represent the SEM of the normalized densitometry values measured in each experiment. A two-way ANOVA was used to determine which groups were statically different. ***$P < 0.001$. (**D**) *TAK1*$^{-/-}$ MEFs were transfected with siRNA targeting murine IKKβ and blotted for the indicated proteins. Blots are representative of three independent experiments. Error bars represent the SEM of the normalized densitometry values measured in each experiment. A ratio paired *t* test was performed to determine whether the control and siIKKβ groups were significantly different. *$P < 0.05$. (**E**) A549 cells were treated for 30 min with either 5z-7-oxozeanol or NG-25 to inhibit TAK1 and immunoblotted with the indicated antibodies. A549 cells were treated with Compound A at the indicated concentrations for 30 min and blotted for phospho-p38 (used as a marker of TAK1 activity) or phospho-p65 (used as a marker of IKKβ activity). Blots are representative of three independent experiments.

(NIK) or MEKK3 (*42*). In contrast to MEFs in which much of the basal AMPK Thr^{172} phosphorylation depended on TAK1 (Fig. 2B), basal AMPK Thr^{172} phosphorylation in LKB1-deficient A549 cancer cells was independent of TAK1. Treatment of A549 cells with the TAK1 inhibitor NG-25 (*43*) or 5z-7-oxozeanol abolished phosphorylation of p38 (a marker of TAK1 pathway activation) (*44*) without affecting either AMPK Thr^{172} phosphorylation or p65 Ser^{536} phosphorylation (a marker of IKK activity; Fig. 2E). These findings indicated that the majority of the basal IKKβ and AMPK activity in these cells was independent of TAK1. In A549 cells, Compound A treatment abolished the phosphorylation of p65 without affecting the ratio of phospho-p38 to total p38 (Fig. 2E), indicating 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 Thr^{172} 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 (*23*). IKKα is also found as a homodimer in the noncanonical IKK complex (*25*, *45*). To determine the relative contributions of the canonical and noncanonical IKK complex in regulation of AMPK Thr^{172} phosphorylation, individual components of IKK were knocked down or inhibited. Knockdown of NEMO decreased AMPK Thr^{172} phosphorylation and downstream ACC Ser^{79} phosphorylation in A549 and MDA-MB-231 cells (Fig. 3A). Two distinct siRNAs targeting NEMO also inhibited AMPK Th^{172} phosphorylation in A549 cells, indicating that the effects of NEMO knockdown were not due to an off-target effect of the siRNA (fig. S2A). Knockdown of either IKKα or IKKβ in A549 cells also led to a decrease in AMPK Thr^{172} phosphorylation (Fig. 3B). However, knockdown of IKKβ decreased AMPK Thr^{172} phosphorylation and ACC Ser^{79} phosphorylation to a greater extent than that of IKKα, because only knockdown of IKKβ caused a statistically significant decrease in AMPK Thr^{172} phosphorylation. These results suggest that the canonical IKK complex plays a more important role in regulating AMPK Thr^{172} phosphorylation than the noncanonical IKK complex in the A549 cells. Similar to the knockdown experiments in A549 cells, *IKKβ*$^{-/-}$ MEFs displayed a greater decrease in AMPK Thr^{172} phosphorylation compared to *IKKα* $^{-/-}$ (Fig. 3C). To corroborate these observations, we used A549 (LKB1-deficient) and MDA-MB-231 (LKB1-positive) cells. Compound A treatment led to a dose-dependent decrease in AMPK Thr^{172} phosphorylation and ACC Ser^{79} phosphorylation in both cell lines (Fig. 3D).

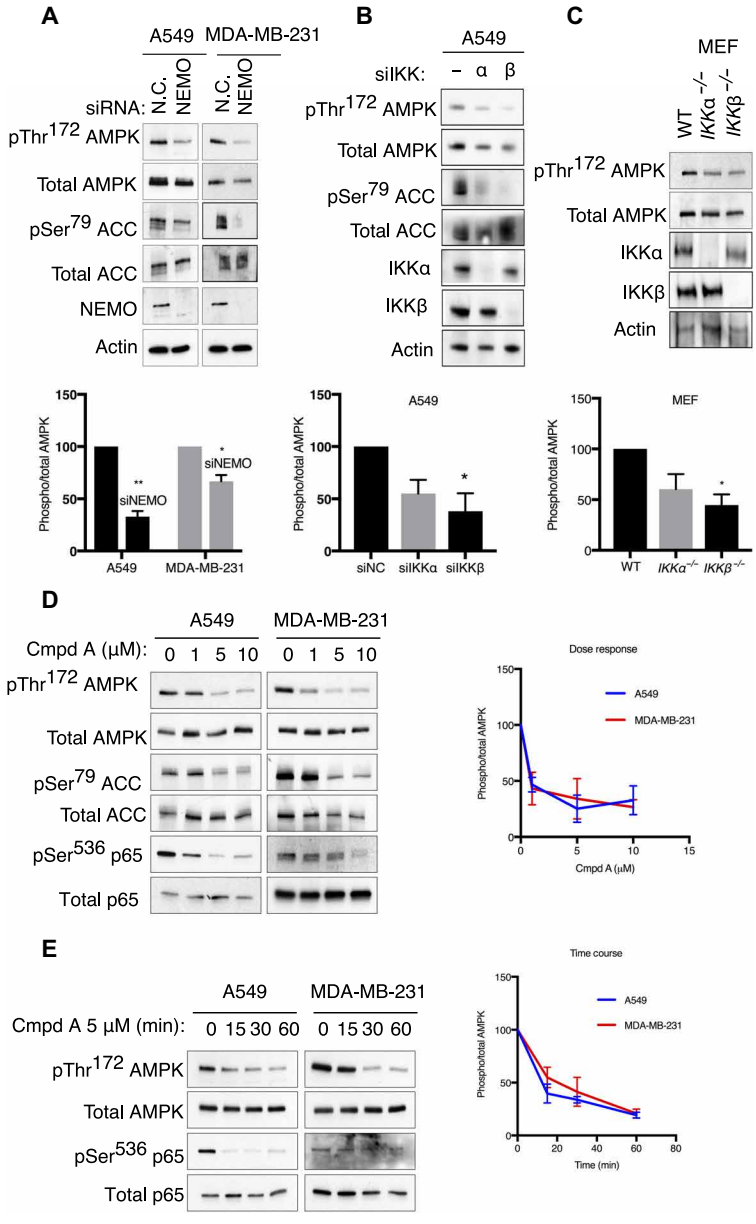

Fig. 3. IKK regulates basal AMPK Thr^{172} phosphorylation in cell lines. (**A**) A549 (LKB1-deficient) and MDA-MB-231 (expressing LKB1) cells were transfected with siRNA targeting NEMO or noncoding siRNA (N.C.) for 48 hours and immunoblotted with the indicated antibodies. Blots are representative of three independent experiments. Error bars represent the SEM of the normalized densitometry values measured for each experiment. A two-way ANOVA was used to determine which groups were statistically different. $^{**}P < 0.01$, $^{*}P < 0.05$. (**B**) A549 cells were treated with siRNA targeting either IKKα or IKKβ for 48 hours and immunoblotted as indicated. Blots are representative of three independent experiments. Error bars represent the SEM of the normalized densitometry values measured in each experiment. A two-way ANOVA was used to determine which groups were statically different. $^{*}P < 0.05$. (**C**) Whole-cell extracts from WT, *IKK*α$^{-/-}$, and *IKK*β$^{-/-}$ MEFs were analyzed by Western blot with antibodies for either AMPK phospho-Thr^{172} or total AMPK. Blots are representative of three independent experiments. Error bars represent the SEM of the normalized densitometry values measured in each experiment. A two-way ANOVA was used to determine which groups were statically different. $^{*}P < 0.05$. (**D**) A549 and MDA-MB-231 cells were grown in full medium [Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS)], treated with the indicated concentrations of Compound A for 1 hour and immunoblotted as indicated. Blots are representative of three independent experiments. Error bars represent the SEM of the normalized densitometry values measured in each experiment. (**E**) A549 and MDA-MB-231 cells were treated with Compound A (5 μM) for the indicated time periods and then analyzed by Western blot for AMPK Thr^{172} phosphorylation. Blots are representative of three independent experiments. Error bars represent the SEM of the normalized densitometry values measured in each experiment.

The effect of Compound A on AMPK Thr^{172} phosphorylation was not due to off-target inhibition of other kinases because Compound A did not affect AMPK Thr^{172} phosphorylation in *IKK*α$^{-/-}$, *IKK*β$^{-/-}$ double-knockout (*DKO*) MEFs (fig. S2B). Another IKK inhibitor, the NEMO-binding domain peptide (NBD peptide), which works through a distinct mechanism from Compound A (*46*), also decreased AMPK Thr^{172} phosphorylation in A549 cells (fig. S2C). In addition, Compound B, an inactive enantiomeric mixture of Compound A, did not affect AMPK Thr^{172} phosphorylation in A549 cells (fig. S2D), further supporting the specificity of Compound A toward AMPK Thr^{172} phosphorylation. Time course experiments demonstrated that AMPK Thr^{172} phosphorylation was decreased as soon as 15 min after Compound A treatment, and maximal inhibition occurred from 30 to 60 min (Fig. 3E). The rapid kinetics of inhibition of AMPK Thr^{172} phosphorylation mediated by Compound A suggested the possibility that IKK directly regulates this phosphorylation site.

IKK regulates AMPK Thr^{172} phosphorylation independently of changes in cellular energy status

AMPK activity is tightly regulated by changes in the ratio of ADP/ATP and AMP/ATP (*5*–*7*). An increase in the ratio of AMP/ATP or ADP/ATP is indicative of energetic stress and is generally associated with an increase in AMPK activity. Because IKK and NF-κB also regulate cellular metabolism in certain contexts (*47*, *48*), we sought to determine whether IKK inhibition affected the ADP/ATP and AMP/ATP ratios. Compound A treatment increased both the ratios of AMP/ATP and ADP/ATP (fig. S3A), which indicates energetic stress and would be expected to increase AMPK Thr^{172} phosphorylation in cells with LBK1 activity. However, at this time and concentration, Compound A decreased AMPK Thr^{172} phosphorylation in A549 cells (Fig. 3E). In addition, Compound A decreased Thr^{172} phosphorylation on an AMPK kinase domain mutant that cannot bind to AMP (fig. S3B) (*49*). These data indicate that the effects of Compound A on AMPK activity are independent of changes in ADP/ATP or AMP/ATP ratio.

IKK phosphorylates AMPK Thr^{172} in cell-free kinase assays and induces its activity

To determine whether IKK regulates AMPK through direct phosphorylation, we performed kinase assays using inactive recombinant AMPK trimers consisting of AMPKα1, AMPKβ1, and AMPKγ1 (*50*) and glutathione *S*-transferase (GST)–IKKβ (1–786). AMPKα1 incorporated γ^{32}P-ATP when incubated with IKKβ (fig. S4A). To map the IKK-dependent phosphorylation sites on AMPK, we subjected a similar reaction performed with cold ATP to mass spectrometry (MS).

Among the phosphopeptides identified was one that corresponded to phosphorylated Thr^{172} (fig. S4A). Mutation of Thr^{172} to alanine reduced incorporation of γ^{32}P-ATP into AMPKα1 in the presence of active IKKβ (fig. S4B). Western blotting confirmed that increasing amounts of IKKβ led to increased Thr^{172} phosphorylation on AMPK trimers (Fig. 4A).

Because phosphorylation of AMPK at Thr^{172} increases the kinase activity of AMPK, we predicted that IKKβ would increase AMPK activity. As measured by the SAMS peptide kinase assay, AMPK activity was increased after incubation with IKKβ (Fig. 4B). IKK also efficiently phosphorylated a KD mutant of AMPK (Fig. 4C), indicating that the IKKβ effect on AMPK Thr^{172} was independent of AMPK autocatalytic activity. We next compared the relative efficacy of IKKα and IKKβ relative to AMPK Thr^{172} phosphorylation. IKKβ phosphorylated about fivefold more AMPK Thr^{172} than IKKα in cell-free kinase assays (Fig. 4D). Moreover, IKKβ bound more effectively to the AMPK kinase domain than did IKKα, as evidenced by coprecipitation experiments in which a GST-tagged AMPK kinase domain was precipitated from HEK293T cells (Fig. 4E). These findings suggested that IKKβ was the dominant kinase for AMPK Thr^{172} within the canonical IKK complex. Compound A effectively inhibited IKKβ-mediated AMPK Thr^{172} phosphorylation but did not prevent either TAK1 or CAMKK2 (two other upstream AMPK kinases) from phosphorylating AMPK Thr^{172} (Fig. 4F). Thus, the effect of Compound A on AMPK Thr^{172} phosphorylation in cells is independent of inhibition of either TAK1 or CAMKK2.

IKK inhibition leads to reduced AMPK Thr^{172} phosphorylation in phenformin-treated LKB1-deficient cells and promotes apoptosis

Phenformin is an inhibitor of complex I of the mitochondria (*51*–*53*) and leads to ATP depletion (*29*). To counteract the action of phenformin, AMPK is activated to conserve ATP (*30*). Phenformin-induced AMPK activity depends largely 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 (*29*, *30*). Because IKK regulated AMPK in LKB1-deficient cells and IKK-mediated AMPK regulation was independent of energy status (fig. S1C), we asked whether IKK inhibition would further sensitize these cells to phenformin-induced apoptosis. We transfected A549 (LKB1-deficient) and MDA-MB-231 (LKB1-positive) cells with siRNA-targeting NEMO to inhibit IKK. Both A549 and MDA-MB-231 cells had lower basal AMPK Thr^{172} phosphorylation when NEMO was knocked down (Figs. 3A and 5A). Phenformin treatment induced AMPK Thr^{172} phosphorylation only in MDA-MB-231 cells, and this effect did not change in cells with NEMO knockdown (Fig. 5A). Accordingly, NEMO knockdown led to phenformin-induced apoptosis as measured by caspase 3/7 activity in A549 cells. In contrast, NEMO knockdown did not alter caspase 3/7 induction by phenformin in MDA-MB-231 cells (Fig. 5B). Similarly, Compound A treatment in combination with phenformin decreased AMPK Thr^{172} phosphorylation in A549 cells but not in MDA-MB-231 (Fig. 5C). This finding was consistent with previous work indicating that LKB1 is required for phenformin to induce AMPK Thr^{172} phosphorylation. These 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 drugs in a panel of LKB1-deficient (HeLa,

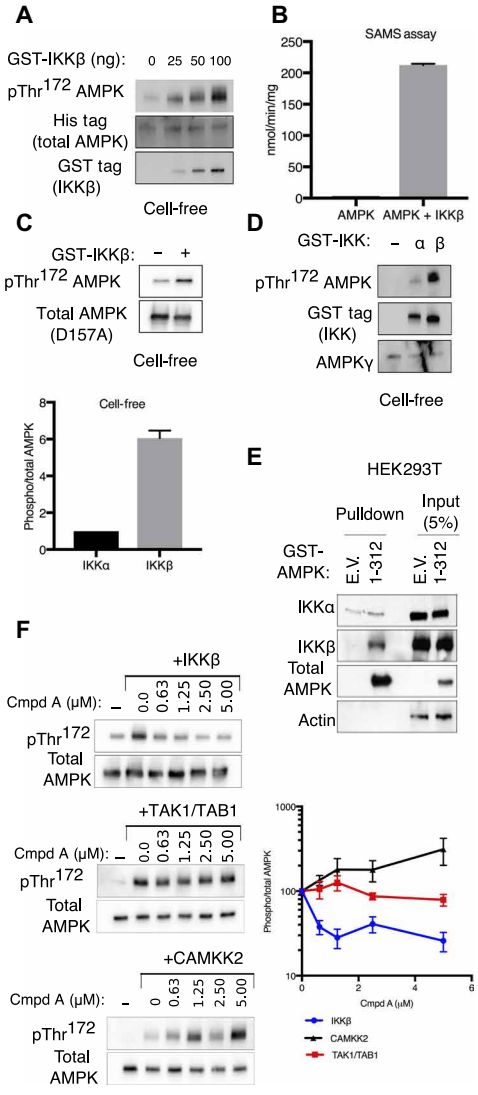

Fig. 4. IKK phosphorylates AMPK Thr^{172} in cell-free kinase assays and induces its activity. (**A**) Increasing amounts of GST-IKKβ were incubated with recombinant AMPKα1/β1/γ1 trimers in cell-free kinase assays and immunoblotted as indicated. Blots are representative of three independent experiments. (**B**) Cell-free kinase assays were performed as in (A) but using SAMS peptide as substrate. AMPK activity was calculated after subtraction of the blank reaction and the reaction with only IKKβ. This experiment was performed on three separate occasions. The graph represents the mean activity calculated from the three experiments, and the error bars represent the SEM. (**C**) A GST-tagged KD mutant of the AMPKα1 kinase domain (AMPK D157A) was purified from human embryonic kidney (HEK) 293T cells and then used as a substrate for cell-free kinase assays with active IKKβ. Blots are representative of three independent experiments. (**D**) Recombinant IKKα and IKKβ were incubated with AMPKα1/β1/γ1 trimers as in (A). The amount of AMPK Thr^{172} phosphorylation by IKKβ was normalized to the amount of AMPK Thr^{172} phosphorylation by IKKα and is displayed in the graph. The blot is representative of three independent experiments. The error bars represent the SEM of the normalized densitometry values measured in each experiment. (**E**) A GST-tagged construct of the AMPK kinase domain (amino acids 1 to 312) or with an empty vector (E.V.) was transfected into HEK293T cells and used to coprecipitate IKKα or IKKβ. Blots are representative of two independent experiments. (**F**) Effect of Compound A on the phosphorylation of AMPK Thr^{172} by recombinant IKKβ, TAK1/TAB1, or CAMKK2. Blots are representative of five independent experiments for each kinase, the ratio of phosphorylated to total AMPK is plotted in the graphs below, and the error bars represent the SEM of the normalized densitometry values measured in each experiment.

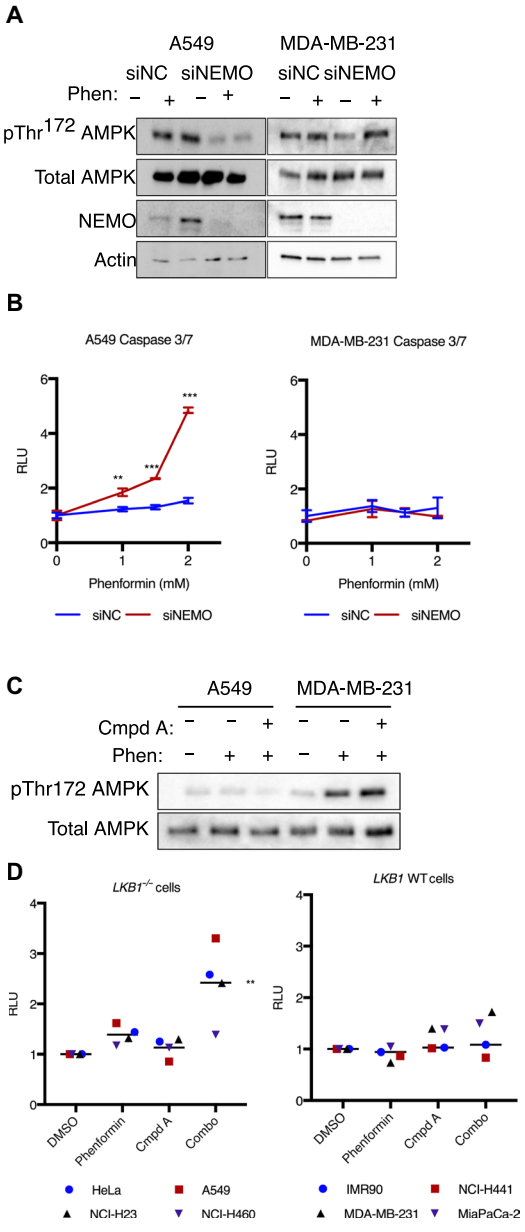

Fig. 5. IKK/AMPK provide resistance to phenformin in *LKB1*$^{-/-}$ cells. (**A**) A549 and MDA-MB-231 cells were transfected with either noncoding siRNA (siNC) or siRNA targeting NEMO (siNEMO), treated with phenformin (Phen.; 1 mM) for 18 hours, and immunoblotted as indicated. Blots are representative of three independent experiments. (**B**) A549 or MDA-MB-231 cells were pretreated with Compound A (5 μM) or DMSO for 15 min and then with phenformin (1 mM) for 45 min and immunoblotted as indicated. Blots are representative of three independent experiments. (**C**) A549 and MDA-MB-231 cells were transfected with either noncoding siRNA (siNC) or siRNA targeting NEMO (siNEMO) and treated with increasing concentrations of phenformin for 18 hours. Caspase 3/7 activity was measured ($n = 3$ biological replicates; the error bars represent the SEM; RLU is relative luminescence units). (**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 (1 mM), Compound A (5 μM), or both (Combo) for 18 hours. Caspase 3/7 activity of each cell line was measured. The average of three independent experiments is shown in the graph (a two-way ANOVA was performed to determine which groups were statistically different). $^{**}P < 0.01$.

A549, NCI-H23, and NCI-H460) or LKB1-positive cell lines (IMR90, NCI-H441, MDA-MB-231, and MiaPaca-2). One millimolar phenformin was used because this concentration was sufficient to induce AMPK Thr^{172} in LKB1-expressing cells but did not induce measurable 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. 5D). Together, these results indicate that IKK inhibition sensitizes LKB1-deficient cells to phenformin-induced apoptosis by decreasing AMPK Thr^{172} phosphorylation.

DISCUSSION

Three kinases phosphorylate AMPK Thr^{172}: LKB1, CAMKK2, and TAK1 (*2*). The molecular mechanisms and the biological context for the phosphorylation of AMPK Thr^{172} mediated by TAK1 are not as well understood as those mediated by LKB1 or CAMKK2. Here, we showed that IKK plays an important role downstream of TAK1 in regulating AMPK Thr^{172}.

Overexpression of TAK1 and the adaptor protein TAB1 is sufficient to promote AMPK phosphorylation. Moreover, TAK1/TAB1 fusion proteins can directly phosphorylate AMPK Thr^{172} in cell-free kinase assays (*16*, *17*, *54*). However, 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 (*54*). Stimuli such as IL-1β, TNF-α, and TRAIL that activate TAK1 lead to the induction of AMPK activity (*16*, *17*, *54*). Here, we provided evidence (Fig. 2A) that inhibition of TAK1 prevented these stimuli from inducing AMPK Thr^{172} phosphorylation. Collectively, these data suggest that cytokine-induced TAK1 activity leads to direct phosphorylation of AMPK Thr^{172} by TAK1. Expression of a constitutively active IKKβ mutant that mimics TAK1 phosphorylation leads to an increase in AMPK Thr^{172} phosphorylation in HeLa cells, which are LKB1-deficient (*27*). This result suggested a potential role for IKKβ downstream of TAK1 in regulating AMPK. Here, we demonstrated that IKK activated AMPK downstream of TAK1 in response to IL-1β or TNF-α.

The canonical IKK complex, which is defined by the presence of the scaffolding protein NEMO (*25*), promoted AMPK activity in response to IL-1β and TNF-α (Figs. 1, A to F, and 2A). In addition to NEMO, the canonical IKK complex consists of two related protein kinases, IKKα and IKKβ, which show variable activity toward different substrates in the NF-κB pathway. For example, IKKβ has higher activity toward IκBα than IKKα, but IKKα has a higher activity toward p100 than IKKβ (*55*). With respect to AMPK, IKKβ had a fivefold greater activity toward AMPK Thr^{172} than IKKα (Fig. 4D). This could be because IKKβ shows more kinase activity toward AMPK Thr^{172} or because IKKβ is directed to and binds AMPK more efficiently than IKKα, as was observed with GST pulldowns in HEK293T cells (Fig. 4E). These findings suggested that it was IKKβ within the canonical complex that directly phosphorylated AMPK Thr^{172}. However, knockdown of IKKα in A549 cells or in knockout of IKKα in MEFs led to a decrease in the phosphorylation of this site. Possible explanations include regulation of the canonical IKK complex by the noncanonical IKK complex or regulation of IKKβ activity within the canonical IKK complex by IKKα, either through phosphorylation of serine residues in the IKKβ activation loop or stabilization of the canonical complex. Future studies are necessary to determine whether stimuli [such as receptor activator of NF-κB (RANK) ligand or CD40 ligand]

that specifically activate the noncanonical IKKα complex promote activation of AMPK. Although IKKα showed less activity toward AMPK Thr^{172} than IKKβ, it is still possible that a stimulus that specifically stimulates IKKα could promote AMPK activity.

The IKK/NF-κB pathway promotes its own feedback to limit the response to IL-1β and TNF-α, notably by inducing the transcription of IκBα (*56*). Activation of AMPK may be another way to limit NF-κB signaling in response to these cytokines, indicated by several studies (*57*, *58*). TNF-α–induced apoptosis is opposed by AMPK (*59*), which 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 promoted autophagosome formation in an IKK-dependent manner in A549 cells, which lack LKB1. IL-1 also promotes autophagy in breast epithelial cells (*16*) and in macrophages (*37*), which both express LKB1. IKK is involved in autophagy induction (*22*) by promoting the transcription of autophagic genes (*28*) and the formation of autophagosomes in response to several autophagy-inducing stimuli. Knockdown of AMPK prevented the induction of autophagosomes by overexpression of constitutively active IKK, suggesting that AMPK is downstream of IKK with respect to autophagy (*27*), specifically, through the direct phosphorylation of AMPK Thr^{172} by IKK.

We also demonstrated that IKK regulated basal AMPK activity independently of TAK1, because inhibition of IKK in *TAK1*$^{-/-}$ MEFs led to a decrease in AMPK Thr^{172} phosphorylation. In addition to TAK1-mediated phosphorylation, IKKβ can also be activated by trans-autophosphorylation, which is induced by forced proximity in the presence of linear ubiquitin molecules (*24*), or another upstream signal, such as from MEKK3 or NIK (*42*). Regardless of the source of the residual IKK activity, inhibiting IKK lowered basal AMPK Thr^{172} phosphorylation in the absence of TAK1 activity (Fig. 2, C and D). This effect was similar to how basal IKK activity in A549 cells was independent of TAK1 activity (Fig. 3E).

The loss of LKB1 in cancer not only promotes tumor growth but also leaves the cells vulnerable to metabolic stress, such as with treatment with the mitochondrial complex I inhibitor phenformin (*29*, *30*). However, little progress has been made in the clinic toward exploiting this vulnerability. This could be because LKB1-deficient cancer cells promote basal AMPK phosphorylation through IKK to buffer against energetic stress. Here, we demonstrated that IKK inhibition in LKB1-deficient cells led to decreased phosphorylation of AMPK Thr^{172}, even when the cells were treated with phenformin (Fig. 5, A and B). This decrease in AMPK Thr^{172} phosphorylation was associated with apoptosis, as measured by caspase 3/7 activity (Fig. 5, C and D). In cells with LKB1, IKK did not affect the ability of phenformin to induce AMPK Thr^{172} phosphorylation. Phenformin alone or in combination with IKK inhibition did not induce apoptosis in cells with LKB1. A future therapeutic strategy might be to combine IKK inhibition with phenformin treatment in LKB1-deficient tumors.

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 1640 (Gico), depending on American Type Culture Collection recommendations, supplemented with 10% FBS (Sigma), penicillin (100 U/ml), streptomycin (100 μg/ml; Gibco), and 1 mM glutamine. Immortalized MEFs of the indicated genotypes were described previously (*28*). 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. Compound A (Bay65-1942) and Compound B (both provided by Bayer) 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. Phenformin hydrochloride was purchased from Sigma-Aldrich. Recombinant IL-1β was purchased from Peprotech, and recombinant TNF-α was purchased from Promega. The NBD peptide was synthesized by American Peptide.

siRNA transfections

ON-TARGETplus SMARTpool siRNA or individual siRNAs (all siRNAS were pooled unless otherwise indicated) and a negative control siRNA were purchased from Dharmacon (Thermo Scientific Pierce). Each pool was reconstituted in 1× siRNA buffer (Dharmacon) and diluted in diethyl pyrocarbonate–treated water to a final concentration of 20 μM. Briefly, 1.5×10^5 cells were plated onto 10-cm 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 min at room temperature and added to the cells in serum-free media. Cells were harvested 48 to 72 hours after transfection for protein extraction preparation.

Plasmid transfections

Plasmids were transfected using the X-tremeGENE Transfection Reagent (Roche Life Science) according to the manufacturer's protocol. Mutations were confirmed by Sanger sequencing at the University of North Carolina (UNC) Genome Analysis Core. The AMPK kinase domain pEBG-AMPKα1(1–312) (Addgene plasmid #27632). KD AMPK 1 to 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 plasmid #82274) into the pDEST-27 to add a GST tag using Gateway Cloning (Invitrogen). Thr^{172} was then mutated to alanine using a quick-change mutagenesis protocol. The WT and KD mutant (K44A) IKKβ, as well as the TAK1 and TAB1 plasmids, were gifts from L. Cantley (Meyer Cancer Center, Weill Cornell Medical College).

Kinase assays

Kinase assays were performed as described previously (*60*). For the kinase assays using recombinant proteins, AMPK trimers purified from *Escherichia coli* were used as previously described (*50*) (a gift from J. Brenman, UNC Chapel Hill). Purified GST-tagged IKKα and IKKβ from Sf9 insect cells were obtained from SignalChem. The kinase buffer contained 1 mM β-glycerolphosphate, 20 mM tris (pH 7.4), 12 mM $MgCl_2$, and 100 μM cold ATP. γ^{32}P-ATP was added where indicated. The reactions were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and either transferred to a nitrocellulose membrane for Western blotting (see below) or Coomassie-stained for MS. The MS 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. Phosphopeptides were enriched using titanium dioxide and then subjected to liquid chromatography–MS (LC-MS) using an LTQ Orbitrap Velos (Thermo Scientific) ion trap mass spectrometer. Peptides were identified using

Mascot Software. For the SAMS peptides assays (*61*), the resulting kinase reactions were mixed with SAMS peptide for 15 min and then spotted on squares of p81 phosphocellulose paper. Each square was washed three times in 1% phosphoric acid and 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 100 μM ATP and 5 μM Compound A (*62*). Active AMPKα1, AMPKβ1, or AMPKγ1, as well as the SAMS peptide and the IκBα peptide were obtained from Medical Research College Protein Phosphorylation and Ubiquitylation Unit Reagents and Services facility [MRC PPU, College of Life Sciences, University of Dundee, Scotland (https://mrcppureagents.dundee.ac.uk)].

Western blotting

Whole-cell protein extraction was performed by scraping the cells in cold 1% NP-40 buffer [20 mM tris (pH 7.6), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and 1% Igepal]. Protein concentrations were determined using the Bradford Assay (Bio-Rad). Protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare Life Sciences), blocked for 1 hour in 1× TBST (tris-buffered saline 1% Tween) 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 Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences), or the Bio-Rad ChemiDoc 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 were 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 pulldowns

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 three times 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 (5 μM) for 30 min. 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 by Johnsen *et al.* (*63*). Known amounts of stable isotopes of ATP, ADP, and AMP (purchased from MilliporeSigma) were added to each sample, the samples were subjected to high-performance LC (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 per 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, and then, the total fluorescence intensity was measured using a 96-well plate reader. The excitation and emission were 360 and 520 nm, respectively.

Caspase assays

The Caspase-Glo reagent was purchased from Promega and used according to 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 and placed on a shaker at room temperature for 30 min, and then, the luminescence was read using a plate reader.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/11/538/eaan5850/DC1

Fig. S1. Further characterization of the effect of IL-1 and IKK on AMPK signaling.

Fig. S2. Validation of Compound A and siRNA specificity.

Fig. S3. IKK regulates AMPK independently of changes in energy status.

Fig. S4. Identification of AMPK Thr^{172} phosphorylation by MS.

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Acknowledgments: We would like to thank J. Brenman for the recombinant AMPK. We would also like to acknowledge the thoughtful discussions and feedback from members of the laboratory, especially H.-C. Hsia, C. Lawrence, and M. Tegowski. We would like to thank X. Tan for his assistance with the statistical analysis. **Funding:** R.J.A. was supported by predoctoral fellowships from the NIH T32 Training Grant (T32CA071341-17) and an American Heart Association predoctoral fellowship. Research funding is derived from NIH/NCI grant R35CA197684 and support from the Waxman Cancer Research Foundation to A.S.B. **Author contributions:** A.S.B. and R.J.A. conceived the study, designed the experiments, and wrote the paper. R.J.A. performed the experiments and analyzed the resulting data. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications database (PRIDE) (*64*) partner repository with the data set identifier PXD009897. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

Submitted 15 May 2017
Accepted 18 June 2018
Published 10 July 2018
10.1126/scisignal.aan5850

Citation: R. J. Antonia, A. S. Baldwin, IKK promotes cytokine-induced and cancer-associated AMPK activity and attenuates phenformin-induced cell death in LKB1-deficient cells. *Sci. Signal.* **11**, eaan5850 (2018).