Published in final edited form as:

Sci Signal. ; 11(538): . doi:10.1126/scisignal.aan5850.

IKK Promotes Cytokine-Induced and Cancer-Associated AMPK Activity and Attenuates Phenformin-Induced Cell Death in LKB1-Deficient Cells

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Abstract

The 5' AMP-activated protein kinase (AMPK) is an energy sensor that is activated upon phosphorylation of Thr¹⁷² in its activation loop by the kinases LKB1, CAMKK2, or TAK1. TAK1-dependent AMPK phosphorylation of Thr¹⁷² is less well-characterized than phosphorylation of this site by LKB1 or CAMKK2. An important target of TAK1 is I κ B kinase (IKK) which controls the activation of the transcription factor NF- κ B. Here, we tested the hypothesis that IKK acted downstream of TAK1 to activate AMPK by phosphorylating Thr¹⁷². IKK was required for phosphorylation of Thr¹⁷² in AMPK in response to treatment with the inflammatory cytokines IL-1 β or TNF- α or upon TAK1 overexpression. Additionally, IKK regulated basal AMPK Thr¹⁷² 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 Thr¹⁷² independently of the tumor suppressor LKB1 or energy stress. Accordingly, in LKB1-deficient cells, IKK inhibition reduced 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.

Editor's summary:

An inflammatory way to activate AMPK

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Author contributions:

A.S.B. and R.J.A. conceived the study and designed the experiments. R.J.A. performed the experiments and analyzed the resulting data. R.J.A. and A.S.B. wrote the paper.

Competing interests: The authors have no competing interests to declare.

Data and materials availability:

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(64) partner repository with the dataset identifier PXD009897.

All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

In its best-known function as a sensor of energy status, the kinase AMPK is activated by phosphorylation mediated by the tumor suppressor LKB1. Antonia and Baldwin characterized a different pathway for AMPK activation involving TAK1, a kinase associated with inflammatory pathways. They found that this process required phosphorylation of AMPK by the TAK1 target IKK and that it occurred independently of LKB1 and, in certain cell lines, independently of TAK1 as well, indicating that TAK1-independent pathways that activate IKK could also stimulate AMPK. Combining an IKK inhibitor with the cancer drug phenformin improved its ability to kill LKB1-deficient cancer cells, highlighting a new potential treatment for cancers lacking this tumor suppressor.

One-sentence summary:

AMPK activity is stimulated by IKK-mediated phosphorylation in cancer cells and downstream of inflammatory cytokines.

Introduction

AMPK (5' AMP-activated protein kinase) is a master energy sensing kinase that phosphorylates various substrates to promote 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 mTOR signaling) to limit tumor growth (3). AMPK activity is regulated by phosphorylation of Thr¹⁷² in the kinase domain activation loop and allosterically by adenosine nucleotide binding (4–6). Binding of AMP to the AMPK γ subunit 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 (5, 6). 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 (4, 7–9). Three kinases 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)(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 Thr¹⁷² 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 Thr¹⁷² (13–15). Additionally, cytokines such as IL-1, TNF and TRAIL lead to AMPK Thr¹⁷² phosphorylation in a TAK1-dependent manner (16, 17), although less is known about the mechanism by which 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- κ B transcription factor pathway, the c-Jun

N-terminal kinase (JNK) pathway, p38 pathway and AMPK (18). To activate p38 and JNK pathways, TAK1 phosphorylates mitogen activated protein kinases (MAPKs). To activate the NF- κ B pathway, TAK1 directly phosphorylates I κ B Kinase (IKK) to promote its activity. TAK1 promotes NF- κ B dependent transcription by directly phosphorylating IKK at Ser¹⁷⁷ and Ser¹⁸¹ which lie within the IKK β activation loop (19). The canonical IKK complex consists of two highly related kinases IKK α and IKK β (20, 21), and a scaffolding protein IKK γ (also known as 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 I κ B α (25). Phosphorylation of I κ B α by IKK leads to its rapid proteasome-mediated degradation, which in turn allows NF- κ B transcription factors to enter the nucleus and promote transcription. Although IKK is best known for its role in promoting the NF- κ B transcription activation pathway, NF- κ B 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- κ B-independent manner (22, 27, 28). Expression in HeLa cells (which are LKB1 deficient) of a form of IKK β with phosphomimetic mutations at Ser¹⁷⁷ and Ser¹⁸¹ is sufficient to induce AMPK Thr¹⁷² 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).

In this study we explored a potential relationship between IKK and AMPK, and demonstrated that IKK was required for basal and cytokine-induced AMPK Thr¹⁷² phosphorylation in various cell lines, regardless of LKB1 status. In response to the inflammatory cytokines IL-1 β and TNF- α , IKK β promoted AMPK Thr¹⁷² 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 Thr¹⁷² 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 Thr¹⁷² 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 Thr¹⁷² phosphorylation induced by IL-1 β

Two inducers of IKK activity, IL-1 and TNF, can increase AMPK Thr¹⁷² phosphorylation in serum-starved HeLa cells, which lack LKB1, the major kinase that phosphorylates AMPK Thr¹⁷² (17). Because IL-1 is a potent inducer of IKK activity, we tested whether IKK is required for AMPK Thr¹⁷² phosphorylation in response to IL-1 β . Pre-treatment with the IKK specific inhibitor Compound A (Bay 65–1942) (31) prevented IL-1 β from inducing AMPK Thr¹⁷² phosphorylation (Fig. 1A). Phosphorylation of AMPK at Thr¹⁷² mirrored the changes in the phosphorylation of IKK and amount of I κ B α protein, which served as markers of IKK activity (Fig 1A). (20). Compound A effectively inhibited IL-1 β -induced AMPK Thr¹⁷² phosphorylation at 1 μ M (Fig. S1A).

To validate the findings with Compound A, we expressed either wild type (WT) or dominant negative, kinase dead (KD, K44A) IKK β in HeLa cells. IL-1 β induced AMPK Thr¹⁷² phosphorylation in cells expressing WT IKK β but not in cells expressing KD IKK β (Fig. 1B). Knockdown of the essential IKK β scaffold protein NEMO by siRNA also blocked IL-1 β -induced AMPK Thr¹⁷² 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-1 β induced AMPK Thr¹⁷² 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-1 β to induce IKK-dependent AMPK Thr¹⁷² phosphorylation. Wild type mouse embryonic fibroblasts (*WT*MEFs) also showed an increase in AMPK Thr¹⁷² in response to IL-1 β that was blocked by deficiency in IKK β (Fig. 1D) or Compound A (Fig. S1B), indicating that LKB1 loss is not required for IL-1 β to induce IKK-dependent AMPK Thr¹⁷² phosphorylation. Although serum starvation have been reported to induce AMPK Thr¹⁷² phosphorylation(32), our data indicate that IL-1 β induced AMPK Thr¹⁷² phosphorylation regardless of whether the cells were grown in complete media or starvation media (Fig. S1C). This finding indicated that IL-1 β can induce AMPK Thr¹⁷² in LKB1-deficient cells experiencing energy stress.

IL-1 β promotes AMPK Thr¹⁷² and ACC Ser⁷⁹ phosphorylation (16, 17). To further validate that IL-1 β induced the phosphorylation of downstream AMPK targets we treated *WT*MEFs or *AMPK α 1*^{-/-}, *AMPK α 2*^{-/-} knockout (*AMPK*^{-/-}) MEFs (33) with IL-1 β . Treatment of *WT*MEFs with IL-1 β for 20 min increased the amount of phosphorylation of the AMPK substrates ACC Ser⁷⁹ and ULK Ser⁵⁵⁵, an effect not seen in *AMPK*^{-/-} MEFs (Fig. 1E). IL-1 β treatment for 30 min also induced ACC Ser⁷⁹ 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 IKK β from inducing autophagy(27). IL-1 β can induce autophagy in macrophage and epithelial cell lines (16, 36, 37). IL-1 β induces AMPK activity in LKB1-deficient A549 cells, and IL-1 β increased the abundance of the autophagosome marker LC3-II (the more quickly migrating LC3 band) within 20 minutes of

treatment in A549 cells (Fig. 1G). Pre-treatment with Compound A (Fig. 1H) or the AMPK inhibitor Dorsomorphin (38, 39) (Fig. S1D) prevented LC3-II induction upon treatment with IL-1 β , indicating that IL-1 β -induced autophagosome formation required IKK and AMPK activity. The changes in LC3 mobility correlated with ACC Ser⁷⁹ phosphorylation, a marker of AMPK activity (Fig. 1F). IL-1 β 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-1 β to induce autophagosome formation. Bafilomycin-A1, an inhibitor of autophagosome acidification(36), also prevented IL-1 β 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 IKK β , but not that of recombinant AMPK (Fig. S1E and Fig. S2B). Together, the increase in phosphorylation of downstream AMPK targets and the increase in autophagosome formation in response to IL-1 β indicate that the increased AMPK Thr¹⁷² phosphorylation in response to IL-1 β 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

Pre-treatment with the TAK1 inhibitor 5z-7-oxozeanol (40) prevented IL-1- or TNF-mediated induction of AMPK Thr¹⁷² phosphorylation to a similar extent as Compound A (Fig. 2A). These results indicated that AMPK activation in response to IL-1 β and TNF- α occurred through TAK1-dependent canonical IL-1 and TNF receptor signaling pathways, respectively. Because 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 functioned downstream of TAK1 with respect to AMPK Thr¹⁷² phosphorylation. Overexpression of TAK1 and the adaptor protein TAB1 in HeLa cells induces AMPK Thr¹⁷² phosphorylation (17). Accordingly, in HeLa cells transfected with TAK1 and TAB1, treatment with Compound A reduced both basal and TAK1/TAB1-induced AMPK Thr¹⁷² phosphorylation (Fig. 2B). To further address the relationship between TAK1, IKK β and AMPK, IKK β was inhibited in *TAK1*^{-/-} MEFs (41). Knockdown of IKK β or treatment with Compound A was sufficient to decrease AMPK Thr¹⁷² phosphorylation in *TAK1*^{-/-} MEFs (Fig. 2C), indicating that IKK β regulated AMPK Thr¹⁷² phosphorylation in the absence of TAK1. *TAK1*^{-/-} MEFs had lower amounts of basal AMPK Thr¹⁷² phosphorylation than their *WT* counterparts and the residual AMPK Thr¹⁷² phosphorylation in *TAK1*^{-/-} MEFs was almost completely ablated by Compound A treatment. Similar to the effect of Compound A treatment, knockdown of IKK β decreased AMPK Thr¹⁷² 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 Thr¹⁷² 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 NIK or MEKK3 (42). In contrast to MEFs in which much of the basal AMPK Thr¹⁷² phosphorylation depended on TAK1 (Fig. 2B), basal

AMPK Thr¹⁷² phosphorylation in LKB1-deficient A549 cancer cells was independent of TAK1. Treatment of A549 cells with the TAK1 inhibitors NG-25 (43) or 5z-7-oxozeanol abolished phosphorylation of p38 (44) (a marker of TAK1 pathway activation) without affecting either AMPK Thr¹⁷² phosphorylation or p65 Ser⁵³⁶ phosphorylation (a marker of IKK activity) (Fig. 2E). These findings indicated that the majority of the basal IKK β 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 Thr¹⁷² phosphorylation in cancer cell lines

The canonical IKK complex is a high molecular weight complex containing the kinases IKK α and IKK β bound to the scaffold protein NEMO (23). IKK α is also found as a homodimer in the non-canonical IKK complex (25, 45). 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 decreased AMPK Thr¹⁷² phosphorylation and downstream ACC Ser⁷⁹ phosphorylation in A549 and MDA-MB-231 cells (Fig. 3A). Two distinct siRNAs targeting NEMO also inhibited AMPK Thr¹⁷² 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 IKK α or IKK β in A549 cells also led to a decrease in AMPK Thr¹⁷² phosphorylation (Fig. 3B). However, knockdown of IKK β decreased AMPK Thr¹⁷² phosphorylation and ACC Ser⁷⁹ phosphorylation to a greater extent than that of IKK α , because only knockdown of IKK β 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. Similar to the knockdown experiments in A549 cells, *IKK β ^{-/-}* MEFs displayed a greater decrease in AMPK Thr¹⁷² phosphorylation compared to *IKK α ^{-/-}* (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 Thr¹⁷² phosphorylation and ACC Ser⁷⁹ phosphorylation in both cell lines (Fig. 3D). The effect of Compound A on AMPK Thr¹⁷² phosphorylation was not due to off-target inhibition of other kinases because Compound A did not affect AMPK Thr¹⁷² 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 Thr¹⁷² phosphorylation in A549 cells (Fig. S2C). In addition, Compound B, an inactive enantiomeric mixture of Compound A, did not affect AMPK Thr¹⁷² phosphorylation in A549 cells (Fig. S2D), further supporting the specificity of Compound A towards AMPK Thr¹⁷² phosphorylation. Time course experiments demonstrated that AMPK Thr¹⁷² phosphorylation was decreased as soon as 15 minutes after Compound A treatment and maximal inhibition occurred from 30 to 60 minutes (Fig. 3E). The rapid kinetics of inhibition of AMPK Thr¹⁷² phosphorylation mediated by Compound A suggested the possibility that IKK directly regulates this phosphorylation site.

IKK regulates AMPK Thr¹⁷² 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, 6) (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- κ B 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 Thr¹⁷² phosphorylation in cells with LKB1 activity. However, at this time and concentration, Compound A decreased AMPK Thr¹⁷² phosphorylation in A549 cells (Fig. 3E). In addition, Compound A decreased Thr¹⁷² phosphorylation on an AMPK kinase domain mutant that cannot bind to AMP (49) (Fig. S3B). 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 through direct phosphorylation, we performed kinase assays using inactive recombinant AMPK trimers consisting of AMPK α 1, AMPK β 1 and AMPK γ 1 (50) and GST-IKK β (1–786). AMPK α 1 incorporated ³²P- γ -ATP when incubated with IKK β (Fig. S4A). To map the IKK-dependent phosphorylation sites on AMPK, a similar reaction performed with cold ATP was subjected to mass spectrometry. Among the phosphopeptides identified was one that corresponded to phosphorylated Thr¹⁷² (Fig. S4A). Mutation of Thr¹⁷² to alanine reduced incorporation of ³²P- γ -ATP into AMPK α 1 in the presence of active IKK β (Fig. S4B). Western blotting confirmed that increasing amounts of IKK β led to increased Thr¹⁷² phosphorylation on AMPK trimers (Fig 4A).

Because phosphorylation of AMPK at Thr¹⁷² increases the kinase activity of AMPK, we predicted that IKK β would increase AMPK activity. As measured by the SAMS peptide kinase assay, AMPK activity was increased after incubation with IKK β (Fig. 4B). IKK also efficiently phosphorylated a kinase-dead mutant of AMPK (Fig. 4C) indicating that the IKK β effect on AMPK Thr¹⁷² was independent of AMPK autocatalytic activity. We next compared the relative efficacy of IKK α and IKK β relative to AMPK Thr¹⁷² phosphorylation. IKK β phosphorylated approximately 5-fold more AMPK Thr¹⁷² than IKK α in cell-free kinase assays (Fig. 4D). Moreover, IKK β bound more effectively to the AMPK kinase domain than did IKK α , as evidenced by coprecipitation experiments in which a GST-tagged AMPK kinase domain was precipitated from HEK293T cells (Fig. 4E). These findings suggested that IKK β was the dominant kinase for AMPK Thr¹⁷² within the canonical IKK complex. Compound A effectively inhibited IKK β -mediated AMPK Thr¹⁷² phosphorylation, but did not prevent either TAK1 or CAMKK2 (two other upstream AMPK kinases) from phosphorylating AMPK Thr¹⁷² (Fig. 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 reduced AMPK Thr¹⁷² 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 because 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 cells (LKB1-positive) with siRNA targeting NEMO to inhibit IKK. Both A549 cells and MDA-MB-231 cells had lower basal AMPK Thr¹⁷² phosphorylation when NEMO was knocked down (Fig 3A and Fig 5A). Phenformin treatment induced AMPK Thr¹⁷² 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 Thr¹⁷² 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 Thr¹⁷² 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, A549, NCI-H23, and NCI-H460) or LKB1-positive cell lines (IMR90, NCI-H441, MDA-MB-231 and MiaPaca-2). 1mM phenformin was used because this concentration was sufficient to induce AMPK Thr¹⁷² 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 Thr¹⁷² phosphorylation.

Discussion

Three kinases phosphorylate AMPK Thr¹⁷²: LKB1, CAMKK2 and TAK1 (2). The molecular mechanisms and the biological context for the phosphorylation of AMPK Thr¹⁷² mediated by TAK1 is not as well understood as that mediated by LKB1 or CAMKK2. In this study, we showed that IKK plays an important role downstream of TAK1 in regulating AMPK Thr¹⁷².

Overexpression of TAK1 and 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 (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-1 β , 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 Thr¹⁷² phosphorylation. Collectively, these data suggest that cytokine-induced TAK1 activity leads to direct phosphorylation of AMPK Thr¹⁷² by TAK1. Expression of a constitutively active IKK β mutant that mimics TAK1 phosphorylation leads to an increase in AMPK Thr¹⁷² phosphorylation in HeLa cells, which are LKB1-deficient (27). This result suggested a potential role for IKK β downstream of TAK1 in regulating AMPK. Here we demonstrated that IKK activated AMPK downstream of TAK1 in response to IL-1 β 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-1 β and TNF- α (Fig. 1A–F, Fig. 2A). In addition to NEMO, the canonical IKK complex consists of two related protein kinases, IKK α and IKK β , which show variable activity towards different substrates in the NF- κ B pathway. For example, IKK β has higher activity towards I κ B α than IKK α , but IKK α has a higher activity towards p100 than IKK β (55). With respect to AMPK, IKK β had a 5-fold greater activity towards AMPK Thr¹⁷² than IKK α (Fig. 4D). This could be because IKK β shows more kinase activity towards AMPK Thr¹⁷² or because IKK β is directed to and binds AMPK more efficiently than IKK α , as was observed with GST-pull downs in HEK293T cells (Fig. 4E). These findings suggested that it was IKK β within the canonical complex that directly phosphorylated AMPK Thr¹⁷². However, knockdown of IKK α in A549 cells or in knockout of IKK α in MEFs led to a decrease in the phosphorylation of this site. Possible explanations include regulation of the canonical IKK complex by the non-canonical IKK complex or regulation of IKK β activity within the canonical IKK complex by IKK α , either through phosphorylation of serine residues in the IKK β activation loop or stabilization of the canonical complex. Future studies are necessary to determine whether stimuli (such as RANK ligand or CD40 ligand) that specifically activate the non-canonical IKK α complex promote activation of AMPK. Although IKK α showed less activity towards AMPK Thr¹⁷² than IKK β , it is still possible that a stimulus that specifically stimulates IKK α could promote AMPK activity.

The IKK/NF- κ B pathway promotes its own feedback to limit the response to IL-1 β and TNF- α , notably by inducing the transcription of I κ B α (56). Activation of AMPK may be another way to limit NF- κ B 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-1 β induces AMPK in an IKK-dependent fashion, IL-1 β 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 Thr¹⁷² 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 Thr¹⁷² phosphorylation. In addition to TAK1-mediated phosphorylation, IKKβ can also be activated by trans-auto-phosphorylation, 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 Thr¹⁷² phosphorylation in the absence of TAK1 activity (Fig. 2C–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 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 towards exploiting this vulnerability. This could be because LKB1-deficient cancer cells promote basal AMPK phosphorylation through IKK to buffer against energetic stress. In this study we demonstrated that IKK inhibition in LKB1-deficient cells led to decreased phosphorylation of AMPK Thr¹⁷², even when the cells were treated with phenformin (Fig. 5A and B). This decrease in AMPK Thr¹⁷² phosphorylation was associated with apoptosis, as measured by Caspase 3/7 activity (Fig. 5C and 5D). In cells with LKB1, IKK did not affect the ability of phenformin to induce AMPK Thr¹⁷² 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 (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 MEFs of the indicated genotypes were described previously (28). All cell lines were maintained in a humidified incubator at 37°C and 5% CO₂.

Antibodies and Compounds

All antibodies were purchased from Cell Signaling Technology (Danvers, Massachusetts). 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 (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). 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) and a 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 μ M. Briefly, 1.5×10^5 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-AMPK α 1(1–312) (Addgene plasmid # 27632). Kinase dead AMPK 1–312 was created by mutating D157 to A in pEBG-AMPK α 1(1–312) using a quick-change mutagenesis protocol. AMPK T172A was created by cloning pDONR223-AMPK α 1 (Addgene plasmid # 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) IKK β , as well as the TAK1 and TAB1 plasmids were kind gifts from Dr. Lewis 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 *E. coli* were used as previously described (50) (a kind gift from Dr. J. Brenman, UNC Chapel Hill). Purified GST- tagged IKK α 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 100 μ M cold ATP. γ -³²P-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 AMPK α 1 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 (61), 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 AMPK α 1 or IKK β activity, peptide based kinase assays were performed as described previously, using 100 μ M ATP and 5 μ M Compound A (62). Active AMPK α 1, AMPK β 1, or AMPK γ 1, the SAMS peptide and the I κ B α 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, Pittsburgh, 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, Pittsburgh, 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 Laemmli 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 (63). 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 total fluorescence intensity was measured using a 96-well plate reader. The excitation and emission were 360 and 520nm 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 well 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

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We would like to thank Dr. Jay Brenman for the recombinant AMPK. We would also like to acknowledge the thoughtful discussions and feedback from members of the laboratory, especially Dr. Hung-Ching Hsia, Dr. Courtney Lawrence and Matthew Tegowski. We would like to thank Dr. Xianming Tan for his assistance with the statistical analysis.

Funding: R.J.A was supported by pre-doctoral fellowships from the National Institutes of Health (NIH) T32 Training Grant (T32CA071341-17) and an American Heart Association pre-doctoral fellowship. Research funding is derived from NIH/NCI grant R35CA197684 and support from the Waxman Cancer Research Foundation to A.S.B.

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Figure 1: AMPK Thr¹⁷² phosphorylation induced by inflammatory signals requires IKK
(A.) HeLa cells were pre-treated with either DMSO or 5 μM Compound A (CmpdA), 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 3 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 minutes. Whole cell lysates were then subjected to western blot analysis using the indicated antibodies. Blots are representative of 2 independent experiments).
(C.) A549 cells were transfected with either non-coding siRNA (siNC) or siRNA targeting NEMO, then stimulated with IL-1β (15 ng/ml) for 5 min and probed with the indicated antibodies. Blots are representative of 3 independent experiments.
(D.) WT or IKKβ^{-/-} MEFs were treated with IL-1β for 5 minutes and were immunoblotted with the indicated antibodies. Blots are representative of 2 independent experiments.
(E.) WT or AMPK^{-/-} MEFs were treated with IL-1β for 30 minutes and then probed with antibodies recognizing ACC and ULK1 phosphorylated at their respective AMPK sites. Blots are representative of 3 independent biological experiments; the error bars represent the

standard error of the mean (S.E.M) of the normalized densitometry value measured for each experiment (n.d = not detected).

(F.) A549 cells were pretreated with either DMSO or Cmpd A for 15 min then treated with IL-1 β for 30 minutes and then probed with antibodies recognizing ACC phosphorylated at the AMPK site. Blots are representative of 3 independent experiments.

(G.) A549 cells were treated with 15ng/ml IL-1 β 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). Repeated measures linear mixed-effect model showed that there was a statistically significant trend of increasing LC3-II with time (p-value = 0.027).

(H.) A549 cells were pre-treated with either with DMSO or 5 μ M Cmpd A for 15 minutes then with IL-1 β for 20 minutes. Whole cell extracts were then prepared and analyzed by western blot for the indicated proteins. Blots are representative of 3 independent experiments. The error bars represent the S.E.M of the densitometry values obtained in each experiment. A two-way ANOVA analysis was used to determine which groups were statistically different, * p<0.05.

(I.) A549 cells were pre-treated with DMSO, Compound A(5 μ M) or Bafilomycin A1 (10nM) for 15 minutes then treated with IL-1 β 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 fluorescence intensity was measured. Error bars represent the S.E.M. of the average total fluorescence measured in 3 independent experiments.

Figure 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 (15ng/ml) for 5 min. Blots are representative of 3 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 Cmpd A (5μM) for 30 minutes. Whole cell lysates were then subjected to western blot analysis with the indicated antibodies. Blots are representative of 3 independent experiments. A two-way ANOVA analysis 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 Cmpd A (5μM) for 1 hour, then whole cell lysates were analyzed by western blot for AMPK Thr¹⁷² phosphorylation and total AMPK. Blots are representative of 3 independent experiments. The error bars represent the S.E.M. of the normalized densitometry values measured in each experiment. 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. Blots are representative of 3 independent experiments. Error bars represent S.E.M of the normalized densitometry values measured in each experiment. 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 for 30 mins with either 5z-7-oxozeanol or NG-25 to inhibit TAK1, and immunoblotted with the indicated antibodies. (n.d. = not detected). A549 cells

were treated with Compound A at the indicated concentrations for 30 minutes and blotted for phospho-p38 (used as a marker of TAK1 activity) or phospho-p65 (used as a marker of IKK β activity) Blots are representative of 3 independent experiments.

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Figure 3.

IKK regulates basal AMPK Thr¹⁷² phosphorylation in cell lines.

(A.) A549 (LKB1-deficient) and MDA-MB-231 (expressing LKB1) cells were transfected with siRNA targeting NEMO for 48 hours and immunoblotted with the indicated antibodies. Blots are representative of 3 independent experiments. Error bars represent the S.E.M of the normalized densitometry values measured for each experiment. A two-way ANOVA analysis 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 3 independent experiments. Error bars represent the S.E.M of the normalized densitometry values measured in each experiment. 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^{-/-} MEFs were analyzed by western blot with antibodies for either AMPK phospho-Thr¹⁷² or total AMPK. Blots are representative of 3 independent experiments. Error bars represent the S.E.M of the normalized densitometry values measured in each experiment. A two-way ANOVA analysis was used to determine which groups were statically different. * p<0.05.

(D.) A549 and MDA-MB-231 cells were grown in full media (DMEM, 10% FBS), treated with the indicated concentrations of Cmpd A for 1 hour, and immunoblotted as indicated. Blots are representative of 3 independent experiments, error bars represent the S.E.M of the normalized densitometry values measured in each experiment.

(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. Blots are

representative of 3 independent experiments. Error bars represent the S.E.M of the normalized densitometry values measured in each experiment.

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Figure 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 cell free kinase assays and immunoblotted as indicated. Blots are representative of 3 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 3 separate occasions. The graph represents the mean activity calculated from the 3 experiments and the error bars represent the S.E.M.

(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 cell-free kinase assays with active IKKβ. Blots are representative of 3 independent experiments.

(D.) Recombinant IKKα and IKKβ were incubated with AMPKα1/β1/γ1 trimers as in (A.) The amount of AMPK Thr¹⁷² phosphorylation by IKKβ was normalized to the amount of AMPK Thr¹⁷² phosphorylation by IKKα and is displayed in the graph. The blot is representative of 3 independent experiments. The error bars represent the S.E.M of the normalized densitometry values measured in each experiment.

(E.) A GST-tagged construct of the AMPK kinase domain (amino acids 1–312) was transfected into 293T cells and used to co-precipitate IKKα or IKKβ. Blots are representative of 2 independent experiments.

(F.) Effect of Compound A on the phosphorylation of AMPK Thr¹⁷² by recombinant IKKβ, TAK1/TAB1 or CAMKK2 Blots are representative of 5 independent experiments for each kinase, the ratio of phosphorylated to total AMPK is plotted in the graphs below and the

error bars represent the S.E.M of the normalized densitometry values measured in each experiment.

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Figure 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), treated with phenformin (1mM) for 18 hours, and immunoblotted as indicated. Blots are representative of 3 independent experiments.

(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, and immunoblotted as indicated. Blots are representative of 3 independent experiments.

(C.) A549 and MDA-MB-231 cells were transfected with either non-coding 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 S.E.M).

(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. 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 statically different (** p < 0.01).