AMP-activated Kinase (AMPK) Promotes Innate Immunity and Antiviral Defense through Modulation of Stimulator of Interferon Genes (STING) Signaling*

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The host protein Stimulator of Interferon Genes (STING) has been shown to be essential for recognition of both viral and intracellular bacterial pathogens, but its regulation remains unclear. Previously, we reported that mitochondrial membrane potential regulates STING-dependent IFN-β induction independently of ATP synthesis. Because mitochondrial membrane potential controls calcium homeostasis, and AMP-activated protein kinase (AMPK) is regulated, in part, by intracellular calcium, we postulated that AMPK participates in STING activation; however, its role has yet to be defined. Addition of an intracellular calcium chelator or an AMPK inhibitor to either mouse macrophages or mouse embryonic fibroblasts (MEFs) suppressed IFN-β and TNF-α induction following stimulation with the STING-dependent ligand 5,6-dimethyl xanthone-4-acetic acid (DMXAA). These pharmacological findings were corroborated by showing that MEFs lacking AMPK activity also failed to up-regulate IFN-β and TNF-α after treatment with DMXAA or the natural STING ligand cyclic GMP-AMP (cGAMP). As a result, AMPK-deficient MEFs exhibit impaired control of vesicular stomatitis virus (VSV), a virus sensed by STING that can cause an influenza-like illness in humans. This impairment could be overcome by pretreatment of AMPK-deficient MEFs with type I IFN, illustrating that de novo production of IFN-β in response to VSV plays a key role in antiviral defense during infection. Loss of AMPK also led to dephosphorylation at Ser-555 of the known STING regulator, UNC-51-like kinase 1 (ULK1). However, ULK1-deficient cells responded normally to DMXAA, indicating that AMPK promotes STING-dependent signaling independent of ULK1 in mouse cells.

Pathogen sensing by innate immune cells is essential for host defense in response to invading microorganisms. Recognition by pattern recognition receptors typically activates signal transduction pathways, leading to up-regulation of cytokines like TNF-α and IFN-β. Although IFN-β has a well-established role in defense against viral infection, intracellular bacterial infections also induce this cytokine. In the past decade, our understanding of the molecular basis for these signaling pathways has expanded greatly and it has been discovered that there is some overlap between proteins responsible for recognition of bacteria and virus alike. One particular example is the mitochondrial and endoplasmic reticulum resident protein stimulator of interferon genes (STING)2 (1–3). STING has been shown to be crucial for recognition of both DNA viruses (4–6) and intracellular bacterial pathogens (7–12). STING senses DNA viruses indirectly, requiring the host enzyme cyclic GMP-AMP synthase (cGAS) to convert the viral double stranded DNA into the compound cGAMP (13–15). This metazoan second messenger, in turn, binds with high affinity to STING dimers, changing the protein conformation (16), such that it leads to activation of the kinase TBK1 and subsequent phosphorylation of the transcription factor IRF3, which is pivotal for IFN-β induction (17). Mouse STING-dependent signaling can also be triggered by the bacteria-specific metabolites cyclic di-AMP (8) and cyclic di-GMP (18), albeit with lesser potency than the endogenously produced cGAMP (16, 19). Although the literature suggests that mouse STING is essential for up-regulation of IFN-β during a number of bacterial infections, it remains unclear whether this is through direct sensing of bacterial-derived dinucleotides or indirect means through cGAS-mediated production of cGAMP.

To study STING signaling in the absence of infection, ligands that activate the STING pathway, such as dsDNA or cGAMP, are routinely delivered into the cytosolic by transfection or digitonin permeabilization, adding additional variables into the study. A synthetic STING agonist, 5,6-dimethyl xanthone-4-acetic acid (DMXAA), is a useful tool with which to study STING-dependent signaling because it is cell permeable and induces IFN-β production through sensing of bacterial-derived dinucleotides, and therefore allows one to dissect the contribution of STING-dependent versus -independent signaling.

2 The abbreviations used are: STING, stimulator of interferon genes; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated kinase; CaMKK, calcium/calmodulin-dependent protein kinase kinase; cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP synthase; DMXAA, 5,6-dimethyl xanthone-4-acetic acid; IRF, interferon regulatory factor; ISG, interferon-stimulated genes; MEFs, mouse embryonic fibroblasts; MTT, methylthiazolyl diphenyl tetrazolium bromide; ULK, UNC-51-like kinase; VSV, vesicular stomatitis virus; m.o.i., multiplicity of infection; ANOVA, analysis of variance; qRT, quantitative RT; BAPTA-AM, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxy methylester.

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**A** IFN-β expression

![Graph showing IFN-β expression](image)

**B** TNF-α expression

![Graph showing TNF-α expression](image)

**Results**

Inhibition of AMPK Disrupts STING-dependent Cytokine Induction in Primary Macrophages—Innate signaling pathways can be regulated at many levels and we have shown previously that the cytokine response to the STING-activating ligand DMXAA is regulated by mitochondrial membrane potential, independent of ATP generation (22). In addition to cellular metabolism, host mitochondria also regulate calcium homeostasis (29), leading us to hypothesize that calcium concentrations alter the response to DMXAA. In support of this assertion, pretreatment of primary murine macrophages with the intracellular calcium chelator BAPTA-AM dose-dependently decreased expression of IFN-β (Fig. 1A) and TNF-α mRNA (Fig. 1B) induced by DMXAA. Because AMPK activity is positively enhanced by calcium downstream of CaMKKβ (37), our findings suggested that AMPK might be the effector protein that mediates this calcium-dependent response. This hypothesis was initially tested by using the AMPK inhibitor Compound C. Compound C dose dependently decreased DMXAA-induced IFN-β (Fig. 2A) and TNF-α (Fig. 2B) in primary macrophages, indicating that AMPK activity is required for this signaling pathway.
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**FIGURE 2.** Inhibition of AMPK activity blocks induction of STING-dependent cytokines. Peritoneal macrophages were treated with the indicated doses of the AMPK inhibitor Compound C or vehicle alone for 30 min. The cells were then treated for an additional 2 h with DMXAA (100 μg/ml). Expression of IFN-β (A) and TNF-α (B) mRNA were determined by qRT-PCR. The data represents the mean fold-induction calculated from 3 independent experiments. The data points represent the individual mean from each experiment. NS, not significantly different. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Inhibition of Cytokine Response by Compound C Is Independent of Host Mitochondria and Is Associated with Decreased AMPK Activation in Primary Macrophages—That the mitochondrial dehydrogenase activity of Compound C-treated macrophages was not disrupted at the doses used in our study as measured by the methylthiazolyldiphenyl tetrazolium bromide (MTT) assay (Fig. 3A) argues against the possibility that the inhibitory effect of Compound C is due to the toxicity or some other effect on mitochondrial physiology. If Compound C were acting at the level of AMPK, the question then becomes whether it is blocking basal levels or ligand-dependent activation of AMPK activity. Treatment of macrophages with the AMP analog AICAR (positive control) increased AMPK phosphorylation at Thr-172, as expected; however, neither DMXAA nor LPS stimulation of macrophages was sufficient to increase AMPK activity in primary macrophages (Fig. 3B). This finding indicates that Compound C possibly influences basal activity of this kinase complex. In agreement with this possibility, addition of Compound C alone decreased basal phosphorylation of AMPK (Fig. 3, C and D).

**Mouse Embryonic Fibroblasts (MEFs) Lacking AMPK Activity Exhibit Impaired STING-dependent Signaling—**To corroborate the finding that the activity of Compound C on macrophages correlated with suppression of AMPK activity, a genetic approach was employed (Fig. 3). Mice deficient for both gene copies of the catalytic AMPKα subunit are embryonic lethal (46), but MEFs from these mice are fully viable (42). Like the primary macrophages examined above, DMXAA-dependent IFN-β expression in WT MEFs was also inhibited in the presence of either BAPTA-AM or Compound C (Fig. 4, A and B). In response to the STING agonist, DMXAA, AMPKα1−/− AMPKα2−/− MEFs exhibit a significantly impaired ability to up-regulate IFN-β and TNF-α mRNA expression (Fig. 4, C and D), showing a remarkably similar phenotype to primary murine macrophages treated with Compound C (Fig. 2). Although DMXAA is an artificial inducer of STING-dependent signaling, AMPKα1−/− AMPKα2−/− MEFs also exhibit impaired induction of IFN-β following transfection of the natural ligand cGAMP (Fig. 4E). Transfection of cells with cGAMP led to significantly less robust induction of IFN-β mRNA compared with DMXAA in AMPK-deficient MEFs (compare Fig. 4, E to C). Transfection of cells with the RNA helicase activating ligand poly(I:C) (47) was also decreased in AMPKα1−/− AMPKα2−/− MEFs (Fig. 4F), albeit to a lesser extent than seen with STING ligands (Fig. 4E).

AMPK has numerous substrate targets in the cell, so it was initially unclear where in the STING signaling pathway inhibition was taking place. Upon STING activation by DMXAA in WT MEFs, TBK1 is rapidly activated by phosphorylation, leading to recruitment and activation of the transcription factor IRF3 (21), which then translocates to the nucleus. TBK1 phosphorylation was detectable by 15 min post-DMXAA treatment and IRF3 phosphorylation by 30 min (Fig. 5), with both increasing significantly by 60 min. Both of these protein modifications were significantly blunted in the AMPKα1−/− AMPKα2−/− MEFs (Fig. 5). This indicates that AMPK promotes the earliest events within the STING-dependent signaling pathway.

**AMPK-deficient MEFs Exhibit Impaired VSV-dependent IFN-β Expression and Impaired Control of Viral Growth—**Defective STING signaling in AMPK-deficient MEFs would be predicted to render these cells more permissive to viruses that are recognized in a STING-dependent fashion. VSV, a STING-dependent virus (1), was used to infect WT and AMPKα1−/− AMPKα2−/− MEFs, and resulted in decreased induction of IFN-β mRNA at 6 h post-infection in AMPKα-deficient MEFs compared with WT MEFs (Fig. 6A). Reduced IFN-β induction is crucial because VSV growth in vitro is restricted in an IFN-β-dependent fashion (48). Thus, reduced IFN-β induction in AMPK-deficient MEFs would be predicted to result in increased susceptibility to virus infection. To test this possibility, we infected WT and AMPKα1−/− AMPKα2−/− MEFs and monitored cell survival. Pretreatment of MEFs with DMXAA to induce IFN-β protected WT MEFs, but not AMPKα1−/− AMPKα2−/− MEFs from VSV-induced cellular cytotoxicity (Fig. 6B). In the absence of DMXAA pretreatment, there was a statistically insignificant difference in survival between WT and AMPKα1−/− AMPKα2−/− MEFs after overnight infection with VSV (Fig. 6B), but the AMPKα1−/− AMPKα2−/− MEFs exhibited signs of cell death as early as 8 h compared with the infected WT cells (Fig. 6C). To confirm that this was caused by an increase in viral replication, VSV-G protein was monitored by Western blot analysis. By 6 h post-infection, VSV-G protein
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FIGURE 3. AMPK inhibition lowers basal and inducible activities of the AMPK signaling complex. Peritoneal macrophages were treated with the indicated doses of the AMPK inhibitor Compound C for 2 h. At the end of this incubation, mitochondrial dehydrogenase activity (A) was quantified by using the MTT assay described under “Experimental Procedures.” B, peritoneal macrophages were treated with AICAR (0.5 mM), DMXAA (100 μg/ml), LPS (100 ng/ml), or media only for 30 min. C, peritoneal macrophages were pre-treated with Compound C (20 μM) for 30 min prior to stimulation with AICAR (0.5 mM) or solvent alone for an additional 30 min. Phosphorylation of the Thr-172 amino acid residue of AMPKα was determined by an ELISA-based assay as described under “Experimental Procedures” in B and C. The data represents the mean ± S.D. for experimental conditions performed in triplicate (A) or duplicate (B and C). A representative of 3 independent experiments is shown. **, p < 0.01; ***, p < 0.001. D, peritoneal macrophages were stimulated for the indicated times with Compound C (20 μM). Lysates were separated by SDS-PAGE. After transfer, proteins were detected by Western blot analysis using antibodies specific for phospho-AMPK (Thr-172) or total AMPK.

was greatly increased in AMPKα1−/−AMPKα2−/− MEFs cells compared with WT MEFs (Fig. 6D). To verify that this phenotype was not due to impaired IFN-β-mediated signaling intrinsic to the AMPK-deficient cells, MEFs were treated with exogenous IFN-β in the absence of infection. AMPKα1−/−AMPKα2−/− MEFs exhibited decreased basal expression of the ISGs IRF7 and ISG56 compared with WT MEFs (Fig. 7, A and B), but showed similar, but somewhat delayed up-regulation of IRF7 and ISG56 gene expression in response to exogenous IFN-β (Fig. 7, C and D). Consistent with this result, pre-treatment of cells with IFN-β protected both WT and AMPKα1−/−AMPKα2−/− MEFs from VSV-induced cell cytotoxicity (Fig. 7E). In toto, our data indicate that in AMPKα1−/−AMPKα2−/− MEFs, the STING signaling defect causes impaired IFN induction leading to loss of control of viral infection. This illustrates a crucial role for AMPK in host antiviral defense mediated by STING signaling.

A Previously Identified Regulator of STING Signaling, ULK1, Is Constitutively Dephosphorylated in AMPK-deficient Cells—ULK1 and ELF4 have been demonstrated recently to regulate STING-dependent signaling in opposing fashions: ELF4 enhances STING-dependent signaling by binding to regulatory sites within the IFN-β promoter, thereby increasing the binding affinity of IRF3 and IRF7 to interferon-sensitive response elements within the IFN-β promoter (49). Conversely, the ULK1 kinase has been reported to inhibit STING-dependent IRF3 phosphorylation by phosphorylating the Ser-366 residue of STING to prevent recruitment of IRF3 (39). Conversely, others have reported that this same Ser-366 residue promotes STING-dependent signaling by facilitating interaction of IRF3 with its kinase TBK1 (40). Because AMPK has been shown to associate physically with ULK1 and to regulate the activity of ULK1 through direct phosphorylation (50), we initially hypothesized that ULK1 mediates repression of STING-dependent signaling in AMPK-deficient MEFs. In untreated WT MEFs, ULK1 Ser-555 was basally phosphorylated and remained so throughout 60 min of DMXAA treatment (Fig. 8). However, in the AMPKα1−/−AMPKα2−/− MEFs, ULK1 Ser-555 remained dephosphorylated throughout the 60-min incubation with DMXAA (Fig. 8). In contrast to ULK1, basal ELF4 protein levels were equivalent in the WT and AMPK-deficient MEFs (data not shown).

The ULK1 Inhibitor SBI-0206965 Represses Activation of STING-dependent Signaling, Whereas ULK1−/− MEFs Behave Like WT—Considering the conflicting role(s) of ULK1 in STING-dependent signaling, we sought to clarify its role in our model system. SBI-0206965 was recently identified as a selective small molecule inhibitor of ULK1 (51). Addition of the ULK1 inhibitor at a concentration used in the prior study (51) led to a decrease in IFN-β expression in WT MEFs treated with DMXAA. Thus, these data indicate that ULK1 is unlikely to be a negative regulator of STING-dependent signaling (Fig. 9A). This decrease in STING-mediated signaling was not associated with a corresponding impairment of mitochondrial dehydrogenase (MTT) activity (Fig. 9B), suggesting that it was not toxic to the cells. To decipher further the role of ULK1 in STING-de-
dependent signaling, WT and ULK1−/− MEFs (Fig. 10A) were treated with two direct agonists of the STING signaling pathway. In stark contrast to the results derived using the ULK1 inhibitor, ULK1-deficient MEFs were not impaired for induction of IFN-β in response to cGAMP (Fig. 10B) or DMXAA (Fig. 10C). ULK1−/− ULK2−/− MEFs also responded like WT MEFs after treatment with DMXAA or cGAMP (data not shown). To investigate this discrepancy further, STING-dependent signaling was induced by DMXAA treatment of WT and ULK1−/− MEFs in the absence or presence of increasing doses of the ULK1 inhibitor. Both the WT and ULK1−/− MEFs exhibited similar dose-dependent decreases in IFN-β induction in response to the ULK1 inhibitor (Fig. 10D). This indicates that SBI-0206965 exerts an off-target effect in the case of STING-dependent signaling. Overall, our data indicate that AMPK plays a crucial role in the regulation of STING-dependent signaling (Fig. 11), but STING signaling is independent of the kinase ULK1.

**Discussion**

AMPK has been previously recognized as being crucial for maintaining metabolic homeostasis of cells. More recently, however, it was also proposed to be involved in immunity to viruses (52, 53). In these more recent studies, the effect of AMPK on metabolism contributed directly to the antiviral effect. However, in our study, we have identified a potentially
new role for AMPK in the host antiviral defense that involves regulation of host innate immune signaling pathways that lead to up-regulation of IFN-β.

The involvement of AMPK in innate immune signaling has been studied less extensively than its role in metabolism, and the results have been conflicting. AMPK activators such as AICAR, metformin, and resveratrol, have been linked to anti-inflammatory activities through inhibition of NF-κB signaling (54–56) and AMPK activation causes up-regulation of the negative regulatory protein Small Heterodimer Partner (also known as NR0B2) (57). From a clinical standpoint, AICAR may be able to mitigate allergy or other lung injuries by reducing airway inflammation (58, 59) and it has also been shown to attenuate the severity of experimental autoimmune encephalitis in mouse models of multiple sclerosis (60, 61). Yet, despite these studies, the role of AMPK in immunity is not exclusively anti-inflammatory. Addition of the AMPK inhibitor Compound C for AMPK. There are published examples of AMPK-independent roles for both of these compounds (63, 64), stressing the importance of using genetic models to corroborate pharmacologic data. Even after taking this caveat into account, the genetic evidence for the role of AMPK is also not always clear-cut, sometimes even when the same pathways are being scrutinized. Macrophages lacking the AMPKα1 subunit have been shown to exhibit an increased response to LPS (65). Yet, human monocytes in which AMPKα1 was knocked down exhibit decreased cytokine induction of NF-κB-dependent genes when treated with LPS (66). Additionally, mice and macrophages that lack one of the activating kinases of AMPK, CaMKKβ, have a diminished response to LPS (67).

The focus of our study was to pinpoint the role of AMPK in the STING signaling network. It was previously demonstrated that the AMPK inhibitor Compound C inhibited cytokine induction in response to transfected dsDNA (39), but there was no corroboration of this finding using a genetic model. In our study, we have shown that perturbations to the AMPK pathway by chelation of calcium, treatment with the AMPK inhibitor Compound C, or absence of AMPK signaling (using AMPKα1−/−AMPKα2−/− MEFs) all lead to a failure to induce cytokine gene expression in response to DMXAA treatment (Fig. 11). This implicates decreased signaling immediately downstream of STING.

ULK1 and ELF4 were recently implicated in the regulation of STING-dependent signaling (39, 49). As AMPK has been shown to interact with ULK1 (50), we hypothesized that, in the absence of AMPK, ULK1 would repress STING-dependent signaling. In AMPK-deficient cells, ULK1 was constitutively dephosphorylated at Ser-555, consistent with the literature that AMPK regulates ULK1 activity through phosphorylation (69–71).

ULK1 possesses at least 16 phosphorylation sites and is the target of multiple kinases (72). Of these different phosphorylation sites, Ser-555 seems to be important for mitochondrial homeostasis (50). AMPK can also phosphorylate ULK1 at Ser-317 and Ser-777 (73). A S555A mutation in ULK1 prevents recruitment of ULK1 to mitochondria during hypoxic conditions, whereas the S555D mutant that mimics constitutive phosphorylation can traffic to the mitochondria even in the absence of AMPK (74). Most importantly, equivalent mutations in human ULK1 (at Ser-556) had a disparate effect on STING-dependent signaling: S556A ULK1 inhibited STING-dependent signaling, whereas S556D ULK1 had no inhibitory effect (39). From these results, the authors reasoned that ULK1 inhibited STING-dependent signaling through direct phosphorylation of STING on Ser-366, whereas concluding that AMPK-dependent phosphorylation of ULK1 repressed its kinase activity (39). This runs counter to the role AMPK exerts on ULK1 kinase activity during autophagy and conflicts with a recent report showing that phosphorylation of STING on Ser-366 helps activate STING-dependent signaling (40). An alternative interpretation is that the S556A mutation in ULK1 prevents its association with STING, preventing a phosphorylation event required for STING activation from occurring. Given that the absence of the autophagic protein ATG9a has been shown to cause excessive activation of STING-dependent signaling (75), it could be theorized that ULK1 initially activates STING-dependent signaling (possibly through direct phosphorylation), but enforces a short window of signal transduction by promoting the eventual degradation of STING protein through autophagy. Our data involving the ULK1 inhibitor initially supported this possibility, but this drug appears to exert an off-target effect in our system. Although the inhibitor-treated cells do appear healthy, as monitored by mitochondrial activity, it is possible that there is a toxic effect that is not reflected within the first 2 h of treatment by our viability assay. Using a genetic model of ULK1 depletion, we showed that ULK1 does not play a major regulatory role in STING-dependent signaling, contrary to previously published findings (39). In that study, ULK1 knockdown was performed in a human fibroblast cell line, with ULK1 knockdown cells exhibiting a modest 2-fold increase in IFN-β protein secretion in response to dsDNA (39). This dis-
crepancy is not easily remedied, but it should be noted that the signaling stimulus used in that study (39) was done by transfection of dsDNA (14). Considering that STING-dependent signaling has already exhibited a species-specific difference related to agonist specificity (20), it is theoretically possible that ULK1 may only play a major regulatory role in human cells or that its role is limited in some way to the secretory pathway. In our study, we have shown that perturbations to the AMPK pathway lead to decreased STING-dependent signaling and that AMPK regulates the phosphorylation of ULK1, but we conclude from our data that the role of AMPK in activation of the STING signaling pathway is independent of ULK1 in mouse cells. It remains to be determined whether AMPK promotes activation of this signaling pathway through direct or indirect means (Fig. 11).

We have shown that the lack of AMPK has a drastic effect on host defense, increasing susceptibility to infection with VSV, whose control during in vitro infection has been shown to require both STING (1) and IFN-β (48). RNA viruses like VSV are also thought to be primarily recognized directly by RNA helicases (76, 77), independently of STING. This likely means that the role of STING in recognition of certain RNA viruses is indirect. Constitutive, low level activation of STING signaling through host or mitochondrial DNA could be required for maintaining expression of the signal transduction machinery that contributes to activation of many pathways, including cytosolic RNA helicases. We predict that this viral susceptibility in the absence of AMPK would be extrapolated to other viruses that signal directly through STING such as HSV-1 (5), adenovirus (6), HIV (4), and Kaposi’s sarcoma-associated herpesvirus (78). This pathway might even be a target for viruses trying to circumvent the innate immune system. In line with this assertion, the Epstein-Barr virus-encoded protein latent membrane protein 1 inhibits liver kinase B1 and inactivates the AMPK signaling network (68). In the future, it will be interesting to study whether viruses may exploit this pathway by modulating the activity of AMPK to enable their growth or to evade the innate immune system.

Experimental Procedures

Antibodies and Reagents—Compound C (BML-EI369-0005) and BAPTA-AM (BML-CA411-0025) were purchased from Enzo Life Sciences (Farmington, NY), dissolved in DMSO, and stored in small volume aliquots. The ULK1 inhibitor SBI-0206965 (number 18477) was purchased from Cayman Chemical Company (Ann Arbor, MI), dissolved in DMSO, and stored in small volume aliquots. Recombinant mouse IFN-β (number 12405-1) was purchased from PBL Interferon Source (Piscataway, NJ). DMXAA (catalog number D5817) and MTT (number 12405-1) was purchased from PBL Interferon Source (Piscataway, NJ). DMXAA (catalog number D5817) and MTT (number 2128) was purchased from Sigma. Protein-free Escherichia coli K235 LPS was isolated by methods previously described (41). The cytosolic immune receptor activators cGAMP (number 0.05; **, p < 0.01. A representative of 3 independent experiments is shown.

FIGURE 6. Decreased IFN-β response to VSV infection increases susceptibility of AMPK-deficient cells. WT and AMPKα1−/−/AMPKα2−/− MEFs were infected with VSV (m.o.i. = 1) for 6 h. Prior to onset of cell death at 6 h post-infection, IFN-β mRNA (A) expression was quantified by qRT-PCR. For A, the data represents the mean fold-induction from 3 independent experiments. B, WT and AMPKα1−/−/AMPKα2−/− MEFs were pretreated with DMXAA (100 μg/ml) or vehicle only for 6 h. Immediately thereafter, cells were infected with VSV (m.o.i. = 10) for 18 h. Viability was determined using the MTT assay and normalized to mock-infected cells to give a cell survival index. C, WT and AMPKα1−/−/AMPKα2−/− MEFs were infected with the indicated m.o.i. of VSV. At 8 h post-infection, viability was calculated as in B. For B and C, the data represents the mean ± S.D. for experimental conditions performed in duplicate in the same experiment. *, p < 0.05; **, p < 0.01. D, WT MEFs and AMPKα1−/−/AMPKα2−/− MEFs were infected with VSV (m.o.i. = 1). At the indicated time points, cell lysates were harvested and separated by SDS-PAGE. After transfer, proteins were detected by Western blot analysis using antibodies specific for VSV G protein or β-tubulin. A representative of 3 independent experiments is shown.
ator AICAR and the following antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-/H9252-Tubulin (number 2146), anti-phospho-AMPK/H9251 (Thr-172) (number 2535), anti-AMPK/H9251 (number 5832), anti-IRF-3 (number 4302), anti-phospho-IRF-3 (number 4947), anti-TBK1 (number 3504), and anti-phospho-ULK1 (Ser-555) (number 5869). Anti-VSV-G antibody (number sc-66180) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). All primary antibodies were used at 1:1000 dilution.

FIGURE 7. Cells deficient for AMPK signaling exhibit normal responses to exogenous type I IFN. Expression of the ISGs IRF7 (A) and ISG56 (B) mRNA in untreated WT MEFs or AMPKα1/−AMPKα2/− MEFs were examined. Each data point represents the pooled results from 3 independent experiments. Induction of the ISGs IRF7 (C) and ISG56 (D) in WT or AMPKα1/−AMPKα2/− MEFs were monitored for 6 h following stimulation with IFN-β (100 units/ml). E, WT and AMPKα1/−AMPKα2/− MEFs were pretreated with IFN-β (100 units/ml) or vehicle only for 6 h. Immediately thereafter, cells were infected with VSV at the indicated m.o.i. for 18 h. Viability was calculated using the MTT assay. The data represents the mean ± S.D. for experimental conditions performed in triplicate for panels C–E and a representative of 3 independent experiments is shown. *, p = 0.0016; **, p = 0.0001.
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FIGURE 8. Cells deficient for AMPK signaling have constitutively dephosphorylated ULK1. WT and AMPKα1−/− AMPKα2−/− MEFs were stimulated for the indicated times with DMXAA (100 μg/ml). Lysates were separated by SDS-PAGE. After transfer, proteins were detected by Western blot analysis using antibodies specific for phospho-ULK1 (Ser-555), total ULK1, and β-Tubulin. A representative of 3 independent experiments is shown.

FIGURE 9. The ULK1 inhibitor SBI-0206965 represses activation of STING-dependent signaling. WT MEFs were treated for 30 min with the ULK1 inhibitor SBI-0206965 (50 μM) or vehicle alone prior to treatment with DMXAA (100 μg/ml). A, expression of IFN-β mRNA was determined by qRT-PCR. The data in A represents the mean fold-induction calculated from 3 independent experiments. B, WT MEFs were treated with the ULK1 inhibitor SBI-0206965 (50 μM) or vehicle alone for 2 h. At the end of this incubation, mitochondrial dehydrogenase activity was quantified by using the MTT assay as described here. C, WT MEFs were genotyped by probing cellular lysates with antibodies specific for ULK1 and β-tubulin. D, cells were treated as in C, but were also pretreated for 30 min with the indicated dose of SBI-0206965. In B–D, expression of IFN-β was determined by qRT-PCR. The data in B–D, represents the mean fold-induction calculated from 3 independent experiments.

Primary Macrophage Isolation and in Vitro Cell Culture—The Institutional Animal Care and Use Committee at the University of Maryland, Baltimore, MD, approved the protocols described below using animals. Primary peritoneal macrophages were isolated and cultured as previously described (22). Briefly, 4 days after i.p. injection of C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) with 2 ml of 3% sterile fluid thioglycollate (catalog number R064710, Remel Products, Lenexa, KS), cells were isolated by peritoneal lavage with fresh culture medium (RPMI 1640 supplemented with 10% FBS, 10 mM HEPES buffer, 1% nonessential amino acids, 2 mM l-glutamine, 1 mM sodium pyruvate, and 1% penicillin/streptomycin). After 2 h of culture, non-adherent cells were removed by aspiration and plate-bound macrophages were replenished with fresh culture medium for overnight incubation. Experiments were performed the day after isolation. Macrophages were treated with DMXAA at a concentration of 100 μg/ml, which was previously demonstrated to be the optimal dose for examining up-regulation of IFN-β (21). Transfection of cGAMP was accomplished using the reagent SuperFect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. In brief, 4 μg of cGAMP was incubated with 10 μl of reagent in serum-free media for 10 min at room temperature. One μg of this master mixture was added to each recipient well. Poly(I:C) conjugated to LyoVec as supplied facilitated its transfection without the use of any additional reagent. In both cases, treated cells were harvested 6 h after transfection for expression analysis. AMPKα1−/− AMPKα2−/− MEFs, which lack both isoforms of the genes that encode the catalytic α-subunit of AMPK, have been described previously and were kindly provided by Dr. Sharon Tooze (44). MEFs were routinely cultured in DMEM containing 5% FBS. Cells were plated in tissue culture dishes the night prior to the experiment.

Western Blotting and AMPK ELISA—Cell lysates were prepared in cell lysis buffer (Cell Signaling Technology) and frozen in single use aliquots at −70 °C. For the ELISA-based assay to detect phosphorylated AMPK (Cell Signaling Technology), cell
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![Diagram](attachment:image.png)

**FIGURE 11. Multiple regulatory pathways are utilized by the cell to control activation of the STING signaling pathway.** A, our data support a model in which the presence of AMPK signaling is required for optimal activation of STING-dependent signaling. Conditions that interfere with AMPK signaling, including chelation of intracellular calcium (1), pharmacologic inhibition of AMPK signaling (2), or genetic ablation of AMPK signaling (3) inhibit STING-dependent signaling. As ULK1−/− and ULK1−/− ULK2−/− MEFs exhibit no defect in response to STING agonists, we conclude that the regulatory role of AMPK on STING is independent of ULK1 and that the ULK1 inhibitor SBI-0206965 has off-target effects leading to signal impairment. B, the exact role of AMPK in the regulation of STING is unknown at present. AMPK might directly promote STING signaling by substrate level phosphorylation (1). Alternatively, it might act indirectly by activating a positive regulator (PR) or inhibiting a negative regulator (NR) of STING-dependent signaling (2). An alternate possibility is that the STING signaling pathway operates efficiently in a narrow metabolic window and is indirectly inhibited in the absence of the catabolic pathways promoted by AMPK (3). Future studies will delineate which of these mechanisms is operative.

Lysates were diluted 1:10 with sample diluent and added to wells containing antibodies specific for AMPKα subunit. The detection antibody was specific for AMPKα phosphorylated at Thr-172. The secondary antibody was conjugated to horseradish peroxidase and the amount of target protein was monitored colorimetrically. Prior to immunoblotting, lysates were mixed with an equal volume of 2× Laemmli sample buffer (Bio-Rad) and heated to 95 °C for 5 min. Samples were separated by SDS-PAGE in a continuous 10% acrylamide precast gel (Bio-Rad). Proteins were transferred from the gel to a nitrocellulose membrane in transfer buffer for 1 h at 4 °C. Afterward, the membrane was washed with TBS and blocked with TBS containing 0.1% (v/v) Tween 20 buffer and 5% (w/v) blotting grade blocker.

**RNA Purification and qRT-PCR**—RNA was isolated as previously described (22) using the TriPure reagent (Roche Applied Science). The purified RNA was then treated with RNase-free DNase (Promega, Madison, WI) at 37 °C for 30 min to remove DNA contamination and reverse transcribed using the SuperScript III first strand synthesis system for real-time PCR (Invitrogen) according to the manufacturer’s supplied instructions with oligo(dT) and random hexamers to prime the reaction. To analyze expression, quantitative PCR were carried out in the 7900HT Fast Real-time PCR system (Applied Biosystems) using the 2× Power SYBR Green PCR master mix (Life Technologies Corp.), and the following primer sequences: 

- **Hprt** sense: 5′-GCTGACCTGCTGGATTACATTAA-3′;
- **Hprt** antisense: 5′-TGATCATCCTAGTGTCTTCAGCT-3′;
- **Ifnb1** sense: 5′-ACTTTGGAAGCTATTACCTGGAAGG-3′;
- **Ifnb1** antisense: 5′-CTCGGACCCCATCCAGG-3′;
- **Tnf** sense: 5′-GACCCTCACTCAGATCATCCTCT-3′;
- **Tnf** antisense: 5′-CCACCTTGTTGTTGTCATACGA-3′.

The total amount of mRNA present was calculated using the comparative C method (45) and expressed as fold-induction compared with untreated or uninfected cells.

**MTT Assay**—A colorimetric assay to measure mitochondrial succinic dehydrogenase activity, a surrogate measure for cell death, was performed as previously described (22). Briefly, at the end of stimulation with the AMPK inhibitor or at time points following viral infection, culture supernatants were removed and replaced with a filter-sterilized solution of MTT (1 mg/ml) dissolved in phosphate-buffered saline (number 21-040-CV, Mediatech, Inc., Herndon, VA). The macrophage cultures were allowed to incubate for 30 min at 37 °C and 5% CO2 in the presence of MTT. The MTT solution was removed and replaced with isopropyl alcohol to dissolve the water-insoluble formazan products (if mitochondrial enzymes were active), yielding a purple solution whose absorbance was quantified at 595 nm on a Biotek microplate reader.

**Viral Infections**—Wild-type (WT) and AMPKα1−/− AMPKα2−/− MEFs were infected with VSV (Indiana strain; m.o.i. = 1 or 10) for 1 h in serum-free DMEM. At the end of this incubation, the media was removed by aspiration and the cells washed three times with PBS before being cultured further in growth medium that contained 5% FBS.

**Statistical Analysis**—Experiments were performed in duplicate for AMPK phosphorylation and MTT assays. The numerical values obtained from these assays were analyzed for significance using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) using either a one-way ANOVA or a two-way ANOVA depending on the number of experimental variables. Significance was accepted if p < 0.05. For the one-way ANOVA, if the data met this standard, a Tukey's post hoc test was used to test for significance between experimental groups in the data set. When only two groups were being compared, an unpaired, two-tailed Student’s t test was performed to determine significance. Significance for gene expression was analyzed from the fold-induction values calculated from two or three independent experiments. The resulting p values between groups are listed in the figure legends for each of the individual experiments.

**Author Contributions**—D. P. designed and coordinated the study, performed and analyzed the experiments, and wrote the manuscript. D. J. P. was integral for studies involving VSV for Figs. 5 and 6 and design of experiments for Fig. 6. S. N. V. contributed to the design and conception of experiments, analysis of the data, and in drafting of the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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