Rutherford, C. et al. (2016) Phosphorylation of Janus kinase 1 (JAK1) by AMP-activated protein kinase (AMPK) links energy sensing to anti-inflammatory signaling. Science Signaling, 9(453), ra109.

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

http://eprints.gla.ac.uk/119360/

Deposited on: 18 November 2016

Phosphorylation of Janus kinase 1 (JAK1) by AMP-activated protein kinase (AMPK) links energy sensing to anti-inflammatory signaling

Authors: Claire Rutherford[1], Claire Speirs[1], Jamie J.L. Williams[2], Marie-Ann Ewart[1$], Sarah J. Mancini[1], Simon A. Hawley[3], Christian Delles[1], Benoit Viollet[4,5,6], Ana P. Costa-Pereira[7], George S. Baillie[1], Ian P. Salt[1*] and Timothy M. Palmer[2*]

Affiliations:

[1]Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK.

[2]School of Pharmacy, University of Bradford, Bradford BD7 1DP, UK.

[3]Division of Cell Signaling and Immunology, University of Dundee, Dundee DD1 5EH, UK.

[4]INSERM, U1016, Institut Cochin, Paris, France.

[5]CNRS, UMR8104, Paris, France.

[6]Université Paris Descartes, Sorbonne Paris Cité, France.

[7]Faculty of Medicine, Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, London W12 0NN, UK.

[$]Current affiliation: AvantiCell Science Ltd., Auchincruive, Ayr KA6 5HW, UK.

*Corresponding author. Email: T.Palmer1@bradford.ac.uk (T.M.P.); Ian.Salt@glasgow.ac.uk (I.P.S.).

Abstract: AMP-activated protein kinase (AMPK) is a pivotal regulator of metabolism at the cellular and organismal levels. AMPK also suppresses inflammation. We found that pharmacological activation of AMPK rapidly inhibited the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway in various cells. In vitro kinase assays revealed that AMPK directly phosphorylated two residues (Ser^{515} and Ser^{518}) within the SH2 domain of JAK1. Activation of AMPK enhanced the interaction between JAK1 and 14-3-3 proteins in cultured vascular endothelial cells and fibroblasts, an effect which required the presence of Ser^{515} and Ser^{518} and was abolished in cells lacking AMPK catalytic subunits. Mutation of Ser^{515} and Ser^{518} abolished AMPK-mediated inhibition of JAK-STAT signaling stimulated either by the sIL-6Rα/IL-6 complex or by expression of a constitutively active V658F-mutant JAK1 in human fibrosarcoma cells. Clinically used AMPK activators metformin and salicylate enhanced the inhibitory phosphorylation of endogenous JAK1 and inhibited STAT3 phosphorylation in primary vascular endothelial cells. Therefore our findings reveal a mechanism by which JAK1 function and inflammatory signaling may be suppressed in response to metabolic stress and provide a mechanistic rationale for the investigation of AMPK activators in a range of diseases associated with enhanced activation of the JAK-STAT pathway.

Introduction

The Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway mediates signaling by a range of cytokines that can stimulate or inhibit survival, proliferation and differentiation responses in diverse cell types. JAKs comprise a family of four cytoplasmic tyrosine kinases (JAK1, JAK2, JAK3, and Tyk2) that function as essential signaling components immediately downstream of receptors for many hematopoietic cytokines, such as granulocyte-macrophage colony-stimulating factor, erythropoietin, leptin, interferons (IFNs) and interleukins such as IL-6 *(1)(2)*. The IL-6 receptor comprises two distinct subunits, an IL-6-binding protein (IL-6Rα) and a 130 kD signal-transducing subunit (gp130) which is shared by all IL-6-family cytokines *(2)*. The gene encoding gp130 subunit is ubiquitously expressed, whereas that encoding IL-6Rα is restricted to hepatocytes, monocytes, neutrophils, and some B and T cell subsets. However, IL-6 can also bind a soluble form of the receptor (sIL-6Rα) generated either by limited proteolytic cleavage of membrane-bound IL-6Rα or alternate splicing of IL-6Rα primary transcripts. The resulting sIL-6Rα/IL-6 "*trans*-signaling" complexes can then associate with gp130 on cells that do not express the membrane-bound IL-6R, thereby widening the spectrum of IL-6-responsive cells *(3)*.

IL-6 binding to the receptor complex results in the activation of gp130-bound JAKs. The activated JAKs are then able to phosphorylate specific Tyr residues within the receptor's cytoplasmic domain, which serve as docking sites for SH2 domain-containing proteins. The major intracellular mediators are the STAT proteins. IL-6 is capable of activating STAT1 and STAT3, although STAT3 activation has been observed to a greater extent than STAT1. Activation of STATs require their recruitment to specific JAK-phosphorylated Tyr residues on gp130 *via* their SH2 domain followed by JAK-mediated phosphorylation of a Tyr residue in the transactivation domain (Tyr^{705} on STAT3, Tyr^{701} on STAT1). Tyr-phosphorylated STATs then form dimers which can translocate to the nucleus to activate target gene transcription. IL-6 can also activate extracellular signal-regulated kinase 1 (ERK1) and ERK2 and phosphatidylinositol 3-kinase pathways following recruitment of the Tyr phosphatase SHP2 to JAK-phosphorylated gp130 *(2)(4)*.

AMP-activated protein kinase (AMPK) is the highly conserved downstream component of a Ser/Thr protein kinase cascade involved in the regulation of metabolism. It was initially characterised as a Ser/Thr protein kinase that is allosterically activated by increases in intracellular AMP concentration resulting from a decline in intracellular ATP that occurs after nutrient deprivation or hypoxia *(5)(6)*. AMPK is a heterotrimeric complex comprised of a catalytic α subunit and regulatory β and γ subunits. In mammals, isoforms of each subunit (α1, α2, β1, β2, γ1, γ2, γ3) are encoded by seven genes. The β subunits are each N-terminally myristoylated, contain carbohydrate-binding domains and interact with both the α and γ subunits, whereas the γ subunits contain the AMP/ADP binding sites responsible for sensing cellular energy status [reviewed in *(5)*]. In addition to the allosteric activation of AMPK by adenine nucleotides, AMP or ADP binding to the regulatory γ subunit of AMPK promotes phosphorylation of the catalytic α subunit at Thr^{172}, which is required for AMPK activity. Binding of AMP and ADP also inhibits dephosphorylation of AMPK, maintaining Thr^{172} phosphorylation by the upstream AMPK Thr^{172} kinase, liver kinase B1 (LKB1) *(5)(6)*. In the absence of changes in adenine nucleotide ratios, AMPK can be activated by increases in

intracellular Ca^{2+} concentrations in cells expressing Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) *(5)(6)*.

The most studied aspect of AMPK function is its role in maintaining cellular energy stores and regulating whole body energy balance. An important aspect of this role is the direct phosphorylation of metabolic enzymes such as acetyl coA carboxylase (ACC) 1 and 2, which catalyze the carboxylation of acetyl coA to malonyl-CoA, a key substrate for fatty acid biosynthesis that also inhibits fatty acid transport into mitochondria *(7)*. AMPK-mediated phosphorylation of ACC1 on Ser^{79} inhibits its activity thereby suppressing fatty acid synthesis and enhancing β-oxidation *(8)*. An increasing body of evidence has revealed that AMPK is also an important regulator of inflammatory responses. For example, AMPK-dependent inhibition of tumor necrosis factor α (TNFα)-, lipopolysaccharide (LPS)- and interleukin-1β (IL-1β)-stimulated leukocyte recruitment to vascular endothelial cells in culture has been observed in multiple studies, and is associated with reduced induction of adhesion molecules (such as VCAM-1 and E-selectin) and chemokines (such as CCL2 and CCL3) *(9)(10)*. Studies using *PRKAA1*$^{-/-}$ and *PRKAA2*$^{-/-}$ mice, which lack AMPKα1 and AMPKα2 catalytic subunits respectively, suggest that both isoforms can contribute to inhibition of post-ischemic leukocyte adhesion to blood vessels by the AMPK activator 5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR) *(11)*. Consistent with a role for AMPK in suppressing inflammation in vivo, aortae from AMPKα1$^{-/-}$ mice have been shown to display enhanced angiotensin II-stimulated VCAM-1 expression *(12)*. Activation of AMPK with AICAR is also associated with reduced infiltration of inflammatory cells in rodent models of acute and chronic colitis *(13)*, lung injury *(14)* and autoimmune encephalomyelitis *(15)*. Furthermore, increased infiltration and activation of macrophages have been observed in adipose tissue from wild type mice transplanted with bone marrow from AMPKβ1$^{-/-}$ mice *(16)*. Therefore, taken as a whole, it has become clear that AMPK can limit inflammatory cell infiltration and activation in multiple settings.

Whereas AMPK's ability to maintain fatty acid oxidation in macrophages has been proposed as an important mechanism by which it limits inflammation in macrophages *(16)*, several mechanisms have also been identified though which AMPK can directly inhibit pro-inflammatory signaling pathways. However, while several potential mechanisms have been proposed to explain AMPK-mediated inhibition of the NF-κB pathway in response to stimuli such as TNFα, LPS and IL-1 *(9)(17)*, relatively few studies have examined how the JAK-STAT pathway is controlled by AMPK. This is a particularly important issue to address given the pathological roles of enhanced JAK-STAT signaling in multiple chronic inflammatory conditions such as atherosclerosis, colitis and rheumatoid arthritis, as well as several cancers and hematological disorders driven by mutational activation of JAK isoforms *(1)(18)(19)(20)*.

In this study, we found that AMPK could rapidly and profoundly inhibit activation of the JAK-STAT pathway in response to multiple stimuli. In addition, we show that this occurs through a previously unappreciated mechanism involving direct AMPK-mediated phosphorylation of JAK1 within its regulatory SH2 domain. Our data therefore reveal how metabolic stress can rapidly inhibit signaling from JAK1, and provide a molecular basis for potential repurposing of AMPK activators for a range of diseases associated with enhanced activation of the JAK-STAT pathway.

Results

AMPK rapidly inhibits IL-6 signaling and function

AMPK plays a largely protective role in vascular endothelial cells (ECs) *via* both nitric oxide (NO)-dependent and –independent mechanisms *(21)(22)*. Incubation of human umbilical vein ECs (HUVECs) with sIL-6Rα/IL-6 *trans*-signaling complexes for 30 min significantly increased the phosphorylation of STAT3 on Tyr[705] (Fig. 1A), an effect that was significantly inhibited in HUVECs that had been pre-incubated with distinct stimuli that each activate AMPK as determined by assessing ACC1 phosphorylation on Ser[79] *(8)*: AICAR, which is phosphorylated intracellularly to the AMP mimetic ZMP *(23)*; A769662, which binds and activates β1-containing AMPK complexes via an AMP/ADP-independent mechanism *(24)*; and a combination of clinically utilized AMPK activators metformin and salicylate, which synergistically activate AMPK by increasing intracellular AMP concentration and directly binding β1-subunits, respectively *(25)(26)(27)*. Under these conditions, AMPK activation on its own had no detectable effect on the phosphorylation of STAT3. Targeted short inhibitory RNA (siRNA)-mediated knockdown of AMPKα1 catalytic subunits reduced the inhibitory effects of both A769662 (Fig. 1B) and AICAR (fig. S1), indicating that these effects were mediated *via* AMPK activation. In addition, compared with controls, adenovirus (AV)-mediated expression of a catalytically inactive dominant negative (DN) AMPKα1 subunit, in which Asp[157] located within subdomain VII of the catalytic domain is mutated to Ala *(28)*, was sufficient to block A769662-mediated inhibition of STAT3 phosphorylation in HUVECs (fig. S1). Together, these data demonstrated that activation of AMPK by multiple stimuli triggered a rapid and significant reduction in the ability of sIL-6Rα/IL-6 to stimulate STAT3 phosphorylation on Tyr[705].

To assess the functional consequences of AMPK-mediated inhibition of IL-6 signaling, we initially examined the effect of A769662 on IL-6-mediated induction of STAT3 target genes suppressor of cytokine signaling 3 (SOCS3) and CCAAT/enhancer binding protein δ (C/EBPδ) *(29)(30)*. Similar to the effect on STAT3 phosphorylation, A769662 significantly inhibited sIL-6Rα/IL-6-stimulated accumulation of SOCS3 and C/EBPδ protein (Fig. 1C) and mRNA (fig. S2) abundance. In addition, we took advantage of the well-described ability of IL-6 to induce chemokine expression and thereby increase monocyte chemotaxis *(20)(31)(32)*. Conditioned media was collected from HUVECs cultured with vehicle, A769662, or sIL-6Rα/IL-6 then applied to a trans-well migration assay to assess its capacity to stimulate monocyte migration. Because the HUVECs were extensively washed to remove A769662 and sIL-6Rα/IL-6 prior to collection of conditioned medium, it is unlikely that there was a substantial residual concentration of either that would contribute to migration directly. We found that the migration of U937 monocytic cells towards conditioned media from sIL-6Rα/IL-6-stimulated HUVECs was significantly greater than that toward conditioned medium from vehicle-treated HUVECs (Fig. 1D). Conditioned medium from HUVECs pre-incubated with A769662 for 30 min before cytokine stimulation elicited significantly less U937 cell migration compared to conditioned media from sIL-6Rα/IL-6-stimulated HUVECs (Fig. 1D).

AMPK inhibits signaling from multiple cytokine receptor complexes

To identify the point in the IL-6 signaling pathway at which AMPK was acting, we examined signaling downstream of multiple cytokines other cytokine receptors. We found that the inhibitory effect of AMPK on sIL-6Rα/IL-6 responses was not restricted to STAT3, given that pre-incubation of HUVECs with A769662 also abrogated sIL-6Rα/IL-6-stimulated phosphorylation of STAT1 on Tyr[701] (Fig. 2A). Whereas IL-6 signals downstream through

interaction with either a membrane-bound or soluble IL-Rα and gp130 homodimers, leukemia inhibitory factor (LIF) and oncostatin M (OSM) signal through cytokine interaction with gp130/LIF receptor (LIFR) and OSM receptor/gp130 heterodimers, respectively *(2)*. Similar to sIL-6Rα/IL-6, pre-treatment of HUVECs with A769662 significantly reduced OSM- and LIF-stimulated phosphorylation of STAT3 and STAT1 (Fig. 2A). In addition, the ability of each cytokine to stimulate STAT3 phosphorylation on Ser[727], which is critical for full transcriptional activation and is mediated by cytokine receptor activation of either the ERK1/2 or phosphatidylinositol 3-kinase (PI3K)–mammalian target of rapamycin (mTOR) pathway, was similarly reduced by A769662 (Fig. 2A). In support of a post-receptor effect of AMPK, A769662 also suppressed STAT3 phosphorylation in response to IFNα, which activates STATs through a distinct IFNα/β receptor 1 (IFNAR1)/IFNAR2 complex that lacks gp130 *(33)* (Fig. 2B). Together, these results would suggest that AMPK exerts its inhibitory effects on multiple cytokine-activated signaling pathways at one or more common loci downstream of both IFNAR1/IFNAR2 and gp130-containing cytokine receptor complexes.

JAK1 is phosphorylated by AMPK in vitro

The IFNAR1/IFNAR2 complex couples to STATs *via* JAK1 and Tyk2 *(33)(34)*. We performed JAK1, JAK2 and Tyk2 siRNA knockdown experiments in HUVECs which demonstrated that JAK1 mediates a substantial proportion of sIL-6Rα/IL-6-stimulated STAT3 activation (fig. S3). Thus JAK1 was identified as a potential common AMPK-regulated post-receptor target. As AMPK is a Ser/Thr protein kinase, and the inhibitory effect of AMPK activator A769662 occurred rapidly, we investigated the possibility that AMPK could directly phosphorylate JAK1. Peptide arrays of overlapping 25-mer peptides sequentially shifted by five amino acids and spanning the full human JAK1 open reading frame (residues 1-1154) were utilized for in vitro kinase assays with AMPK purified from rat liver and $[\gamma^{32}P]$ATP *(35)(36)*. These experiments identified a sequence within the JAK1 SH2 domain (R[506]YSLHGSDRSFPSLGDLMSHLKKQI) as a potential substrate whose phosphorylation on the array was greater than that of the so-called "SAMS" peptide typically used as an in vitro substrate for assaying AMPK activity and which is based on the primary AMPK phosphorylation site (Ser[79]) in rat ACC1 *(35)* (Fig. 3A). To define which of the five potential phospho-acceptor sites within the JAK1 peptide were phosphorylated in vitro by AMPK, kinase assays were then performed on additional arrays containing the wild-type peptide sequence and mutant peptides in which each Ser residue was replaced with a non-phosphorylatable Ala residue either individually or in combination (Fig. 3B). Simultaneous replacement of all five Ser residues with Ala abolished AMPK-mediated phosphorylation of the peptide. Individual replacement of each Ser residue with Ala either did not alter or enhanced AMPK phosphorylation of the resulting peptides. However, simultaneous replacement of Ser[515] and Ser[518] with Ala abrogated peptide phosphorylation to the same extent as mutating all five Ser residues. In addition, a peptide in which Ser[508], Ser[512] and Ser[524] were all replaced with Ala was phosphorylated to the same extent as the wild-type JAK1 sequence (Fig. 3B). Together, these observations identified Ser[515] and Ser[518] in human JAK1 as potential sites of phosphorylation by AMPK in vitro.

Peptide library screening, mutagenesis and molecular modelling studies *(37)(38)* have all been used to identify optimal AMPK substrate motifs. Bioinformatic analysis of JAK1 orthologues revealed that phosphorylation sites equivalent to Ser[518] in human JAK1 are conserved in multiple species (Fig. 3C). Residues at positions +3 and +4 downstream of the phospho-acceptor sites that are known to be critical for substrate recognition are also well conserved (Fig. 3C). Ser[518] is

located at the start of a specific α-helix that is conserved amongst all JAK isoforms (Fig. 3D) *(39–41)*. In contrast, Ser[515] is unique to human JAK1 and would be predicted to only form a weak consensus for AMPK substrate recognition if Ser[518] became negatively charged by phosphorylation (Fig. 3C). However other examples of AMPK substrates that only loosely follow the optimized consensus [such as Ser[633] in endothelial constitutive nitric oxide synthase *(42)*] have also been documented. Thus we examined a potential role for both Ser[515] and Ser[518] in more detail.

AMPK mediates the phosphorylation of JAK1 in intact cells

A common mechanism for phosphorylation-mediated regulation of target protein function is phosphorylation-dependent binding to members of the 14-3-3 family of proteins *(43)*. To assess whether phosphorylation of Ser[515] and Ser[518] could facilitate JAK1 interaction with 14-3-3 proteins in vitro, overlay assays were performed with horseradish peroxidase (HRP)-conjugated 14-3-3ζ (HRP-14-3-3ζ)and peptide arrays comprising 25-mer peptides from the identified JAK1 region in which either or both of Ser[515] and Ser[518] were phosphorylated (Fig. 4A). These demonstrated that phospho-Ser[515] and phospho-Ser[518] peptides strongly interacted with HRP-14-3-3ζ in vitro, whereas no binding was detectable with either non-phosphorylated peptide or peptide in which both Ser[515] and Ser[518] were phosphorylated (Fig. 4A).

A phosphorylation-dependent interaction with 14-3-3 proteins has been reported for several AMPK substrates, including autophagy-related protein 9 (ATG9), Raptor, and Unc-51-like autophagy activating kinase 1 (ULK1) *(44)(38)(45)*. We tested our ability to detect such interaction using two approaches. First, we compared the ability of bacterially expressed and affinity purified glutathione S-transferase (GST)/14-3-3 fusion proteins to capture AMPK-phosphorylated JAK1 in pull-down assays from soluble extracts prepared from intact cells. Initial experiments in *JAK1*-null U4C human fibrosarcoma cells stably expressing recombinant JAK1 *(46)(47)* demonstrated that distinct GST/14-3-3 isoforms (14-3-3ζ and 14-3-3τ) but not GST alone could specifically isolate JAK1 (fig. S4). To assess the importance of Ser[515] and Ser[518] in mediating this interaction, we used a different approach in which wild-type and S515A/S518A (SSAA)-mutated human JAK1 were transiently expressed in U4C cells before JAK1 was immunoprecipitated and resulting blots were overlaid with HRP-14-3-3ζ. Consistent with the peptide array overlays, these experiments demonstrated that whereas wild-type JAK1 could be specifically identified, the SSAA-mutated JAK1 could not, despite equivalent abundances of JAK1 in the immunoprecipitates (fig. S4). Finally, to assess the role of AMPK activation on JAK1 phosphorylation in intact cells, wild-type and SSAA-mutated human JAK1 were transiently expressed in U4C cells before treatment with A769662 and preparation of cell extracts for pull-down assays with GST/14-3-3ζ and immunoblotting for JAK1 (Fig. 4B). These demonstrated that A769662 treatment increased 14-3-3ζ interaction with JAK1 but not SSAA-mutant JAK1, despite an apparently equivalent activation of AMPK and expression of wild-type and mutant JAK1 in transfected cells (Fig. 4B).

To further examine the AMPK dependence of this effect, GST/14-3-3ζ pull downs were performed in wild-type murine embryonic fibroblasts (MEFs) and MEFs with no detectable AMPK activity due to homozygous deletion of AMPKα1 and α2 catalytic subunits *(48)*. AMPK activation with A769662 in wild-type MEFs transiently increased interaction of endogenous JAK1 with GST/14-3-3ζ but this effect was absent in cells devoid of AMPK activity as determined by the loss of ACC phosphorylation on Ser[79] (Fig. 4C). Because murine JAK1 lacks

Ser^{515} or an equivalent residue (Fig. 3C), this suggests that phosphorylation of Ser^{517} alone on murine JAK1 is required for interaction with GST/14-3-3ζ. Finally, we examined the effects of combined metformin and salicylate treatment on AMPK-mediated JAK1 phosphorylation (as determined by interaction with GST/14-3-3ζ) in HUVECs, a condition which we had found significantly inhibited sIL-6Rα/IL-6-stimulated STAT3 phosphorylation (Fig. 1A). Combined treatment of HUVECs with metformin and salicylate significantly increased ACC phosphorylation and triggered a transient increase in the amount of endogenous JAK1 that was captured by GST/14-3-3ζ, which peaked approximately1 hour after stimulation (Fig. 4D).

AMPK-mediated inhibition of JAK1-dependent signaling requires Ser^{515} and Ser^{518}

To investigate any contribution of AMPK-mediated JAK1 phosphorylation on Ser^{515} and Ser^{518} towards regulating signaling, we utilized *JAK1*-null U4C human fibrosarcoma cells. Consistent with previous work *(46)* and our own siRNA experiments in HUVECs, loss of JAK1 was associated with no detectable sIL-Rα/IL-6-mediated phosphorylation of STAT3. However, transient expression of wild-type human JAK1 restored sIL-Rα/IL-6-mediated STAT3 phosphorylation over GFP-expressing controls, and this was inhibited by pre-treatment with A769662 similar to the phenomenon observed in HUVECs (Fig. 5A). Expression of SSAA-mutant JAK1 in U4C cells was also able to restore sIL-Rα/IL-6-mediated STAT3 phosphorylation, but the ability of A769662 to inhibit this effect was lost despite maintaining its ability to activate AMPK as measured by ACC phosphorylation (Fig. 5B).

We then examined the effects of AMPK activation on signaling downstream of a constitutively active V658F-mutant version of JAK1 found in patients with acute lymphoblastic leukemia (ALL) *(49)*. Analogous to the V617F mutation in JAK2, which is implicated in the etiology of several myeloproliferative neoplasms, this mutation within the JAK1 JH2 pseudokinase domain is thought to relieve an auto-inhibitory interaction with the catalytic JH1 domain, thereby increasing Tyr kinase activity independently of cytokine stimulation *(1)(50)*. In contrast to wild-type JAK1, transient expression of V658F-mutant JAK1 in *JAK1*-null U4C cells was sufficient to trigger detectable Tyr phosphorylation of both STAT1 and STAT3 in the absence of added cytokine (Fig. 5C). Exposing cells to A769662 increased ACC phosphorylation and inhibited STAT1 and STAT3 Tyr phosphorylation. Expression of V658F-mutant JAK1 incorporating the S515A/S518A (SSAA) mutations also resulted in increased basal STAT phosphorylation, although this effect was less than that caused by V658F-mutant JAK1 expression. However, A769662 treatment failed to significantly inhibit Tyr phosphorylation of STAT1 and STAT3 despite increasing ACC phosphorylation (Fig. 5C). Together, these observations suggest that Ser^{515} and Ser^{518} in JAK1 are required for effective AMPK-mediated inhibition of JAK1-mediated downstream signaling to STAT1 and STAT3.

Discussion

It is becoming increasingly apparent that cells can couple inflammatory status to the availability of nutrients *(51)(52)*. Conversely, the mechanisms by which nutrient availability can regulate inflammation remain unclear. In this study we demonstrate that direct phosphorylation of JAK1, a key signaling intermediate utilized by a variety of cytokines, can occur in response to stimulation of AMPK, a key determinant of cellular energy status, by multiple activators. In conjunction with other observations, this would suggest that energy stress triggers an LKB1-

dependent activation of AMPK that can inhibit the JAK-STAT pathway through several distinct mechanisms. For example, others have shown that chronic AMPK activation in hepatocytes with metformin for 12 hr is able to increase levels of the orphan nuclear receptor small heterodimer partner (SHP), which reduces STAT3 phosphorylation on Tyr705 and co-localizes with nuclear STAT3 to reduce its binding to target gene promoters *(53)(54)*. Similarly, others have demonstrated that chronic treatment of hepatocytes with AICAR suppresses IL-6 signaling, and was thought to involve an AMPK-mediated inhibition of IL-6-stimulated Tyr phosphorylation and activation of JAK1 and JAK2, and that depletion of AMPKα1 and α2 subunits could block the inhibitory effects of AMPK activators on cytokine-stimulated signaling without significantly altering basal activation *(55)(56)*. However the underlying mechanisms were not investigated. Finally, a role for AMPK-mediated induction of mitogen-activated protein kinase phosphatase-1 (MKP-1) has been reported to play a critical role in suppressing STAT1 activation in aortic smooth muscle cells *(57)*. In contrast, we have demonstrated that AMPK-mediated phosphorylation of JAK1 within its regulatory SH2 domain is a rapid event mediated by direct AMPK activators (A769662, salicylate) and mitochondrial inhibitors (metformin) and is required for AMPK to inhibit STAT3 activation by IL-6 in multiple cell systems. The location of the conserved Ser518 residue at the start of the αB helix within the JAK1 SH2 domain *(39)* suggests that phosphorylation may disrupt SH2 domain structure sufficiently to uncouple cytokine receptors from JAK1 activation, although this has yet to be tested. We also found that either AMPKα1 knockdown or AV-mediated expression of dominant negative AMPKα1 specifically blocked the ability of AMPK activators to suppress STAT, thereby ruling out a constitutive suppressive effect of AMPK on JAK-STAT signaling under our conditions. Finally, the existence of several inhibitory mechanisms by which AMPK can suppress JAK-STAT signaling mirrors the multi-faceted impact of AMPK on the mTOR signaling pathway, which it inhibits through direct phosphorylation of two components [tuberous sclerosis complex 2 (TSC2) on Ser^{1387} *(58)(59)* and Raptor on Ser^{722} and Ser^{792} *(38)*]. These observations suggest that, like the mTOR pathway, AMPK-mediated regulation of JAK-STAT signaling has also evolved such that multiple mechanisms and targets are used to limit its activation.

Given the importance of AMPK and JAK-STAT signaling pathways in either inhibiting or stimulating processes such as cell proliferation, longevity, angiogenesis and inflammation, AMPK-mediated JAK1 phosphorylation could potentially influence these events. Several studies have reported that LKB1, the major kinase responsible for activation loop phosphorylation of AMPKα subunits in response to energy stress, exerts a suppressive effect on STAT3 phosphorylation and function, such that loss of LKB1 increases STAT3 phosphorylation *(60)(61)(62)*. LKB1 is a tumor suppressor and is frequently inactivated in human cancers *(63)*, while hyperactivation of STAT3 within tumor cells and the tumor microenvironment has been extensively described *(18)*. The identification of JAK1 as a target would suggest that AMPK activation might be a useful strategy for limiting STAT3-mediated tumor progression, in addition to its well described effects on cell growth, proliferation and autophagy *(45)(38)(64)*. The ability of AMPK to inhibit STAT phosphorylation following expression of a constitutively active V658F-mutant of JAK1 through a mechanism requiring Ser^{515} or Ser^{518} would also support clinical studies to evaluate AMPK activators such as metformin as potential treatment options for patients with ALL caused by activating JAK1 mutations *(49)(65)*.

Materials and Methods

8

Materials

Antibodies against JAK1 were from BD Transduction Laboratories (cat. no. 610232). HRP-conjugated antibodies against GST were from Abcam (cat. no. ab3416). Cytokines and HRP-conjugated 14-3-3ζ were from R&D Systems. Other antibodies used have been described elsewhere *(66)(67)(68)(21)(69)*. Peptide arrays were synthesized on glass slides by automatic SPOT synthesis using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments). AMPK preparations used for in vitro kinase assays were purified from rat liver as previously described *(35)*. Ad5 adenoviruses expressing either GFP (AV.GFP) or a dominant negative mutant AMPKα1 (AV.DN-AMPKα1) were prepared, titered and used to infect HUVECs as described previously*(28)(68)(70)*

Cell culture and transfections

SV40-immortalised wild-type and *PRKAA1$^{-/-}$/PRKAA2$^{-/-}$* MEFs lacking AMPKα1 and AMPKα2 catalytic subunits, HUVECs, U937 promonocytic cells and the human fibrosarcoma cell lines 2C4, U4C and U4C.JAK1 were cultured as previously described *(34)(47)(48)(21)(71)*. Transient expression of wild type and mutated JAK1 constructs in U4C cells was performed using PolyFect transfection reagent (Qiagen) in accordance with the manufacturer's instructions. Transfection of HUVECs with siRNAs was performed as we have previously described *(71)(72)*.

Expression constructs

pGEX4T/14-3-3ζ was obtained from Professor Graeme Milligan (University of Glasgow, UK). C-terminally Flag- and myc epitope-tagged human JAK1 in pCMV6 was obtained from Origene (cat. no. RC213878). QuikChange mutagenesis (Agilent Technologies) was used to introduce JAK1 mutations at Ser^{515} and Ser^{518} to Ala, and Val^{658} to Phe. All constructs were sequenced in their entirety to ensure that no additional unanticipated mutations had been introduced.

SDS-PAGE, immunoblotting and overlays

Cells were washed twice with ice-cold PBS and lysed by scraping into lysis buffer [50 mM HEPES pH 7.4, 150 mM sodium chloride, 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 mM sodium fluoride, 5mM EDTA, 10 mM sodium phosphate, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, 2% (w/v) EDTA-free complete protease inhibitor cocktail (Sigma)]. After 30 min on ice, lysates were vortexed and cleared by centrifugation. Equivalent amounts of protein, as determined by bicinchoninic acid protein assay, were fractionated by 8 to 12% SDS-PAGE gels depending on the application, transferred to a nitrocellulose membrane and analyzed either by immunoblotting or overlay with HRP-14-3-3ζ as previously described *(68)(71)*. Peptide arrays were blocked in 5% (w/v) BSA in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) prior to overlay with HRP-14-3-3ζ (1:500 in BSA/TBST) overnight at 4°C, washing and visualization by enhanced chemiluminescence.

Peptide array AMPK assays

Peptide arrays were synthesized as previously described *(73)* AMPK phosphorylation of immobilized peptide arrays spotted in duplicate was undertaken with either vehicle or 0.5 units/ml of purified rat liver AMPK *(35)* diluted in phosphorylation buffer (50 mM HEPES, pH7.4, 0.01% (v/v) Brij-35, 1 mM DTT, 1 mM ATP, 0.2 mM AMP, 25 mM MgCl$_2$, 1% (w/v)

BSA) containing 1 μCi [γ^{32}P]ATP followed by incubation at 30°C for 30 min. Phosphorylated peptides were visualized by autoradiography.

Pull-down assays and immunoprecipitations

Recombinant GST, GST/14-3-3τ and GST/14-3-3ζ were produced following induction in *E. coli* BL21 cells and purified from clarified bacterial extracts using glutathione-Sepharose beads. 20 μg of GST, GST/14-3-3τ or GST/14-3-3ζ immobilized on glutathione-Sepharose beads were added to protein-equalized clarified cell extracts prepared in pull down assay buffer (50 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, supplemented with phosphatase inhibitors 5mM sodium fluoride, 1mM sodium orthovanadate, and protease inhibitors 10 μg/ml benzamidine, 0.1mM PMSF, 10 μg/ml soybean trypsin inhibitor, 2% (w/v) EDTA-free complete protease inhibitor cocktail) and incubated overnight with rotation at 4°C. After washing three times in pull down assay buffer, samples were eluted in SDS-PAGE sample buffer by incubation at 65°C for 30 min prior to fractionation on 8% SDS-PAGE gels and immunoblotting with an antibody against JAK1. GST fusion proteins were identified either by probing with HRP-conjugated antibody against GST or Ponceau staining of nitrocellulose blots.

Flag-tagged wild type and mutated human JAK1 were immunoprecipitated from transfected U4C cells solubilized in immunoprecipitation buffer using Flag M2-Sepharose beads prior to analysis by SDS-PAGE and immunoblotting as previously described *(73)*.

Analysis of conditioned medium for chemotactic activity

Conditioned medium was obtained from HUVECs pre-incubated in the presence or absence of 100 μM A769662 for 30 min prior to incubation with 25 ng/ml sIL-6Rα and 5 ng/ml IL-6 for 2 h. HUVECs were washed prior to collection of conditioned medium for 1h (such that residual sIL-6Rα/IL-6 and A769662 were not in conditioned medium). Conditioned medium from each treatment was then added to the bottom wells of a Boyden chamber and equal numbers of U937 promonocytic cells added to the top chamber, which was separated from the bottom by a collagen-coated membrane. After 5 hr, migrated U937 cells were collected from the lower well and counted in urinalysis glasstic slides (Stratagene, Cambridge, UK).

Statistical Analysis

Results are expressed as mean ± SEM. Statistically significant differences were determined using a two-tail t-test, or one or two-way ANOVA where appropriate, with $p < 0.05$ as significant.

Supplementary Materials

Fig. S1. AMPK-mediated inhibition of STAT3 phosphorylation in HUVECs.

Fig. S2. AMPK-mediated inhibition of sIL-6Rα/IL-6-stimulated *SOCS3* and *CEBPD* mRNA induction.

Fig. S3. Contributions of JAK1, JAK2 and Tyk2 in mediating sIL-6Rα/IL-6-mediated STAT3 phosphorylation in HUVECs.

Fig. S4. Specific interaction of JAK1 with 14-3-3ζ and 14−3−3τ.

References and Notes

1. J. J. O'Shea, D. M. Schwartz, A. V Villarino, M. Gadina, I. B. McInnes, A. Laurence, The JAK-STAT pathway: impact on human disease and therapeutic intervention., *Annu. Rev. Med.* **66**, 311–28 (2015).

2. C. Garbers, H. M. Hermanns, F. Schaper, G. Müller-Newen, J. Grötzinger, S. Rose-John, J. Scheller, Plasticity and cross-talk of interleukin 6-type cytokines., *Cytokine Growth Factor Rev.* **23**, 85–97 (2012).

3. S. A. Jones, J. Scheller, S. Rose-John, Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling., *J. Clin. Invest.* **121**, 3375–83 (2011).

4. R. Eulenfeld, A. Dittrich, C. Khouri, P. J. Müller, B. Mütze, A. Wolf, F. Schaper, Interleukin-6 signalling: more than Jaks and STATs., *Eur. J. Cell Biol.* **91**, 486–95 (2012).

5. D. G. Hardie, AMP-activated protein kinase: maintaining energy homeostasis at the cellular and whole-body levels., *Annu. Rev. Nutr.* **34**, 31–55 (2014).

6. G. R. Steinberg, B. E. Kemp, AMPK in Health and Disease., *Physiol. Rev.* **89**, 1025–78 (2009).

7. L. Tong, Structure and function of biotin-dependent carboxylases, *Cell. Mol. Life Sci.* **70**, 863–891 (2013).

8. K. Marcinko, G. R. Steinberg, The role of AMPK in controlling metabolism and mitochondrial biogenesis during exercise, *Exp. Physiol.* **99**, 1581–1585 (2014).

9. E. Bess, B. Fisslthaler, T. Frömel, I. Fleming, Nitric oxide-induced activation of the AMP-activated protein kinase α2 subunit attenuates IκB kinase activity and inflammatory responses in endothelial cells., *PLoS One* **6**, e20848 (2011).

10. B. Gongol, T. Marin, I.-C. Peng, B. Woo, M. Martin, S. King, W. Sun, D. A. Johnson, S. Chien, J. Y.-J. Shyy, AMPKα2 exerts its anti-inflammatory effects through PARP-1 and Bcl-6., *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3161–6 (2013).

11. F. S. Gaskin, K. Kamada, M. Y. Zuidema, A. W. Jones, L. J. Rubin, R. J. Korthuis, Isoform-selective 5'-AMP-activated protein kinase-dependent preconditioning mechanisms to prevent postischemic leukocyte-endothelial cell adhesive interactions., *Am. J. Physiol. Heart Circ. Physiol.* **300**, H1352–60 (2011).

12. S. Schuhmacher, M. Foretz, M. Knorr, T. Jansen, M. Hortmann, P. Wenzel, M. Oelze, A. L. Kleschyov, A. Daiber, J. F. Keaney, G. Wegener, K. Lackner, T. Münzel, B. Viollet, E. Schulz, α1AMP-activated protein kinase preserves endothelial function during chronic angiotensin II treatment by limiting Nox2 upregulation., *Arterioscler. Thromb. Vasc. Biol.* **31**, 560–6 (2011).

13. A. Bai, M. Yong, A. G. Ma, Y. Ma, C. R. Weiss, Q. Guan, C. N. Bernstein, Z. Peng, Novel anti-inflammatory action of 5-aminoimidazole-4-carboxamide ribonucleoside with protective effect in dextran sulfate sodium-induced acute and chronic colitis., *J. Pharmacol. Exp. Ther.* **333**, 717–25 (2010).

14. X. Zhao, J. W. Zmijewski, E. Lorne, G. Liu, Y.-J. Park, Y. Tsuruta, E. Abraham, Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung

injury., *Am. J. Physiol. Lung Cell. Mol. Physiol.* **295**, L497–504 (2008).

15. R. Prasad, S. Giri, N. Nath, I. Singh, A. K. Singh, 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates experimental autoimmune encephalomyelitis via modulation of endothelial-monocyte interaction., *J. Neurosci. Res.* **84**, 614–25 (2006).

16. S. Galic, M. D. Fullerton, J. D. Schertzer, S. Sikkema, K. Marcinko, C. R. Walkley, D. Izon, J. Honeyman, Z.-P. Chen, B. J. van Denderen, B. E. Kemp, G. R. Steinberg, Hematopoietic AMPK β1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity., *J. Clin. Invest.* **121**, 4903–15 (2011).

17. Y. Zhang, J. Qiu, X. Wang, Y. Zhang, M. Xia, AMP-activated protein kinase suppresses endothelial cell inflammation through phosphorylation of transcriptional coactivator p300., *Arterioscler. Thromb. Vasc. Biol.* **31**, 2897–908 (2011).

18. H. Yu, H. Lee, A. Herrmann, R. Buettner, R. Jove, Revisiting STAT3 signalling in cancer: new and unexpected biological functions, *Nat. Rev. Cancer* **14**, 736–746 (2014).

19. W. Vainchenker, S. N. Constantinescu, JAK/STAT signaling in hematological malignancies., *Oncogene* **32**, 2601–13 (2013).

20. G. Ortiz-Muñoz, J. L. Martin-Ventura, P. Hernandez-Vargas, B. Mallavia, V. Lopez-Parra, O. Lopez-Franco, B. Muñoz-Garcia, P. Fernandez-Vizarra, L. Ortega, J. Egido, C. Gomez-Guerrero, Suppressors of cytokine signaling modulate JAK/STAT-mediated cell responses during atherosclerosis., *Arterioscler. Thromb. Vasc. Biol.* **29**, 525–31 (2009).

21. M.-A. Ewart, C. F. Kohlhaas, I. P. Salt, Inhibition of tumor necrosis factor alpha-stimulated monocyte adhesion to human aortic endothelial cells by AMP-activated protein kinase., *Arterioscler. Thromb. Vasc. Biol.* **28**, 2255–7 (2008).

22. B. Enkhjargal, S. Godo, A. Sawada, N. Suvd, H. Saito, K. Noda, K. Satoh, H. Shimokawa, Endothelial AMP-activated protein kinase regulates blood pressure and coronary flow responses through hyperpolarization mechanism in mice., *Arterioscler. Thromb. Vasc. Biol.* **34**, 1505–13 (2014).

23. J. M. Corton, J. G. Gillespie, S. A. Hawley, D. G. Hardie, 5-Aminoimidazole-4-Carboxamide Ribonucleoside. A Specific Method for Activating AMP-Activated Protein Kinase in Intact Cells?, *Eur. J. Biochem.* **229**, 558–565 (1995).

24. J. W. Scott, B. J. W. van Denderen, S. B. Jorgensen, J. E. Honeyman, G. R. Steinberg, J. S. Oakhill, T. J. Iseli, A. Koay, P. R. Gooley, D. Stapleton, B. E. Kemp, Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes., *Chem. Biol.* **15**, 1220–30 (2008).

25. M. R. Owen, E. Doran, A. P. Halestrap, Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain., *Biochem. J.* **348 Pt 3**, 607–14 (2000).

26. S. A. Hawley, M. D. Fullerton, F. A. Ross, J. D. Schertzer, C. Chevtzoff, K. J. Walker, M. W. Peggie, D. Zibrova, K. A. Green, K. J. Mustard, B. E. Kemp, K. Sakamoto, G. R. Steinberg, D. G. Hardie, The ancient drug salicylate directly activates AMP-activated protein kinase., *Science* **336**, 918–22 (2012).

27. R. J. Ford, M. D. Fullerton, S. L. Pinkosky, E. A. Day, J. W. Scott, J. S. Oakhill, A. L. Bujak,

B. K. Smith, J. D. Crane, R. M. Blümer, K. Marcinko, B. E. Kemp, H. C. Gerstein, G. R. Steinberg, Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity., *Biochem. J.* **468**, 125–32 (2015).

28. A. Woods, D. Azzout-Marniche, M. Foretz, S. C. Stein, P. Lemarchand, P. Ferre, F. Foufelle, D. Carling, Characterization of the Role of AMP-Activated Protein Kinase in the Regulation of Glucose-Activated Gene Expression Using Constitutively Active and Dominant Negative Forms of the Kinase, *Mol. Cell. Biol.* **20**, 6704–6711 (2000).

29. J. Yang, J. Huang, M. Dasgupta, N. Sears, M. Miyagi, B. Wang, M. R. Chance, X. Chen, Y. Du, Y. Wang, L. An, Q. Wang, T. Lu, X. Zhang, Z. Wang, G. R. Stark, Reversible methylation of promoter-bound STAT3 by histone-modifying enzymes., *Proc. Natl. Acad. Sci. U. S. A.* **107**, 21499–504 (2010).

30. C. A. Cantwell, E. Sterneck, P. F. Johnson, Interleukin-6-Specific Activation of the C/EBPδ Gene in Hepatocytes Is Mediated by Stat3 and Sp1, *Mol. Cell. Biol.* **18**, 2108–2117 (1998).

31. M. Jougasaki, T. Ichiki, Y. Takenoshita, M. Setoguchi, Statins suppress interleukin-6-induced monocyte chemo-attractant protein-1 by inhibiting Janus kinase/signal transducers and activators of transcription pathways in human vascular endothelial cells., *Br. J. Pharmacol.* **159**, 1294–303 (2010).

32. M. Romano, M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, F. Bussolino, V. Poli, G. Ciliberto, A. Mantovani, Role of IL-6 and Its Soluble Receptor in Induction of Chemokines and Leukocyte Recruitment, *Immunity* **6**, 315–325 (1997).

33. E. C. Borden, G. C. Sen, G. Uze, R. H. Silverman, R. M. Ransohoff, G. R. Foster, G. R. Stark, Interferons at age 50: past, current and future impact on biomedicine., *Nat. Rev. Drug Discov.* **6**, 975–90 (2007).

34. M. C. Gauzzi, L. Velazquez, R. McKendry, K. E. Mogensen, M. Fellous, S. Pellegrini, Interferon- -dependent Activation of Tyk2 Requires Phosphorylation of Positive Regulatory Tyrosines by Another Kinase, *J. Biol. Chem.* **271**, 20494–20500 (1996).

35. S. A. Hawley, M. Davison, A. Woods, S. P. Davies, R. K. Beri, D. Carling, D. G. Hardie, Characterization of the AMP-activated Protein Kinase Kinase from Rat Liver and Identification of Threonine 172 as the Major Site at Which It Phosphorylates AMP-activated Protein Kinase, *J. Biol. Chem.* **271**, 27879–27887 (1996).

36. K. M. Brown, L. C. Y. Lee, J. E. Findlay, J. P. Day, G. S. Baillie, Cyclic AMP-specific phosphodiesterase, PDE8A1, is activated by protein kinase A-mediated phosphorylation., *FEBS Lett.* **586**, 1631–7 (2012).

37. J. W. Scott, D. G. Norman, S. A. Hawley, L. Kontogiannis, D. G. Hardie, Protein kinase substrate recognition studied using the recombinant catalytic domain of AMP-activated protein kinase and a model substrate., *J. Mol. Biol.* **317**, 309–23 (2002).

38. D. M. Gwinn, D. B. Shackelford, D. F. Egan, M. M. Mihaylova, A. Mery, D. S. Vasquez, B. E. Turk, R. J. Shaw, AMPK phosphorylation of raptor mediates a metabolic checkpoint., *Mol. Cell* **30**, 214–26 (2008).

39. R. Ferrao, H. J. A. Wallweber, H. Ho, C. Tam, Y. Franke, J. Quinn, P. J. Lupardus, The

Structural Basis for Class II Cytokine Receptor Recognition by JAK1., *Structure* **24**, 897–905 (2016).

40. R. McNally, A. V. Toms, M. J. Eck, T. J. Boggon, Ed. Crystal Structure of the FERM-SH2 Module of Human Jak2, *PLoS One* **11**, e0156218 (2016).

41. H. J. A. Wallweber, C. Tam, Y. Franke, M. A. Starovasnik, P. J. Lupardus, Structural basis of recognition of interferon-α receptor by tyrosine kinase 2., *Nat. Struct. Mol. Biol.* **21**, 443–8 (2014).

42. Z. Chen, I.-C. Peng, W. Sun, M.-I. Su, P.-H. Hsu, Y. Fu, Y. Zhu, K. DeFea, S. Pan, M.-D. Tsai, J. Y.-J. Shyy, AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser633., *Circ. Res.* **104**, 496–505 (2009).

43. D. Bridges, G. B. G. Moorhead, 14-3-3 proteins: a number of functions for a numbered protein., *Sci. STKE* **2005**, re10 (2005).

44. V. K. Weerasekara, D. J. Panek, D. G. Broadbent, J. B. Mortenson, A. D. Mathis, G. N. Logan, J. T. Prince, D. M. Thomson, J. W. Thompson, J. L. Andersen, Metabolic-stress-induced rearrangement of the 14-3-3ζ interactome promotes autophagy via a ULK1- and AMPK-regulated 14-3-3ζ interaction with phosphorylated Atg9., *Mol. Cell. Biol.* **34**, 4379–88 (2014).

45. H. I. D. Mack, B. Zheng, J. M. Asara, S. M. Thomas, AMPK-dependent phosphorylation of ULK1 regulates ATG9 localization., *Autophagy* **8**, 1197–214 (2012).

46. M. Müller, J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. G. Harpur, G. Barbieri, B. A. Witthuhn, C. Schindler, The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction., *Nature* **366**, 129–35 (1993).

47. D. Guschin, N. Rogers, J. Briscoe, B. Witthuhn, D. Watling, F. Horn, S. Pellegrini, K. Yasukawa, P. Heinrich, G. R. Stark, A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6., *EMBO J.* **14**, 1421–9 (1995).

48. K. R. Laderoute, K. Amin, J. M. Calaoagan, M. Knapp, T. Le, J. Orduna, M. Foretz, B. Viollet, 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments., *Mol. Cell. Biol.* **26**, 5336–47 (2006).

49. C. G. Mullighan, J. Zhang, R. C. Harvey, J. R. Collins-Underwood, B. A. Schulman, L. A. Phillips, S. K. Tasian, M. L. Loh, X. Su, W. Liu, M. Devidas, S. R. Atlas, I.-M. Chen, R. J. Clifford, D. S. Gerhard, W. L. Carroll, G. H. Reaman, M. Smith, J. R. Downing, S. P. Hunger, C. L. Willman, JAK mutations in high-risk childhood acute lymphoblastic leukemia., *Proc. Natl. Acad. Sci. U. S. A.* **106**, 9414–8 (2009).

50. O. Silvennoinen, S. R. Hubbard, Molecular insights into regulation of JAK2 in myeloproliferative neoplasms., *Blood* **125**, 3388–92 (2015).

51. L. A. J. O'Neill, D. G. Hardie, Metabolism of inflammation limited by AMPK and pseudo-starvation., *Nature* **493**, 346–55 (2013).

52. M. Dandapani, D. G. Hardie, AMPK: opposing the metabolic changes in both tumour cells and inflammatory cells?, *Biochem. Soc. Trans.* **41**, 687–93 (2013).

53. Y. D. Kim, Y. H. Kim, Y. M. Cho, D. K. Kim, S. W. Ahn, J. M. Lee, D. Chanda, M. Shong, C. H. Lee, H. S. Choi, Metformin ameliorates IL-6-induced hepatic insulin resistance via induction of orphan nuclear receptor small heterodimer partner (SHP) in mouse models., *Diabetologia* **55**, 1482–94 (2012).

54. Y. D. Kim, Y.-H. Kim, S. Tadi, J. H. Yu, Y.-H. Yim, N. H. Jeoung, M. Shong, L. Hennighausen, R. A. Harris, I.-K. Lee, C.-H. Lee, H.-S. Choi, Metformin inhibits growth hormone-mediated hepatic PDK4 gene expression through induction of orphan nuclear receptor small heterodimer partner., *Diabetes* **61**, 2484–94 (2012).

55. A. Nerstedt, E. Cansby, M. Amrutkar, U. Smith, M. Mahlapuu, Pharmacological activation of AMPK suppresses inflammatory response evoked by IL-6 signalling in mouse liver and in human hepatocytes., *Mol. Cell. Endocrinol.* **375**, 68–78 (2013).

56. A. Nerstedt, A. Johansson, C. X. Andersson, E. Cansby, U. Smith, M. Mahlapuu, AMP-activated protein kinase inhibits IL-6-stimulated inflammatory response in human liver cells by suppressing phosphorylation of signal transducer and activator of transcription 3 (STAT3), *Diabetologia* **53**, 2406–2416 (2010).

57. C. He, H. Li, B. Viollet, M.-H. Zou, Z. Xie, AMP-activated Protein Kinase Suppresses Vascular Inflammation in vivo by Inhibiting Signal Transducer and Activator of Transcription-1., *Diabetes* (2015), doi:10.2337/db15-0107.

58. K. Inoki, T. Zhu, K.-L. Guan, TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival, *Cell* **115**, 577–590 (2003).

59. R. J. Shaw, N. Bardeesy, B. D. Manning, L. Lopez, M. Kosmatka, R. A. DePinho, L. C. Cantley, The LKB1 tumor suppressor negatively regulates mTOR signaling., *Cancer Cell* **6**, 91–9 (2004).

60. A. Mukhopadhyay, K. C. Berrett, U. Kc, P. M. Clair, S. M. Pop, S. R. Carr, B. L. Witt, T. G. Oliver, Sox2 cooperates with Lkb1 loss in a mouse model of squamous cell lung cancer., *Cell Rep.* **8**, 40–9 (2014).

61. Y. Wang, W. Dai, X. Chu, B. Yang, M. Zhao, Y. Sun, Downregulation of LKB1 suppresses Stat3 activity to promote the proliferation of esophageal carcinoma cells, *Mol. Med. Rep.* **9**, 2400–2404 (2014).

62. D. W. Kim, H. K. Chung, K. C. Park, J. H. Hwang, Y. S. Jo, J. Chung, D. V Kalvakolanu, N. Resta, M. Shong, Tumor suppressor LKB1 inhibits activation of signal transducer and activator of transcription 3 (STAT3) by thyroid oncogenic tyrosine kinase rearranged in transformation (RET)/papillary thyroid carcinoma (PTC)., *Mol. Endocrinol.* **21**, 3039–49 (2007).

63. M. Momcilovic, D. B. Shackelford, Targeting LKB1 in cancer - exposing and exploiting vulnerabilities., *Br. J. Cancer* **113**, 574–84 (2015).

64. D. F. Egan, D. B. Shackelford, M. M. Mihaylova, S. Gelino, R. A. Kohnz, W. Mair, D. S. Vasquez, A. Joshi, D. M. Gwinn, R. Taylor, J. M. Asara, J. Fitzpatrick, A. Dillin, B. Viollet, M. Kundu, M. Hansen, R. J. Shaw, Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy., *Science* **331**, 456–61 (2011).

65. E. Flex, V. Petrangeli, L. Stella, S. Chiaretti, T. Hornakova, L. Knoops, C. Ariola, V. Fodale, E. Clappier, F. Paoloni, S. Martinelli, A. Fragale, M. Sanchez, S. Tavolaro, M. Messina, G.

Cazzaniga, A. Camera, G. Pizzolo, A. Tornesello, M. Vignetti, A. Battistini, H. Cavé, B. D. Gelb, J.-C. Renauld, A. Biondi, S. N. Constantinescu, R. Foà, M. Tartaglia, Somatically acquired JAK1 mutations in adult acute lymphoblastic leukemia., *J. Exp. Med.* **205**, 751–8 (2008).

66. W. A. Sands, H. D. Woolson, S. J. Yarwood, T. M. Palmer, Exchange protein directly activated by cyclic AMP-1-regulated recruitment of CCAAT/enhancer-binding proteins to the suppressor of cytokine signaling-3 promoter., *Methods Mol. Biol.* **809**, 201–14 (2012).

67. S. J. Yarwood, G. Borland, W. A. Sands, T. M. Palmer, Identification of CCAAT/enhancer-binding proteins as exchange protein activated by cAMP-activated transcription factors that mediate the induction of the SOCS-3 gene., *J. Biol. Chem.* **283**, 6843–53 (2008).

68. M. M. A. Safhi, C. Rutherford, C. Ledent, W. A. Sands, T. M. Palmer, Priming of signal transducer and activator of transcription proteins for cytokine-triggered polyubiquitylation and degradation by the A 2A adenosine receptor., *Mol. Pharmacol.* **77**, 968–78 (2010).

69. J. A. Reihill, M.-A. Ewart, I. P. Salt, The role of AMP-activated protein kinase in the functional effects of vascular endothelial growth factor-A and -B in human aortic endothelial cells., *Vasc. Cell* **3**, 9 (2011).

70. W. A. Sands, A. F. Martin, E. W. Strong, T. M. Palmer, Specific Inhibition of Nuclear Factor- B-Dependent Inflammatory Responses by Cell Type-Specific Mechanisms upon A2A Adenosine Receptor Gene Transfer, *Mol. Pharmacol.* **66**, 1147–1159 (2004).

71. H. D. Woolson, V. S. Thomson, C. Rutherford, S. J. Yarwood, T. M. Palmer, Selective inhibition of cytokine-activated extracellular signal-regulated kinase by cyclic AMP via Epac1-dependent induction of suppressor of cytokine signalling-3., *Cell. Signal.* **21**, 1706–15 (2009).

72. C. Rutherford, S. Childs, J. Ohotski, L. McGlynn, M. Riddick, S. MacFarlane, D. Tasker, S. Pyne, N. J. Pyne, J. Edwards, T. M. Palmer, Regulation of cell survival by sphingosine-1-phosphate receptor S1P1 via reciprocal ERK-dependent suppression of Bim and PI-3-kinase/protein kinase C-mediated upregulation of Mcl-1., *Cell Death Dis.* **4**, e927 (2013).

73. Y. Y. Sin, T. P. Martin, L. Wills, S. Currie, G. S. Baillie, Small heat shock protein 20 (Hsp20) facilitates nuclear import of protein kinase D 1 (PKD1) during cardiac hypertrophy., *Cell Commun. Signal.* **13**, 16 (2015).

74. B. Wang, H. Yang, Y.-C. Liu, T. Jelinek, L. Zhang, E. Ruoslahti, H. Fu, Isolation of High-Affinity Peptide Antagonists of 14-3-3 Proteins by Phage Display †, *Biochemistry* **38**, 12499–12504 (1999).

Figure Legends:

Fig. 1. **AMPK rapidly inhibits IL-6 signaling and function.** (**A**) Immunoblotting of HUVECs pre-incubated with vehicle (V), AICAR (1 mM, 2 hours), A769662 (100 μM, 40 min), or a combination of metformin and salicylate (Met+Sal; 3 mM and 5 mM,1 hour) then stimulated with sIL-6Rα/IL-6 (25 ng/ml, 5 ng/ml) for a further 30 min. Densitometry of phosphorylated STAT3 was normalized to that of total protein. Data are mean ±SEM, N=3 independent experiments. (**B**) Immunoblotting of HUVECs transfected with either control or AMPKα1 transcript-targeted siRNA as indicated. Cells were then pre-incubated with vehicle or A769662 then stimulated with sIL-6Rα/IL-6 as in (A). Data were analyzed as in (A), N=3 independent experiments. (**C**) Immunoblotting of HUVECs pre-treated with vehicle or A769662 (100 μM, 40 min) then stimulated with sIL-6Rα/IL-6 (25 ng/ml, 5 ng/ml) for 5 hours. Densitometry of SOCS3 and C/EBPδ were normalized to GAPDH; N=3 independent experiments. (**D**) Chemotaxis of U937 promonocytic cells cultured in conditioned medium from HUVECs that were pre-treated with vehicle or A769662 (100 μM, 30 min) then stimulated with sIL-6Rα/IL-6 (25 ng/ml, 5 ng/ml) for 2 hours, washed and incubated in fresh medium for conditioning for a further 1 hour. Data are mean ±SEM, N=3 independent experiments. *p<0.05 and **p<0.01 using a one-way ANOVA with Bonferroni post hoc test.

Fig. 2. AMPK inhibits STAT1 and STAT3 activation by IL-6, LIF, OSM and IFNα. (**A**) Immunoblotting of HUVECs pre-incubated with vehicle (V) or A769662 (100 μM, 30 min) then stimulated with vehicle (-), sIL-6Rα/IL-6 (25 ng/ml, 5 ng/ml), leukemia inhibitory factor (LIF; 10 ng/ml), or oncostatin M (OSM; 10 ng/ml) for 30 min. Densitometry of phosphorylated protein was normalized to that of the respective total fraction. Data are mean ±SEM from N=4 independent experiments. **p<0.01 and ***p<0.001 using a one-way ANOVA with Bonferroni post hoc test. (**B**) Immunoblotting of HUVECs pre-incubated with vehicle (V) or A769662 (100 μM, 30 min) then stimulated with vehicle, sIL-6Rα/IL-6 (25 ng/ml/5 ng/ml, 30 min), or IFNα (1000U/ml,
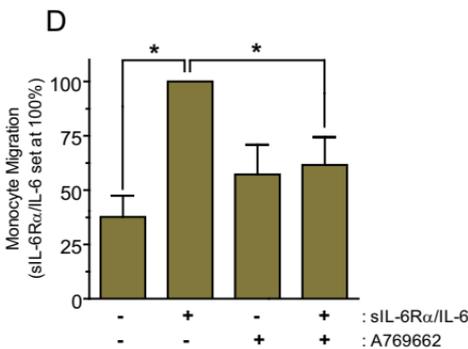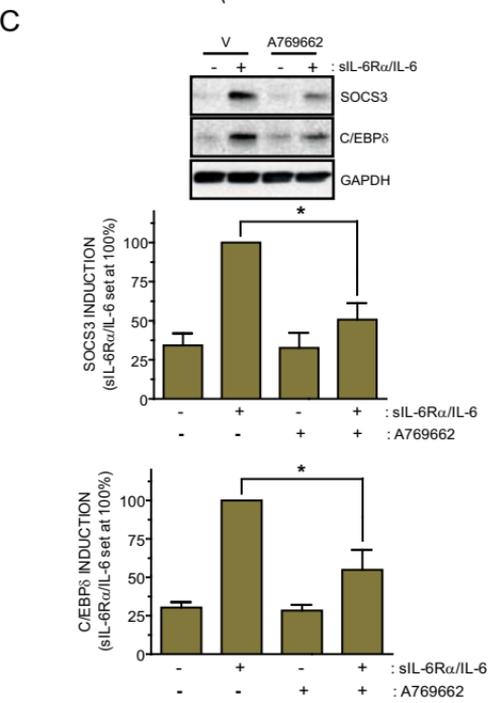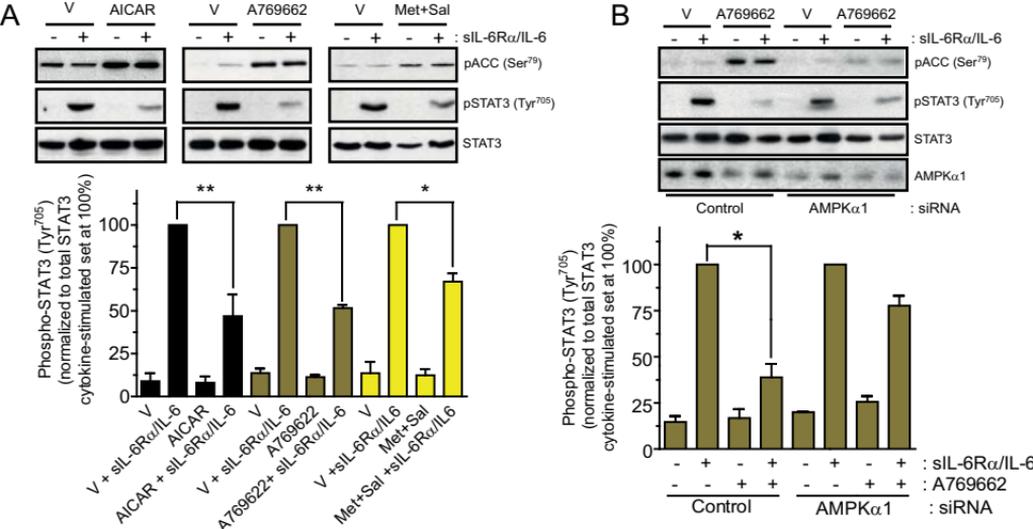
15 min). Data were analyzed as in (A); N=3 independent experiments. ***p<0.001 using a one-way ANOVA with Bonferroni post hoc test.

Fig. 3. Identification of Ser[515] and Ser[518] within the JAK1 SH2 domain as sites of AMPK phosphorylation in vitro. (A) Overlapping peptide arrays spanning the human *JAK1* open reading frame were incubated with [γ-^{32}P]ATP in the absence (-) or presence (+) of AMPK purified from rat liver. Shown are the region of the array in which AMPK-dependent phosphorylation was detectable (bottom) and the location of the sequence within the domain structure of JAK1 (top). Immobilized SAMS peptide on the same array served as a control. Data are representative of three experiments. **(B)** Kinase array as in (A) using immobilized comprising wild-type (WT) and S515A/S518A (*) mutant JAK1 peptides. Italics indicate peptides with no detectable phosphorylation by AMPK. N=3 experiments. **(C)** Optimal and secondary selections for AMPK substrate recognition from *(38)* alongside the sequence of rat ACC1 that is phosphorylated by AMPK at Ser[79]. Below: The conformity of the sequence surrounding Ser[518] on human JAK1 to the AMPK consensus and its evolutionary conservation are shown (color coded), as is the sequence surrounding Ser[515] in human JAK1. The underlined Ser residue (equivalent to Ser[518]) indicates that this sequence would potentially conform to a weak AMPK consensus site after the acquisition of negative charge upon its phosphorylation. **(D)** Alignment of SH2 domains from the four human JAK isoforms using T-Coffee then modified to match the structural data available for JAK1, JAK2 and Tyk2 *(39–41)*. For clarity, only the alignment of the indicated regions within each SH2 domain is shown. The lettering above the alignment indicates secondary structural elements; the βG1 and βG2 regions present in the JAK2 and Tyk2 SH2 domains are moderately disordered in the only available JAK1 SH2 domain structure *(39)*. The residues corresponding to Ser[515] and Ser[518] in JAK1 are underlined and bolded, and the conserved Ser residues in JAK2, JAK3 and Tyk2 equivalent to Ser[518] are bolded and marked with an asterisk (*). Box highlights the sequence surrounding the conserved Ser residue in each JAK isoform, color coded to show conformity to the AMPK consensus. Bottom right: structure of the human JAK1 FERM-SH2 fusion (PDB: 5IXD) generated using PyMol *(39)*. Zoom: location of Ser[518] within the αB helix immediately downstream of the EF loop in the SH2 domain. The αA and αB loops, present in all SH2 domains, are also indicated.

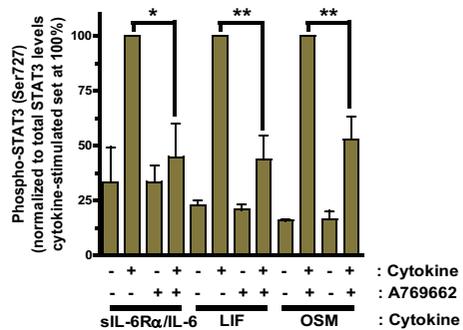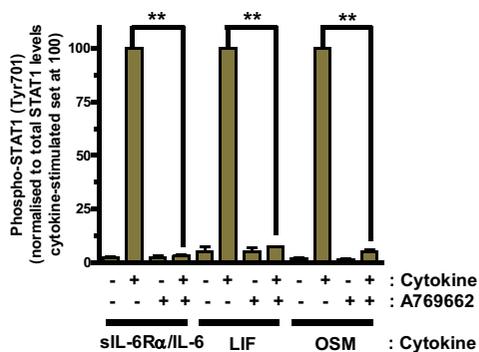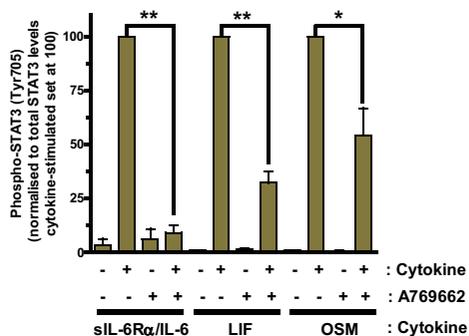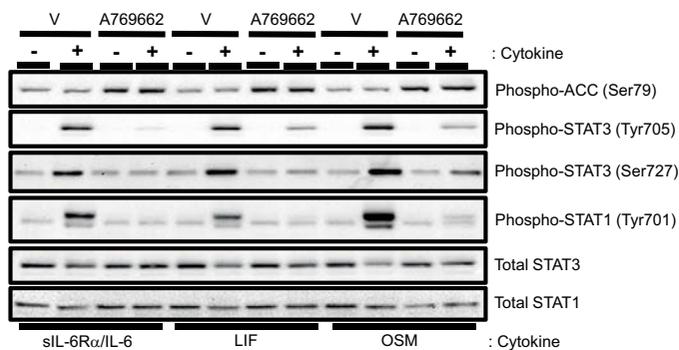**Fig. 4 AMPK-mediated phosphorylation of JAK1 in intact cells. (A)** Immobilized peptides comprising the indicated JAK1 sequence (top) and variants with phosphorylated (p) and non-phosphorylated versions of Ser[515] and Ser[518] were overlaid with HRP-conjugated 14-3-3ζ as indicated. Positive and negative control peptides for 14-3-3ζ interaction *(74)* are also shown. Blot is representative of N=3 experiments. **(B)** *JAK1*-null U4C human fibrosarcoma cells were transiently transfected with wild-type (WT) JAK1, S515A/S518A (SSAA) mutant JAK1 constructs, or a control and treated with vehicle (-) or A769662 (100 μM) for 40 min. 10% of each total cell extract was immunoblotted as indicated; the remainder was incubated with GST/14-3-3ζ and glutathione-Sepharose beads, and immunoprecipitates were immunoblotted for JAK1. Densitometry of

immunoprecipitated JAK1 was normalized to that in the total extract. Data are mean ±SEM for N=3 independent experiments. *p<0.05 using a one-way ANOVA with Bonferroni post hoc test. (**C**) Pulldown and immunoblotting as described in (B) in immortalized WT and AMPKα1$^{-/-}$:AMPKα2$^{-/-}$ MEFs treated with A769662 (100 μM) for up to 20 min. Data were analyzed as described in (B). *p<0.05 and **p<0.01 using a one-way ANOVA with Bonferroni post hoc test. (**D**) Pulldown and immunoblotting as described in (B) in HUVECs treated for the indicated times with metformin and salicylate (Met+Sal; 3 and 5 mM, respectively). Data were analyzed as described in (B). *p<0.05 using a one-way ANOVA with Dunnett post hoc test.
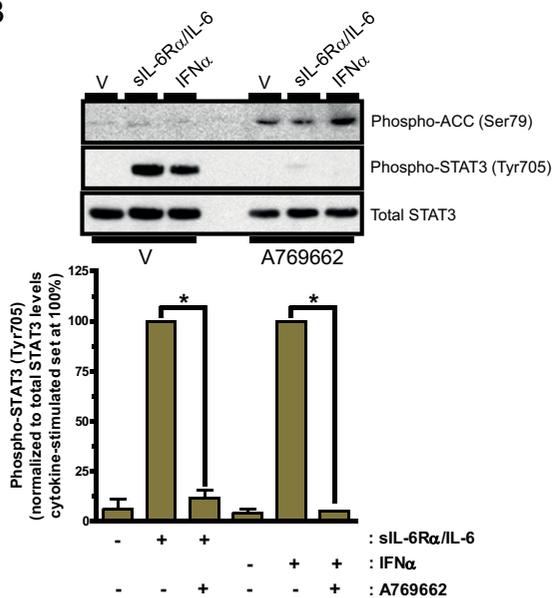
Fig. 5 AMPK-mediated inhibition of JAK1-dependent signaling requires Ser^{515} and Ser^{518}.
(**A**) Immunoblotting in *JAK1*-null U4C cells transiently transfected with either control GFP or wild-type (WT) JAK1 constructs and treated with vehicle (-) or sIL-6Rα/IL-6 (25 ng/ml, 5 ng/ml) for 30 min after pre-incubation with vehicle (V) or A769662 (100 μM) for 40 min. Blots are representative of N=4 experiments. (**B**) Immunoblotting in U4C cells transiently transfected with either WT JAK1 or SSAA-mutant JAK1 and treated with vehicle (-) or sIL-6Rα/IL-6 complex (25 ng/ml, 5 ng/ml) for 30 min after pre-incubation with vehicle or A769662 (100 μM) for 30 min, as indicated. Line in top blot marks where unrelated lanes were removed. Densitometry of phosphorylated STAT3 was normalized to total STAT3. Data are mean ±SEM for N=3 independent experiments. ***p<0.001 using a one-way ANOVA with Bonferroni post hoc test. (**C**) U4C cells were subject to a mock transfection (M) or transiently transfected with WT, V658F, or V658F SSAA JAK1 construct then treated with vehicle or A769662 (100 μM) for 30 min as indicated.. Data were analyzed as in (B); **p<0.01.
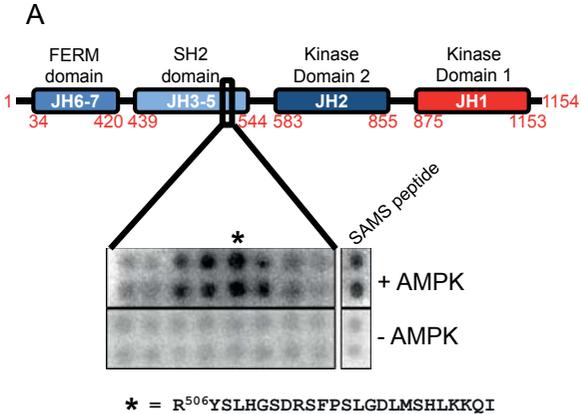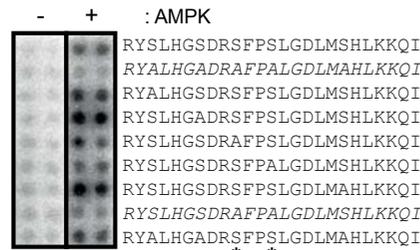
A

	V	AICAR	V	A769662	V	Met+Sal
sIL-6Rα/IL-6	− +	− +	− +	− +	− +	− +

pACC (Ser^{79})
pSTAT3 (Tyr^{705})
STAT3

Phospho-STAT3 (Tyr^{705})
(normalized to total STAT3 cytokine-stimulated set at 100%)

**
V
V + sIL-6Rα/IL-6
AICAR
AICAR + sIL-6Rα/IL-6

**
V
V + sIL-6Rα/IL-6
A769622
A769622+ sIL-6Rα/IL-6

*
V
V +sIL-6Rα/IL6
Met+Sal
Met+Sal +sIL-6Rα/IL6

B

	V	A769662	V	A769662	
sIL-6Rα/IL-6	− +	− +	− +	− +	

pACC (Ser^{79})
pSTAT3 (Tyr^{705})
STAT3
AMPKα1

Control · · · · · · · · · · AMPKα1 : siRNA

Phospho-STAT3 (Tyr^{705})
(normalized to total STAT3 cytokine-stimulated set at 100%)

*

: sIL-6Rα/IL-6	−	+	−	+	−	+	−	+
: A769662	−	−	+	+	−	−	+	+

Control | AMPKα1 : siRNA

C

	V		A769662	
sIL-6Rα/IL-6	−	+	−	+

SOCS3
C/EBPδ
GAPDH

SOCS3 INDUCTION
(sIL-6Rα/IL-6 set at 100%)

*

: sIL-6Rα/IL-6	−	+	−	+
: A769662	−	−	+	+

C/EBPδ INDUCTION
(sIL-6Rα/IL-6 set at 100%)

*

: sIL-6Rα/IL-6	−	+	−	+
: A769662	−	−	+	+

D

Monocyte Migration
(sIL-6Rα/IL-6 set at 100%)

*   *

: sIL-6Rα/IL-6	−	+	−	+
: A769662	−	−	+	+

A

B

A

FERM domain   SH2 domain   Kinase Domain 2   Kinase Domain 1

JH6-7   JH3-5   JH2   JH1

1   34   420   439   544   583   855   875   1153   1154

SAMS peptide

*   + AMPK   − AMPK

* = R^{506}YSLHGSDRSFPSLGDLMSHLKKQI

B

−   +   : AMPK

RYSLHGSDRSFPSLGDLMSHLKKQI
RYALHGADRAFPALGDLMAHLKKQI
RYALHGSDRSFPSLGDLMSHLKKQI
RYSLHGADRSFPSLGDLMSHLKKQI
RYSLHGSDRAFPSLGDLMSHLKKQI
RYSLHGSDRSFPALGDLMSHLKKQI
RYSLHGSDRSFPSLGDLMAHLKKQI
RYSLHGSDRAFPALGDLMSHLKKQI
RYALHGADRSFPSLGDLMAHLKKQI
         *   *

C

        -5 -4 -3 -2 -1 0 +1 +2 +3 +4

Optimal		LRRVXSXXNL
Secondary		MKKSXSXXDV
Additional		IXHRXSXXEI
Rat ACC1	Ser79	MRSSMSGLHL

Ser518 JAK1 site

Human JAK1	Ser518	DRSFPSLGDL
Mouse JAK1	Ser517	MDHFPSLRDL
Xenopus tropicalis JAK1	Ser504	DRGFDSLKDL
Danio rerio JAK1	Thr514	DTFRPTLKEL

Ser515 JAK1 site

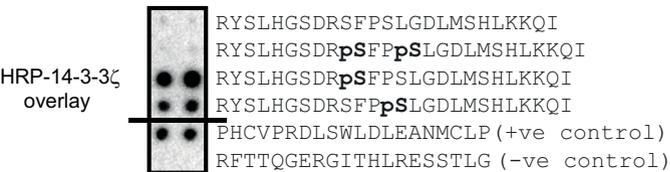|		
Human JAK1	Ser515	HGSDRSFPSL

D

     EF   αB   βG1   βG2

Human JAK1 (505-546)   ...GRYSLHGS**DRSFPSLGDL**MSHLKKQILRTDNISFMLKRCCQP...
Human JAK2 (460-501)   ...EEYNLSGT**KKNFSSLKDI**LNCYQMETVRSDNIIFQFTKCCPP...
Human JAK3 (436-477)   ...GTFLLVGL**RPHSSLKEI**LATCWDGGLHVDVGVAVTLTSCCIP...
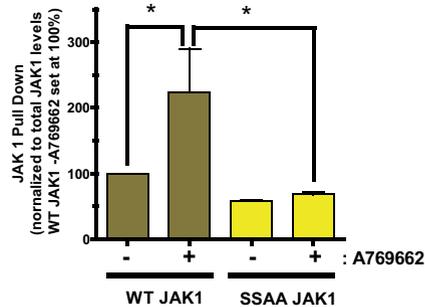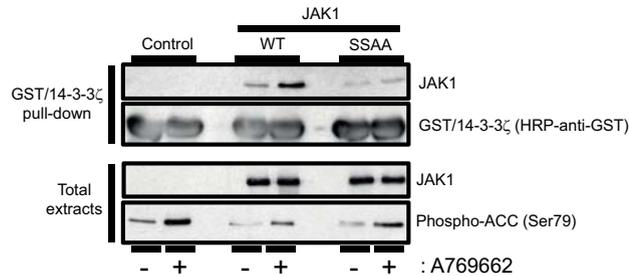Human Tyk2 (512-553)   ...GAFVLEGW**RSFPSVREI**LAALQGCLLRAGDDCFSLRRCCLP...
          *

EF   Ser518   αB   αA   SH2   FERM

A

HRP-14-3-3ζ overlay

RYSLHGSDRSFPSLGDLMSHLKKQI
RYSLHGSDR**pS**FP**pS**LGDLMSHLKKQI
RYSLHGSDR**pS**FP**pS**LGDLMSHLKKQI
RYSLHGSDRSFP**pS**LGDLMSHLKKQI
PHCVPRDLSWLDLEANMCLP (+ve control)
RFTTQGERGITHLRESSTLG (−ve control)

B

GST/14-3-3ζ pull-down

JAK1
GST/14-3-3ζ (HRP-anti-GST)

Total extracts

JAK1
Phospho-ACC (Ser79)

Control | JAK1 WT | SSAA
− + − + − + : A769662

JAK 1 Pull Down (normalized to total JAK1 levels WT JAK1 -A769662 set at 100%)

− + − + : A769662
WT JAK1 | SSAA JAK1

C

0 10 20 0 10 20 : A769662 (min)

GST/14-3-3ζ pull-down

JAK1
GST/14-3-3ζ (Ponceau)

Total extracts

JAK1
AMPKα1
Phospho-ACC (Ser79)

WT | α1⁻/⁻α2⁻/⁻ : AMPK MEFs

JAK1 Pull Down (normalized to total JAK1 levels, WT t=0 set at 100%)

0 10 20 0 10 20 : A769662 (mins)
WT | α1⁻/⁻α2⁻/⁻ : AMPK MEFs

D

0 0.5 1 2 : Met+Sal (hr)

GST/14-3-3ζ pull-down

JAK1
GST/14-3-3ζ (Ponceau)

Total extracts

JAK1
Phospho-ACC (Ser79)

JAK1 Pull Down (normalized to total JAK1 levels, t=0 set at 100%)

0 0.5 1 2
Met+Sal (hr)

A

V | A769662 | V | A769662
\- + | \- + | \- + | \- + : sIL-6Rα/IL-6

Phospho-ACC (Ser79)

Phospho-STAT3 (Tyr705)

Total STAT3

JAK1

GFP control | WT JAK1

B

V | A769662 | V | A769662
\- + | \- + | \- + | \- + : sIL-6Rα/IL-6

Phospho-ACC (Ser79)

Phospho-STAT3 (Tyr705)

Total STAT3

WT | SSAA : JAK1

STAT3 phosphorylation (+sIL-6Rα/IL-6 set at 100%)

\- + \- + \- + \- + : sIL-6Rα/IL-6
\- \- + + \- \- + + : A769662

WT JAK1 | SSAA JAK1

C

M | WT | V658F | SSAA V658F : JAK1

Phospho-STAT1 (Tyr701)

Total STAT1

Phospho-STAT3 (Tyr705)

Total STAT3

JAK1

Phospho-ACC (Ser79)

\- + \- + \- + \- + : A769662

Phospho-STAT1 (Tyr701) (normalized to total STAT1 levels V658F JAK1 -A769662 set at 100%)

\- + \- + \- + \- + : A769662

M | WT | V658F | V658F SSAA : JAK1

S1

A

	V		A769662		V		A769662

: sIL-6Rα/IL-6

Phospho-ACC (Ser79)

Phospho-STAT3 (Tyr705)

Total STAT3

AMPKα1

GFP

GFP (control)   DN-AMPKα1   : AV

B

	V		AICAR		V		AICAR

: sIL-6Rα/IL-6

Phospho-ACC (Ser79)

Phospho-STAT3 (Tyr705)

Total STAT3

Control   AMPKα1   : siRNA

Phospho-STAT3 (Tyr705)
(normalized to total STAT3 levels
cytokine-stimulated set at 100%)

: sIL-6Rα/IL-6
: A769662

AV. GFP   AV.DN-AMPKα1

S2

S3

S4

A

GST | GST/14-3-3ζ | Total extracts

JAK1

HRP-anti-GST

GST/14-3-3ζ →

GST →

Pull downs | 10% of input

B

GST | GST/14-3-3ζ | GST/14-3-3τ

JAK1

HRP-anti-GST

GST/14-3-3ζ
GST/14-3-3τ

GST →

Pull downs

C

Control | WT | SSAA | : JAK1

IP: JAK1
(Flag)

HRP-14-3-3ζ

JAK1