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AMPK directly activates mTORC2 to promote cell survival during acute energetic stress

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Abstract

AMP-activated protein kinase (AMPK) senses energetic stress and, in turn, promotes catabolic and suppresses anabolic metabolism coordinately to restore energy balance. We found that a diverse array of AMPK activators increased mTOR complex 2 (mTORC2) signaling in an AMPK-dependent manner in cultured cells. Activation of AMPK with the type 2 diabetes drug metformin (Glucophage) also increased mTORC2 signaling in liver in vivo and in primary hepatocytes in an AMPK-dependent manner. AMPK-mediated activation of mTORC2 did not result from AMPK-mediated suppression of mTORC1 and thus reduced negative feedback on PI3K flux. Rather, AMPK associated with and directly phosphorylated mTORC2 (mTOR in complex with rictor). As determined by two-stage in vitro kinase assay, phosphorylation of mTORC2 by recombinant AMPK was sufficient to increase mTORC2 catalytic activity toward Akt. Hence, AMPK phosphorylated mTORC2 components directly to increase mTORC2 activity and downstream

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SUPPLEMENTARY MATERIALS

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signaling. Functionally, inactivation of AMPK, mTORC2, and Akt increased apoptosis during acute energetic stress. By showing that AMPK activates mTORC2 to increase cell survival, these data provide a potential mechanism for how AMPK paradoxically promotes tumorigenesis in certain contexts despite its tumor-suppressive function through inhibition of growth-promoting mTORC1. Collectively, these data unveil mTORC2 as a target of AMPK and the AMPK-mTORC2 axis as a promoter of cell survival during energetic stress.

INTRODUCTION

AMPK [adenosine monophosphate (AMP)–activated protein kinase] functions as an ancestral energy sensor [reviewed in (1–4)]. During conditions of low cellular energy caused by glucose or nutrient deprivation, exercise, or hypoxia, increased levels of AMP and ADP (adenosine diphosphate) activate AMPK. AMPK functions in a heterotrimeric complex composed of one catalytic α subunit (a serine/threonine kinase), one scaffolding β subunit, and one regulatory γ subunit. Vertebrates contain multiple α ($\alpha 1$ and $\alpha 2$), β ($\beta 1$ and $\beta 2$), and γ ($\gamma 1$ to $\gamma 3$) subunits and thus express 12 potential AMPK $\alpha\beta\gamma$ complexes whose distinct functions remain poorly defined. Upon energetic stress, AMP and ADP bind directly to the γ subunit, causing an allosteric conformational change that activates AMPK by an incompletely defined mechanism involving increased ability of LKB1 or CaMKK β to phosphorylate the activation loop site (Thr¹⁷²) on the AMPK α subunit, decreased dephosphorylation of the activation loop, and/or allosteric activation of phosphorylated AMPK [reviewed in (1–4)]. Upon activation, AMPK phosphorylates a diverse set of targets that redirect cell metabolism toward ATP (adenosine triphosphate)–generating pathways (such as fatty acid oxidation, autophagy, glucose utilization, and mitochondrial biogenesis) and away from ATP-consuming anabolic pathways (such as ribosome biogenesis; fatty acid, lipid, and protein synthesis; gluconeogenesis; and cell growth and proliferation) to restore energy balance.

The evolutionarily conserved kinase mTOR (mechanistic target of rapamycin) functions as an environmental sensor that responds to diverse cues to control fundamental cellular processes [reviewed in (5–8)]. mTOR forms the catalytic core of two signaling complexes with distinct regulation and function, mTOR complex 1 (mTORC1) and mTORC2. The mTOR partner raptor defines mTORC1 (a rapamycin-sensitive complex) (9, 10), whereas the mTOR partner rictor defines mTORC2 (a rapamycin-insensitive complex) (11, 12). Upon activation by hormones such as insulin and growth factors, mTORC1 promotes anabolic cell metabolism (including ribosome biogenesis; lipid, nucleotide, and protein synthesis; and cell growth) and suppresses catabolic cell metabolism (such as autophagy) (5–7). Activation of mTORC1 requires sufficient levels of amino acids, which localize mTORC1 to lysosomal membranes near an important upstream activator [the guanosine triphosphatase (GTPase) Rheb] through the action of the regulator/LAMTOR complex and Rag GTPases (13–15) [reviewed in (16, 17)]. Insulin-mediated activation of PI3K (phosphatidylinositol 3-kinase) generates PIP₃ (phosphatidylinositol 3,4,5-trisphosphate), which enables PDK1 (phosphoinositide-dependent kinase 1) to activate Akt through phosphorylation of its activation loop site (Thr³⁰⁸). In turn, Akt phosphorylates Tsc2 to inhibit the tumor-suppressive Tsc1/Tsc2 complex (TSC), whose GTPase activating protein (GAP) activity

inhibits the GTPase Rheb on lysosomal membranes [reviewed in (18–20)]. Thus, insulin-PI3K-Akt signaling promotes Rheb-mediated activation of mTORC1 by suppressing TSC function. The AGC kinase family member S6K1 (ribosomal protein S6 kinase 1) is a well-defined mTORC1 substrate [reviewed in (5, 18, 21)]. mTORC1-mediated phosphorylation of the hydrophobic motif site (Thr³⁸⁹), together with PDK1-mediated phosphorylation of the activation loop site (Thr²²⁹), activates S6K1. Various types of cell stress suppress mTORC1 function [reviewed in (22)]. For example, AMPK inhibits mTORC1 during energetic stress through at least two mechanisms involving AMPK-mediated phosphorylation of Tsc2 on an activating site and raptor on inhibitory sites (23, 24).

The regulation and function of mTORC2 remains less well defined than mTORC1. Growth factors activate mTORC2 in a PI3K-dependent manner [reviewed in (7, 8)]. PI3K-generated PIP₃ binds to the pleckstrin homology (PH)-domain on mSin1, an mTORC2 binding partner. PIP₃ binding to the mSin1 PH-domain allosterically relieves its suppressive effect on the mTOR kinase domain (25). Beyond PI3K, upstream regulation of mTORC2 remains elusive. Curiously, TSC was reported to associate with and activate mTORC2 (26–28). These data suggest that TSC inhibits mTORC1 but activates mTORC2. A distinct set of AGC kinases (Akt, SGK1, and PKC α) represent substrates of mTORC2, with Akt representing the best-defined target (7, 8, 19). During growth factor signaling, mTORC2 phosphorylates Akt on its hydrophobic motif site (Ser⁴⁷³) (29). Although Akt Thr³⁰⁸ phosphorylation alone is sufficient to activate Akt, additional Ser⁴⁷³ phosphorylation maximally activates Akt and modulates substrate specificity, directing Akt to some substrates (such as FoxO) but not others (such as Tsc2, PRAS40, and GSK3) (30, 31). In many cell lines, knockdown of the mTORC2 components rictor or mSin1, and thus ablation of Akt Ser⁴⁷³ phosphorylation, does not inhibit mTORC1 signaling (30, 31). Thus, mTORC1 and mTORC2 signal in parallel in many cellular contexts. Constitutive mTORC1 signaling (such as upon inactivation of TSC) suppresses mTORC2 signaling through well-established negative feedback on PI3K flux mediated by inhibitory phosphorylation on IRS-1 (32, 33). mTORC2 also phosphorylates Akt on its turn motif site (Thr⁴⁵⁰) in a growth factor-insensitive manner during the translation of nascent Akt on ribosomes, which facilitates Akt folding and stability (34–36). mTORC2 promotes cell survival and metabolic homeostasis by increasing glucose uptake in fat and muscle and reducing hepatic glucose production [reviewed in (6, 7, 37)].

Here, we identify a previously unknown for activation of mTORC2, demonstrating that AMPK interacts with and phosphorylates mTORC2, resulting in its activation in response to energetic stress. This finding was unexpected, because mTORC2 has traditionally been thought to mediate insulin/PI3K signaling and because AMPK inhibits mTORC1. A diverse array of AMPK-activating agonists increased mTORC2 signaling in cultured cells, and the AMPK-activating type 2 diabetes drug metformin (GlucoPhage) increased mTORC2 signaling in primary hepatocytes and in liver in vivo in an AMPK-dependent manner. Our data show that the mechanism by which AMPK increases mTORC2 signaling is not through AMPK-mediated suppression of mTORC1 signaling and thus reduced negative feedback on IRS-1 and PI3K flux. We found that AMPK phosphorylated mTOR and rictor in vitro, which was sufficient to increase mTORC2 catalytic activity toward Akt. AMPK interacted with mTORC2 by coimmunoprecipitation and increased mTOR phosphorylation in cultured

cells and in vivo. During acute energetic stress, AMPK, mTORC2, and Akt suppressed apoptosis to promote cell survival. Our finding that AMPK activates mTORC2 directly may provide a mechanistic basis for the paradoxical role of AMPK as a tumor promoter (rather than a tumor suppressor) in certain contexts and its beneficial role in metabolism and glycemic control [reviewed in (38, 39)].

RESULTS

AMPK phosphorylates mTOR within mTORC2, associates with mTORC2, and promotes mTORC2 signaling

While investigating regulation of cellular mTOR signaling networks, we unexpectedly found that AICAR (5-aminoimidazole-4-carboxamide ribonucleotide), an AMP-mimetic compound that activates AMPK, increased the phosphorylation of Akt Ser⁴⁷³ and the Akt downstream target FoxO1/3A Thr^{24/32} in serum-starved mouse embryonic fibroblasts (MEFs). These data indicate that AICAR increases mTORC2 and Akt signaling (Fig. 1A). Akt Ser⁴⁷³ phosphorylation was sensitive to the mTOR inhibitor torin1 (Fig. 1A) and the class I PI3K α inhibitor BYL719 (Fig. 1B), indicating that AICAR increases Akt Ser⁴⁷³ phosphorylation in a manner requiring the activity of mTOR and PI3K. Although we serum-starved the MEFs overnight, measurable PI3K activity remained, because BYL719 reduced Akt Ser⁴⁷³, Akt Thr³⁰⁸, and S6K1 Thr³⁸⁹ phosphorylation further after serum starvation (fig. S1A). Thus, basal PI3K activity is required for AICAR to increase mTORC2 signaling. To confirm that the ability of AMPK to increase Akt Ser⁴⁷³ phosphorylation required intact mTORC2 function, we used *riCTOR*^{-/-} MEFs stably reconstituted with vector control or hemagglutinin (HA)-riCTOR. AICAR increased phosphorylation of Akt Ser⁴⁷³ and the SGK1 target NDRG1 Thr³⁴⁶ in *riCTOR*^{-/-} MEFs reconstituted with HA-riCTOR but not with vector control (Fig. 1C). Note that SGK1 is another mTORC2 substrate. These data indicate that intact mTORC2 is required for AICAR to increase mTORC2 signaling to Akt and SGK1. As expected, AICAR increased phosphorylation of AMPK on its activation loop site (Thr¹⁷²), phosphorylation of raptor on Ser⁷⁹² [a site phosphorylated directly by AMPK (24)], and reduced mTORC1 signaling, as monitored by phosphorylation of S6K1 on Thr³⁸⁹ (Fig. 1A). Consistent with mTORC2-mediated Akt Ser⁴⁷³ phosphorylation stabilizing Thr³⁰⁸ phosphorylation (29, 40), AICAR increased the phosphorylation of Akt on Thr³⁰⁸ in a torin1-sensitive manner (Fig. 1A).

By comparing MEFs lacking both AMPK α 1 and AMPK α 2 [double knockout (DKO)] to wild-type counterparts, we found that both basal and AICAR-stimulated phosphorylation of Akt Ser⁴⁷³ was reduced in DKO MEFs (Fig. 1A). When normalized to total Akt levels, which were slightly lower in AMPK DKO MEFs, AICAR increased the phosphorylation of Akt Ser⁴⁷³ 2.3-fold in wild-type MEFs and 1.9-fold in DKO MEFs (Fig. 1A, graph). These data indicate that AICAR, which acts both dependently and independently of AMPK (41–44), increases mTORC2 signaling in a manner partially dependent on AMPK (Fig. 1A). Consistent with mTORC2 also functioning as the Akt Thr⁴⁵⁰ kinase that facilitates Akt stability (34, 35), Akt Thr⁴⁵⁰ phosphorylation was reduced modestly in AMPK DKO MEFs relative to wild-type MEFs, which likely explains the reduction in total Akt levels (Fig. 1A).

These data suggest that AMPK-dependent and AMPK-independent targets of AICAR promote mTORC2 signaling to Akt in a manner that requires PI3K activity.

Finding that the AMPK-activating agonist AICAR increased mTORC2 signaling was intriguing because we had found, through an in vitro human kinome screen, that recombinant AMPK α 1 β 1 γ 1 and AMPK α 2 β 1 γ 1 phosphorylated mTOR on Ser¹²⁶¹ (fig. S1B), a site whose phosphorylation promotes mTORC1 signaling (45). We confirmed the kinome screen with classic in vitro kinase (IVK) assays. Recombinant active AMPK α 1 β 1 γ 1 and AMPK α 2 β 1 γ 1 phosphorylated a bacterially produced glutathione *S*-transferase (GST)–mTOR fragment in vitro (fig. S1C) and phosphorylated full-length Myc-mTOR but not an S1261A mutant immunoprecipitated from transfected cells, thus confirming the phospho- and site-specificity of our phospho-mTOR Ser¹²⁶¹ antibody (Fig. 1D) [see also (45)]. AMPK α 1 β 1 γ 1 and AMPK α 2 β 1 γ 1 also phosphorylated rictor-associated mTOR, indicating that AMPK phosphorylates mTOR within mTORC2 (Fig. 1E). The peptide sequence surrounding mTOR Ser¹²⁶¹ resembles an AMPK consensus phosphorylation motif and the sequence surrounding raptor Ser⁷⁹², a site phosphorylated by AMPK (Fig. 1F) (1,24). Coimmunoprecipitation assays demonstrated that HA-AMPK α 1 and HA-AMPK α 2 associated with Flag-rictor when coexpressed in human embryonic kidney (HEK) 293 cells (Fig. 1G). These data demonstrate that AMPK directly phosphorylates mTOR on Ser¹²⁶¹ within mTORC2 in vitro and associates with mTORC2 in intact cells.

We next asked whether AMPK mediates mTOR Ser¹²⁶¹ phosphorylation in intact cells. To monitor mTOR Ser¹²⁶¹ phosphorylation specifically within mTORC2, we immunoprecipitated rictor from serum-starved wild-type and AMPK DKO MEFs stimulated with AICAR. AICAR increased mTOR Ser¹²⁶¹ phosphorylation within mTORC2 in wild-type but not AMPK DKO MEFs (Fig. 1H). mTOR Ser¹²⁶¹ phosphorylation, either on rictor-associated mTOR (Fig. 1H) or on total mTOR when immunoblotted directly (Fig. 1H; see also Fig. 1A), was virtually undetectable in AMPK DKO MEFs. Quantitative Western blot analysis indicated that AICAR-stimulated phosphorylation of mTOR Ser¹²⁶¹ required AMPK (fig. S1D), similar to phosphorylation of raptor Ser⁷⁹² as previously published (24). As expected, AICAR decreased mTORC1 signaling to S6K1 in an AMPK-dependent manner (Fig. 1, A and H). Analysis of *rictor*^{-/-} MEFs reconstituted with vector control or HA-rictor indicated that intact mTORC2 was not required for AICAR to increase mTOR Ser¹²⁶¹ phosphorylation (Fig. 1C). Unexpectedly, we found that AICAR also increased mTOR Ser¹²⁶¹ phosphorylation on raptor-associated mTOR (mTORC1) in an AMPK-dependent manner (Fig. 1H). Similar to AICAR, activation of AMPK by glucose withdrawal and phenformin, an analog of the type 2 diabetes drug metformin, also increased phosphorylation of Ser¹²⁶¹ on rictor-associated mTOR in an AMPK-dependent manner (fig. S1, E and F). Treatment of MEFs with the AMPK inhibitor compound C reduced the phosphorylation of mTOR Ser¹²⁶¹ to the level found in AMPK DKO MEFs (fig. S1G), and AMPK α 1 overexpression increased mTOR Ser¹²⁶¹ phosphorylation in HEK293 cells (fig. S1H). While analyzing mTOR Ser¹²⁶¹ phosphorylation, we also analyzed mTOR Ser²⁴⁸¹ autophosphorylation, which monitors mTOR catalytic activity (46). We found that AICAR increased rictor-associated but decreased raptor-associated mTOR Ser²⁴⁸¹ autophosphorylation, suggesting increased catalytic activity of mTORC2 but decreased catalytic activity of mTORC1, which both occurred in an AMPK-dependent manner (Fig.

1H). Glucose withdrawal also increased AMPK-dependent mTOR Ser²⁴⁸¹ autophosphorylation (fig. S1E). Together, these data indicate that AMPK phosphorylates mTOR on Ser¹²⁶¹ within mTORC2, an event that correlates with increased mTORC2 autophosphorylation and downstream signaling.

AMPK promotes mTORC2 signaling in response to energetic stress induced by diverse agonists in cultured cells

Because the cellular effects of AICAR have been reported to occur in both an AMPK-dependent and AMPK-independent fashion (41–44), we analyzed the effect of various AMPK-activating agents on mTORC2 signaling in serum-deprived cells. We continued to study serum-deprived cells to reduce the activating effect of serum growth factors on mTORC2 signaling and the potential contribution of mTORC1-mediated negative feedback on mTORC2 activation. Glucose withdrawal increased the phosphorylation of Akt Ser⁴⁷³, mTOR Ser¹²⁶¹, and raptor Ser⁷⁹² in wild-type but not AMPK DKO MEFs (Fig. 2A), suggesting that glucose withdrawal activates AMPK and promotes mTORC2 signaling and mTOR phosphorylation in an AMPK-dependent manner. We next treated wild-type and AMPK DKO MEFs with several different mitochondrial electron transport chain inhibitors, specifically the complex I inhibitors phenformin (Fig. 2B) and rotenone (Fig. 2C) and the complex III inhibitor antimycin A (fig. S2A). As expected, these inhibitors activated AMPK, as monitored by phosphorylation of raptor Ser⁷⁹² and/or AMPK Thr¹⁷². Moreover, they all increased the phosphorylation of Akt Ser⁴⁷³ and mTOR Ser¹²⁶¹ in an AMPK-dependent manner (Fig. 2, B and C, and fig. S2A). Quantitative Western blot analysis demonstrated that phenformin increased mTORC2 signaling (Fig. 2B) and mTOR Ser¹²⁶¹ and raptor Ser⁷⁹² phosphorylation (fig. S2B) in a manner dependent on AMPK. As for AICAR, PI3K activity was required for glucose withdrawal, phenformin, and rotenone to increase mTORC2 signaling (fig. S2C). Stable reconstitution of wild-type AMPK α 1 in AMPK α 1/ α 2 DKO MEFs restored AMPK function (as monitored by raptor Ser⁷⁹² and mTOR Ser¹²⁶¹ phosphorylation) and rescued mTORC2 signaling (as monitored by Akt Ser⁴⁷³ and NDRG1 Thr³⁴⁶ phosphorylation) in response to rotenone (fig. S2D).

The small-molecule AMPK activator A769662 binds preferentially to the β 1 subunit to activate β 1-containing, but not β 2-containing, AMPK complexes (47, 48). Acute treatment of MEFs (Fig. 2D) and HEK293 cells (fig. S2E) with A769662 increased AMPK and mTORC2 signaling rapidly without reducing mTORC1 signaling. To determine whether AMPK was required for mTORC2 signaling in HEK293 cells, we knocked down either AMPK α 1 and AMPK α 2 together or AMPK β 1 in HEK293 cells with small interfering RNA (siRNA) before A769662 treatment. Knockdown of either subunit reduced mTORC2 signaling and expression of the other AMPK subunit (Fig. 2E). Together, these data demonstrate that AMPK is required for various AMPK-activating agonists to promote mTORC2 signaling in MEFs and HEK293 cells.

AMPK promotes mTORC2 signaling independently of mTORC1-mediated negative feedback

Chronic elevation (through TSC inactivation) or reduction (through raptor knockdown) of mTORC1 signaling decreases or increases PI3K-mediated signaling, respectively (32, 33)

[reviewed in (19)]. Observations such as these led to the identification of a negative feedback loop whereby mTORC1-S6K1 signaling mediates inhibitory phosphorylation on IRS-1 to reduce insulin/insulin-like growth factor 1 (IGF-1)–mediated PI3K activation and signaling. Because AMPK suppresses mTORC1 signaling (19, 23, 24), we designed experiments to dissociate AMPK-mediated suppression of mTORC1 from AMPK-mediated activation of mTORC2. That is, because mTORC2 signaling requires PI3K, AMPK-mediated suppression of mTORC1 could increase mTORC2 signaling indirectly through increased PI3K activity rather than through a direct mechanism proposed here. We first compared how AICAR and rapamycin affected mTORC1 and mTORC2 signaling over time (10 to 240 min) in serum-starved MEFs. Although basal S6K1 Thr³⁸⁹ phosphorylation was quite low in serum-starved MEFs, AICAR and rapamycin reduced this level further (Fig. 3A). Across the time course, AICAR increased Akt Ser⁴⁷³ phosphorylation, whereas rapamycin did not. Moreover, AICAR failed to increase S6K1 phosphorylation on its PI3K-PDK1–dependent activation loop site, Thr²²⁹, suggesting that AICAR treatment of serum-deprived cells did not increase PI3K activity. Note that PI3K-PDK1–mediated phosphorylation of S6K1 Thr²²⁹ occurs independently of mTORC1-mediated phosphorylation of S6K1 Thr³⁸⁹ (49) and thus represents an appropriate readout for PI3K activity. The time course with which AICAR increased Akt Ser⁴⁷³ phosphorylation correlated well with increased mTOR Ser¹²⁶¹ phosphorylation and activation of AMPK, as monitored by phosphorylation of raptor Ser⁷⁹² and AMPK Thr¹⁷² (Fig. 3A). Thus, under conditions lacking serum growth factors, AICAR and rapamycin each reduced mTORC1 signaling to a similar extent, yet AICAR but not rapamycin increased mTORC2 signaling. These data suggest that AICAR does not increase mTORC2 signaling indirectly through AMPK-mediated suppression of mTORC1 signaling and subsequent reduction of negative feedback on PI3K. Note that in addition to an AICAR time course (Fig. 3A), other time courses examining effects of glucose withdrawal (fig. S3A), phenformin (fig. S3B), rotenone (fig. S3C), and A769662 (Fig. 2D and fig. S2E) on Akt Ser⁴⁷³, mTOR Ser¹²⁶¹, raptor Ser⁷⁹², and/or AMPK Thr¹⁷² phosphorylation were conducted. The shortest treatment times that increased AMPK activity with subsequent activation of mTORC2 signaling and without substantial cell death were chosen for this work. For example, although 12-hour glucose withdrawal increased Akt Ser⁴⁷³ phosphorylation to a greater extent than 8 hours, it caused cell death (fig. S3A).

We next tested whether several other AMPK-activating agonists promote mTORC2 signaling independently of mTORC1 function by treating MEFs without and with rapamycin before AMPK activation. AICAR, glucose withdrawal, phenformin, or rotenone all increased Akt Ser⁴⁷³ phosphorylation in both the absence and presence of rapamycin in MEFs (Fig. 3B). We obtained similar results in serum-starved HEK293 cells treated with A769662 (Fig. 3B). Because rapamycin is an allosteric inhibitor that suppresses mTORC1 signaling to various degrees depending on the substrate, we sought to exclude the possibility that AMPK-mediated mTORC1 suppression increases mTORC2 signaling through a rapamycin-insensitive mechanism. For example, although rapamycin completely blocks mTORC1-mediated phosphorylation of S6K1 Thr³⁸⁹, it only partially blocks the phosphorylation of 4EBP1 and some, but not all, of the mTORC1 sites on Grb10, an adaptor protein that mediates negative feedback on insulin/IGF-1–PI3K signaling (50–53) [reviewed in (54, 55)]. We therefore treated serum-starved HEK293 cells with siRNA-mediated raptor

knockdown without or with A769662. As expected, because of chronic mTORC1 inactivation and thus reduced negative feedback, knockdown of raptor increased Akt Ser⁴⁷³ phosphorylation in untreated cells relative to scrambled controls. Despite increased Akt Ser⁴⁷³ phosphorylation in raptor knockdown cells, A769662 increased Akt Ser⁴⁷³ phosphorylation further (Fig. 3C). Last, A769662 increased mTORC2 signaling without reducing mTORC1 signaling in MEFs (Fig. 2E) and HEK293 cells (fig. S2E). Together, these data dissociate AMPK-mediated mTORC1 suppression from AMPK-mediated mTORC2 activation.

We also noted that AICAR and insulin promoted mTORC2 signaling with different kinetics: Insulin increased mTORC2 signaling quickly, reaching a maximal level at 10 in, whereas AICAR increased mTORC2 signaling more slowly (Fig. 3A). AICAR and insulin increased mTORC2 signaling additively over either agonist alone (Fig. 3D). These data suggest that AMPK and insulin promote mTORC2 signaling by different mechanisms. Moreover, they raise the intriguing idea that AMPK activators function as insulin sensitizers in vivo when used to treat type 2 diabetes by activating mTORC2 in parallel to and independently of insulin signaling.

AMPK promotes mTORC2 signaling in primary hepatocytes in culture and in tissue in vivo and ex vivo

To confirm that AMPK promotes mTORC2 signaling beyond immortalized cells in culture, we analyzed primary mouse hepatocytes and liver tissue isolated from mice expressing or lacking AMPK α 1 and AMPK α 2 catalytic subunits through Cre-mediated excision of floxed AMPK α alleles (56). To generate these mice, we injected mice bearing floxed alleles of AMPK α 1 and AMPK α 2 with adeno-associated virus (AAV)–green fluorescent protein (GFP) or AAV-Cre viruses. Fourteen days after AAV infection, we isolated primary hepatocytes, serum-starved them overnight, and treated them without or with metformin, an analog of phenformin that activates AMPK and used clinically to treat type 2 diabetes, or AICAR. Alternately, 14 days after AAV infection, we dissected liver tissue from mice that had been fasted overnight and then injected acutely with saline control or metformin. Metformin (Fig. 4A) and AICAR (Fig. 4B) increased Akt Ser⁴⁷³ phosphorylation in wild-type but not AMPK α 1/ α 2 DKO hepatocytes. Metformin also increased Akt Thr³⁰⁸ phosphorylation in an AMPK α -dependent manner (Fig. 4A), again consistent with Akt Ser⁴⁷³ phosphorylation promoting and/or stabilizing Akt Thr³⁰⁸ phosphorylation. Metformin and AICAR increased mTOR Ser¹²⁶¹ phosphorylation and activated AMPK, as monitored by raptor Ser⁷⁹² phosphorylation (Fig. 4, A and B). We also found that metformin and insulin additively increased mTORC2 signaling in an AMPK α -dependent manner in hepatocytes (Fig. 4C), similar to the additive effect of AICAR and insulin on mTORC2 signaling in MEFs (Fig. 3D).

We next tested whether activation of AMPK promotes mTORC2 signaling in tissue in vivo and ex vivo. Metformin administration to mice increased Akt Ser⁴⁷³ and Thr³⁰⁸ phosphorylation in liver expressing AMPK α 1/ α 2 (AAV-GFP) but not in liver with excised AMPK α 1/ α 2 (AAV-Cre) (Fig. 4D and fig. S4A). In fasted liver from control mice, S6K1 Thr³⁸⁹ phosphorylation was almost undetectable, indicating low basal mTORC1 signaling,

and metformin was unable to reduce mTORC1 signaling further. This observation is consistent with the increase in mTORC1 signaling induced by feeding after fasting (57). Moreover, these data indicate that suppression of mTORC1 cannot explain how metformin increases mTORC2 signaling. Metformin administration to mice increased mTOR Ser¹²⁶¹ phosphorylation in an AMPK-dependent manner and activated AMPK, as monitored by raptor Ser⁷⁹² phosphorylation. Last, treatment of rat skeletal muscle (soleus) ex vivo with A769662 or AICAR also increased phosphorylation of Akt Ser⁴⁷³, mTOR Ser¹²⁶¹, and AMPK Thr¹⁷² (fig. S4B). Together, these data demonstrate that AMPK promotes mTORC2 signaling in primary cells in culture and in tissue in vivo and ex vivo.

AMPK is sufficient to increase mTORC2 catalytic activity directly

We next sought to define the mechanism by which AMPK promotes mTORC2 signaling by asking whether AMPK phosphorylates mTORC2 to increase its intrinsic catalytic activity. We first performed a classic IVK assay by monitoring the ability of immunoprecipitated rictor/mTOR (mTORC2) to phosphorylate recombinant Akt substrate. We treated serum-starved MEFs without or with AICAR after torin1 pretreatment and found that AICAR increased mTORC2-mediated phosphorylation of His-Akt1 on Ser⁴⁷³ in a torin1-sensitive manner (Fig. 5A). Moreover, stimulation of MEFs with insulin increased mTORC2 catalytic activity in our assay conditions. AICAR also increased mTOR Ser²⁴⁸¹ autophosphorylation and Ser¹²⁶¹ phosphorylation within mTORC2, and both AICAR and insulin increased mTORC2 signaling to Akt in intact cells. These data demonstrate that AICAR increases mTORC2 intrinsic catalytic activity toward itself and downstream substrate.

To ask whether AMPK per se was sufficient to increase mTORC2 catalytic activity directly, we developed a two-stage mTORC2 IVK assay. First, active recombinant AMPK α 1/ β 1/ γ 1 was incubated in vitro with rictor-associated mTOR (mTORC2) (stage 1), and an IVK reaction was performed with ATP. Next, the mTORC2-containing beads were washed (to remove AMPK α 1/ β 1/ γ 1) and incubated in vitro with recombinant His-Akt1 and ATP (stage 2). Under our IVK conditions, incubation of mTORC2 with AMPK α 1/ β 1/ γ 1 in stage 1 increased mTORC2-mediated phosphorylation of His-Akt in stage 2 (Fig. 5B). Compound C provided in vitro reduced AMPK-mediated mTOR phosphorylation in stage 1 and ablated mTORC2 activity toward Akt. Torin1 provided in vitro did not affect AMPK-mediated mTOR phosphorylation in stage 1 but ablated mTORC2 catalytic activity in stage 2. mTORC2 immunoprecipitated from insulin-stimulated cells maintained an increased level of catalytic activity over unstimulated cells throughout the two-stage IVK assay (Fig. 5B). We noted that incubation of mTORC2 with AMPK α 1/ β 1/ γ 1 during the stage 1 IVK reduced the electrophoretic mobility of rictor (Fig. 5C). Because phosphorylation of rictor reduces its electrophoretic mobility (11, 58), this finding suggests that AMPK phosphorylates rictor. Together, these data indicate that the catalytic activity of AMPK is sufficient to increase mTORC2 catalytic activity, likely through phosphorylation of mTOR and/or its partner proteins (such as rictor).

Because AMPK-mediated mTOR Ser¹²⁶¹ phosphorylation and mTORC2 signaling correlate tightly in cultured cells, primary cells, and in vivo, we asked whether mTOR Ser¹²⁶¹ is required for AMPK-mediated mTORC2 signaling. To test this question, we used CRISPR-

Cas9-mediated genome editing to generate a mouse knock-in allele bearing nonphosphorylatable Ala at mTOR Ser¹²⁶¹ (*mTOR^{A/A}*) (fig. S5, A and B). In primary MEFs, AICAR treatment (Fig. 6A) and glucose withdrawal (Fig. 6B) increased Akt Ser⁴⁷³ phosphorylation similarly in wild-type (*mTOR^{+/+}*) and S1261A (*mTOR^{A/A}*) MEFs. AICAR and glucose withdrawal increased mTOR Ser¹²⁶¹ in wild-type but not *mTOR^{A/A}* MEFs, thus validating our *mTOR^{A/A}* mouse knock-in model (Fig. 6, A and B). These agents activated AMPK to a similar extent in wild-type and *mTOR^{A/A}* MEFs, as monitored by increased raptor Ser⁷⁹² phosphorylation. In hepatocytes, metformin also similarly increased Akt Ser⁴⁷³ phosphorylation in both genotypes, whereas it increased mTOR Ser¹²⁶¹ phosphorylation only in wild-type but not *mTOR^{A/A}* hepatocytes (Fig. 6C). These data reveal that mTOR Ser¹²⁶¹ phosphorylation per se is not required for mTORC2 signaling upon AMPK activation. These data do not rule out the possibility, however, that this phosphorylation event cooperates with other sites to promote mTORC2 signaling. Because AMPK phosphorylates mTORC2 components (such as mTOR and rictor) and its enzymatic activity is required for mTORC2 to phosphorylate Akt in vitro, we propose a model in which AMPK activates mTORC2 through multisite phosphorylation of mTOR and/or mTORC2 partner proteins.

The AMPK-mTORC2 axis promotes cell survival during acute energetic stress

Because AMPK promotes cell survival during energetic stress (59), we next asked whether mTORC2 and Akt are required for suppression of cell death during energetic stress. We induced energetic stress using either AICAR treatment or glucose withdrawal (23, 59) and assessed apoptosis by measuring the cleavage of caspase 3 and poly(ADP-ribose) polymerase (PARP) by immunoblotting. AICAR treatment (Fig. 7A) and glucose withdrawal (Fig. 7B) increased apoptosis in AMPK α 1/ α 2 DKO MEFs to a greater extent than wild-type MEFs. Inhibition of mTOR with torin1 increased apoptosis induced by these agents in wild-type MEFs under energetic stress. These data indicate that both AMPK and mTOR are required to suppress apoptosis during acute energetic stress. As expected, AICAR and glucose withdrawal activated AMPK (as monitored by mTOR Ser¹²⁶¹ phosphorylation) and increased mTORC2 signaling (as monitored by Akt Ser⁴⁷³ phosphorylation) in wild-type but not AMPK α 1/ α 2 DKO MEFs. Akt inhibits the proapoptotic protein Bad through Ser¹³⁶ phosphorylation, and mTORC2 function is required for Bad Ser¹³⁶ phosphorylation and cell survival (19, 31, 60). Consistently, we found that AICAR (Fig. 7A) and glucose withdrawal (Fig. 7B) increased Bad Ser¹³⁶ phosphorylation in an AMPK- and mTOR-dependent manner. Moreover, we confirmed that reintroduction of HA-AMPK α 1 into AMPK α 1/ α 2DKO MEFs reduced energetic stress-induced apoptosis and rescued mTOR Ser¹²⁶¹ phosphorylation, Akt Ser⁴⁷³ phosphorylation (mTORC2 signaling), and Bad Ser¹³⁶ phosphorylation (as a measure of Akt signaling) (Fig. 7C).

We next tested whether mTORC2 and Akt functions were required to suppress energetic stress-induced apoptosis. Apoptosis in response to AICAR or glucose withdrawal was higher in *rictor^{-/-}* MEFs expressing vector control relative to those reconstituted with HA-rictor (Fig. 7, D and E). Treatment of wild-type MEFs with the Akt inhibitor MK2206 increased energetic stress-induced apoptosis and concomitantly reduced the phosphorylation of Akt Ser⁴⁷³ and Bad Ser¹³⁶ in response to AICAR (fig. S6A) or glucose withdrawal (fig.

S6B). We also confirmed that AMPK and mTORC2 suppress cell death upon energetic stress induced by AICAR by live cell fluorescent microscopy using Ready Probes reagents. Consistent with our analysis of apoptosis by Western blotting, we found that in response to energetic stress, inhibition of mTOR with torin1 (Fig. 7, F and G) or inactivation of mTORC2 by rictor knockout increased cell death (indicated by green cells; Fig. 7G). Together, these data demonstrate that during energetic stress, activation of AMPK, mTORC2, and Akt promotes cell survival. Because the level of apoptosis was higher in AMPK α 1/ α 2 DKO MEFs relative to wild-type MEFs treated with torin1, we propose a model in which AMPK promotes cell survival during acute energetic stress by both mTORC2-Akt-dependent and mTORC2-Akt-independent pathways (Fig. 7H). In summary, our work demonstrates that AMPK phosphorylates mTOR and/or partner proteins to directly activate mTORC2, which functions to promote cell survival during acute energetic stress at least partially through Akt (Fig. 7I).

DISCUSSION

Metabolic homeostasis requires that cells sense and integrate a diverse array of signals that fluctuate dynamically depending on environmental conditions, the disruption of which contribute to pathologic conditions. AMPK and mTOR function as critical metabolic sensors [reviewed in (1, 3, 6–8)]. AMPK activation in response to low-energy conditions augments cell survival and promotes ATP-generating metabolic processes to restore energy balance (23, 59). mTOR within mTORC1 promotes anabolic and suppresses catabolic metabolism during nutrient and growth factor sufficiency, whereas mTOR within mTORC2 promotes cell survival and controls metabolism, both through Akt (7, 19, 31, 60). AMPK-mediated inhibition of mTORC1 during energetic stress thus facilitates coordination between catabolic and anabolic cell metabolism. Here, we report the unexpected finding that AMPK, like the Tsc1/2 complex, activates mTORC2 directly, likely through multisite phosphorylation on mTOR and its mTORC2 partner proteins.

Several AMPK activators increased the phosphorylation of Akt Ser⁴⁷³ in serum-starved cells in a manner that required AMPK, mTOR, and mTORC2 function. Other groups have observed increased Akt Ser⁴⁷³ phosphorylation upon AMPK activation (59, 61, 62), although the mechanism was not investigated. Because inhibition of PI3K blocked AMPK-mediated activated mTORC2 signaling in serum-starved cells, these data indicate that a minimal, basal level of PI3K activity is required for mTORC2 activation by AMPK. Because we could dissociate AMPK-mediated suppression of mTORC1 from AMPK-mediated activation of mTORC2, mTORC1-mediated negative feedback on PI3K flux does not underlie the effect of AMPK on mTORC2 activation described here. Concomitant with increased Akt Ser⁴⁷³ phosphorylation, cellular AMPK activation increased the phosphorylation of mTOR Ser¹²⁶¹ within mTORC2 (and within mTORC1; see below) in an AMPK-dependent manner. AMPK coimmunoprecipitated with mTORC2 in intact cells, phosphorylated mTOR (on Ser¹²⁶¹) and rictor (on unknown sites) within mTORC2 in vitro, and was sufficient to increase mTORC2 catalytic activity. These data support a model in which AMPK phosphorylates and activates mTORC2. Functionally, AMPK, mTOR, mTORC2, and Akt were all required to suppress apoptosis in response to acute energetic stress. Because we found that AMPK activation and insulin increased Akt Ser⁴⁷³

phosphorylation additively in both MEFs and primary hepatocytes, an effect observed by others (61, 63), we propose that insulin and AMPK signal in parallel to increase mTORC2 function (Fig. 7I). Thus, mTORC2 functions as an effector not only of insulin/PI3K signaling but also of AMPK.

Analysis of primary fibroblasts and hepatocytes isolated from CRISPR-generated mTOR S1261A knock-in mice (*mTOR^{A/A}*) indicated that the phosphorylation of mTOR Ser¹²⁶¹ (within the centrally located mTOR FAT-domain) was not essential for mTORC2 signaling in response to AMPK activation. However, this phosphorylation event reflected the action of AMPK on mTORC2, and we speculate that it contributed to mTORC2 activation in cooperation with other sites. We found that recombinant AMPK phosphorylated rictor in vitro. Moreover, a quantitative phosphoproteomic study predicted that rictor is an AMPK substrate (64). It will be important in the future to identify the critical set of AMPK phosphorylation sites on mTOR and/or partner proteins responsible for mTORC2 activation. Because of tight coupling between AMPK activation and mTOR Ser¹²⁶¹ phosphorylation across our experiments, we propose that the phosphorylation of mTOR Ser¹²⁶¹ represents a new biomarker to monitor AMPK activity in intact cells, similar to the phosphorylation of raptor Ser⁷⁹² or ACC (acetyl-coenzyme A carboxylase) Ser⁷⁹. Determining where in the cell AMPK activates mTORC2 remains an unresolved question. Because late endosomal/lysosomal membranes serve as a subcellular site important for AMPK activation (65), AMPK-mediated mTORC2 activation may occur on this membrane platform. Consistent with this idea, analysis of a reporter construct engineered to monitor Akt Ser⁴⁷³ phosphorylation localized active mTORC2 to early and late endosomes, as well as to the plasma membrane and mitochondria (66). Consistent with mitochondria as a potential site important for mTORC2 regulation and/or function, fluorescent microscopy localized rictor to mitochondria-associated endoplasmic reticulum (ER) membranes (67).

Although our data may initially present a paradox—AMPK inhibits mTORC1 but activates mTORC2—they fit with emerging data revealing a complex interplay between AMPK and mTOR in the control of cell and animal physiology. Huang *et al.* demonstrated that the mTORC1 inhibitory Tsc1/2 complex interacts with mTORC2 and is required for insulin and other growth factors to increase mTORC2-mediated Akt Ser⁴⁷³ phosphorylation in cells (26, 68). Like our work on AMPK-mTORC2, Tsc1/2-mediated activation of mTORC2 occurs independently of mTORC1-mediated negative feedback (26). Moreover, recombinant Tsc1/2 stimulates the IVK activity of mTORC2 directly, similar to our work with AMPK (27). Thus, both AMPK and Tsc1/2 inhibit mTORC1 but activate mTORC2. Moreover, the v-ATPase-ragulator/LAMTOR complex, which enables mTORC1 activation on lysosomal membranes during amino acid sufficiency by driving Rag-GTP loading (13–15, 69), also activates AMPK on lysosomal membranes during glucose deprivation (65) [reviewed in (70, 71)]. Dual activation of AMPK and mTORC1 by v-ATPase-ragulator/LAMTOR has been proposed to control a switch between anabolic and catabolic cell metabolism. Because conventional wisdom holds that AMPK inhibits mTORC1 during energetic stress, these data reveal a more complicated relationship between AMPK and mTOR than previously appreciated. In addition, glucose deprivation activates AMPK independently of AMP and ADP levels by a glucose-sensing mechanism involving fructose-1, 6-bisphosphate, aldolase, and v-ATPase-ragulator/LAMTOR (72), thus adding to the emerging complexity of AMPK

regulation. Because AMPK inhibits mTORC1 signaling (in response to energetic stress) (23, 24) and because our previous work indicated that mTOR Ser¹²⁶¹ phosphorylation promotes mTORC1 signaling (in the absence of energetic stress) (45), we were surprised to find here that AMPK increases Ser¹²⁶¹ phosphorylation on raptor-associated mTOR (mTORC1) [as well as on rictor-associated mTOR (mTORC2)]. Perhaps an AMPK-related kinase phosphorylates mTOR Ser¹²⁶¹ within mTORC1 to promote mTORC1 activation during energy sufficiency. Our kinome screen found that AMPK-related kinases called SIKs (salt-inducible kinases) and MARKs (microtubule affinity-regulating kinases) phosphorylate mTOR Ser¹²⁶¹ in vitro (fig. S1A). We speculate that perhaps in response to energetic stress, AMPK-mediated inhibitory phosphorylation of Tsc2 and raptor dominantly suppresses mTORC1 signaling despite activating phosphorylation of mTOR Ser¹²⁶¹ within mTORC1. Future work should investigate roles for AMPK, SIK, and MARK in mTORC1 regulation in response to mTOR Ser¹²⁶¹ phosphorylation.

Our finding that AMPK activated mTORC2 and the prosurvival kinase Akt fits well with defined roles for these kinases in the promotion of cell survival and tumorigenesis. Traditionally, AMPK has been thought to function as a tumor suppressor due to its phosphorylation (on Thr¹⁷²) and activation by LKB1, the tumor suppressor protein inactivated in the Peutz-Jeghers benign tumor syndrome (59, 73, 74). In addition, AMPK inhibits mTORC1, a driver of cell growth and proliferation. Consistent with this tumor-suppressive role, retrospective meta-analysis of type 2 diabetic patients treated with metformin indicates reduced cancer incidence (75, 76). However, AMPK can also function as a tumor promoter that promotes cell survival during metabolic stress (59, 77, 78). Mutational inactivation of LKB1 renders non-small cell lung carcinoma cells and tumors bearing oncogenic K-Ras more susceptible to apoptosis caused by phenformin-induced metabolic stress (78). These data suggest that metabolic drugs may function as selective anticancer agents by driving apoptosis in the absence of sufficient survival signaling. Moreover, they suggest that AMPK functions as a tumor promoter in certain contexts, perhaps by promoting survival of cancer cells deep within solid tumors experiencing metabolic stress (such as glucose deprivation and hypoxia) (38, 79–82). Our data demonstrate that AMPK, mTORC2, and Akt promote cell survival during energetic stress.

AMPK and mTORC2 control glucose metabolism in similar ways. AMPK activation produces insulin-sensitizing and antihyperglycemic effects in cultured cells and in vivo. This knowledge has made AMPK an attractive therapeutic target for treatment of insulin resistance and type 2 diabetes, thus spurring the ongoing development of AMPK-activating small molecules such as A769662, 991, MT 63–78, MK-8722, and PF-739 (47, 83–86). The AMPK-activating drug metformin (trademarked as GlucoPhage) is the most widely prescribed drug worldwide for treatment of type 2 diabetes because of its ability to suppress hepatic glucose production and improve systemic glycemic control. However, metformin mediates beneficial metabolic effects independently of AMPK as well (76, 87). Our finding that AICAR and insulin increase mTORC2 signaling additively may explain, in part, how AMPK activation produces insulin-mimetic effects in vivo. In the liver, both AMPK and mTORC2 suppress gluconeogenesis and thus hepatic glucose production, whereas in muscle and fat, they enhance insulin sensitivity and promote glucose uptake (4, 7, 86, 88–92). Thus, AMPK and mTORC2 functionally align in controlling glucose metabolism. Together, our

data support the concept that although the AMPK-mTORC2 axis may improve metabolic control, it may also promote tumorigenesis in certain contexts. The identification of mTORC2 as a new target of AMPK that promotes cell survival and possibly glucose homeostasis advances and shifts our understanding of the complex relationship between AMPK and mTOR and their roles in health and disease.

MATERIALS AND METHODS

Materials

Chemicals were from Fisher Scientific or Sigma. Protein A- and G-Sepharose Fast Flow and glutathione-sepharose beads were from GE Healthcare; CHAPS was from Pierce; Immobilon-P polyvinylidene difluoride (PVDF) membrane (0.45 μ M) was from Millipore; reagents for enhanced chemiluminescence (ECL) were from either Millipore [Immobilon Western Chemiluminescent Horseradish Peroxidase (HRP) Substrate], Advansta (WesternBright Sirius HRP Substrate), or Alkali Scientific (BrightStar). Recombinant AMPK α 1/ β 1/ γ 1 (#PV4672) was from Thermo Fisher Scientific/Invitrogen.

Antibodies

Myc-9E10 (#MMS-150P) and HA.11 (#MMS-101P) monoclonal antibodies for immunoprecipitation and immunoblotting were from Covance (now BioLegend). Flag antibodies were from either Sigma (#F3165) or Cell Signaling Technology (CST; #2368). The following antibodies were from CST: AMPK P-Thr¹⁷² (#4188), pan-AMPK α (#2532), AMPK β 1 (#12063), Akt P-Ser⁴⁷³ (#4060), Akt P-Thr³⁰⁸ (#4056), Akt P-Thr⁴⁵⁰ (#9267), Akt (#9272), NDRG1 P-Thr³⁴⁶ (#3217), NDRG1 (#5196), Bad P-Ser¹³⁶ (#4366), mTOR (#2972), raptor P-Ser⁷⁹² (#2083), raptor (#2280), S6K1 P-Thr³⁸⁹ (#9234), GST (#2625), cleaved caspase 3 (#9664), cleaved PARP (#9544), Erk1/Erk2 (#9102), β -actin (#4967), and α -tubulin (#2144). mTOR P-Ser²⁴⁸¹ was from Millipore (#09-343). S6K1 P-Thr²²⁹ antibody was from Abcam (#ab59208). The following custom polyclonal anti-peptide antibodies were generated by us with the aid of Covance, as described previously (45): mTOR P-Ser¹²⁶¹ (amino acids 1256 to 1266; rat), rictor (amino acids 6 to 20; human), and S6K1 (amino acids 485 to 502; rat 70-kDa isoform). Donkey anti-rabbit-HRP secondary antibody was from Jackson ImmunoResearch (#711-095-152), and sheep anti-mouse-HRP was from GE Healthcare (#NA931V).

Plasmids

HA-AMPK α 1, HA-AMPK α 1, Myc-AMPK β 1, and Myc-AMPK γ 1 expression plasmids were obtained from K. Inoki (University of Michigan, Ann Arbor, MI). pRK5/Myc-mTOR plasmid was from Addgene (#186) (originally from D. Sabatini, MIT and the Whitehead Institute); pCMV/Flag-rictor plasmid was purchased from the Medical Research Council Protein Phosphorylation and Ubiquitylation Unit (University of Dundee). pCI/HA-rictor was from E. Jacinto (Rutgers University, New Brunswick, NJ).

Generation of recombinant GST-mTOR for IVK assays

A fragment of mTOR encoding amino acids 1223 to 1271 (rat) was subcloned via polymerase chain reaction (PCR) into vector pGEX-20T for production of GST fusion

proteins in the bacterial strain BL21(DE3) LysS. The following primers were used to PCR amplify the mTOR fragment: primer 1, 5'-gacgggattcgctgatgaagaagaagaccctt-3'; primer 2, 5'-gattgaattcgacccttctggcagctcc-3'. GST-mTOR was affinity-purified on glutathione-sepharose beads via a standard protocol and dialyzed against 10 mM tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, dithiothreitol (DTT; 154 mg/liter), and 5% glycerol.

In vitro kinome screen

The in vitro kinome screen was performed in collaboration with Invitrogen/Life Technologies. About 300 recombinant human kinases arrayed on a 384-well plate were incubated with GST-mTOR substrate (0.125 mg/ml) in reactions containing 25 nM recombinant kinases and 1 mM ATP [50 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, and 0.01% Brij-35]. Reactions were incubated at room temperature for 1 hour. Dot blots of the kinase reactions were imaged after incubation with P-mTOR-Ser¹²⁶¹ primary antibody and Alexa Fluor 488 anti-rabbit secondary antibody.

IVK assays

IVK assays were performed by incubating recombinant GST-mTOR (~100 ng) or immunoprecipitated Myc-mTOR substrate with ATP (250 μM) and recombinant (100 ng/reaction) AMPK α1/β1/γ1 or AMPK α2/β1/β1 in 15 μl of kinase buffer containing 10 mM tris (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, and 1 mM DTT. Reactions were incubated at 30°C for 30 min and stopped by addition of sample buffer followed by incubation at 95°C for 10 min. Samples were resolved on SDS-polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membrane, and immunoblotted with mTOR Ser¹²⁶¹ antibodies. For drug pretreatments, recombinant kinases were preincubated (30 min) with torin1 (5 μM; from D. Sabatini), with compound C (0.5 mM; #171261, Millipore/Calbiochem), or in kinase buffer on ice for 30 min.

mTORC2 IVK assays were performed as described (26). Briefly, rictor was immunoprecipitated from serum-starved MEFs either unstimulated or stimulated with AICAR (2.5 mM) (2 hours) or insulin (100 nM; 30 min) (about one 10-cm plate for each immunoprecipitate). Immunoprecipitates were preincubated with torin1 (5 μM) on ice (30 min) and then incubated with ATP (250 μM) and recombinant Akt1 (100 ng/reaction) in 15 μl of kinase buffer containing 25 mM Hepes, 100 mM potassium acetate, and 1 mM MgCl₂ at 30°C for 30 min and stopped by addition of sample buffer followed by incubation at 95°C for 10 min.

To perform two-stage mTORC2 IVK assays, rictor was immunoprecipitated from serum-starved MEFs, washed three times with CHAPS lysis buffer and once with kinase buffer A [10 mM tris (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, and 1 mM DTT], and incubated with recombinant AMPK α 1 β1 γ1 (100 ng/reaction) in 15 μl of kinase buffer A for 30 min at 30°C (stage 1). Immediately after the first IVK reactions, tubes were placed on ice and washed twice with kinase buffer B (25 mM Hepes, 100 mM potassium acetate, and 1 mM MgCl₂), incubated with ATP (250 μM) and recombinant Akt1 (100 ng/reaction) in 15 μl of kinase buffer B at 30°C for 30 min, and stopped by addition of sample buffer followed by incubation at 95°C for 10 min (stage 2).

Cell culture, transfection, and drug treatments

Wild-type and AMPK $\alpha 1/2$ DKO MEFs were from B. Viollet(Inserm, Paris, France); HEK293 cells were from the American Type Culture Collection. All cell lines were cultured in DMEM that contained high glucose (4.5 g/liter), glutamine (584 mg/liter), and sodium pyruvate (110 mg/liter; Life Technologies/Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. HEK293 cells were transfected according to the manufacturer's directions using TransIT-LT1 (Mirus Bio). Cells were lysed ~24 to 48 hours after transfection. To effect serum starvation, cells were washed twice with DMEM/20 mM Hepes (pH 7.2) and cultured in this medium for ~20 hours.

Cells were then stimulated with AICAR (2.5 mM; 1 to 2 hours; #9944, CST), phenformin (2 mM; 90 min; #P7045, Sigma), rotenone (250 ng/ml; 60 min; #150154, MP Biomedical), A769662 (100 μ M; 5 to 30 min; #3336, Tocris Bioscience), antimycin A (10 mM; 60 min; #A8674, Sigma), oligomycin A (10 μ g/ml; 60 min; #75351, Sigma), metformin (2 mM; 2 hours; #PHR1084, Sigma), and insulin (100 nM; 30 min; #12585, Invitrogen). The following additional drugs not described above were used: rapamycin (20 ng/ml; #553210, Calbiochem), torin1 (100 nM; shared by D. Sabatini), compound C (25 μ M; #171261, Calbiochem), BYL719 (#1020, Selleck), and MK2206 (10 μ M; #1078, Selleck).

Cell lysis, immunoprecipitation, and immunoblotting

Unless indicated otherwise, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold buffer A containing 0.5% NP-40 and 0.1% Brij-35, as described (45). To maintain the detergent-sensitive mTOR-raptor interaction, cells were lysed in ice-cold buffer A containing 0.3% CHAPS. Lysates were spun at 13,200 rpm for 5 min at 4°C, and the post-nuclear supernatants were collected and incubated on ice (15 min). The Bradford assay was used to normalize protein levels for immunoprecipitation and immunoblot analysis. For immunoprecipitation, whole-cell lysates were incubated with antibodies for 2 hours at 4°C, followed by incubation with protein G- or A-Sepharose beads for 1 hour. Sepharose beads were washed three times in lysis buffer and resuspended in 1× sample buffer. Samples were resolved on SDS-PAGE and transferred to PVDF membranes in Towbin transfer buffer containing 0.02% SDS, as described (45). Immunoblotting was performed by blocking PVDF membranes in tris-buffered saline (TBS; pH 7.5) with 0.1% Tween 20 (TBST) containing 3% nonfat dry milk, as described (45), and incubating the membranes in TBST with 2% bovine serum albumin (BSA) containing primary antibodies or secondary HRP-conjugated antibodies. Blots were developed by ECL and detected digitally with a ChemiDoc-It System (UVP).

Viral transduction

riCTOR^{-/-} MEFs stably expressing HA-riCTOR and AMPK $\alpha 1/\alpha 2$ DKO MEFs stably expressing HA-AMPK $\alpha 1$ were generated by lentiviral transduction. HA-tagged rictor cDNA or HA-AMPK $\alpha 1$ complementary DNA (cDNA) was subcloned into a modified lentiviral vector, pHAGE-Puro-MCS (pPPM; modified by A. Hudson, Medical College of Wisconsin) (93). Lentivirus particles were packaged in HEK293T cells by cotransfection with empty pPPM vector, pPPM/HA-riCTOR, or pPPM/HA-AMPK $\alpha 1$ together with pRC/Tat, pRC/Rev,

pRC/gag-pol, and pMD/VSV-G using Mirus TransIT-LT1 transfection reagent. Supernatants containing viral particles were collected 48 hours after transfection and filtered through a 0.45- μ m filter. *riCTOR*^{-/-} MEFs or AMPK α 1/ α 2 DKO MEFs were infected with fresh viral supernatants containing polybrene (8 μ g/ml). Twenty-four hours after infection, cells were selected in DMEM/10% FBS supplemented with puromycin (3 μ g/ml).

RNA interference

AMPK β 1, AMPK α 1/ α 2, and raptor were knocked down in HEK293 cells by transient transfection with ON-TARGETplus SMARTpool siRNAs (20 nM; Dharmacon-GE Healthcare) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions [human PRKAB1 (AMPK α 1), #M-007675-00; human AMPK α 1/ α 2, #sc-45312; human raptor, #L-004107-00-0005; nontargeting, #D-001810-10-05].

Generation of genetically modified mice bearing a germline mTOR knock-in S1261A allele using CRISPR-Cas9 genome editing technology

A 20-nucleotide (nt) guide sequence targeting genomic mTOR upstream of Ser¹²⁶¹ was subcloned into pX330. Forward sequence: 5'-caccgacctgccttttgaggttga; reverse sequence: 5'-aaactcaactccaaaaggcaggac. The following single-stranded oligonucleotide served as repair template, which includes the targeted sequence (underlined), left and right homology arms, and the S1261A mutation (also underlined; AGC to GCG): 5'-gaccctttgattaccagcatcgaatgctaaggagcagccagggagatgccctggccagtggaccagttgagacaggacccatgaagaaactgcatgtcGCGaccatAaaTctTcaaaaggcaggtccattgtttccaggggattgggaagcagggtctgtttttttctctcattca-3'.

The repair template also included several silent mutations (capitalized) to prevent retargeting of edited genomic signals and a new Nru I restriction site ([tcGCGa]) to facilitate genotyping. The guide RNA (gRNA) targeting plasmid (pX330-mTOR) and the repair template were co-microinjected into single-cell fertilized mouse oocytes and implanted into a pseudopregnant mouse. Heterozygous founders were identified through Nru I restriction of genomic DNA. To confirm co-recombination of both the Nru I site and the S1261A mutation, the genomic region was TOPO-cloned and sequenced.

The following PCR primers were used for TOPO cloning: 5'-GTTGAAATCCTGGCTCTTGC-3' (forward) and 5'-GCAGGATTACACGTTTAA-3' (reverse).

To generate *mTOR*^{A/A} homozygous mice, mice heterozygous for mTOR S1261A were mated (*mTOR*^{+/mTOR}^A × *mTOR*^{+/mTOR}^A). These mice were generated with the assistance of the Molecular Genetics Core of the MDRC (Michigan Diabetes Research Center) and the University of Michigan Transgenic Core. For genotyping, a ~600-nt fragment of genomic DNA surrounding the mTOR Ser¹²⁶¹ locus was PCR-amplified and digested with Nru I. The following PCR primers were used for genotyping: 5'-GTTGAAATCCTGGCTCTTGC-3' (forward) and 5'-GCAGGATTACACGTTTAA-3' (reverse).

Isolation of MEFs

MEFs were isolated as described previously (94).

Cre-mediated excision of floxed AMPK α 1 and AMPK α 2 in vivo

Mice (C57BL6) were housed in a specific pathogen-free facility with a 12-hour light/12-hour dark cycle and given free access to food and water. All animal use was in compliance with the Institute of Laboratory Animal Research *Guide for the Care and Use of Laboratory Animals* and approved by the University Committee on Use and Care of Animals at the University of Michigan. Mice bearing floxed AMPK α 1 and AMPK α 2 (AMPK α *f/f*), generated by S. Morrison (UT Southwestern Medical Center) as described (56) and shared by K. Inoki (University of Michigan Medical School), were injected through the tail vein with AAV-GFP (AAV8-TBG.PI.eGFP.WPRE. bGH) or AAV-Cre (AAV8.TBG.PPI.Cre.rBG) (1.5×10^8 to 10^9 plaque-forming units per mouse) (Penn Vector Core). After 14 days, primary mouse hepatocytes were isolated or mice were administered metformin, as described below.

Isolation of primary mouse hepatocytes

Primary mouse hepatocytes were isolated from 9- to 12-week-old mice, as described previously (95). Briefly, the liver was perfused with Earle's balanced salt solution (Invitrogen) containing 0.5 mM EGTA for 5 min and perfused with type I collagenase (200 U/ml; Worthington) via the inferior vena cava for 5 min. After dissection, hepatocytes were released by scattering, passed through a 100- μ m cell strainer, and then spun at 50g for 1 min. The pellet was resuspended in DMEM and then spun at 50g for 10 min in a Percoll gradient to remove dead hepatocytes. Viable cells were washed with DMEM at 50g for 10 min and checked by trypan blue staining. Primary mouse hepatocytes were plated in DMEM with 10% FBS.

In vivo metformin treatment and analysis

Male mice (8 weeks old) were injected intraperitoneally with PBS or metformin (250 mg/kg body weight; 1 hour). Livers were immediately flash-frozen in liquid nitrogen and homogenized in radioimmuno-precipitation assay (RIPA) lysis buffer [50 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM NaF] for Western blot analysis.

Ex vivo incubation of rat soleus muscle

After an overnight fast, male Wistar rats were anesthetized [with an intraperitoneal injection of sodium pentobarbital (50 mg/kg)], and their soleus muscles were isolated. The muscles were longitudinally dissected into strips that underwent a two-step incubation (step 1 = 20 min; step 2 = 120 min) in vials containing oxygenated Krebs-Henseleit buffer (KHB) along with BSA (0.1%) and glucose (8 mM) that were placed in a heated (35°C) water bath. During step 2, muscle strips were transferred to a second flask including KHB/BSA/glucose along with AICAR (2 mM), A769662 (200 μ M), or neither compound. After step 2, muscles were immediately freeze-clamped using aluminum tongs cooled to the temperature of liquid nitrogen and homogenized in RIPA lysis buffer, as described above.

Cell viability assay

Cells were seeded on 35-mm polylysine-coated glass bottom plates, serum-starved (20 hours), pretreated with 100 nM torin1, and treated with 2.5 mM AICAR (5 hours). Cell viability was assessed by adding Ready Probes reagents (Thermo Fisher Scientific; catalog no. R37609) for the last 15 min of incubation, according to the manufacturers' instructions. NucBlue Live reagent (Hoechst 33342) stains the nuclei of all cells, whereas NucGreen Dead stains the nuclei of dead cells with compromised plasma membranes. Images were collected on an inverted epifluorescence microscope (Nikon TE2000E) equipped with a Photometrics CoolSnap HQ camera and analyzed using ImageJ (National Institutes of Health).

Image editing

Adobe Photoshop was used for image preparation using only levels, brightness, and contrast and changing these parameters equivalently over the entire image.

Statistical analysis

Results are presented as means $\pm$ SD. Significance of the difference between two conditions was determined by unpaired Student's *t* test. To determine significance of the difference between multiple conditions, one-way ANOVA followed by Tukey's post hoc tests was used. Values of $P < 0.05$ were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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REFERENCES AND NOTES

1. Hardie DG, Schaffer BE, Brunet A, AMPK: An energy-sensing pathway with multiple inputs and outputs. *Trends Cell Biol* 26, 190–201 (2016). [PubMed: 26616193]
2. Carling D, AMPK signalling in health and disease. *Curr. Opin. Cell Biol* 45, 31–37(2017). [PubMed: 28232179]
3. Garcia D, Shaw RJ, AMPK: Mechanisms of cellular energy sensing and restoration of metabolic balance. *Mol. Cell* 66, 789–800 (2017). [PubMed: 28622524]
4. Herzig S, Shaw RJ, AMPK: Guardian of metabolism and mitochondrial homeostasis. *Nat. Rev. Mol. Cell Biol* 19, 121–135 (2018). [PubMed: 28974774]
5. Huang K, Fingar DC, Growing knowledge of the mTOR signaling network. *Semin. Cell Dev. Biol* 36, 79–90 (2014). [PubMed: 25242279]

6. Kennedy BK, Lamming DW, The mechanistic target of rapamycin: The grand ConducTOR of metabolism and aging. *Cell Metab* 23, 990–1003 (2016). [PubMed: 27304501]
7. Saxton RA, Sabatini DM, mTOR signaling in growth, metabolism, and disease. *Cell* 169, 361–371 (2017).
8. Gaubitz C, Prouteau M, Kusmider B, Loewith R, TORC2 structure and function. *Trends Biochem. Sci* 41, 532–545 (2016).
9. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM, mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110, 163–175 (2002). [PubMed: 12150925]
10. Hara K, Maruki Y, Long X, Yoshino KI, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K, Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 110, 177–189 (2002). [PubMed: 12150926]
11. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM, Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol* 14, 1296–1302 (2004). [PubMed: 15268862]
12. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN, Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell Biol* 6, 1122–1128 (2004). [PubMed: 15467718]
13. Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL, Regulation of TORC1 by Rag GTPases in nutrient response. *Nat. Cell Biol* 10, 935–945 (2008). [PubMed: 18604198]
14. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM, The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320, 1496–1501 (2008). [PubMed: 18497260]
15. Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM, Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* 141, 290–303 (2010). [PubMed: 20381137]
16. Wolfson RL, Sabatini DM, The dawn of the age of amino acid sensors for the mTORC1 pathway. *Cell Metab* 26, 301–309 (2017). [PubMed: 28768171]
17. Nguyen TP, Frank AR, Jewell JL, Amino acid and small GTPase regulation of mTORC1. *Cell Logist* 7, e1378794 (2017). [PubMed: 29296509]
18. Dibble CC, Cantley LC, Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol* 25, 545–555 (2015). [PubMed: 26159692]
19. Manning BD, Toker A, AKT/PKB signaling: Navigating the network. *Cell* 169, 381–405 (2017). [PubMed: 28431241]
20. Yang H, Jiang X, Li B, Yang HJ, Miller M, Yang A, Dhar A, Pavletich NP, Mechanisms of mTORC1 activation by RHEB and inhibition by PRAS40. *Nature* 552, 368–373 (2017). [PubMed: 29236692]
21. Magnuson B, Ekim B, Fingar DC, Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. *Biochem. J* 441, 1–21 (2012). [PubMed: 22168436]
22. Reiling JH, Sabatini DM, Stress and mTOR signaling. *Oncogene* 25, 6373–6383 (2006). [PubMed: 17041623]
23. Inoki K, Zhu T, Guan KL, TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115, 577–590 (2003). [PubMed: 14651849]
24. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ, AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* 30, 214–226 (2008). [PubMed: 18439900]
25. Liu P, Gan W, Chin YR, Ogura K, Guo J, Zhang J, Wang B, Blenis J, Cantley LC, Toker A, Su B, Wei W, PtdIns(3,4,5)P₃-dependent activation of the mTORC2 kinase complex. *Cancer Discov* 5, 1194–1209 (2015). [PubMed: 26293922]
26. Huang J, Dibble CC, Matsuzaki M, Manning BD, The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. *Mol. Cell. Biol* 28, 4104–4115 (2008). [PubMed: 18411301]

27. Huang J, Wu S, Wu CL, Manning BD, Signaling events downstream of mammalian target of rapamycin complex 2 are attenuated in cells and tumors deficient for the tuberous sclerosis complex tumor suppressors. *Cancer Res* 69, 6107–6114 (2009). [PubMed: 19602587]
28. Huang J, Manning BD, A complex interplay between Akt, TSC2 and the two mTOR complexes. *Biochem. Soc. Trans* 37, 217–222 (2009). [PubMed: 19143635]
29. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307, 1098–1101 (2005). [PubMed: 15718470]
30. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM, Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC α , but not S6K1. *Dev. Cell* 11, 859–871 (2006). [PubMed: 17141160]
31. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B, SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 127, 125–137 (2006). [PubMed: 16962653]
32. Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP, Lamb RF, The TSC1–2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J. Cell Biol* 166, 213–223 (2004). [PubMed: 15249583]
33. Shah OJ, Wang Z, Hunter T, Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr. Biol* 14, 1650–1656 (2004). [PubMed: 15380067]
34. Facchinetti V, Ouyang W, Wei H, Soto N, Lazorchak A, Gould C, Lowry C, Newton AC, Mao Y, Miao RQ, Sessa WC, Qin J, Zhang P, Su B, Jacinto E, The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J* 27, 1932–1943 (2008). [PubMed: 18566586]
35. Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL, Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J* 27, 1919–1931 (2008). [PubMed: 18566587]
36. Oh WJ, Wu CC, Kim SJ, Facchinetti V, Julien LA, Finlan M, Roux PP, Su B, Jacinto E, mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide. *EMBO J* 29, 3939–3951 (2010). [PubMed: 21045808]
37. Hung CM, Garcia-Haro L, Sparks CA, Guertin DA, mTOR-dependent cell survival mechanisms. *Cold Spring Harb. Perspect. Biol* 4, a008771 (2012). [PubMed: 23124837]
38. Hardie DG, Alessi DR, LKB1 and AMPK and the cancer-metabolism link ten years after. *BMC Biol* 11, 36 (2013). [PubMed: 23587167]
39. Hardie DG, Targeting an energy sensor to treat diabetes. *Science* 357, 455–456 (2017). [PubMed: 28774917]
40. Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, Davis JG, Salmon AB, Richardson A, Ahima RS, Guertin DA, Sabatini DM, Baur JA, Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* 335, 1638–1643 (2012). [PubMed: 22461615]
41. Guigas B, Bertrand L, Taleux N, Foretz M, Wiernsperger N, Vertommen D, Andreelli F, Viollet B, Hue L, 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glucokinase translocation. *Diabetes* 55, 865–874 (2006). [PubMed: 16567505]
42. Guigas B, Taleux N, Foretz M, Demaille D, Andreelli F, Viollet B, Hue L, AMP-activated protein kinase-independent inhibition of hepatic mitochondrial oxidative phosphorylation by AICA riboside. *Biochem. J* 404, 499–507 (2007). [PubMed: 17324122]
43. Santidrian AF, Gonzalez-Girones DM, Iglesias-Serret D, Coll-Mulet L, Cosialls AM, de Frias M, Campas C, Gonzalez-Barca E, Alonso E, Labi V, Viollet B, Benito A, Pons G, Villunger A, Gil J, AICAR induces apoptosis independently of AMPK and p53 through up-regulation of the BH3-only proteins BIM and NOXA in chronic lymphocytic leukemia cells. *Blood* 116, 3023–3032 (2010). [PubMed: 20664053]

44. Kirchner J, Brüne B, Namgaladze D, AICAR inhibits NFκB DNA binding independently of AMPK to attenuate LPS-triggered inflammatory responses in human macrophages. *Sci. Rep* 8, 7801 (2018). [PubMed: 29773845]
45. Acosta-Jaquez HA, Keller JA, Foster KG, Ekim B, Soliman GA, Feener EP, Ballif BA, Fingar DC, Site-specific mTOR phosphorylation promotes mTORC1-mediated signaling and cell growth. *Mol. Cell. Biol* 29, 4308–4324 (2009). [PubMed: 19487463]
46. Soliman GA, Acosta-Jaquez HA, Dunlop EA, Ekim B, Maj NE, Tee AR, Fingar DC, mTOR Ser-2481 autophosphorylation monitors mTORC-specific catalytic activity and clarifies rapamycin mechanism of action. *J. Biol. Chem* 285, 7866–7879 (2010). [PubMed: 20022946]
47. Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, Dickinson R, Adler A, Gagne G, Iyengar R, Zhao G, Marsh K, Kym P, Jung P, Camp HS, Frevert E, Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab* 3, 403–416 (2006). [PubMed: 16753576]
48. Scott JW, van Denderen BJW, Jorgensen SB, Honeyman JE, Steinberg GR, Oakhill JS, Iseli TJ, Koay A, Gooley PR, Stapleton D, Kemp BE, Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes. *Chem. Biol* 15, 1220–1230 (2008). [PubMed: 19022182]
49. Keshwani MM, von Daake S, Newton AC, Harris TK, Taylor SS, Hydrophobic motif phosphorylation is not required for activation loop phosphorylation of p70 ribosomal protein S6 kinase 1 (S6K1). *J. Biol. Chem* 286, 23552–23558 (2011). [PubMed: 21561857]
50. Choo AY, Yoon SO, Kim SG, Roux PP, Blenis J, Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc. Natl. Acad. Sci. U.S.A* 105, 17414–17419 (2008). [PubMed: 18955708]
51. Yu Y, Yoon SO, Poulgiannis G, Yang Q, Ma XM, Villen J, Kubica N, Hoffman GR, Cantley LC, Gygi SP, Blenis J, Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science* 332, 1322–1326 (2011). [PubMed: 21659605]
52. Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, Peterson TR, Choi Y, Gray NS, Yaffe MB, Marto JA, Sabatini DM, The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science* 332, 1317–1322 (2011). [PubMed: 21659604]
53. Kang SA, Pacold ME, Cervantes CL, Lim D, Lou HJ, Ottina K, Gray NS, Turk BE, Yaffe MB, Sabatini DM, mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin. *Science* 341, 1236566 (2013). [PubMed: 23888043]
54. Choo AY, Blenis J, Not all substrates are treated equally: Implications for mTOR, rapamycin-resistance and cancer therapy. *Cell Cycle* 8, 567–572 (2014).
55. Thoreen CC, Sabatini DM, Rapamycin inhibits mTORC1, but not completely. *Autophagy* 5, 725–726 (2014).
56. Nakada D, Saunders TL, Morrison SJ, Lkb1 regulates cell cycle and energy metabolism in haematopoietic stem cells. *Nature* 468, 653–658 (2010). [PubMed: 21124450]
57. Howell JJ, Hellberg K, Turner M, Talbott G, Kolar MJ, Ross DS, Hoxhaj G, Saghatelian A, Shaw RJ, Manning BD, Metformin inhibits hepatic mTORC1 signaling via dose-dependent mechanisms involving AMPK and the TSC complex. *Cell Metab* 25, 463–471 (2017). [PubMed: 28089566]
58. Boulbes D, Chen CH, Shaikenov T, Agarwal NK, Peterson TR, Addona TA, Keshishian H, Carr SA, Magnuson MA, Sabatini DM, Sarbassov DD, Rictor phosphorylation on the Thr-1135 site does not require mammalian target of rapamycin complex 2. *Mol. Cancer Res* 8, 896–906 (2010). [PubMed: 20501647]
59. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC, The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc. Natl. Acad. Sci. U.S.A* 101, 3329–3335 (2004). [PubMed: 14985505]
60. Yang Q, Inoki K, Ikenoue T, Guan KL, Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev* 20, 2820–2832 (2006). [PubMed: 17043309]

61. Tao R, Gong J, Luo X, Zang M, Guo W, Wen R, Luo Z, AMPK exerts dual regulatory effects on the PI3K pathway. *J. Mol. Signal* 5, 1 (2014).
62. Choudhury Y, Yang Z, Ahmad I, Nixon C, Salt IP, Leung HY, AMP-activated protein kinase (AMPK) as a potential therapeutic target independent of PI3K/Akt signaling in prostate cancer. *Oncoscience* 1, 446–456 (2014). [PubMed: 25594043]
63. Lee JH, Budanov AV, Talukdar S, Park EJ, Park HL, Park HW, Bandyopadhyay G, Li N, Aghajani M, Jang I, Wolfe AM, Perkins GA, Ellisman MH, Bier E, Scadeng M, Foretz M, Viollet B, Olefsky J, Karin M, Maintenance of metabolic homeostasis by Sestrin2 and Sestrin3. *Cell Metab* 16, 311–321 (2012). [PubMed: 22958918]
64. Schaffer BE, Levin RS, Hertz NT, Maures TJ, Schoof ML, Hollstein PE, Benayoun BA, Banko MR, Shaw RJ, Shokat KM, Brunet A, Identification of AMPK phosphorylation sites reveals a network of proteins involved in cell invasion and facilitates large-scale substrate prediction. *Cell Metab* 22, 907–921 (2015). [PubMed: 26456332]
65. Zhang C-S, Jiang B, Li M, Zhu M, Peng Y, Zhang Y-L, Wu Y-Q, Li TY, Liang Y, Lu Z, Lian G, Liu Q, Guo H, Yin Z, Ye Z, Han J, Wu J-W, Yin H, Lin S-Y, Lin S-C, The lysosomal v-ATPase-Ragulator complex is a common activator for AMPK and mTORC1, acting as a switch between catabolism and anabolism. *Cell Metab* 20, 526–540 (2014). [PubMed: 25002183]
66. Ebner M, Sinkovics B, Szczygieł M, Ribeiro DW, Yudushkin I, Localization of mTORC2 activity inside cells. *J. Cell Biol* 216, 343–353 (2017). [PubMed: 28143890]
67. Betz C, Stracka D, Prescianotto-Baschong C, Frieden M, Demareux N, Hall MN, Feature Article: mTOR complex 2-Akt signaling at mitochondria-associated endoplasmic reticulum membranes (MAM) regulates mitochondrial physiology. *Proc. Natl. Acad. Sci. U.S.A* 110, 12526–12534 (2013). [PubMed: 23852728]
68. Huang J, Manning BD, The TSC1-TSC2 complex: A molecular switchboard controlling cell growth. *Biochem. J* 412, 179–190 (2008). [PubMed: 18466115]
69. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM, mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H⁺-ATPase. *Science* 334, 678–683 (2011). [PubMed: 22053050]
70. Hardie DG, Lin S-C, AMP-activated protein kinase—Not just an energy sensor. *F1000Res* 6, 1724 (2017). [PubMed: 29034085]
71. Lin S-C, Hardie DG, AMPK: Sensing glucose as well as cellular energy status. *Cell Metab* 27, 299–313 (2018). [PubMed: 29153408]
72. Zhang C-S, Hawley SA, Zong Y, Li M, Wang Z, Gray A, Ma T, Cui J, Feng J-W, Zhu M, Wu Y-Q, Li TY, Ye Z, Lin S-Y, Yin H, Piao H-L, Hardie DG, Lin S-C, Fructose-1,6-bisphosphate and aldolase mediate glucose sensing by AMPK. *Nature* 548, 112–116 (2017). [PubMed: 28723898]
73. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR, Hardie DG, Complexes between the LKB1 tumor suppressor, STRADA/β and MO25α/β are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol* 2, 28 (2003). [PubMed: 14511394]
74. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LGD, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D, LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr. Biol* 13, 2004–2008 (2003). [PubMed: 14614828]
75. Evans JMM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD, Metformin and reduced risk of cancer in diabetic patients. *BMJ* 330, 1304–1305 (2005). [PubMed: 15849206]
76. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B, Metformin: From mechanisms of action to therapies. *Cell Metab* 20, 953–966 (2014). [PubMed: 25456737]
77. Jeon S-M, Chandel NS, Hay N, AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature* 485, 661–665 (2012). [PubMed: 22660331]
78. Shackelford DB, Abt E, Gerken L, Vasquez DS, Seki A, Leblanc M, Wei L, Fishbein MC, Czernin J, Mischel PS, Shaw RJ, LKB1 inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin. *Cancer Cell* 23, 143–158 (2013). [PubMed: 23352126]
79. Laderoute KR, Amin K, Calaoagan JM, Knapp M, Le T, Orduna J, Foretz M, Viollet B, 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. *Mol. Cell. Biol* 26, 5336–5347 (2006). [PubMed: 16809770]

80. Jeon S-M, Hay N, The dark face of AMPK as an essential tumor promoter. *Cell Logist* 2, 197–202 (2012). [PubMed: 23676995]
81. Svensson RU, Shaw RJ, Cancer metabolism: Tumour friend or foe. *Nature* 485, 590–591 (2012). [PubMed: 22660317]
82. Hardie DG, The LKB1-AMPK pathway—Friend or foe in cancer? *Cancer Cell* 23, 131–132 (2013). [PubMed: 23410967]
83. Xiao B, Sanders MJ, Carmena D, Bright NJ, Haire LF, Underwood E, Patel BR, Heath RB, Walker PA, Hallen S, Giordanetto F, Martin SR, Carling D, Gamblin SJ, Structural basis of AMPK regulation by small molecule activators. *Nat. Commun* 4, 3017 (2013). [PubMed: 24352254]
84. Zadra G, Photopoulos C, Tyekucheva S, Heidari P, Weng QP, Fedele G, Liu H, Scaglia N, Priolo C, Sicinska E, Mahmood U, Signoretti S, Birnberg N, Loda M, A novel direct activator of AMPK inhibits prostate cancer growth by blocking lipogenesis. *EMBO Mol.Med* 6, 519–538 (2014). [PubMed: 24497570]
85. Myers RW, Guan H-P, Ehrhart J, Petrov A, Prahalada S, Tozzo E, Yang X, Kurtz MM, Trujillo M, Gonzalez Trotter D, Feng D, Xu S, Eiermann G, Holahan MA, Rubins D, Conarello S, Niu X, Souza SC, Miller C, Liu J, Lu K, Feng W, Li Y, Painter RE, Milligan JA, He H, Liu F, Ogawa A, Wisniewski D, Rohm RJ, Wang L, Bunzel M, Qian Y, Zhu W, Wang H, Bennet B, LaFranco Scheuch L, Fernandez GE, Li C, Klimas M, Zhou G, van Heek M, Biftu T, Weber A, Kelley DE, Thornberry N, Erion MD, Kemp DM, Sebat IK, Systemic pan-AMPK activator MK-8722 improves glucose homeostasis but induces cardiac hypertrophy. *Science* 357, 507–511 (2017). [PubMed: 28705990]
86. Cokorinos EC, Delmore J, Reyes AR, Albuquerque B, Kjøbsted R, Jørgensen NO, Tran J-L, Jatkar A, Cialdea K, Esquejo RM, Meissen J, Calabrese MF, Cordes J, Moccia R, Tess D, Salatto CT, Coskran TM, Opsahl AC, Flynn D, Blatnik M, Li W, Kindt E, Foretz M, Viollet B, Ward J, Kurumbail RG, Kalgutkar AS, Wojtaszewski JFP, Cameron KO, Miller RA, Activation of skeletal muscle AMPK promotes glucose disposal and glucose lowering in non-human primates and mice. *Cell Metab* 25, 1147–1159.e10 (2017). [PubMed: 28467931]
87. Foretz M, Hébrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, Sakamoto K, Andreelli F, Viollet B, Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J. Clin. Invest* 120, 2355–2369 (2010). [PubMed: 20577053]
88. Kumar A, Harris TE, Keller SR, Choi KM, Magnuson MA, Lawrence JC Jr., Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity. *Mol. Cell. Biol* 28, 61–70(2007). [PubMed: 17967879]
89. Kumar A, Lawrence JC Jr., Jung DY, Ko HJ, Keller SR, Kim JK, Magnuson MA, Harris TE, Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism. *Diabetes* 59, 1397–1406(2010).
90. Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Rüegg MHMA, Hall MN, Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metab* 15, 725–738 (2012). [PubMed: 22521878]
91. Kjøbsted R, Munk-Hansen N, Birk JB, Foretz M, Viollet B, Bjørnholm M, Zierath JR, Treebak JT, Wojtaszewski JFP, Enhanced muscle insulin sensitivity after contraction/exercise is mediated by AMPK. *Diabetes* 66, 598–612 (2017). [PubMed: 27797909]
92. Friedrichsen M, Mortensen B, Pehmøller C, Birk JB, Wojtaszewski JFP, Exerciseinduced AMPK activity in skeletal muscle: Role in glucose uptake and insulin sensitivity. *Mol. Cell. Endocrinol* 366, 204–214 (2013). [PubMed: 22796442]
93. Mostoslavsky G, Fabian AJ, Rooney S, Alt FW, Mulligan RC, Complete correction of murine Artemis immunodeficiency by lentiviral vector-mediated gene transfer. *Proc. Natl. Acad. Sci. U.S.A* 103, 16406–16411 (2006). [PubMed: 17062750]
94. Todaro GJ, Green H, Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol* 17, 299–313(1963). [PubMed: 13985244]
95. Zhang D, Tong X, VanDommelen K, Gupta N, Stamper K, Brady GF, Meng Z, Lin J, Rui L, Omary MB, Yin L, Lipogenic transcription factor ChREBP mediates fructoseinduced metabolic adaptations to prevent hepatotoxicity. *J. Clin. Invest* 127, 2855–2867 (2017). [PubMed: 28628040]

Fig. 1. AMPK associates with and phosphorylates mTOR within mTORC2.

(A) Wild-type (WT) and AMPK α 1/ α 2 DKO MEFs were serum-starved (20 hours), pretreated without or with torin1 (T; 100 nM; 30 min), and treated without (–) or with (+) AICAR (2.5 mM; 2 hours). Whole-cell lysates were immunoblotted (IB) as indicated. Graph represents quantification of the mean ratio $\pm$ SD of Akt^{S473} phosphorylation over total Akt levels. Each bar represents the mean of five independent experiments performed in duplicate $\pm$ SD; thus, $n = 10$. *** $P < 0.001$, analysis of variance (ANOVA), WT compared to DKO. (B) MEFs were serum-starved (20 hours), pretreated with torin1 (100 nM) or BYL719 (10 μ M; 30 min), and treated without (–) or with (+) AICAR (2.5 mM; 2 hours). Whole-cell lysates were immunoblotted as indicated. Blots are representative of three independent experiments. (C) *rictor*^{–/–} MEFs stably expressing vector control (V) or HA-rictor were serum-starved (20 hours), pretreated without or with torin1 (100 nM; 30 min), and treated without (–) or with (+) AICAR (2.5 mM; 1 hour). Whole-cell lysates were immunoblotted as indicated. Blots are representative of three independent experiments. (D) Myc-mTOR WT

and Myc-mTOR S1261A were immunoprecipitated (IP) with anti-Myc antibodies from transfected human embryonic kidney (HEK) 293 cells and incubated without (–) or with (+) recombinant active AMPK α 1/ β 1/ γ 1 (α 1) or AMPK α 2/ β 1/ γ 1 (α 2) (100 ng; 30 min at 30°C). IVK reactions and input were analyzed as indicated. Blots are representative of three independent experiments. (E) Rictor was immunoprecipitated and incubated with recombinant active AMPK α 1/ β 1/ γ 1 or AMPK α 2/ β 1/ γ 1 in vitro as described in (D). Blots are representative of five independent experiments. (F) mTOR Ser¹²⁶¹ peptide sequence compared to the AMPK consensus phosphorylation motif and the raptor Ser⁷⁹² peptide sequence. (G) HEK293 cells were cotransfected with Flag-rictor, HA-AMPK α 1, or HA-AMPK α 2. Anti-Flag immunoprecipitates (IP) and whole-cell lysates (WCL) were immunoblotted as indicated. Blots are representative of six independent experiments. (H) WT and AMPK α 1/ α 2 DKO MEFs were serum-starved (20 hours), pretreated with torin1 (100 nM; 30 min), and treated without (–) or with (+) AICAR as in (A). Rictor or raptor was immunoprecipitated, and immunoprecipitates and whole-cell lysates were immunoblotted as indicated. Blots are representative of four independent experiments.

Fig. 2. AMPK promotes mTORC2 signaling in response to energetic stress induced by diverse agents in cultured cells.

(A) WT and AMPK α 1/ α 2 DKO MEFs were serum-starved (20 hours) and refed with Dulbecco's modified Eagle's medium (DMEM) without (–) and with (+) glucose withdrawal (Glc W/D) for 8 hours to induce glucose deprivation without or with torin1 (100 nM). Graph represents quantification of the mean ratio $\pm$ SD of Akt^{S473} phosphorylation over total Akt levels. $n = 3$ samples from three independent experiments. * $P < 0.05$ by unpaired t test. (B) WT and AMPK α 1/ α 2 DKO MEFs were serum-starved (20 hours),

pretreated without or with torin1 (100 nM; 30 min), and treated without (–) or with (+) phenformin (2 mM; 90 min). Graph represents quantification of the mean ratio $\pm$ SD of Akt^{S473} phosphorylation over total Akt levels. $n = 6$ samples from three independent experiments. *** $P < 0.001$ by unpaired t test. (C) WT and AMPK α 1/ α 2 DKO MEFs were treated as in (B) except without (–) or with (+) rotenone (2.5 μ g/ml; 60 min). Graph represents quantification of the mean ratio $\pm$ SD of Akt^{S473} phosphorylation over total Akt levels. $n = 4$ samples from four independent experiments. ** $P < 0.01$ by unpaired t test. (D) MEFs were serum-starved (20 hours), pretreated without or with torin1 as above, and treated without (–) or with (+) A769662 (100 μ M) for a time course (0 to 30 min). Blots are representative of three independent experiments. (E) HEK293 cells were transiently transfected with scrambled (Scr), AMPK β 1 (siAMPK β 1), or pan-AMPK α 1/ α 2 (siAMPK α) siRNAs (96 hours), serum-starved (20 hours), pretreated without or with torin1 (100 nM; 30 min), and then stimulated without (–) or with (+) A769662 (100 μ M; 10 min). Blots are representative of three independent experiments.

Fig. 3. AMPK promotes mTORC2 signaling independently of mTORC1-mediated negative feedback.

(A) MEFs were serum-starved (20 hours), pretreated without or with torin1 (100 nM; 30 min), and treated without (–) or with (+) AICAR (2.5 mM), rapamycin (20 ng/ml), or insulin (100 nM) for the indicated times (0 to 240 min). Whole-cell lysates were immunoblotted as indicated. Blots are representative of three independent experiments. (B) Left: MEFs were serum-starved (20 hours), pretreated without (–) or with (+) rapamycin (20 ng/ml) or torin1 (100 nM; 30 min), and treated without (–) or with (+) AICAR (2.5 mM; 2 hours), phenformin (2 mM; 1 hour), glucose withdrawal (8 hours), or rotenone (2.5 µg/ml; 1 hour). Right: HEK293 cells were serum-starved and treated as in (A) except without (–) or with (+) A769662 (100 µM; 2 hours). Blots are representative of three independent experiments. (C) HEK293 cells were transfected with scrambled (Scr) or raptor (siRaptor) siRNAs (96 hours), serum-starved (20 hours), pretreated with torin1 (100 nM; 30 min), and treated without (–) or with (+) A769662 (100 µM; 10 min). Blots are representative of three independent experiments. (D) MEFs were serum-starved and torin1-treated as in (A) except without (–) or with (+) AICAR (2.5 mM; 2 hours), insulin (100 nM; 2 hours), or both. Blots are representative of three independent experiments.

Fig. 4. AMPK promotes mTORC2 signaling in cultured primary hepatocytes and in liver in vivo. (A) Primary hepatocytes expressing (AAV-GFP) or lacking AMPK α 1 and α 2 (AAV-Cre) were isolated from male AMPK α 1/ α 2 floxed mice injected with AAVs for 14 days. The cells were then placed in vitro, serum-starved (20 hours), and treated without (–) or with (+) metformin (2 mM; 2 hours). Whole-cell lysates were immunoblotted as indicated. Blots are representative of two independent experiments. (B) Primary hepatocytes expressing (AAV-GFP) or lacking AMPK α 1 and α 2 (AAV-Cre) were serum-starved and treated without (–) or with (+) AICAR (2.5 mM; 2 hours). Blots are representative of two independent experiments. (C) Primary hepatocytes expressing (AAV-GFP) or lacking (AAV-Cre) AMPK α 1 and α 2 were serum-starved and treated with metformin (2 mM), insulin (100 nM), or both (2 hours). SE, short exposure; LE, long exposure. Blots are representative of

two independent experiments. **(D)** Male AMPK α 1/ α 2 floxed mice administered AAV-GFP viruses (control) or AAV-Cre viruses to excise AMPK α 1/ α 2 (14 days) were fasted overnight (O/N) and injected intraperitoneally with saline or metformin (250 mg/kg; 1 hour). Liver lysates were immunoblotted as indicated. Blot is representative of one independent experiment using 3 to 5 individual mice per treatment group (14 mice total).

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Fig. 5. AMPK directly increases mTORC2 catalytic activity.

(A) AMPK α 1/ α 2 DKO MEFs were serum-starved (20 hours), pretreated with torin1 (100 nM; 30 min), and treated without (–) or with (+) AICAR (2.5 mM; 2 hours) or insulin (INS; 100 nM; 30 min). Immunoprecipitations with anti-rictor antibodies or beads only (b/o) were incubated with recombinant His-Akt1 substrate (100 ng; 30 min) at 30°C. IVK reactions, immunoprecipitates, and whole-cell lysates were immunoblotted as indicated. Graph represents the mean ratio $\pm$ SD of Akt^{S473} phosphorylation over total Akt levels. $n = 3$ samples from three independent experiments. * $P < 0.05$ by unpaired t test. (B) AMPK α 1/ α 2 DKO MEFs were serum-starved (20 hours) and stimulated without (–) (lanes 1 to 7) or with (+) (lanes 8 and 9) insulin (100 nM; 30 min). Immunoprecipitations with anti-rictor antibodies (+) or beads only (–) were preincubated without or with compound C (CC; 0.5 mM) or torin1 (5 μ M; 30 min) on ice, incubated without (–) or with (+) recombinant GST-AMPK α 1 β 1 γ 1 (100 ng; 30 min) at 30°C (stage 1), washed twice, and incubated without (–) or with (+) His-Akt1 (100 ng; 30 min) at 30°C (stage 2). IVK reactions were immunoblotted as indicated. Graph represents the mean ratio $\pm$ SD of Akt^{S473} phosphorylation over total Akt levels. $n = 4$ samples from four independent experiments. ** $P < 0.01$ by unpaired t test. (C) Rictor (+) or beads-only (–) immunoprecipitates were incubated without (–) or with (+) recombinant active GST-AMPK α 1/ β 1/ γ 1 (100 ng) and without (–) or with (+) compound C (25 μ M; 30 min) at 30°C. Blots are representative of four independent experiments.

Fig. 6. AMPK-mediated mTORC2 signaling does not require mTOR Ser¹²⁶¹ phosphorylation. (A) Two littermate-matched pairs of WT (*mTOR*^{+/+}) and mTOR S1261A (*mTOR*^{A/A}) primary MEFs were serum-starved (20 hours) and treated without (–) or with (+) AICAR (2.5 mM; 2 hours). Whole-cell lysates were immunoblotted as indicated. Blots are representative of four independent experiments. (B) Primary MEFs from *mTOR*^{+/+} or *mTOR*^{A/A} littermates were serum-starved (20 hours) and refed with DMEM containing (–) or lacking (+) glucose (8 hours) to induce glucose deprivation (Glc W/D; glucose withdrawal) without or with torin1 (100 nM). Blots are representative of three independent experiments. (C) Primary hepatocytes from *mTOR*^{+/+} or *mTOR*^{A/A} littermates were serum-starved and treated without (–) or with (+) metformin (2 mM; 2 hours). Blots are representative of four independent experiments.

Fig. 7. AMPK, mTORC2, and Akt promote cell survival during acute energetic stress.

(A and B) WT and AMPKα1/α2 DKO MEFs were serum-starved (20 hours), pretreated without (–) or with (+) torin1 (100 nM; 30 min), and treated without (–) or with (+) AICAR (2.5 mM; 5 hours) (A) or subjected to glucose withdrawal (Glc W/D) (+) or not (–). (B) Whole-cell lysates were immunoblotted as indicated. Blots are representative of three independent experiments. (C) AMPKα1/α2 DKO MEFs stably expressing vector control (V) or HA-AMPKα1 were serum-starved and treated with AICAR as in (A). Blots are representative of three independent experiments. (D and E) *rictor*^{–/–} MEFs stably expressing vector control or HA-rictor were serum-starved and treated with AICAR as in (A) or subjected to glucose withdrawal (24 hours). Blots are representative of three independent experiments. (F) WT and AMPKα1/α2 DKO MEFs were treated with AICAR (5 hours) as in (A). ReadyProbes cell viability reagents were added to the culture medium, and live cells were imaged using blue [4',6-diamidino-2-phenylindole (DAPI)] and green [fluorescein isothiocyanate (FITC)/GFP] filters on an inverted epifluorescence microscope. Cell death (%) was calculated using the ratio of green, nonviable cells over total blue cells (includes green cells). Graph represents the mean ± SD of *n* = 4 experiments, in which *n* = 300 cells were counted per treatment in each experiment. Statistical significance was tested by ANOVA followed by pairwise Tukey's post hoc tests (***P* < 0.001). (G) *rictor*^{–/–} MEFs rescued with HA-rictor or vector control (Vector) were treated with AICAR (5 hours) as in (A). ReadyProbes cell viability reagents were added to the culture medium, and cells were imaged as in (F). Cell death (%) was calculated as in (F). Graph represents the mean ± SD of

$n = 3$ experiments, in which $n = 250$ to 300 cells were counted per treatment in each experiment. Statistical significance was tested by ANOVA (** $P < 0.001$) followed by pairwise Tukey's post hoc tests. **(H)** AMPK promotes cell survival during acute energetic stress by both mTORC2-dependent and mTORC2-independent pathways. **(I)** AMPK phosphorylates mTOR and/or partner proteins directly to increase mTORC2 catalytic activity, which functions to promote cell survival during acute energetic stress through Akt.