Regulation of the Mechanistic Target of Rapamycin by Cellular Stress

by

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2DG	2-deoxyglucoses
4EBP1	eIF4E binding protein 1
Acc1	acetyl-CoA carboxylase 1
AGC	PKA, PKG, PKC (kinase family)
AICAR	5-aminoimidizole-4-carboxamide riboside
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ATM	ataxia telangiectasia mutated
Bad	Bcl-2-associated death promoter
BCAA	branched-chain amino acid
Bcl-2	B-cell lymphoma 2
BRCA1	breast cancer type 1 susceptibility protein
CaMK	calcium/calmodulin-dependent protein kinase
CaMKKβ	calcium/calmodulin-dependent kinase kinase β
CBM	carbohydrate-binding module
CBS	cystathione-β-synthase (domain)
deptor	DEP-domain containing mTOR interacting protein
DKO	double-knockout
DNA-PK	DNA-dependent protein kinase
eEF2K	eukaryotic elongation factor 2 kinase
eIF4B/4E	eukaryotic initiation factor 4B/4E
FKBP12	FK506-binding protein 12
FoxO	forkhead box O
FRB	FKBP12-rapamycin binding (domain)
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GLUT4	Glucose transporter type 4
Grb10	growth factor receptor-bound protein 10
GS	glycogen synthase
GSK3β	glycogen synthase kinase 3β
GST	glutathione S-transferase
hAtg13	human homolog to autophagy related 13
HDAC1/5	histone deacetylase 1/5
HEAT	Huntingtin, Elongation factor 3, PP2A A-subunit, and TOR1
HEK293	human embryonic kidney 293
HM	hydrophobic motif

HMGR	HMG-CoA reductase
IGF	insulin-like growth factor
IGFR	IGF receptor
IKKε	IκB kinase ε
ILK	integrin-linked kinase
IVK	*in vitro* kinase
IR	insulin receptor
IRAK	interleukin-1 receptor associated kinase
IRS1	insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LRS	leucyl-tRNA synthetase
LKB1	liver kinase B1
MARK	MAP/microtubule-affinity protein kinases
MEF	mouse embryonic fibroblast
MEK	MAP kinase kinase
mLst8	mammalian lethal with SEC13 8
MO25	mouse protein 25
mTOR	mechanistic (or mammalian) target of rapamycin
mTORC	mTOR Complex
NDRG1	N-myc downstream regulated gene 1
NEK	NimA-related kinase
PDCD4	programmed cell death protein 4
PDK1	phosphoinositide-dependent protein kinase 1
PKB	protein kinase B
PKC	protein kinase C
PH	pleckstrin homology (domain)
PI3K	phosphatidylinositol-3'OH kinase
PIKK	PI3K-like kinase
PPARγ	peroxisome proliferator-activated receptor γ
PRAS40	proline-rich Akt substrate of 40 kDa
PRR5L	proline rich protein 5-like
Rheb	Ras homolog enriched in brain
ROS	reactive oxygen species
rpS6	ribosomal protein S6
RSK	(p90) ribosomal S6 kinase
S6K1	(p70) ribosomal protein S6 kinase 1
SKO	single-knockout
SGK1	serum- and glucocorticoid-activated protein kinase 1
SNF1	sucrose non-fermenting 1
SREBP	sterol regulatory element binding protein
STRAD	Ste20-related adaptor protein
TBC1D1	TBC1 domain family member 1
TBK1	TANK-binding protein 1
Tel2	telomere maintenance 2

Tg	thapsigargin
TM	turn motif
Tm	tunicamycin
TOS	TOR signaling (motif)
Tsc	tuberous sclerosis complex
Tti1	Tel2 interacting protein 1
TZD	thiazolidinedione
ULK	Unc-51-like kinase

ABSTRACT

In complex eukaryotes, cell, tissue, and organismal homeostasis requires proper sensing of growth factors and nutrients. The mechanistic target of rapamycin (mTOR) functions as a central integrator of diverse cellular stimuli to regulate fundamental cellular processes. mTOR, a serine/threonine protein kinase, forms the catalytic core of at least two distinct signaling complexes, the raptor-associated mTOR complex 1 (mTORC1) and the rictor-associated mTOR complex 2 (mTORC2). Growth factors and nutrients activate mTORC1 to promote anabolic processes including protein and lipid biosynthesis, cell growth, and cell proliferation. mTORC1 dysregulation contributes to insulin resistance, type 2 diabetes, tumorigenesis, and neurodegenerative disorders. mTORC2 responds to insulin signaling and is important for glucose homeostasis, cell survival, and actin cytoskeleton organization. The regulation and function of mTORC2 remains poorly understood. Our group previously identified a novel phosphorylation site on mTOR (S1261). To identify the mTOR S1261 kinase we performed an *in vitro* kinome screen. My analysis identified the AMP-activated protein kinase (AMPK) as a bona fide mTOR S1261 kinase in intact cells. The discovery of AMPK as an mTOR kinase was paradoxical, as AMPK is best known as an energy sensor that suppresses anabolic pathways, including mTORC1 signaling, during energy stress, yet our published work demonstrated that mTOR S1261 phosphorylation promotes mTORC1 signaling. As expected,

AMPK suppressed mTORC1 signaling. I therefore investigated a role for AMPK in regulation of mTORC2 function. I found that AMPK phosphorylated mTOR S1261 within mTORC2 and promoted mTORC2 signaling, even in the absence of serum growth factors. Pharmacological activation of AMPK increased mTORC2-associated mTOR autophosphorylation, an indicator of mTORC2 activity in intact cells. Furthermore, AMPK and mTORC2 cooperatively protected cells from energy stress-induced apoptosis. Taken together, these data identify AMPK as a novel mTOR kinase and mTORC2 activator. This novel connection between AMPK and mTORC2 has two significant implications. First, healthy tissues may depend on mTORC2 signaling in the absence of insulin signaling to manage glucose metabolism and cell survival during energy stress. Second, this model provides insight into the poorly understood mechanism of clinically prescribed anti-diabetic compounds, such as metformin and rosiglitazone, which activate AMPK to restore insulin sensitivity and glucose homeostasis. AMPK may therefore bypass defects in signaling caused by insulin resistance through the direct activation of mTORC2.

CHAPTER 1

Introduction

Homeostasis, a term derived from the Greek meaning “staying the same”, implies unmoving or unchanging objects. In biology, however, this literal definition belies the fact that homeostasis is about continual change. Multicellular eukaryotes inherited the ability to sense nutrients from their unicellular ancestors, but have also evolved mechanisms to tightly regulate growth, proliferation, death, and differentiation of cells at both near and far distances through the secretion of signaling molecules, or hormones. The mechanistic target of rapamycin (mTOR) integrates nutrient and hormonal signals to regulate diverse cellular processes and it critical for proper cellular and organismal homeostasis.

Imbalances in homeostasis lead to numerous human pathologies, ranging from cancer to type 2 diabetes. Risk of these diseases increases with age as the body’s capacity to maintain homeostasis naturally deteriorates[1–3]. Type 2 diabetes in turn increases the risk for neuropathic, cardiovascular, and hepatic diseases[1,4,5]. Morbidity and mortality associated with diabetes is largely a result of such complications. Furthermore, both genetic and environmental factors contribute to pathologies of homeostasis and thus the etiology of these diseases is complex [6]. Therapeutic strategies depend on the understanding of regulation of metabolism by nutrients and hormones within individual cells, yet the

molecular mechanisms underlying these regulatory systems remain poorly understood.

Research in recent years has indicated that two signal transducers, mTOR and the AMP-activated protein kinase (AMPK), are critical regulators of cellular metabolism. mTOR functions within two multiprotein complexes, mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). mTORC1 responds to growth factors and nutrients to promote protein synthesis, lipid synthesis, and cell growth and is suppressed by cellular stresses[7,8]. mTORC1 hyperactivation contributes to insulin resistance, tumorigenesis, and neurodegeneration. The regulation and function of mTORC2 remains poorly understood, however recent studies have demonstrated its importance in maintaining glucose homeostasis and insulin sensitivity, as well as survival pathways required by some cancers[9–11]. The AMP-activated protein kinase (AMPK) downregulates energy-costly processes in response to energy stress and has garnered considerable attention in recent years after it was discovered to be a key player in the mechanism of some anti-diabetic drugs, including the widely prescribed metformin and rosiglitazone, which increase insulin sensitivity[12].

AMPK and mTORC2 signaling both promote glucose metabolism and insulin sensitivity, but whether they act together or independently remains unclear. In this dissertation, I propose and test the hypothesis that AMPK functions as a novel, direct upstream activator of mTORC2, thus placing these two important signaling kinases within a linear pathway. The implication of such a relationship is that cellular response to stress, through AMPK, promotes

mTORC2 signaling, which could explain how AMPK activation enhances insulin sensitivity in diabetic patients, as mTORC2 is an important component of insulin signaling. This mechanistic insight would help explain the action of current anti-diabetic drugs that activate AMPK, as well as rationalize the targeting of both AMPK and mTORC2 in future treatment strategies.

1-1. The mechanistic target of rapamycin

The mechanistic target of rapamycin (mTOR) is a conserved and ubiquitously-expressed serine/threonine protein kinase that receives input from a variety of cellular stimuli and regulates numerous cellular processes[7,8]. Through the use of its namesake drug rapamycin, a naturally occurring macrolide antibiotic, much has been learned about mTOR regulation and function in unicellular fungi, invertebrates, and mammals, including the original identification of TOR1/2 in *S. cerevisiae* [13,14] In fact, rapamycin has found therapeutic use due to its immunosuppressive and antiproliferative properties[15–18]. We have only begun to understand mTORC2, which was only discovered in 2002 primarily because it is resistant to acute rapamycin treatment[19–21]. The Fingar laboratory has demonstrated that novel phosphorylation of mTOR augments mTORC1 signaling [22,23]. To better understand how phosphorylation regulates mTOR, we have been engaged in identifying the kinases for these mTOR phosphorylation sites and characterizing mTOR phosphorylation within mTORC1 and mTORC2. This dissertation describes the identification of AMPK as an mTOR S1261 kinase and tests the hypothesis that AMPK represents a novel upstream activator of mTORC2 (see Chapter 2).

mTOR domain features and structure

mTOR is a member of the phosphatidylinositol 3'-kinase (PI3K)-like kinase (PIKK) family, a group of large, atypical kinases[24]. The domain structure of these proteins is similar, comprised of a kinase domain situated between two co-conserved domains called FAT and FAT C-terminal (FATC), all of which reside near the C-terminus (illustrated in Figure 1-1). The FAT/FATC domains were named after three PIKK family members FKBP12-rapamycin associated protein (FRAP; also known as mTOR), ataxia telangiectasia mutated (ATM), and transformation/transcription domain-associated protein (TRRAP). The precise function of the FAT and FATC domains remains unclear, but they may regulate kinase activity through direct interactions with the kinase domain[25,26]. Throughout the N-terminal region of mTOR, including the FAT domain, are tandem HEAT repeats. The HEAT motif (named after the HEAT repeat proteins huntingtin, elongation factor 3, PP2A A-subunit, and TOR1) is a sequence of 30-50 amino acids which form short units of two α-helices, which stack together, forming an α-superhelix. The less-conserved N-terminal region of PIKKs, including mTOR, contain numerous HEAT repeats, which are fairly diverse in sequence and length and this region likely plays a key role in binding partner specificity[27]. A unique domain in mTOR, called the FKBP12-rapamycin-binding (FRB) domain, lies between the FAT and kinase domains. Rapamycin interacts with mTOR in a bimolecular fashion, first binding to FKBP12, forming a complex that then binds to the mTOR FRB domain. The mechanism of mTORC1 inhibition by rapamycin remains incompletely understood, but may be through allosteric reduction of

mTOR kinase activity and/or destabilization of protein-protein interactions within mTORC1[20,28].

The mTOR kinase forms the catalytic core of mTORC1 and mTORC2, each composed of several protein subunits (see Figure 1-2). mTOR depends on its binding partners for regulation by upstream factors as well as for recognition of downstream substrates. The unique protein composition of mTORC1 and mTORC2 is the basis for the functional differences between these complexes. mTORC1 and mTORC2 share a number of subunits. mTOR interacts with the scaffolding protein mammalian lethal with SEC13 8 (mLst8)[21,29]. DEP domain-containing mTOR-interacting protein (deptor) binds to the mTOR FAT domain and inhibits both mTORC1 and mTORC2[30]. Tti1 and Tel2 form a scaffolding structure that was reported to be important for mTORC1 and mTORC2 complex stability[31]. The mTOR complexes are distinguished primarily through two proteins: regulatory-associated protein of mTOR (raptor), specific to mTORC1, and rapamycin-insensitive companion of mTOR (rictor) specific to mTORC2[20,21,29]. Raptor and rictor association with mTOR is mutually exclusive, a property utilized to biochemically isolate mTORC1 and mTORC2. Raptor interacts with the N-terminal HEAT-repeat region of mTOR and is required for the recruitment of mTORC1 targets such as ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP) via the TOR-signaling (TOS) motif located on these substrates [32,33]. The proline-rich Akt substrate of 40 kDa (PRAS40) interacts with raptor and inhibits mTORC1 function[34–38]. Rictor is classified as a scaffold with no known catalytic function

and is essential for mTORC2 function, though it does play mTOR-independent roles in the cell[39]. Sin1 is a protein that interacts primarily with rictor and is both exclusive to mTORC2 and essential for mTORC2 function [40,41]. Genetic ablation of either rictor or Sin1 is sufficient to inhibit mTORC2 function through disruption of mTORC2 assembly and/or stability[40–42]. A third mTORC2-specific component has been described, protein observed with rictor 1 and 2 (protor1/2; also called proline rich protein 5-like [PRR5L]), but its role in mTORC2 function is unclear[37,43,44].

An x-ray crystal structure of mTOR currently does not exist, due to the large size of mTOR (~300 kDa), which make it difficult to purify and crystallize[28]. What little we know of its secondary structure derives from three sources: the structure of the FRB domain, homology modeling studies of the kinase domain, and a cryo-electron microscopic structure of mTORC1. The binding of rapamycin to the FRB domain is greatly enhanced by its interaction with FKBP12, and this ternary complex was crystallized in 1996[28,45–47]. More recently, it was demonstrated that FKBP12 and the FRB domain do not interact appreciably in the absence of rapamycin, and that FKBP12-rapamycin bound to the FRB domain with 2000-fold greater affinity than rapamycin alone, which agrees with previous theories[48,49]. The FKBP12-rapamycin-FRB structure has been utilized for rational design of rapamycin analogs which are used both clinically and as research tools[50]. The FRB domain is only about 100 amino acids in length, however, and thus its structure reveals very little about the overall secondary structure of mTOR. Homology modeling techniques have been used to generate

a structural model of the mTOR kinase domain based on the PI3K structure[45]. PI3K and mTOR kinase domains share ancestry (the PIKK kinase family was, after all, named for its homology to PI3K), however, sequence alignment alone is insufficient for making conclusions about structural similarities (PI3K and mTOR kinase domains share only 17% identity and 40% similarity)[45]. The crystal structures of PI3Kα and PI3Kγ have been solved, however, justifying the use of structural homology modeling[51–53].

Based on structural studies of other HEAT domain-containing proteins, mTOR is probably highly flexible[54] . Flexibility and size of mTOR (~280 kDa) contribute to the difficulty of purification and crystallization required for high-resolution structural analysis. In lieu of a crystal structure, a low-resolution structure of mTORC1 was obtained by cryoelectron microscopy, revealing interesting features of the complex[28]. First, the mTOR binding sites of raptor and mLst8 were determined to be N-terminal and C-terminal, respectively. mLst8 distinctly protrudes from mTOR, and the mLst8-mTOR interface is relatively small. Second, purified mTORC1 complexes were found to interact with each other with two-fold symmetry, supporting previous reports that mTORC1 dimerizes[55–58]. Third, FKBP12-rapamycin was found to disrupt the mTORC1 complex, as fewer identifiable mTORC1 structures were found after adding FKBP12 and rapamycin *in vitro*, thus clarifying the inhibitory mechanism of rapamycin[28]. Interestingly, this structure study may indirectly support the hypothesis that chronic rapamycin inhibits mTORC2 by interfering with mTORC2 assembly[59–61]. It is currently unclear whether mTORC2 dimerizes[39].

Rapamycin and its analogs are used clinically to prevent renal transplant rejection, restenosis (a complication of angioplasty), and to treat late-stage renal cell carcinoma [15,16,62]. The recent advent of ATP-competitive (catalytic site) mTOR inhibitors (e.g. Torin1, PP242, Ku-0063794, and WAY600) inhibit both mTORC1 and mTORC2, and have been critical in understanding rapamycin-resistant mTORC1 functions as well as mTORC2 functions, as there are currently no mTORC2-specific inhibitors[17].

Regulation of mTORC1

Growth factors. Growth factor-mediated PI3K signaling activates mTORC1 (see Figure 1-3)[7,63]. Insulin and insulin-like growth factor (IGF) initiate signaling at the cell surface through their cognate receptor tyrosine kinases (RTKs), insulin receptor (IR) and IGF receptor (IGFR). Activated receptors phosphorylate insulin receptor substrate (IRS), which recruits PI3K to the plasma membrane. PI3K, a lipid kinase, generates phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3), which recruits pleckstrin homology (PH) domain-containing proteins to the plasma membrane[64]. These include 3'-phosphoinositide-dependent protein kinase 1(PDK1) and Akt (also known as protein kinase B [PKB]). Activation of Akt is achieved through phosphorylation of its activation loop at T308 (*H. sapiens*) by PDK1[65] and phosphorylation of its hydrophobic motif (HM) at S473 (*H. sapiens*) by mTORC2[61]. Other AKT HM kinases have been proposed, including integrin-linked kinase (ILK), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase 2), DNA-dependent protein kinase (DNA-PK), and, more recently, IκB kinase ε (IKKε) and TANK-binding kinase 1 (TBK1) [66–69]. It is

currently believed that mTORC2 is the predominant Akt HM kinase during growth factor stimulation; the fact that other kinases can compensate for loss of mTORC2, however, demonstrates that the Akt HM kinase remains a controversial topic.

Akt phosphorylates many cellular targets, including tuberous sclerosis complex 2 (Tsc2) and PRAS40, which both negatively regulate mTORC1. Hereditary mutations causing loss of Tsc1 or Tsc2 leads to tuberous sclerosis complex, a hamartoma syndrome[70]. Tsc2 is a GTPase-activating protein (GAP) that inhibits the small GTPase Ras-homolog enriched in brain (Rheb) [71,72]. Akt-mediated Tsc2 phosphorylation may inhibit Tsc1/2 function by suppressing Tsc2 GAP activity, disrupting the Tsc1-Tsc2 interaction, altering Tsc2 sub-cellular localization, or targeting Tsc1 and/or Tsc2 for degradation. Hence, the exact mechanism of Tsc1/2 inhibition by Akt-mediated phosphorylation remains controversial[73,74]. Hypophosphorylated PRAS40 binds to raptor and suppresses mTORC1 signaling. Phosphorylation of PRAS40 by Akt induces its dissociation from mTORC1[34,35] . Thus, through Tsc2 and PRAS40, Akt activates mTORC1 by inhibiting negative regulators of mTORC1. Rheb putatively binds to the mTOR kinase domain to enhance substrate recruitment in a GTP-dependent manner[75,76]. Phospholipase D (PLD) is a serum-activated lipase, whose product, phosphatidic acid (PA), binds to mTORC1 and is important for its activation[77]. GTP-Rheb is also required for PLD1 signaling to mTORC1, representing another role for Rheb in activating mTORC1[78,79]. No guanine nucleotide exchange factor

(GEF) for Rheb has yet been identified and this missing detail could shed light on the mechanism of Rheb-mediated activation of mTORC1.

In addition to PI3K/Akt signaling, the Ras/ERK pathway also activates mTORC1[80]. Epidermal growth factor (EGF) and phorbol esters stimulate mTORC1 through several mechanisms involving the Ras/Raf/MEK/ERK cascade. Both ERK and its substrate RSK phosphorylate and inhibit Tsc2, thus activating mTORC1[81,82]. ERK and RSK also phosphorylate raptor in a manner that promotes mTORC1 signaling[83,84]. This two-pronged mechanism is reminiscent of the action of Akt, which targets both Tsc2 and mTORC1 (via PRAS40). mTORC1, therefore, is cross-activated by non-insulin/IGF growth factor pathways.

Nutrients: amino acids and energy. Amino acids are fundamental cellular nutrients that regulate mTORC1 function. Monitoring of amino acids is crucial in balancing metabolic demand with nutrient availability and sufficient amino acid levels are required for mTORC1 activation by growth factors in multicellular eukaryotes[63]. mTORC1 signaling is highly sensitive to branched-chain amino acids—leucine, valine, and isoleucine— and (to a lesser degree) glutamine. It is unclear how amino acids trigger a given signaling pathway, as the direct sensing mechanism is not well understood. Two recent studies suggest that leucyl-tRNA synthetase (LRS) acts as an amino acid sensor that leads to mTORC1 activation[85,86], though the role for tRNAs or tRNA-charging enzymes as amino acid signaling “receptors” remains controversial. Tight control of leucine import is a rate-limiting step in mTORC1 activation, conferred through cooperation

between the amino acid transporter SLC1A5, which imports glutamine, and the SLC7A5/SLC3A2 transporter, which couples glutamine export with leucine import[87]. Amino acid signaling to mTORC1 depends on the Class III PI3K Vps34, which is involved in membrane trafficking[88,89]. MAPK kinase kinase kinase 3 (MAP4K3) and RalA have both been implicated in amino acid signaling to mTORC1, though the mechanisms linking them to mTORC1 are unclear[90,91].

The Rag family GTPases (RagA, B, C, and D) have recently been studied extensively as activators of mTORC1 in response to amino acids. Rags associate with MP1, p14, and p18 (a complex called Ragulator) which localizes to endolysosomal membranes and recruits mTORC1 to these membranes[92,93]. Ragulator was recently found to have guanine nucleotide exchange factor (GEF) activity towards RagA and RagB, thus in addition to tethering Rags to the membrane, it also regulates their GTPase activity[94]. GTP-bound RagA or B heterodimerizes with GDP-bound RagC or D and binds to raptor upon amino acid stimulation, causing translocation of mTORC1 to endolysosomal membranes[92,93]. A recent study found that the vacuolar ATPase (v-ATPase), a lysosomal proton pump, interacts with Ragulator and is necessary for transmitting amino acid signaling to mTORC1 via the Rags, revealing that the amino acid signal may originate in the lysosome[95]. Leucyl-tRNA synthetase, however is a cytosolic enzyme, suggesting the signal is of cytosolic origin. Thus further investigation is needed to resolve this discrepancy[96].

Homeostasis is a corrective and adaptive mechanism, adjusting cellular activities to changing metabolic demand of the cell or external influences. The

onset of stress is often rapid and requires an effective triggering mechanism in order to protect cells from necrosis or programmed cell death. Stress-activated suppression of mTORC1 has been studied in great detail in recent years. Stresses that inhibit mTORC1 signaling include energy depletion, hypoxia, DNA damage, reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, hyperosmolarity, mechanical strain, injury, and inflammation[63,97–99]. We understand less about the regulation of mTORC2 in stress response pathways, though recent studies have suggested that ER stress, redox status, and hypoxia may regulate mTORC2 signaling[100–102].

Energy stress induced by glucose-withdrawal, glycolysis inhibitors, and inhibitors of mitochondrial energy production suppress mTORC1 signaling. Suppression of mTORC1 is mediated by AMPK in response to cellular ATP depletion (and the concomitant rise in AMP and ADP levels). AMPK suppresses mTORC1 signaling by two known mechanisms. First, AMPK phosphorylates Tsc2 on S1345, which activates the Tsc1/2 complex, an upstream negative regulator of mTORC1[103]. Second, AMPK phosphorylates raptor on S792, which conveys Tsc1/2-independent energy stress directly to mTORC1[104]. AMPK also promotes autophagy by phosphorylating and activating ULK1 in direct opposition of mTORC1, which phosphorylates and suppresses ULK1[105]. Autophagy is an important survival mechanism during cellular stress and the fact that AMPK activates this process by several pathways, including inhibition of mTORC1, underscores the importance of AMPK in metabolic rebalancing during energy stress[106]. The role of energy stress in regulating mTORC2 is poorly understood.

The study presented in Chapter 2 tests the hypothesis that energy stress signaling through AMPK, already known to inhibit mTORC1 signaling, actually promotes mTORC2 signaling and that this may be part of a survival mechanism to cope with acute stress conditions.

Regulation of mTORC2

While much is known about the upstream signals that regulate mTORC1, surprisingly little is known of the upstream regulation of mTORC2. *In vitro* kinase assays have demonstrated that mTORC2 phosphorylates Akt on S473 and mTORC2 kinase activity is enhanced by treatment of cells with insulin and inhibited by PI3K inhibitors, such as wortmannin [40,42,61,74]. Furthermore, the addition of PI-3,4,5-P_3 enhances mTORC2 activity *in vitro*[107]. The Fingar laboratory has also shown that insulin-PI3K signaling enhances mTORC2-associated mTOR S2481 autophosphorylation, which correlates to mTORC2 kinase activity in intact cells[108]. In addition to insulin-PI3K signaling, the Tsc1-Tsc2 complex has been shown to interact with and promote mTORC2 activity and signaling—an interesting observation, given that Tsc1 and Tsc2 are critical negative regulators of mTORC1[74].

Regulation of mTOR by phosphorylation

Reversible phosphorylation regulates many signaling molecules, both positively and negatively, and kinase-mediated regulation of downstream kinases enables signal amplification. Recent work from the Fingar laboratory and others has shown that phosphorylation of mTOR and its binding partners, raptor and rictor, regulates mTORC1 and mTORC2 function.

Raptor phosphorylation at S863 is mediated by mTORC1 itself, promotes mTORC1 kinase activity *in vitro*, and is required for phosphorylation of S859 and S855[109,110]. Raptor S8, S696, and S863 were also found to be phosphorylated by ERK in response to Ras signaling and upon mutation of all three sites to alanine, mTORC1 signaling was impaired[84]. Raptor is also phosphorylated by cdc2 during mitosis on S696 and T706, promoting mTORC1 signaling during this phase of the cell cycle[111,112]. Recently, c-Jun N-terminal kinase (JNK) was found to phosphorylate raptor on three sites (S696, T706, and S863) in response to osmotic stress and promoted mTORC1 signaling[113]. Another recent study reported a novel phosphorylation site on raptor (T908), which was found to be phosphorylated by intestinal cell kinase (ICK) and promoted mTORC1 signaling[114]. Together, these reports suggest that different kinases can utilize similar sites on raptor to activate the mTORC1 pathway. Raptor phosphorylation events also suppress mTORC1 signaling. AMPK inhibits mTORC1 signaling through raptor S792 phosphorylation in response to energy stress[104]. ULK1 was reported to phosphorylate raptor on multiple sites, including S855, S859, S863, and S792, though phosphorylation was strongest at S855 and S859[115].

Very little is known about the regulation of mTORC2 by phosphorylation of its component proteins. Rictor is highly phosphorylated in the presence of serum and most of these sites lie in the C-terminal half of the protein[116,117]. Rictor T1135 is phosphorylated by S6K1 in a rapamycin-sensitive manner. Two studies on rictor T1135 phosphorylation found that mutation of this site has no discernible phenotype[118,119], but two other studies reported that a rictor T1135A mutant

slightly increases Akt S473 phosphorylation[116,117]. Thus the role of this site in regulation of mTORC2 remains controversial. Glycogen synthase kinase 3 β (GSK3β) was found to phosphorylate rictor on S1235, inhibiting mTORC2 signaling to Akt during endoplasmic reticulum stress[100]. Sin1 is also phosphorylated in intact cells and phosphorylation is sensitive to chronic rapamycin treatment, which reduces its ability to bind to mTOR[42]. However, these sites on Sin1 have neither been characterized nor demonstrated to regulate mTORC2 function.

mTOR is highly phosphorylated *in vivo* and indeed undergoes autophosphorylation[120,121]. S2481 autophosphorylation and S2448 phosphorylation (which is mediated by S6K1) have been used extensively as markers of mTORC1 activation, and S2481 phosphorylation reflects on mTORC1 and mTORC2 catalytic activity in intact cells[108,122,123]. Despite their usefulness as analytic biomarkers, no known function has been attributed to these phosphorylation sites (see Figure 1-1).

The Fingar laboratory recently identified three novel mTOR phosphorylation sites—S1261, S2159, and T2164—all of which promote mTORC1 signaling (see Figure 1-1)[22,23]. mTOR S1261 is conserved among animals, but not in fungi or plants. S1261 phosphorylation in both mTORC1 and mTORC2 was found to be regulated by insulin in 3T3-L1 adipocytes, but not in HEK293 cells. mTORC1-associated mTOR S1261 phosphorylation is elevated in $Tsc1^{-/-}$ mouse embryonic fibroblasts (MEFs) compared to wild-type MEFs. Furthermore, mTOR S1261 phosphorylation within mTORC1 was inhibited in

$Tsc1^{-/-}$ MEFs upon knockdown of Rheb. Expression of an mTOR S1261 alanine (S1261A) mutant reduced mTORC1-associated S2481 autophosphorylation and reduced signaling towards the mTORC1 substrates S6K1 and 4EBP1. Importantly, a rapamycin-resistance version of this mutant failed to rescue rapamycin-induced reduction of cell size as well as rapamycin-resistant wild-type mTOR, thus suggesting S1261 phosphorylation promotes cell growth. Expression of an mTOR S1261D mutant did not increase S6K1 phosphorylation, thus this mutant may not behave phosphomimetically[22]. As described in Chapter 2, mTOR S1261 phosphorylation may also promote mTORC2 function, as AMPK phosphorylates S1261, increases mTORC2 autophosphorylation, and promotes mTORC2 downstream signaling.

mTOR S2159 and T2164 lie at the extreme N-terminus of the conserved mTOR kinase domain[23,124]. Sequence alignments reveal S/T conservation of S2164 in yeast TOR1 and TOR2 as well as mammalian PI3K (no such conservation is evident at S2159)[23,45]. According to the structure homology model proposed by Sturgill *et al.*, mTOR S2165 lies between two beta sheets, kβ3/kβ4, and is in close proximity to ATP in the kinase active site[124]. The putative phosphorylation sites S2159 and T2164 are both located on kβ3 and thus phosphorylation of these residues could directly affect kinase activity[124]. The Fingar laboratory found that S2159 and T2164 phosphorylation weakens the interaction of raptor with mTOR and PRAS40, promoting mTORC1 autophosphorylation and signaling to S6K1 and 4EBP1. Utilizing mTOR S2159A/S2164A ("mTOR-AA") phosphodefective and mTOR S2159D/T2164E

("mTOR-DE") phosphomimetic mutants, the Fingar laboratory found that expression of mTOR-AA reduces mTORC1 signaling to S6K1 and 4EBP, while expression of mTOR-DE increases mTORC1 signaling. S2159/T2164 phosphorylation also promoted cell growth and cell cycle progression[23].

mTORC1 substrates and functions

S6 kinase 1. Perhaps the best-studied mTORC1 target is S6K1, which promotes anabolic processes including protein synthesis, lipid synthesis, and cell growth[125,126]. Global knockout of S6K in *D. melanogaster* and loss of S6K1 in *M. musculus* caused developmental delay characterized by smaller cell and body size[127–129]. The ribosomal protein S6, a subunit of the 40S ribosome, is the best-studied target of S6K1. Its phosphorylation is important for maintenance of cell size, though, strangely, cells derived from phosphodefective knockin mice ($S6^{P-/-}$) proliferate more rapidly than their wild-type counterparts, suggesting that S6 phosphorylation may be important for balancing growth and proliferation in the presence of mitogens[130]. S6K1 has been reported to positively regulate protein translation initiation through phosphorylation of eukaryotic initiation factor 4B (eIF4B)[131,132] and programmed cell death protein 4 (PDCD4)[133]. S6K1 also promotes lipid synthesis by activating sterol regulatory element binding proteins 1 and 2 (SREBP1/2), which are transcription factors[134,135]. S6K1 participates in a negative feedback loop that represses insulin-PI3K signaling through phosphorylation of IRS1 on S636/639, which leads to subsequent degradation of IRS1 by the proteasome. This negative feedback loop is important in maintaining cellular sensitively to insulin/IGF, however pathological mTORC1 hyperactivation

leads to insulin resistance (at least in part) through this mechanism. S6K1 also targets other cellular processes including transcription, mRNA processing, protein folding, and cell survival[125].

mTORC1 directly phosphorylates S6K1 on its hydrophobic motif site, T389, which is critical for S6K1 activity[136,137]. S6K1 is a member of the AGC kinase family (named after three of its members: protein kinase A [PKA], PKG, and PKC). A common theme among AGC kinases is that phosphorylation of the HM, the activation loop, and in some cases the turn motif (TM) coordinate to activate the kinase. S6K1 is activated in a stepwise fashion by phosphorylation on its TM, HM, activation loop, and C-terminal autoinhibitory domain[125]. The activation loop site, T229, is phosphorylated by PDK1, which is also the primary activation loop kinase for other AGC kinases and often regarded as the "master regulator" of AGC kinases[138,139]. mTORC1 signaling to S6K1 depends on the TOS motif, which is a conserved N-terminal sequence (FDIDL) in S6K1[140]. The TOS motif is critical for substrate docking to mTORC1 facilitated by raptor and is also found in other mTORC1 substrates, including 4EBP[32,33].

4EBP. 4EBP is phosphorylated by mTORC1 on T37 and T46[136,141]. In the absence of mTORC1 signaling, 4EBP binds to eIF4E, a translation initiation factor that binds directly to the 7-methylguanosine (m^7-GTP) cap structure located at the 5' end of mRNA transcripts. 4EBP-eIF4E binding prevents assembly of the pre-initiation complex, blocking translation at this step. mTORC1-dependent phosphorylation of 4EBP induces its dissociation from eIF4E, leading to pre-initiation complex formation and ultimately recruitment of

the 40S ribosome. Loss of 4EBP1/2 in mouse cells increases cell proliferation and cell cycle progression, but not cell growth[142]. Because S6K1-deficiency impairs cell growth, but not proliferation, it is believed that in mammals, mTORC1-mediated cell growth and cell proliferation are separable functions. Indeed, the Fingar laboratory found that mTOR S2159/T2164 phosphorylation differentially regulates a 4EBP/cell cycle progression axis and a S6K1/ cell growth axis, supporting this hypothesis[23].

ULK and other mTORC1 targets. In mammals, mTORC1 suppresses autophagy, a process of recycling old and damaged proteins and organelles that provides nutrients and energy to the cell[143,144]. mTORC1 inhibits autophagy through a complex containing Unc-51-like kinase 1 or 2 (ULK1/2), hAtg13, and focal adhesion kinase family-interacting protein of 200 kDa (FIP200), which is analogous to the complex found in *S. cerevisiae* composed of Atg1, Atg13, and Atg17[145,146]. mTORC1 phosphorylates both ULK1/2 and hAtg13, preventing phosphorylation of FIP200 (the Atg17 homolog) by ULK1/2, which would otherwise initiate autophagy. Two recent, independent screens for mTOR substrates revealed a novel mTORC1 target, growth factor receptor-bound protein 10 (Grb10). Grb10 is phosphorylated by mTORC1 on S501 and S503 and mediates the inhibition of IRS1 (along with S6K1), thus contributing to the mTORC1 negative feedback loop[147,148]. The remaining mTOR substrates identified in these screens require further study, as our current set of *bona fide* mTORC1 and mTORC2 substrates are likely insufficient to mediate the numerous cellular functions controlled by these mTOR complexes.

mTORC2 substrates and functions

Cellular functions attributed to mTORC2 remain poorly understood, but we have learned that mTORC2 plays a role in survival and growth signaling through its known cellular targets, Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and conventional protein kinase C (PKC)[39]. Like S6K1, these three proteins are AGC kinase family members. PDK1 is not only critical for mTORC1 signaling but also for mTORC2 signaling, for it phosphorylates the activation loops of these kinases: T308 in Akt, T256 in SGK1, and T500 in PKC [65,149–151]. PKC1, a PKC homolog in *S. cerevisiae*, was the first TORC2 effector identified[19]. At that time, PKCα was hypothesized to be an mTORC2 substrate in *H. sapiens*[21], but the first mTORC2 target to be demonstrated was Akt, phosphorylated on its hydrophobic motif at S473[61,152]. mTORC2 is also important for Akt turn motif (TM; T450) phosphorylation. While mTORC2 phosphorylates Akt T450 *in vitro*, it is unclear whether mTORC2 directly phosphorylates this site in intact cells[153,154]. Akt TM phosphorylation promotes protein stability and is believed to occur co-translationally[153,154]. PKCα HM (S657) and TM (T638) phosphorylation are also mTORC2-regulated. Although direct PKCα TM phosphorylation was not reported *in vitro,* PKCα instability was readily evident in mTORC2-deficient cells (mLst8$^{-/-}$, rictor$^{-/-}$, and Sin1$^{-/-}$ MEFs)[21,153–155]. Presumably, stability is mediated through TM site phosphorylation, as the TM phosphodefective mutant (T631A/T638A) is highly unstable, associates with insoluble fractions, and co-localizes with the aggresome[153]. SGK1 represents the

third known mTORC2 substrate. This protein is phosphorylated on its HM (S422) by mTORC2[156].

mTORC2 functions include regulation of the actin cytoskeleton. A pathway involving mTORC2 and PKC1 regulates yeast actin polarization, identified by lack of actin patches accumulating in the bud[19]. mTORC2 in mammals was also identified as a factor in cytoskeletal function, which was important for cell spreading in cultured cells[21,29]. Rictor knockdown inhibits neutrophil chemotaxis through loss of actin polarization in a PKC-dependent manner[157]. The physiological role of mTORC2-mediated cytoskeletal regulation remains poorly understood [39].

Akt functions as an oncogenic kinase that promotes cell survival and antagonizes apoptosis; nearly 100 Akt targets have been identified[158,159]. Akt phosphorylates several pro-apoptotic proteins, including Bcl2-family proteins, such as Bad, and forkhead box O (FoxO) transcription factors. Akt phosphorylation inhibits these proteins by creating a docking site for 14-3-3 proteins, leading to their sequestration and proteasomal degradation. Akt also promotes glucose transporter type 4 (GLUT4)-dependent glucose uptake through phosphorylation and inhibition of Akt substrate of 160 kDa (AS160) and TBC1D1, an AS160 paralog [160,161]. Despite the apparent upstream location of mTORC2 relative to mTORC1, loss of mTORC2 components do not significantly impair activation of mTORC1 and Akt T308 phosphorylation correlates more with Akt-mediated Tsc2 and PRAS40 phosphorylation than does Akt S473

phosphorylation[162]. The differential contribution of T308 and S473 phosphorylation to Akt activities remains controversial.

The significance of SGK signaling is not well understood as knockout of SGK1, SGK3, or both does not lead to any severe phenotype[163–165]. SGK1 regulates ion channels[166], phosphorylation of proteins involved in transcriptional regulation and antiapoptotic functions[167,168]. SGK1 shares a phosphorylation consensus motif with Akt and indeed they share some substrates, including FoxO transcription factors[167,169]. SGK1 phosphorylates N-myc downregulated genes 1 and 2 (NDRG1/2), which are currently the only known exclusive SGK1 substrates. The cellular function of NDRG1/2 remains unclear[170].

1-2. The AMP-activated protein kinase

Energy usage in tissues and individual cells changes continually as they alter metabolic activity to suit their needs and adapt to stimuli. The majority of available energy in eukaryotic cells is stored in the form of adenosine triphosphosphate (ATP), and the relative amounts of ATP and its hydrolyzed forms, adenosine di- and mono-phosphate (ADP and AMP), are closely monitored and controlled. Tight regulation of energy usage shields cells from potentially harmful metabolic strain brought on by energy starvation (e.g. ischemia) or high demand (e.g. muscle contraction). The AMP-activated protein kinase (AMPK) is central to this sensory/response program, directly sensing cellular energy levels through adenine nucleotide binding. When energy is depleted, AMPK phosphorylates metabolic enzymes, signal transducers, and transcription factors to restore energy balance within the cell[171,172].

AMPK was described as early as 1973 as a cellular activity that senses energy status and maintains ATP levels through regulation of fatty acid synthesis enzymes[173–175]. Following its molecular characterization in the early 1990s, AMPK was quickly billed as a master regulator of energy status through carbohydrate and lipid metabolism. The discoveries that AMPK activity correlates with exercise-induced glucose uptake and that pharmacological AMPK activation was sufficient to induce glucose uptake lead to the hypothesis that impaired AMPK signaling may contribute to type 2 diabetes[176–178]. Soon it was discovered that the widely prescribed anti-diabetic, metformin, activates AMPK, giving teeth to this theory[179].

Structure and regulation of AMPK

For a graphical representation of AMPK and its major upstream and downstream components, see Figure 1-4. AMPK is a heterotrimeric serine/threonine protein kinase, consisting of one catalytic α subunit and two regulatory subunits (β and γ) and is highly conserved across *Eukaryota*[172]. In the brewer's yeast *S. cerevisiae*, the sucrose non-fermenting 1 (SNF1) was first identified via a mutant yeast strain incapable of adapting to glucose deprivation [180]. SNF1 is composed of one α-subunit (Snf1), one γ-subunit (Snf4), and one of three possible β-type subunits (Gal83, Sip1, and Sip2). The SNF1 holoenzyme, therefore, has three distinct isoforms. Mammalian AMPK genes encode two α-subunits (AMPKα1/2), two β-subunits (AMPKβ1/2), and three γ-subunits (AMPKγ1/2/3), of which any $\alpha/\beta/\gamma$ combination is theoretically possible, implying the existence of 12 distinct AMPK heterotrimers[171]. Expression of the mammalian

AMPK component genes across tissues may shed light on isoform-specific functions, though at this time little is known on this topic[171,181]. AMPKα1, β1, and γ1 are more ubiquitously expressed than AMPKα2, β2, and γ2, which are more highly expressed in skeletal muscle, heart, and brain. AMPKγ3 has the most specific expression profile with preferential expression in fast-twitch muscle fibers (white and red muscle), preferring white muscle, which are more glycolytic than red muscle fibers[182]. Overall, expression studies suggest that AMPKα1/β1/γ1 represents the most common AMPK isoform and probably best represents AMPK's most generic "housekeeping" role in cells.

AMPK is activated by processes that consume ATP (e.g. muscle contraction) or prevent ATP production, such as glucose and oxygen starvation and compounds that inhibit glycolysis or mitochondrial metabolism. AMPK directly senses rises in AMP and ADP levels through adenine nucleotide binding on its γ-subunit[183]. Exchange of ATP with AMP or ADP at the nucleotide-binding sites directly controls access to the activation loop site (T172 in mammalian AMPKα) by upstream kinases and phosphatases. AMP and ADP binding promotes phosphorylation of T172 and ATP-binding promotes dephosphorylation, though nucleotide binding is not strictly required for T172 phosphorylation[184]. Allosteric nucleotide-binding is competitive and achieved via four conserved cystathione-β-synthase (CBS) domains located on the γ-subunit[185,186]. AMP has a binding affinity four-fold higher than ATP, and binding is positively cooperative between at least two of the CBS domains. These facts provide the molecular basis for the exquisite sensitivity of AMPK to changes in

cellular AMP levels. Recent work demonstrated that ADP also competes with ATP for binding and promotes AMPKα T172 phosphorylation[187], suggesting that AMPK senses relative amounts of all three adenine nucleotides. Interestingly, these recent studies provide direct evidence for an older concept known as the "adenylate charge hypothesis," in which ATP, ADP, and AMP are all implicit charge carriers that couple with metabolic pathways[188–190]. This system implies the ability to sense the levels of both ATP hydrolysis products (AMP and ADP) in order to appropriately regulate recharging (ATP production) and AMPK serves this purpose[187].

Primary control of AMPK activity lies in activation loop phosphorylation of the α-subunit. In *S. cerevisiae*, PAK1, ELM1, and TOS3 are thought to phosphorylate this site[191]; in mammals the AMPK T172 kinases are liver kinase B1 (LKB1) and calcium/calmodulin-activated kinase kinase β (CaMKKβ)[192–197]. LKB1 is a "master kinase" for numerous AMPK-related kinases in addition to AMPK itself, phosphorylating the conserved activation loop sites on these kinases[192,198]. AMPK can be stimulated by changes in calcium flux, demonstrated by using A23187, a calcium ionophore, and STO-609, a drug that inhibits CaMKKβ but not LKB1[195,196]. LKB1 is likely the predominant AMPKα T172 kinase, at least in peripheral tissues, however physiological roles for CaMKKβ in AMPK regulation have recently been described in adipocytes[199,200].

AMPK is regulated by three other mechanisms: autoregulation via the β-subunit, glycogen binding, and acetylation. AMPKβ is myristoylated on its N-terminal glycine[201]. When AMPK is inactive (sufficient energy levels), this myristic

acid restricts AMPKα T172 phosphorylation. During energy stress, however, this same moiety is required for maximum AMPK activation, thus AMPKβ may function as an AMPK switch[202]. The β-subunit also includes a putative glycogen-binding domain (called the carbohydrate-binding module [CBM]). Glycogen-binding to the CBM allosterically inhibits AMPK activity *in vitro* though the physiological significance remains unclear[203]. Acetylation of AMPKα was recently reported (residues K31,K33, and K71 on *H. sapiens* AMPKα) and is regulated by HDAC1 (a deacetylase) and p300 (an acetyltransferase). Deacetylation correlated with increased T172 phosphorylation and hence the role of acetylation is inhibitory[204].

A partial x-ray crystal structure of the AMPK αβγ heterotrimer has been solved[205–207]. An interesting fact drawn from these studies is that the overall secondary structure of the complex is relatively unchanged between ATP and AMP binding, suggesting that allosteric conformational alterations are conservative. The four nucleotide-binding sites (CBS domains) exhibit variable occupancy: CBS4 is always occupied by AMP, CBS2 is almost always empty, and CBS1 and 3 are the most dynamically occupied[183,205]. CBS1 binding by AMP has the greatest allosteric effect, while AMP or ADP binding of CBS3 promotes T172 phosphorylation[207]. The linker region between the N-terminal domain and the C-terminal domain of the α-subunit is far away from CBS1 (the allosteric site) but is adjacent to CBS3 (the T172 phosphorylation regulating site), which may explain why CBS3 regulates tT172 phosphorylation[207]. AMPKα T172 is sandwiched between the C-terminal domain and the N-terminal lobe of the

kinase domain, dynamically poised for a pro-kinase or pro-phosphatase conformational change[207].

A number of compounds have been shown to activate AMPK *in vivo*: antidiabetics (e.g. metformin and rosiglitazone), several plant-derived compounds (e.g. resveratrol, capsaicin, and berberine), the AMP analog AICAR, and the unique, small molecule activator A769662[171]. Biguanine anti-diabetics (metformin and phenformin) accumulate in mitochondria and inhibit Complex I of the electron transport chain, impairing ATP production and thus activating AMPK [208,209]. Thiazolidinediones (TZDs; i.e. rosiglitazone) stimulate AMPK, coincident with rises in cellular AMP levels[210–214]. AICAR, a purine biosynthetic metabolite, is converted in the cell to the AMP analog ZMP and is the most commonly used AMPK activator[215]. ZMP binds directly to and activates AMPK, but also modulates other AMP-sensitive proteins, such as fructose-1,6-bisphosphatase, and induces a state of energy stress independent of the AMPK pathway[215,216]. A769662 is a direct activator of AMPK whose mechanism of action, which is incompletely understood, may involve binding to the β-subunit[217–221], though AMPK-independent effects of this drug have been reported[220,222]. When administered to a mouse model for obesity, A769662 caused weight loss, enhanced glucose and triglyceride clearance, reduced expression of gluconeogenic enzymes, and reduced expression of fatty acid synthetase, all hallmarks of improved metabolic physiology[217]. It is uncertain whether this drug will provide clinical benefit (early reports suggest poor oral availability), however there is no doubt that it is an important research tool[217]. The best characterized

inhibitor of AMPK is a pyrrazolopyrimidine called Compound C, which reversibly competes with ATP in the catalytic domain, but may not inhibit AMPK in all conditions and off-target effects of this drug have been reported[223].

AMPK substrates and functions

AMPK was first described as a protein kinase activity responsible for phosphorylating and inhibiting two metabolic enzymes: acetyl-CoA carboxylase 1 (Acc1)[173], a critical (rate-limiting) fatty acid synthetic enzyme, and HMG-CoA reductase (HMGR)[174], a rate-limiting cholesterol synthetic enzyme. AMPK phosphorylates Acc1 on S79 and HMGR on S871[224,225]. Glycogen synthase was also identified early on as an AMPK substrate, phosphorylated on S7 to inhibit glycogen production[226]. Since its molecular characterization in the early 1990s, a number of other direct substrates have been reported, the majority of which are inhibited by AMPK because they promote ATP-consuming processes such as protein, lipid, and carbohydrate biosynthesis. Other substrates are activated by AMPK because they promote ATP-generating processes including glucose uptake/metabolism, fatty acid oxidation, and autophagy[171].

Glucose transport. AMPK promotes glucose uptake in peripheral tissues [227]. AMPK phosphorylates AS160, on S588, S341, and T642 and TBC1D1 on S237. AS160 and TBC1D1, which are Rab GAPs, inhibit vesicular translocation of glucose transporters to the plasma membrane. AMPK-mediated phosphorylation of AS160 and TBC1D1 inhibit their function through enhanced 14-3-3 binding and thus AMPK promotes glucose transport through this

mechanism[227–229]. Additionally, AMPK promotes GLUT4 expression through phosphorylation of histone deacetylase 5 (HDAC5)[227,230].

Gluconeogenesis. AMPK regulates the expression of a number of proteins that regulate hepatic glucose generation and mitochondrial biogenesis (an important organelle in this process) by phosphorylation and inactivation of at least three mammalian transcription factors/coactivators: PGC-1α (T177 and S538)[231], HNF4α (S304)[232], CREB-regulated transcription coactivator 2 (CRTC2)[233], and p300 (S89)[234]. Through these transcription factors, AMPK suppresses the expression of gluconeogenic enzymes including L-type pyruvate kinase (L-PK), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G-6-Pase)[235–237].

Protein synthesis. Protein synthesis is one of the most energy-costly processes in a cell. In order for AMPK to effectively regulate energy homeostasis during low-energy states, it regulates protein synthesis primary through two mechanisms: suppression of translation elongation via eukaryotic elongation factor 2 kinase (eEF2K) and suppression of translation initiation via mTORC1. AMPK phosphorylates and activates eEF2K (S398), leading to phosphorylation and inhibition of eEF2. eEF2 is responsible for translocation of mRNA-engaged ribosomes to the next codon during protein elongation, and phosphorylation on T56 by eEF2K causes its dissociation from ribosomes[238–241]. AMPK inhibits mTORC1 signaling in two distinct ways. First, It phosphorylates and activates Tsc2 (S1345), which promotes energy stress-induced suppression of mTORC1[103], a process that may partially depend on GSK3, which also

phosphorylates Tsc2[242]. AMPK also inhibits mTORC1 signaling independent of Tsc1/2 through direct phosphorylation of the complex itself (raptor S792)[104]. Attenuation of protein synthesis is conferred through subsequent dephosphorylation of mTORC1 effectors, including S6K1 and 4EBP.

Autophagy. AMPK promotes autophagy, a process of recycling cytosolic contents and organelles. Autophagy is a catabolic process that correlates highly with cell survival during energy stress and nutrient starvation[106]. Recently AMPK was found to phosphorylate and activate ULK1, the mammalian homolog of yeast Atg1, and activate autophagy. Both AMPK and ULK1 were necessary for maintaining normal autophagic flux in mammalian cells and *C. elegans* hypodermal seam cells and AMPK was found to interact with and phosphorylate ULK1[243,244]. S317 and S777 on ULK1 (*H. sapiens*) were minimally required for AMPK to promote ULK1 activity and autophagy. As mentioned previously, mTORC1 inhibits autophagy through phosphorylation and inactivation of ULK1 and therefore AMPK promotes autophagy in part through its suppression of mTORC1[145,146]. Furthermore, phosphorylation of ULK1 on S757 by activated mTORC1 disrupts the AMPK-ULK1 interaction and reduces phosphorylation of ULK1 on AMPK target sites, illustrating the opposing roles of mTORC1 and AMPK in regulating autophagy[244].

Cell proliferation, survival, and longevity. While AMPK has been implicated in the regulation of apoptosis and tissue growth, few direct mechanisms have been described. AMPK inhibits lipogenic enzymes, such as Acc1 and fatty acid synthase, which are critical for cancer cell proliferation[245]. AMPK also

phosphorylates p53 on S15, stabilizing this tumor suppressor, and phosphorylates the cyclin dependent kinase inhibitor p27[246,247], though the physiological significance of these phosphorylation events is still debated[183]. AMPK phosphorylates several novels sites on mammalian FoxO3 and DAF-16/FoxO in *C. elegans*, promoting expression of a subset of its target genes[248,249]. Recent evidence suggests, however, that AMPK signaling also inhibits FoxO3, through activation of Akt following expression of sestrins[250]. Another recent study found that AMPK promotes cell survival by maintain NADPH levels during energy stress[251]. Thus AMPK may both prevent cell proliferation and promote cell survival. Genetic studies in invertebrate models have provided insight into the role of AMPK in regulating lifespan. Knockout of the AMPKα homolog in *D. melanogaster* is lethal due do developmental defects, akin to AMPKα1/2 double knockout in mice[252,253]. Overexpression of Lkb1 (the AMPK upstream kinase) promoted *D. melanogaster* longevity[254]. In *C. elegans*, overexpression of AAK-2 (an AMPKα homolog) in low energy conditions increased longevity[255].

1-3. mTOR and AMPK in health and disease

A large body of research indicates that mTOR (primarily in the form of mTORC1) plays important roles in pathological conditions, including metabolic disease, age-related pathologies, cancer and tumor syndromes, neurological disorders, and cardiovascular disease[256–258]. Dysregulation of mTOR has broad effects on cell growth and proliferation, metabolism, and both cellular and whole-

body homeostasis. It has recently emerged that the less-understood mTORC2 is important for regulating glucose homeostasis, insulin signaling, and may promote tumorigenesis, but our understanding of the mechanistic aspects of mTORC2 in the context of metabolic disease and cancer remains in its infancy. AMPK activity is critical for both cellular and whole-body maintenance of energy homeostasis. Not only does AMPK play a role in metabolic pathologies (where its activity is beneficial), but it may also be important in neurodegenerative diseases (such as Huntington's disease) and cancer[183].

Cancer

mTOR. Some hereditary cancer syndromes are caused by mutations in upstream negative regulators of mTORC1, including tuberous sclerosis complex (Tsc1/2 mutated), Peutz-Jegher syndrome (LKB1 mutated) and Cowden syndrome (PTEN mutated)[259]. Though these tumor syndromes are relatively rare, mutations of the above tumor suppressors are found in many sporadic cancers and broadly deregulate mTORC1. Additionally, loss of p53 enhances mTORC1 signaling[260]. mTORC1 contributes to tumorigenesis by promoting cell proliferation, *de novo* lipid biogenesis, and defective autophagy[8]. The mTORC2 substrate Akt has been studied extensively as a protooncogene, however the role of mTORC2 itself in cancer development has not been studied extensively. The mTORC2 component rictor is overexpressed in gliomas and is required for tumor formation in PTEN-deficient prostate cancer, suggesting that mTORC2 is critical for Akt signaling in cancer[9,261]. Rapamycin and its analogs have limited efficacy in treating cancer, however ATP-competitive mTOR inhibitors as well as

dual mTOR/PI3K inhibitors (e.g. PI-103 and NVP-BEZ235) are promising alternatives and some have entered clinical trials[17,262,263].

AMPK. The AMPK kinase LKB1 is a tumor suppressor that is mutated in Peutz-Jeghers syndrome, an autosomal dominant heritable disorder noted for development of hamartomas in the gastrointestinal (GI) tract and predisposing patients to G!-related cancer as they age. Somatic LKB1 inactivation has been reported in lung and endometrial cancers[264–267]. AMPK mediates at least some of LKB1-dependent tumor suppressor functions, as metformin reduces cancer risk in diabetic patients more than other therapies, suggesting the intriguing possibility of treating two diseases via one target[268]. Fatty acid synthesis plays a significant role in cancer pathogenesis and AMPK inhibits lipogenic enzymes such as fatty acid synthase and Acc1[245]. The breast cancer type 1 susceptibility protein (BRCA1), which is associated with breast and ovarian cancers, binds to Acc1 and prevents its dephosphorylation, thus synergizing with AMPK[269,270]. Recent evidence suggests that AMPK promotes cell survival in both normal and tumor cells, thus whether AMPK opposes or promotes cancer remains controversial[183,250,251].

Type 2 diabetes

The metabolic syndrome is essentially a cluster of clinical disorders, including insulin resistance, impaired glucose regulation, obesity, hypertension, and dyslipidemia that predispose patients to develop type 2 diabetes[271,272]. In addition to environment factors, such as high-calorie/high-fat diets and sedentary lifestyle, poorly understood genetic components also drive the emergence of type

2 diabetes[6]. Untreated type 2 diabetes leads to cardiovascular and liver diseases as well as blindness and peripheral neuropathy[4,256].

mTORC1. mTORC1 is a critical component of normal insulin signaling, as its loss can contribute to hypoinsulinemia, glucose intolerance, and reduced pancreatic β cell size[128,256]. Chronically activated mTORC1, on the other hand, causes insulin resistance through the S6K1-mediated negative feedback loop, which suppresses insulin signaling[273–275]. Furthermore, lipid overproduction contributes to type 2 diabetes and mTORC1 promotes lipid synthesis in adipose, liver, and muscle tissues through the peroxisome proliferator-activated receptor γ (PPARγ) and SREBP transcription factors[134,245,276]. In the liver, insulin resistance promotes hyperglycemia through increased gluconeogenesis[277]. The mechanism by which chronic mTORC1 signaling is maintained during insulin resistance remains unclear. A recent study profiling blood metabolites in obese and lean human subjects, discovered that obese subjects carried significantly higher levels of branched chain amino acids (BCAAs)[278]. mTORC1 signaling depends on amino acids (particularly BCAAs) and thus overnutrition may support chronic mTORC1 signaling. Insulin resistance may also be the product of chronic inflammation, as inflammatory cytokines, such as tumor necrosis factor α (TNFα), activate mTORC1 independent of insulin signaling [279].

mTORC2. The role of mTORC2 in glucose homeostasis has largely been determined through tissue-specific rictor knockout studies. Deletion of rictor in muscle caused mild glucose intolerance due to defective glucose uptake[280]. In pancreatic β cells, mTORC2 signaling is important for β cell proliferation and

survival and β cell-specific rictor loss leads to hyperglycemia, glucose intolerance, and insulin secretion defects[281]. Additionally, chronic rapamycin treatment (which interferes with mTORC2 assembly) and liver-specific rictor knockout caused insulin resistance characterized by deregulated hepatic gluconeogenesis[10,60]. mTORC2 ostensibly promotes glucose metabolism through Akt, which suppresses gluconeogenesis in the liver through FoxO phosphorylation[5,282]. Sirtuin 1 (Sirt1) has also been implicated in regulation of glucose metabolism and insulin sensitivity[283]. Recently, Sirt1 was found to promote the expression of rictor and inhibit gluconeogenesis through mTORC2/Akt[284]. Loss of Sirt1 was found to lead to insulin resistance[284].

AMPK. AMPK activity counteracts metabolic disorders by increasing insulin sensitivity and glucose uptake/utilization while reducing gluconeogenesis and fatty acid synthesis. Genetic models for obesity exhibit reduced AMPK activity in peripheral tissues. AMPK signaling defects are most apparent in advanced metabolic conditions, such as insulin resistance and frank type 2 diabetes[171]. AMPK responds to muscle contraction/exercise and increases glucose clearance in peripheral ttissues[285], two physiological processes critical for prevention and treatment of type 2 diabetes. AMPK activity prevents hyperglycemia by improving glucose uptake but also through inhibition of gluconeogenesis in the liver. Dyslipidemia is also a hallmark of type 2 diabetes and AMPK activity prevents this condition by inhibiting lipogenic enzymes and by promoting lipid oxidation, ultimately reducing triglyceride storage[12].

The AMPKα2 global knockout mouse has a mild insulin-resistance phenotype, whereas the AMPKα1 global knockout exhibits little to no metabolic phenotype[286]. AMPKα2 loss results in compensatory increase in protein levels and overall activity of AMPKα1 in skeletal muscle during exercise, which may explain why the metabolic phenotype in this animal is mild[287]. Germ line AMPKα1/2 double-knockout (DKO) is embryonic lethal (e10.5), precluding its physiological study. Liver-specific AMPKα1/2 DKO mice were found to have reduced mitochondrial biosynthesis and overall lower hepatic ATP levels[288]. AMPKα2 knockout mice on a high-fat diet exhibit increased lipid storage/adiposity and body weight compared to control mice[289]. Similarly, loss of AMPKα1 exacerbates high fat diet-induced obesity, insulin resistance, and inflammation[290].

AMPK plays a special role in regulating food intake from within the brain. AMPK is regulated hormonally; for example, leptin and adiponectin, secreted from adipose tissue, promote AMPK activity in muscle and liver tissue[291–295]. AMPK activation in the hypothalamus correlates with increased feeding behavior. The gut hormone ghrelin activates AMPK in the hypothalamus and both ghrelin-mediated and pharmacological AMPK activation induced feeding and weight gain[296,297]. Contrary to its effect in liver and muscle, leptin *inhibits* AMPK signaling in the hypothalamus, demonstrating that AMPK regulation by hormones is tissue- and organ-specific[296,297]. Furthermore, the anti-diabetic drug metformin, which activates AMPK in other tissues, inhibits AMPK in the hypothalamus[298]. Further work is required to understand the molecular basis for this hormone-

derived control of AMPK and how the same hormone can have opposite effects in different tissues.

1-4. Regulation of Akt phosphorylation by AMPK: is mTORC2 the missing link?

An important aspect of AMPK signaling in the context of glucose homeostasis is that it enhances insulin sensitivity. While the underlying mechanism is not fully understood, a logical assumption is that AMPK converges at some point upon the insulin signaling pathway and activates it. While mTORC2 is similarly important for insulin sensitivity and glucose homeostasis, it has been unclear whether these two signal transducers are dependent on one another, however studies have indicated that AMPK-activating proteins and drugs act upstream of Akt and promote its phosphorylation and downstream function. AMPK activators, such as AICAR and metformin, promote Akt S473 phosphorylation[299–301]. Signaling by the AMPK kinase LKB1 promotes Akt S473 phosphorylation and anti-apoptotic functions mediated in part by Akt[300,302,303]. Recently, expression of sestrin2, which promotes AMPK signaling, was found to promote Akt S473 phosphorylation and Akt-related cell survival in an AMPK-dependent fashion[250]. Shaw *et al.* found that energy stress induced by AICAR caused apoptosis in LKB1$^{-/-}$ cells, relative to wild-type cells, and noted (but did not explain) an LKB1-dependent increase in Akt S473 phosphorylation following AICAR treatment [300]. Lee *et al.* proposed that AMPK inhibits mTORC1 signaling, enhancing Akt phosphorylation through relief of the mTORC1 negative feedback loop, though they did not prove this point[250]. Together these studies indicate that AMPK converges on the insulin pathway upstream of Akt. The signaling

intermediates have not been identified, however. mTORC2, an Akt S473 kinase, represents a previously-untested link between AMPK and Akt.

The study presented in Chapter 2 demonstrated that AMPK phosphorylates mTOR on S1261. This was a surprising result, given that the Fingar laboratory previously showed that phosphorylation of this site promotes mTORC1 signaling yet it is widely accepted that mTORC1 signaling is inhibited by AMPK. Though this paradox has not yet been resolved, it prompted me to test the idea that AMPK regulates mTORC2 to promote signaling to Akt. Such a mechanism would reveal novel regulation of the mTORC2-Akt axis by a growth factor-independent pathway. This work not only sheds light on the poorly understood regulation of mTORC2 but defines it as an effector of AMPK, the significance of which is a better understanding of how AMPK potentiates insulin signaling during cellular stress and pathological insulin resistance.

1-5. Figures

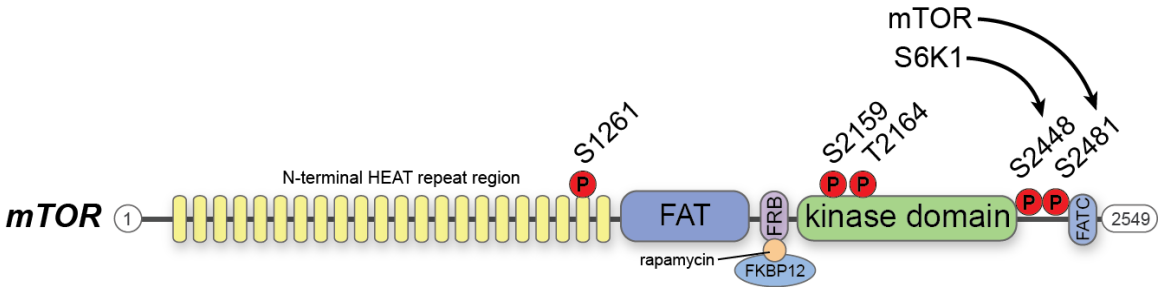


Figure 1-1. mTOR domain structure and phosphorylation sites

mTOR contains a tandem series of HEAT-repeats thought to mediate protein-protein interactions that extends from the N-terminus through the FAT domain. The conserved FAT and FATC domains flank the kinase domain. The rapamycin-FKBP12 complex binds the FKBP12-rapamycin binding (FRB) domain. Upon activation, mTOR autophosphorylates on S2481. Upon activation by mTORC1, S6K1 phosphorylates mTOR S2448. mTOR S1261, S2159, and T2164 are novel phosphorylation sites identified by the Fingar laboratory that promote mTORC1 signaling. **Abbreviations:** mTOR: mechanistic target of rapamycin; mTORC1: mTOR Complex 1; HEAT: huntingtin, elongation factor 3, PP2A A-subunit, and TOR1; FAT: FRAP, ATM, TTRAP domain; FATC: FAT C-terminal domain; FKBP12: FK506-binding protein 12; FRB: FKBP12-rapamycin-binding domain; S6K1: ribosomal protein S6 kinase 1.

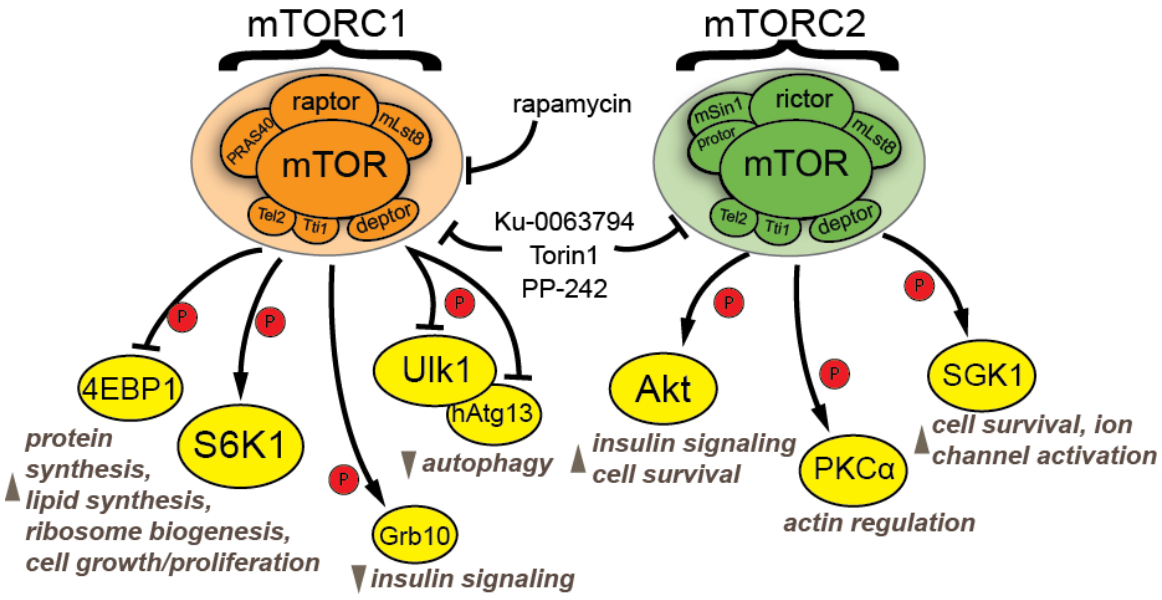


Figure 1-2. mTOR complex components and downstream substrates

mTOR Complex 1 (mTORC1) contains mTOR, raptor, PRAS40, mLst8, deptor, Tel2, and Tti1. Its substrates include 4EBP1, S6K1, Grb10, Ulk1 and hAtg13. 4EBP1 inhibits translation initiation and cell cycle progression and phosphorylation by mTORC1 inhibits 4EBP1. S6K1 is phosphorylated and activated by mTORC1, promoting cell growth, lipid synthesis, and ribosome function. Grb10 phosphorylation suppresses insulin signaling. mTORC1 inhibits autophagy through Ulk1 and hAtg13 phosphorylation. mTOR Complex 2 (mTORC2) contains mTOR, rictor, mSin1, protor, mLst8, deptor, Tel2, and Tti1. mTORC1 phosphorylates Akt, SGK1, and PKCα, which promote cell survival, growth, ion channel activation, and calcium signaling. Rapamycin acutely inhibits mTORC1 functions. Ku-0063794, Torin1, and PP-242 are ATP-competitive mTOR kinase inhibitors which inhibit both mTORC1 and mTORC2.
Abbreviations: mTOR: mechanistic target of rapamycin; mTORC: mTOR Complex; raptor: regulatory associated protein of mTOR; PRAS40: proline-rich Akt substrate of 40 kDa; mLst8: mammalian homolog of lethal with SEC13 8; rictor: rapamycin-insensitive companion to mTOR; protor: protein observed with rictor; deptor: DEP-domain containing mTOR-interacting protein; Tel2: telomere maintenance 2; Tti1: Tel2 interacting protein 1; 4EBP1: eIF4E binding protein 1; S6K1: ribosomal protein S6 kinase 1; Grb10: growth factor receptor-bound protein 10; Ulk1: Unc-51-like kinase 1; hAtg13: human homolog of autophagy related 13; PKCα: protein kinase Cα; SGK1: Serum- and glucocorticoid-activated kinase 1.

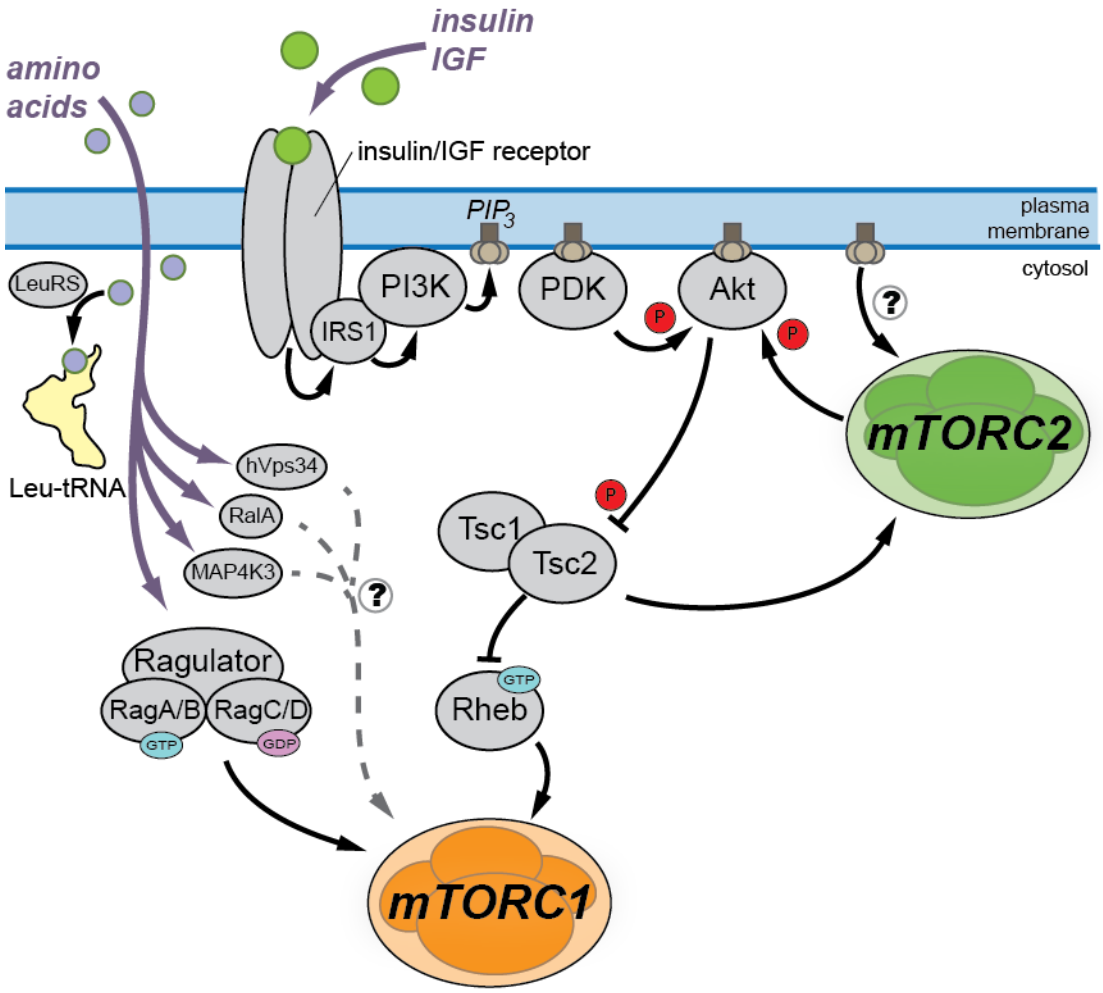
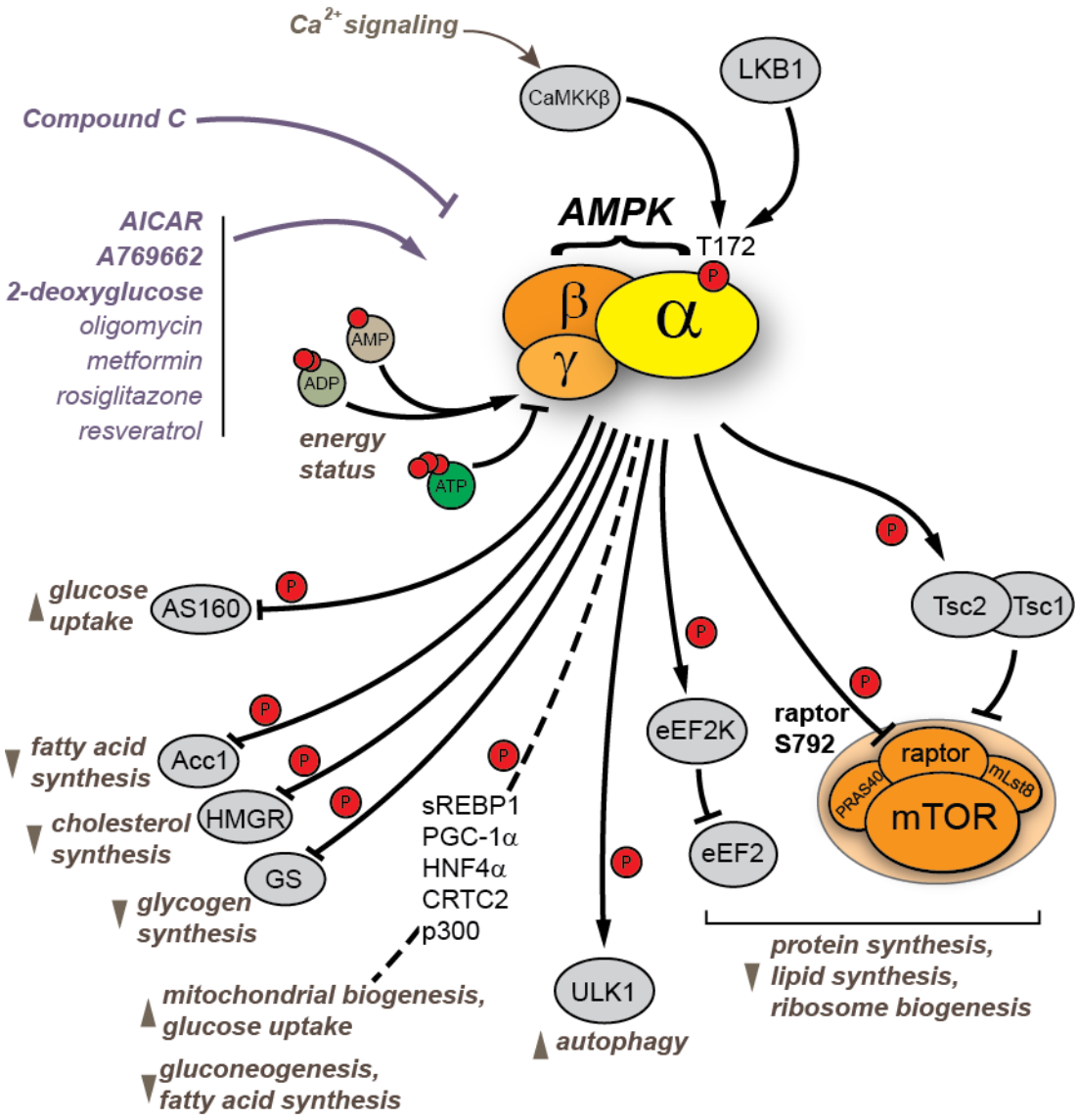


Figure 1-3. Upstream signaling to mTORC1 and mTORC2

mTORC1 is stimulated by insulin or IGF through cognate receptors. Receptor tyrosine kinase activity recruits and activates IRS1. IRS1 recruits the lipid kinase PI3K to the plasma membrane where it produces the lipid signaling molecule PI-3,4,5-P_3. PI-3,4,5-P_3 recruits PH-domain containing proteins PDK1 and Akt to the plasma membrane nad may be important in regulating mTORC2. PDK1 and mTORC2 cooperatively phosphorylate and activate Akt. Akt phosphorylates and inhibits Tsc2. Tsc1 and Tsc2 form a complex that inhibits Rheb via Tsc2 GAP activity. mTORC1 is activated by Rheb. Amino acid signaling activates mTORC1 through Ragulator and Rag GTPases which bind to raptor and recruit mTORC1 to endolysosomal membranes. hVps34, RalA, and MAP4K3 also regulate amino acid signaling to mTORC1. **Abbreviations.** mTORC: mTOR Complex; IGF: insulin-like growth factor; IRS1: insulin receptor substrate 1; PI3K: PI-3'OH-kinase; PI: phosphatidylinsolitol; PDK1: 3'-phosphoinositide-dependent kinase 1; Tsc: tuberous sclerosis complex; Rheb: Ras homolog enriched in brain; GAP: GTPase-activating protein; MAP4K3: MAPK kinase kinase 3.

Figure 1-4. AMPK upstream and downstream

AMPK is a heterotrimer consisiting of α, β, and γ subunits. AMPKα, the catalytic subunit, is phosphorylated on T172 by LKB1 or CaMKKβ. AMPKγ senses AMP, ADP, and ATP allosterically. Compound C inhibits AMPK. Several drugs activate AMPK, including: AICAR, which is metabolized into ZMP, an AMP-mimetic; A769662, a small molecule inhibitor of AMPK; 2-deoxyglucose, a glycolytic poison; oligomycin, a mitochondrial poison; metformin and rosiglitazone, used to treat type 2 diabetes; resveratrol, a plant phenol derived from grape skins. AMPK inhibits protein synthesis through eEF2K, Tsc2 and raptor phosphorylation. AMPK inhibits lipid synthesis through phosphorylation of numerous substrates, including Acc1 and HMGR. AMPK also phosphorylates several transcription factors to promote mitochondrial biogenesis, suppress gluconeogensis, and suppress lipid synthesis. See Chapter 1-2 for more details. **Abbreviations:** AMPK: AMP-activated protein kinase; LKB1: liver kinase B1; AICAR: 5-aminoimidazole-4-carboxamide ribotide; Acc1: acetyl-CoA carboxylase 1; HMGR: HMG-CoA reductase; GS: glycogen synthase.


Ca2+ signaling
CaMKKβ
LKB1
Compound C
AICAR
A769662
2-deoxyglucose
oligomycin
metformin
rosiglitazone
resveratrol
AMPK
T172
β
α
γ
AMP
ADP
ATP
energy status
glucose uptake
AS160
fatty acid synthesis
Acc1
cholesterol synthesis
HMGR
glycogen synthesis
GS
sREBP1
PGC-1α
HNF4α
CRTC2
p300
mitochondrial biogenesis, glucose uptake
gluconeogenesis, fatty acid synthesis
ULK1
autophagy
eEF2K
eEF2
protein synthesis, lipid synthesis, ribosome biogenesis
Tsc2
Tsc1
raptor S792
raptor
mLst8
PRAS40
mTOR

CHAPTER 2

Novel regulation of mTOR Complex 2 by AMPK

2-1. Introduction

Cell, tissue, and whole-body health requires proper maintenance of energy homeostasis. Two key signal transducers, the mechanistic target of rapamycin (mTOR) and the AMP-activated protein kinase (AMPK), coordinately regulate cellular metabolism in response to changing endocrine, nutrient, and energy status[97,295]. AMPK suppresses gluconeogenesis and lipid synthesis, and promotes insulin sensitivity and glucose uptake[304]. Certain anti-diabetic drugs, such as metformin and rosiglitazone, depend on AMPK signaling for their clinical benefits, though the underlying mechanisms remain poorly understood[183]. Understanding the molecular basis of type 2 diabetes is a major un-met need in developing therapies for this modern health epidemic.

mTOR is a conserved serine/threonine protein kinase that functions as the catalytic core of two multiprotein complexes, mTORC1 and mTORC2, which have distinct composition and cellular functions. mTORC1 (composed of mTOR, raptor, PRAS40, mLst8, and deptor) promotes protein and lipid synthesis, cell growth, and cell proliferation[8]. Chronic activation of mTORC1 contributes to insulin resistance. The regulation and function of mTORC2 (composed of mTOR, rictor, Sin1, protor, mLst8, and deptor) remains poorly understood. Recent work

suggests that it is important in maintaining proper glucose homeostasis and insulin sensitivity[10,60,280,281].

Insulin-stimulated Akt promotes mTORC1 signaling by phosphorylating and inhibiting Tsc2 (an upstream inhibitor of mTORC1) and PRAS40 (an mTORC1 component)[34–38,305]. Sufficient levels of amino acids are required for growth factor stimulation of mTORC1, and amino acid-sensing is facilitated by hVps34 and Rag GTPases[88,89,92,93]. mTORC1 promotes lipid synthesis and Cap-dependent protein translation through phosphorylation of its substrates S6K1 and 4EBP. In response to insulin signaling, S6K1 phosphorylates S636/639 on IRS1, inducing its degradation and uncoupling insulin/IGF receptors from PI3K, a pathway known as the mTORC1 negative feedback loop[273,274,306]. PI3K activity suppressed by this negative feedback loop leads to reduced Akt phosphorylation and activity.

Upstream regulation of mTORC2, in contrast to mTORC1, is poorly understood[39]. mTORC2 is regulated by insulin-PI3K signaling, as insulin treatment increases mTORC2 *in vitro* kinase activity and mTORC2-assiciated mTOR S2481 autophosphorylation[61,107,108]. Consistent with regulation by the insulin signaling pathway, mTORC2 kinase activity is sensitive to PI3K inhibitors, such as wortmannin[61]. The Tsc1-Tsc2 complex was reported to interact with and promotes mTORC2-mediated substrate phosphorylation, in opposition to the role of Tsc1/2 as negative regulators of mTORC1[74]. The mTORC2 substrates Akt, PKCα, and SGK1 are all members of the AGC kinase family, phosphorylated by mTORC2 on their hydrophobic motifs (S473, S657, and S422, respectively)

[21,153,154]. mTORC2 also regulates co-translational phosphorylation of Akt and PKCα on their turn motifs (T450 and T638, respectively), which stabilizes the nascent polypeptides[153,154]. By phosphorylating numerous proteins, such as FoxO transcription factors, Akt promotes cell survival and antagonizes apoptotic pathways[307].

AMPK is an evolutionarily conserved, heterotrimeric serine/threonine protein kinase composed of a catalytic α-subunit and regulatory β- and γ-subunits[183,295]. AMPK functions as an energy sensor through binding of ATP, AMP, and ADP to its γ-subunit[183]. AMP and ADP binding promote allosteric activation of AMPK and α-subunit activation loop phosphorylation (T172), which is mediated by LKB1 and CaMKKβ[192,193,195,196,308]. Activated by energy stress, AMPK inhibits ATP-consuming processes—protein, lipid, and carbohydrate synthesis—and promotes ATP-generating processes—glucose uptake/utilization, fatty acid oxidation, and autophagy[295]. AMPK suppresses protein synthesis through phosphorylation of eEF2K, which inhibits translation elongation[240,241] and through inhibition of mTORC1. Upstream of mTORC1, AMPK phosphorylates Tsc2, activating the Tsc1/2 complex[103]. At the level of mTORC1, AMPK phosphorylates the mTOR-binding partner raptor on S792, reducing mTORC1 signaling[104]. AMPK promotes autophagy through phosphorylation and activation of ULK1[243,244] and through suppression of mTORC1, which phosphorylates and inhibits ULK1[145,146]. AMPK has been implicated in the regulation of Akt. AMPK activators LKB1 and sestrin2 have been shown to promote Akt phosphorylation

and anti-apoptotic signaling[250,300], however the role of mTORC2 in AMPK-dependent regulation of these pathways has not been defined.

The Fingar laboratory previously identified mTOR S1261 as a phosphorylation site that promotes mTORC1 function, though its role within mTORC2 was unclear[22]. Our collaborator Steve Riddle (Life Technologies, Madison, WI) conducted an *in vitro* screen the human kinome to identify candidate mTOR S1261 kinases. AMPKα1 and AMPKα2 were identified by this screen. I subsequently demonstrated that AMPK promotes mTOR S1261 in intact cells and regulates this site within both mTORC1 and mTORC2. The fact that AMPK both inhibits mTORC1 signaling and phosphorylates mTOR S1261 (this study), yet mTOR S1261 phosphorylation promotes mTORC1 signaling[22], remains an unresolved conundrum. AMPK promoted Akt S473 phosphorylation in the absence of growth factors in an mTOR- and PI3K-dependent manner. This AMPK-mediated Akt phosphorylation occurred independently of the mTORC1-S6K1 negative feedback loop and thus AMPK promotes mTORC2 signaling directly, rather than indirectly through this negative feedback loop. Furthermore, pharmacological activation of AMPK promotes mTORC2-associated mTOR S2481 autophosphorylation, suggesting that AMPK promotes mTORC2 activity in intact cells in addition to phosphorylation of Akt. Finally, I found that AMPKα1/2 double-knockout MEFs were more sensitive to energy stress-induced apoptosis than wild-type MEFs. Taken together, this data suggests a model whereby AMPK functions as a novel, positive regulator of mTORC2. AMPK signaling promotes

mTORC2-associated mTOR autophosphorylation, mTORC2 signaling to downstream substrates, and cell survival during acute energy stress.

2-2. Materials and Methods

Materials

Protein A-Sepharose CL4B (#17-0780-01), protein G-Sepharose Fast Flow (#17-0618-01), and glutathione-Sepharose 4B (#17-0756-01) beads were from GE Healthcare. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was from Pierce. Polyvinylidene difluoride (PVDF) membrane (0.45 μm) was from EMD Millipore (Immobilon-P). Enhanced chemiluminescense (ECL) reagents were from EMD Millipore (Immobilon Western). All chemicals were from either Fisher Scientific or Sigma-Aldrich, unless otherwise specified.

Antibodies

The following rabbit polyclonal antibodies were custom generated as described previously[22]: mTOR (used for IB only), P-mTOR S1261, raptor (used for IP in all cells and IB in HEK293 and U2OS cells), rictor, and S6K1. Mouse monoclonal HA (HA.11, #MMS-101P) and Myc (9E10, #MMS-150P) epitope tag antibodies (used for IP and IB) were from Covance. Rabbit AMPKα1 (used for IP) was from Abcam (#ab32047). The following antibodies were from Cell Signaling Technology: GST (#2622), mTOR (#2972; used for IP only), P-mTOR S2481 (#2974), raptor (#2280; used for IB in MEFs), P-raptor S792 (#2083), AMPKα (#2532), AMPKα1 (#2795; used for IB only), AMPKα2 (#2757; used for IP and IB), P-AMPKα T172 (#4188), Acc1 (#3676), P-Acc1 S79 (#3661), Akt (#9272), P-

Akt S473 (#4060), P-Akt T308 (#9275), P-Akt T450 (#9267), P-S6K1 T389 (#9234), P-S6 S240/244 (#2215), S6 (#2217), P-FoxO1/3a (T24/T32, # 9464), FoxO3a (#2497), IRS1 (#2382), P-IRS1 S636/639 (#2388), PKCα (#2056), cleaved-Caspase 3 (#9664), cleaved-PARP (#5625). Abbreviations - IB: immunoblotting; IP: immunoprecipitation.

Plasmid DNA

HA-AMPKα, myc-AMPKβ, and myc-AMPKγ expression plasmids were from K. Inoki (University of Michigan, Ann Arbor, MI). pRK5/Myc-mTOR was from D. Sabatini (MIT, Cambridge, MA) via Addgene (#1861). pcDNA3 (empty vector) was used as control DNA in transfections. Plasmid cDNA details:

pcDNA3/3xHA-AMPKα1 (*H. sapiens* PRKAA1, NCBI RefSeq: NM_006251.5)

pcDNA3/3xHA-AMPKα2 (*H. sapiens* PRKAA2, NCBI RefSeq: NM_006252.3)

pRK5/myc-mTOR (*R. Norvegicus* MTOR [formerly FRAP1,RAFT1], NCBI RefSeq: NM_019906.1)

Plasmid Mutagenesis

Kinase dead mutants were generated from pcDNA3/3xHA-AMPKα1 (K56R) and α2 (K45R) using QuikChange II XL (Stratagene) and fully sequenced. The following primer pairs were used for mutagenesis (capital letters indicate mismatch, relevant codons are underlined):

AMPKα1 K56R Primer 1:

5′-cataaagtagctgtgaGGatactcaatcgacag-3′

AMPKα1 K56R Primer 2:

5′-ctgtcgattgagtatcCtcacagctactttatg-3′

AMPKα2 K45R Primer 1:

5′-ggccataaagtggcagttaGaatcttaaatagacag-3′

AMPKα2 K45R Primer 2:

5′-ctgtctatttaagattCtaactgccactttatggcc-3′

Generation of GST-mTOR 1223-1271

A DNA fragment was amplified from pcDNA3/AU1-mTOR (*R. norvegicus*) using the following primers (underlined nucleotides represent the annealing region, capitalized nucleotides indicate restriction sites for subcloning) :

Primer 1: 5′-gacgGGATCCgctgatgaagaagaagacccttt-3′

Primer 2: 5′-gattGAATTCgaccttctggcagctcc-3′

This amplification product was digested with BamHI/EcoRI and subcloned into the pGEX-20T vector, which encodes glutathione S-transferase (GST) and a flexible linker 5' of the insert. Protein production was induced in transformed, log-growth phase *E. coli* (BL21) with IPTG (1mM; Invitrogen) for 4 h at 25°C in a shaker incubator. Bacteria were pelleted at 4200rpm in a Beckman J6-MC centrifuge at 4°C for 15' and resuspended in TEN buffer (50mM Tris pH 7.5, 0.5mM EDTA, 0.3M NaCl) at 1/20th of the original culture volume. Cells were lysed on ice in 1mg/mL lysozyme (Invitrogen), 4mM DTT, and 0.2% NP-40 with protease inhibitors. 1.5 volumes of NaCl-Mg (1.5M NaCl, 12mM $MgCl_2$) and 4μg/mL of DNAseI were then added, followed by incubation on a nutator for 1-2 h at 4°C. Lysates were homogenized by sonication (3x 10 sec bursts at 60%

amplitude), then cleared of insoluble material by centrifugation at 15,000 x *g* in a Sorvall centrifuge with an SS-34 rotor for 20 min at 4°C. Supernatants were mixed with 0.5 mL of a glutathione-Sepharose bead slurry containing PBS and incubated on a nutator for 1 h at 4°C. The bead-lysate mixture was then added to a disposable, fritted column. Gravity flow-through was discarded and beads were washed once with 10mL ice-cold 1% Triton X-100 in PBS and a second time with 10mL ice-cold PBS. Protein was eluted with elution buffer (50mM Tris pH 8.0, 2mM EDTA, 10mM reduced glutathione [Sigma-Aldrich #G4251]) and 0.5mL fractions were collected. Following dot-blot analysis (Ponceau-S or imido black on nitrocellulose membrane), fractions of similar concentrations were pooled, placed in 7000 MWCO dialysis tubing (Pierce Snakeskin), and dialyzed overnight at 4°C in 2L dialysis buffer (10mM Tris pH 7.4, 100mM NaCl, 1mM EDTA, 5% glycerol, 154mg/L dithiothreitol [DTT]). Dialyzed protein was diluted in dialysis buffer as needed and stored at -80°C.

In vitro kinome screen

Small-scale reactions (10μL) were performed in 96-well plates for 1 h at room temperature. Each well contained 1mM ATP, 25 nM kinase, and 0.25 mg/mL GST-mTOR 1223-1271 (substrate) in kinase buffer A (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1mM EGTA, 0.01% Brij-35). Samples were analyzed by dot blot, using a phospho-specific P-mTOR S1261antibody and an Alexa Fluor 488 labeled anti-rabbit -secondary antibody. Phospho-signal intensity was compared to a positive control (HEK293 cell lysates) and positive results determined qualitatively.

In vitro kinase assays

Recombinant AMPKα1/β1/γ1 ("AMPKα1") and AMPKα2/β1/γ1 ("AMPKα2") were obtained from Life Technologies (#PV4672 and #PV4674). 10 ng AMPKα1/β1/γ1 or 30 ng AMPKα2/β1/γ1 were used per reaction. These quantities (approximately) normalize the kinase activity units per μL as reported in the lot analysis for each kinase. *In vitro* kinase (IVK) reactions using GST-mTOR 1223-1271 as substrate were performed in 30μL volumes containing 1μL diluted kinase, 100 ng substrate, and 250 μM ATP (Roche) in kinase buffer B (10 mM $MgCl_2$, 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM DTT). AMPK IVK reactions using mTOR immunoprecipitates were performed in 60 μL volumes (excluding bead volumes) containing 1 μL diluted kinase per reaction. ATP and $MgCl_2$ concentrations and reaction conditions were as above, resuspending beads every 5-10 min during incubation by lightly flicking the tubes. AMPK IVK reactions using HEK293 cell lysates were performed by adding 20 μL 3x kinase buffer containing 1 μL diluted kinase, 750 mM ATP, and 30 mM $MgCl_2$ to 40 μL lysate (60 μL total volume). All AMPK IVK reactions were incubated for 30 min at 30°C and the reaction was quenched by placing test tubes on ice, adding one reaction volume of 2x sample loading buffer, and heating at 95°C for 5 min.

Tissue culture, transfection, and drug treatment

All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco #11995) containing glutamine (584 mg/L), sodium pyruvate (110 mg/L) and high glucose (4.5 g/L) and supplemented with 10% fetal bovine serum (FBS) from

HyClone (#10437). TSC2-/- (+EV) MEFs and TSC2-/- (+TSC2) MEFs were maintained in DMEM/FBS with 100μg/L hygromycin B (Invitrogen #10687010). To serum-starve cells, they were first grown to 75-80% confluency, washed once with starve media (DMEM plus sterile-filtered 20mM HEPES pH 7.2), then incubated in starve media for 20 h prior to any drug treatments. For transfection experiments, HEK293 cells were grown on 10 cm plates to 50-60% confluency and transfected with 8 μg total plasmid DNA using TransIT-LT1 (Mirus). Plasmid quantities for specific experiments are indicated in the figure legends. 16-20 h post-transfection, cells were put in serum-free media for 20 h prior to drug treatments. 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR; Tocris #2840) was prepared fresh on treatment day (200mM in starve media or PBS) and added to cells at a 2.5mM final concentration. 2-deoxyglucose (2-DG; Sigma #D6134) was also prepared fresh on treatment day (500mM in DMEM) and used at a 50mM final concentration. A769662 (Tocris #3336) was added to cells at 100 nM. Rapamycin (Calbiochem #553210) was used at 20 ng/mL. Wortmannin (EMD Millipore #681675) was used at 100 nM. Compound C (EMD Millipore #171261) was used at 25 μM. Ku-0063794 (Tocris #3725) was used at (1 μM). AMPKα1/2 DKO MEFs were from B. Viollet (Inserm, Paris, France). AMPKα1 and AMPKα2 SKO MEFs were from Reuben Shaw (Salk Institute, San Diego, CA). $Tsc1+^{/+}$ and $Tsc1^{-/-}$ MEFs were from D. Kwiatkowski (Brigham and Women's Hospital, Boston, MA). $Tsc2^{-/-}$ MEFs stably expressing Tsc2 or empty vector were from B. Manning (Harvard School of Public Health, Boston, MA).

Stable cell lines

AMPKα1/2 DKO MEFs stably expressing HA-AMPKα1 were generated by retroviral infection. To generate virus, the pQCXIH/HA-AMPKα1 was transfected into Phoenix retroviral packaging HEK293P cells and culture media containing virus was harvested after 48 h. AMPKα1/2 DKO MEFs were infected with this virus in the presence of polybrene (8 μg/mL) and selected with hygromycin (100 μg/mL) 24 h post-infection. This cell line was maintained in DMEM +10% FBS with hygromycin (100 μg/mL). The AMPKα1/2 DKO MEFs used for stable expression were from S. Morrison (University of Michigan, Ann Arbor, MI)[309].

Cell lysis, immunoprecipitation, and immunoblotting

Cells were washed once with ice-cold PBS and scraped into ice-cold lysis buffer (10 mM KPO_4, pH 7.2, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate [Na_3VO_4], 5 μg/ml pepstatin A, 10 μg/mL leupeptin, 40 μg/mL phenylmethylsulfonyl fluoride [PMSF]) containing 0.5% NP-40 and 0.1% Brij35. CHAPS (0.3%) is substituted for NP40 and Brij35 where indicated, to preserve protein interactions during immunoprecipitation. Lysates were cleared by centrifugation at 13,200 rpm for 5 min at 4°C. Supernatants were then collected and immediate placed on ice. In all experiments, protein levels were normalized by the Bradford protein assay using a BioRad iMark microplate absorbance reader. For immunoprecipitation, whole-cell lysates (WCL) at 1-3 mg/mL protein concentrations were incubated end-over-end with 1-2μg antibodies for 1-1.5 h at 4°C, then incubated end-over-end with

Protein A or G sepharose beads (10μL of a 1:1 slurry per 100μL of WCL) for 1-1.5 h at 4°C. Beads were pelleted by centrifugation at 3000rpm for 1 min at 4°C, the supernatant aspirated, and in this fashion washed three times in ice-cold lysis buffer. Beads were resuspended in 1x sample loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.02% bromophenol blue) and heated at 95°C for 5 min. For *in vitro* kinase reactions, beads were washed one additional time in kinase buffer (described in more detail below) prior to the reaction. Samples were resolved by SDS-PAGE and transferred to methanol-activated polyvinylidene difluoride (PVDF) membranes in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, 0.02% SDS). Immunoblotting was performed by blocking PVDF membranes in Tris-buffered saline with Tween 20 (TBST; 40 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween 20) containing 3% nonfat dry milk and incubating the membranes in TBST with 2% bovine serum albumin (BSA) containing primary antibodies overnight at 4°C. Blots were washed 3 times in TBST, incubated in HRP-conjugated secondary antibodies for 30-60 min, followed by 3 more washes (all at room temperatures). Blots were developed by ECL using HyBlot CL autoradiography film (Denville) or imaged after ECL using the Chemi-DocIt HR 410 system (UVP).

Densitometry, statistical analyses, and image production

Immunoblot bands were quantified in some experiments by densitometry using VisionWorks LS (UVP). A 2-tailed, paired *t* test was used to determine significance; *P* values were calculated using Microsoft Excel and $P < 0.05$ was considered significant. Immunoblot images not acquired by the Chemi-DocIt

system were scanned from autoradiography film at 300dpi. Immunoblot images were levels-adjusted in Adobe Photoshop by setting white and black points only. Irrelevant lanes were removed from images of some immunoblots and indicated by a vertical, dashed line in the figure.

2-3. Results

AMPK phosphorylates mTOR S1261

The Fingar laboratory found previously that mTOR S1261 phosphorylation promotes mTORC1 signaling[22]. To identify the unknown mTOR S1261 kinase, our collaborator Steve Riddle (Life Technologies, Madison, WI) employed an *in vitro* screen of the human kinome that covered ~280 purified, active kinases. For substrate, I generated a small mTOR fragment in which amino acids 1223-1271 were fused to GST and expressed in *E. coli*. This fragment consists of mTOR HEAT domain 25T, which contains S1261 flanked by at least 14 residues on either side (see illustration in Figure 2-1A). *In vitro* phosphorylation was determined using a phospho-specific antibody against P-S1261 that we generated and described previously[22]. The results of this screen reveal three distinct classes of candidate kinases: the calcium/calmodulin-regulated kinase (CaMK)/AMPK-like kinase family, the NimA-related kinase (NEK) family, and the interleukin-1 receptor associated kinase (IRAK) family. Among these candidates, AMPKα1 and α2 (gene names PRKAA1 PRKAA2) were of particular interest, as mTOR S1261 fits the AMPK consensus phosphorylation motif (Figure 2-1A). As previously shown, mTOR S1261 is conserved in vertebrates[22]. Multiple sequence

alignment also showed that the residues within the AMPK consensus motif are also 100% conserved in vertebrates.

I first confirmed that AMPK phosphorylates the GST-mTOR 1223-1271 *in vitro*. Recombinant AMPKα1/β1/γ1 and AMPKα2/β1/γ1 holoenzymes both phosphorylated S1261 compared to the bovine serum albumin (BSA) control (Figure 2-1A). To determine whether AMPK promotes mTOR S1261 phosphorylation in intact cells, I examined mTOR phosphorylation status in AMPK-deficient cells. I found that AMPKα1/2 double-knockout (DKO) mouse embryonic fibroblasts (MEFs), cultured in full-serum conditions (DMEM +10% FBS) exhibited strongly reduced mTOR S1261 phosphorylation, compared to wild-type (WT) MEFs (Figure 2-1B). As expected, AMPKα1/2 DKO MEFs lacked AMPKα activation loop (T172) phosphorylation as well as S79 phosphorylation of acetyl-CoA carboxylase 1 (Acc1), a direct substrate of AMPK, together confirming the lack of AMPK signaling in these cells. I next confirmed that, in addition to the GST-mTOR fragment, AMPK phosphorylated full-length mTOR *in vitro*. Because mTOR S1261 phosphorylation was very low in AMPKα1/2 DKO MEFs, we immunoprecipitated mTOR from these cells to use as a substrate in an *in vitro* kinase assay. Compared to the control (BSA), both AMPKα1/β1/γ1 and AMPKα2/β1/γ1 phosphorylated full-length mTOR (Figure 2-1C, compare lane 1 to lanes 2 and 3). Pre-incubation of AMPKα1/β1/γ1 or AMPKα2/β1/γ1 with Compound C, a small molecule which has been shown previously to inhibit AMPK in kinase assays[179], blocked its ability to phosphorylation mTOR (Figure 2-1C, compare lanes 2 and 3 to lanes 4 and 5).

I then asked whether an AMPK inhibitor was sufficient to reduce mTOR S1261 phosphorylation in intact cells. Compound C added to the culture medium inhibited mTOR S1261 phosphorylation in both WT MEFs (Figure 2-1D) and HEK293 cells (Figure 2-1F). AMPKα T172 phosphorylation is also reduced by Compound C, consistent with published work. Because Compound C has been reported to have AMPK-independent effects and does not inhibit activation of AMPK in all contexts[223], I did not rely upon its use in subsequent signaling experiments.

The *in vitro* kinase assays indicated that both AMPKα1 and AMPKα2 phosphorylate mTOR S1261. I asked whether depletion of AMPK from a cell extract also reduced lysate-mediated mTOR S1261 phosphorylation *in vitro*. Control (non-depleted) HEK293 whole cell lysate phosphorylated the GST-mTOR fragment, while BSA alone did not (Figure 2-1E, compare IVK lanes 1 and 2). Immunodepletion of AMPKα1, but not AMPKα2, reduced the capacity of cell lysates to phosphorylate the GST-mTOR fragment (Figure 2-1E, compare IVK lane 1 to lanes 5 and 6). Some activity still remained in the AMPKα1-depleted lysate, which could be explained by the incomplete depletion of AMPKα1. This experiment suggests that AMPKα1 may predominantly phosphorylate mTOR S1261 kinase in these cells, as AMPKα2 depletion did not alter the lysate activity, though the expression and activity of AMPKα1 *versus* AMPKα2 in these cells was not determined. Together, the *in vitro* phosphorylation of mTOR and both the inhibition and the genetic ablation of AMPKα1 and AMPKα2 support the hypothesis that AMPK is a direct and cell-physiological mTOR S1261 kinase.

mTOR S1261 is phosphorylated within both mTORC1 and mTORC2

I next determined whether AMPK phosphorylates mTOR S1261 in mTORC1, mTORC2, or both, as differential phosphorylation could define mTOR complex-specific functions. To address the question of mTOR S1261 complex specificity, I isolated mTORC1 and mTORC2 by raptor and rictor IP, respectively, from WT and AMPKα1/2 DKO MEFs, as well as AMPKα1 and AMPKα2 single-knockout (SKO) MEFs. In full-serum conditions ("steady-state"; Figure 2-2A) or after serum-deprivation for 24 h (Figure 2-2B), S1261 was phosphorylated in both mTORC1 and mTORC2 isolated from WT MEFs, but not AMPKα1/2 DKO MEFs. Similar to AMPKα1/2 DKO MEFs, AMPKα1 SKO MEFs lacked mTOR S1261 phosphorylation in both mTORC1 and mTORC2, however AMPKα2 SKO MEFs did not exhibit defective S1261 phosphorylation in either mTORC1 or mTORC2. Both AMPKα1/2 DKO and AMPKα1 SKO MEFs showed dramatic reduction in AMPK signaling, as evidenced by phosphorylation of two substrates, Acc1 S79 and raptor S792. AMPKα1 may function as the predominant AMPK catalytic subunit in these MEFs, given that Acc1 and raptor phosphorylation appear normal in AMPKα2 SKO MEFs. This may explain why mTOR S1261 phosphorylation is not defective in this cell line.

To corroborate the effects of AMPK loss and mTOR complex specificity in a different cell line, mTOR S1261 phosphorylation was assayed by Hugo Acosta-Jaquez (Fingar laboratory) in HEK293 cells treated with three pharmacological AMPK activators (2-deoxyglucose [2DG], AICAR, and A769662) and one AMPK inhibitor (Compound C). 2DG inhibits ATP production through inhibition of

glycolysis [310]. AICAR, a compound metabolized within the cell into ZMP, an AMP mimetic, is a commonly used AMPK agonist. Though AICAR activates AMPK by direct binding, it also elicits cellular energy stress and thus has AMPK-independent effects[311]. A769662 is a unique small molecule activator of AMPK that acts directly without altering cellular AMP or ADP levels[217–219]. mTORC1 and mTORC2 were isolated from serum-deprived HEK293 cells by raptor and rictor IP, respectively. Both 2DG and A769662 enhanced mTOR S1261 phosphorylation in whole cell lysates as well as in both mTOR complexes (Figure 2-2C). The AMPK inhibitor Compound C slightly inhibited mTOR P-S1261 in both mTORC1 and mTORC2 (Figure 2-2C lane 6). AICAR only weakly increased mTOR P-S1261 and AMPK signaling readouts Acc1 P-S79 and raptor P-S792 (Figure 2C lane 4) in this cell line. Taken together, the MEF and HEK293 cell data demonstrate that both pharmacological AMPK activation and genetic ablation of AMPK affects mTOR S1261 phosphorylation in both mTORC1 and mTORC2, consistent with AMPK signaling towards its known downstream substrates.

AMPK promotes mTORC2 signaling

AMPK phosphorylates Tsc2 and raptor, inhibiting mTORC1 signaling in response to energy stress[103,104]. While this mechanism is well-established, whether AMPK contributes to the regulation of mTORC2 remains unclear. I postulated that AMPK may promote mTORC2 signaling because LKB1 (an AMPK activation loop kinase) and sestrin2 (which activates AMPK through an unknown mechanism) have been shown to promote phosphorylation of Akt on

S473[250,300]. To test the hypothesis that AMPK promotes mTORC2 signaling, I first compared WT MEFs to AMPKα1/2 DKO MEFs cultured in steady-state conditions and found that both Akt S473 phosphorylation and PKCα protein levels were reduced in DKO MEFs (Figure 2-3A). PKCα is stabilized by mTORC2 and its levels thus serve as a marker for mTORC2 signaling[41,42,312]. The bar graph shows total PKCα levels relative to a control protein (Akt) in WT vs. AMPKα1/2 DKO MEFs, indicating a 32% reduction in PKCα levels in AMPK-deficient MEFs. In contrast to mTORC2 signaling, basal mTORC1 signaling to S6K1 (P-T389) and S6 (P-S240/244) was unchanged in AMPKα1/2 DKO MEFs, indicating that mTORC1 signaling is not affected by loss of AMPK and that reduced basal mTORC2 signaling is likely not due to altered mTORC1 signaling. AMPK suppression of mTORC1 signaling to S6K1 was not observed in this experiment, likely due to lack of energy stress or AMPK activation; S6K1 P-T389 reduction was indeed observed in MEFs after AMPK activation (e.g. Figure 2-3C, compare lanes 1 and 2). These data suggest that basal AMPK signaling promotes Akt P-S473 and PKCα expression and, because mTORC2 is an Akt S473 kinase, they also suggest that AMPK activity promotes basal mTORC2 signaling.

To perform the converse experiment, to ask whether AMPK activation increases Akt P-S473 in MEFs, I treated WT and AMPKα1/2 DKO MEFs for 2 h with AICAR (2.5 mM). The cells were cultured in serum-free media for 20 h prior to AICAR treatment, because I found that AMPK signaling was slightly higher in this condition, but also so I could analyze AMPK-mediated phosphorylation of Akt

independent of serum growth factors. AICAR stimulates both mTOR S1261 and Akt S473 phosphorylation in WT MEFs (Figure 2-3B). In AMPKα1/2 DKO MEFs, mTOR P-S1261 was not stimulated by AICAR and Akt P-S473 was weakly stimulated. As indicated by the bar graph, AICAR increased Akt P-S473 in WT MEFs more than twofold, whereas, in AMPK DKO MEFs, AICAR induced a much smaller increase in Akt P-S473 (~60%). It is important to note that AMPK induces a state of energy stress and affects cellular processes other than AMPK signaling, which could explain this small rise in Akt phosphorylation in AMPK-deficient cells[215]. A PCR analysis of the AMPKα1/2 DKO MEFs did not detect the presence of WT AMPKα1 or AMPKα2 alleles in these MEFs, suggesting that this effect is not due to cross-contamination of the cell lines (data not shown).

Although mTORC2 is considered the major Akt S473 kinase, other kinases have been reported to phosphorylate this site, including DNA-PK, ILK, MAPKAP kinase 2, IKKε, and TBK1[66–69 66,69]. Therefore, I asked whether AICAR-induced Akt phosphorylation was sensitive to the mTOR ATP-competitive inhibitor Ku-0063794 (Ku), which blocks mTOR catalytic activity in both mTORC1 and mTORC2[313,314]. Indeed, AICAR-induced Akt P-S473 was inhibited by Ku (Figure 2-3C, compare lanes 2 and 3). Consistent with global mTOR inhibition, the mTORC1 substrate S6K1 P-T389 was also inhibited by Ku (Figure 2-3C, compare lanes 1 and 3). The generation of PI-3,4,5-P_3 by PI3K is critical for localizing Akt to the plasma membrane, the putative location where it is phosphorylated by PDK1 (T308) and mTORC2 (S473). As mTORC2 activity has also been shown to depend on PI3K activity[107], we asked whether AICAR-

induced P-Akt was sensitive to the PI3K inhibitor wortmannin (Wm). Indeed, I found that Wm blocked AICAR-induced Akt P-S473 (Figure 2-3C, compare lanes 2 and 4). S6K1 P-T389 was reduced by AICAR, consistent with AMPK-mediated mTORC1 suppression. Wortmannin also induced dephosphorylation of S6K1 T389, consistent with the dependence of basal mTORC1 signaling on PI3K. These data show that AMPK activation promotes Akt S473 phosphorylation in an mTOR- and PI3K-dependent manner.

If AMPK activation mediates both mTOR S1261 and Akt S473 phosphorylation then AICAR should induce these phosphorylation events in a manner temporally similar to AMPK signaling. To test this hypothesis, I serum-deprived WT and AMPK DKO MEFs and treated them with 2.5mM AICAR for 15', 30', 1 h, 2 h, and 4 h (Figure 2-3D). Indeed, both Akt P-S473 and mTOR P-S1261 increased with a similar time course, as did AMPK T172 and Acc1 S79 phosphorylation, in WT but not AMPKα1/2 DKO MEFs. Also, as expected, AICAR-induced AMPK activation suppresses mTORC1 signaling (S6K1 P-T389) in WT but not AMPKα1/2 DKO MEFs.

The data presented thus far indicate that AMPK activation increases Akt P-S473 in the absence of serum. As serum growth factors such as insulin acutely activate Akt, I tested the idea that insulin and AICAR act synergistically. Serum-starved WT and AMPK DKO MEFs were treated with 100nM insulin for 30 minutes in the absence or presence of AICAR (Figure 2-3E). In WT MEFs, insulin induced both Akt S473 and S6K1 T389 phosphorylation (Figure 2-3E, compare lanes 1 and 2) in a wortmannin- and Ku-sensitive manner (lanes 3 and 6).

Double treatment with both AICAR and insulin was additive for Akt S473 phosphorylation (Figure 2-3E, compare lanes 2 and 4). In AMPKα1/2 DKO MEFs, insulin-activated Akt S473 phosphorylation is lower overall, though AICAR slightly increases it. These results show that insulin action does not dominate or oppose the ability of AICAR to induce Akt S473 phosphorylation in MEFs. Interestingly, insulin-activated S6K1 T389 phosphorylation was unaffected by AICAR treatment, suggesting that insulin-mediated mTORC1 signaling is resistant to AMPK activation (Figure 2-3E, compare lanes 2 and 4). Together, these data demonstrate that pharmacological AMPK activation strongly promotes mTOR S1261 and Akt S473 phosphorylation in WT MEFs but not AMPK-deficient MEFs in a manner dependent on mTOR and PI3K.

To show that these observations hold true in different cell lines, Hugo Acosta-Jaquez asked whether AMPK activation promotes Akt S473 and mTOR S1261 phosphorylation in cell lines other than MEFs. In human osteosarcoma U20S cells, AICAR indeed stimulated mTOR S1261 and Akt S473 phosphorylation in both serum-starved and steady-state cells (Figure 2-4A). AICAR activated AMPK in these cells, as indicated by increased raptor P-S792, AMPKα P-T172, and upward mobility shifting of AMPKα. As I observed in MEFs, AMPK activation in U2OS cells increased Akt P-S473 in a Ku- and wortmannin-sensitive manner, indicating the requirement for mTORC2 and PI3K (Figure 2-4A, lanes 1-4). AICAR also increased Akt on its activation loop site (P-T308) and turn motif (TM) site (P-T450) in serum-starved U2OS cells, though phosphorylation of these sites is not clearly regulated by AMPK activation in

steady-state conditions (Figure 2-4A, lanes 5-8). In serum-starved HEK293 cells, the AMPK agonist A769662 increased Akt S473 and mTOR S1261 phosphorylation in a Ku- and wortmannin-sensitive manner (Figure 2-4B, lanes 1-4). Consistent with promotion of Akt P-S473, Hugo Acosta-Jaquez observed that AMPK activation promoted phosphorylation of the Akt substrate FoxO3a on T32 in HEK293 cells. FoxO3a phosphorylation was not detected in U2OS cells (data not shown). In HEK293 cells cultured in steady-state conditions, A769662 did not noticeably regulate AMPK signaling, mTOR P-S1261, Akt P-S473, or FoxO3a P-T32 (Figure 2-4B, compare changes in lanes 1 and 2 to lanes 5 and 6). In HEK293 cells, A769662 promoted AMPKα T172 phosphorylation and signaling to raptor S792 better in serum-starved cells than in steady-state conditions. As noted earlier, AICAR does little to activate AMPK in our HEK293 cells (Figure 2-2C compare lanes 1 and 4). Taken together, these experiments demonstrate that pharmacological activation of AMPK in U2OS and HEK293 cells promotes mTOR S1261 and Akt S473 phosphorylation in a manner that depends on mTOR and PI3K activity, consistent with our results in MEFs.

AMPK activation mediates Akt phosphorylation independently of the mTORC1 negative feedback loop

Because AICAR suppresses mTORC1 signaling to S6K1 (e.g. Figure 2-3D) and increases both S473 and T308 phosphorylation in a wortmannin-sensitive manner, I tested the possibility that AICAR indirectly promotes Akt phosphorylation through suppression of mTORC1 and found that it does not. mTORC1 negatively regulates the insulin/PI3K signaling pathway (and Akt

phosphorylation), through a mechanism known as the mTORC1 negative feedback loop (NFL)[273,274,306]. NFL activation is caused by mTORC1-activated S6K1, which phosphorylates insulin receptor substrate 1 (IRS1) on S636 and S639, leading to IRS1 degradation by the proteasome and reduced PI3K signaling flux[273,274]. Grb10 was also recently reported as an mTORC1 effector that promotes the NFL[147,148] and both S6K1 and Grb10 promote the NFL in a rapamycin-sensitive manner. Because we observed that AMPK activation promoted both Akt S473 and T308 phosphorylation, both of which depend on PI3K signaling, the root cause of AMPK-mediated Akt phosphorylation could be through inhibition of mTORC1. In the scenario whereby the NFL is suppressed by AMPK-activation, we would expect rapamycin, which inhibits mTORC1 (and the NFL), to boost Akt T308 and S473 phosphorylation without any further increase following AMPK stimulation. To test this, I pretreated MEFs with rapamycin to completely block the mTORC1 NFL. I serum-starved MEFs as per my previous experiments and treated them with or without AICAR for 2 h and with or without a 30' rapamycin pre-treatment (Figure 2-5A). AICAR and rapamycin both reduced S6K1 T389 phosphorylation, as expected (rapamycin more so than AICAR, also as expected). Rapamycin did not induce Akt P-S473 or P-T308 in either WT or AMPK DKO MEFs (Figure 2-5A, compare lane 1 to lane 5 and lane 3 to lane 7). This suggests either that mTORC1 signaling (and NFL activity) is already quite low in serum-starved MEFs or that the effect of inhibiting the NFL on Akt phosphorylation requires a substantially longer time course, consistent with previous reports [273,274]. Hugo Acosta-Jaquez also tested

the effect of rapamycin pre-treatment on AMPK-mediated Akt phosphorylation in serum-starved U2OS and HEK203 cells (Figure 2-5B and C). In agreement with my data in MEFs, Akt S473 and T308 phosphorylation were induced by AICAR but not rapamycin in U2OS cells (Figure 2-5B). Akt T308 phosphorylation was difficult to detect in serum-starved HEK293 cells, however rapamycin did not boost Akt S473 phosphorylation in these cells, consistent with MEFs and U2OS cells (Figure 2-5C). Importantly, AMPK activation in these cell lines does not correlate with reduced mTORC1 signaling to S6K1. In U20S cells, AICAR did not inhibit S6K1 P-T389 (Figure 2-5B), and phosphorylation of this site was difficult to detect in serum-starved HEK293 cells (Figure 2-5C). Thus these data demonstrate that AMPK-mediated Akt phosphorylation is not explained through mTORC1-dependent feedback inhibition.

In addition to these rapamycin experiments, I observed that in AMPKα1/2 DKO MEFs cultured in steady-state (full-serum) conditions, S6K1 and S6 are unchanged compared to WT MEFs (Figure 2-3A). Furthermore, IRS1 S636/S639 phosphorylation and IRS1 protein levels remained unchanged with AMPK activation and rapamycin treatments, indicating no detectable changes in the NFL. Taken together, the MEF, U2OS, and HEK293 data strongly suggest a novel role for AMPK in mTORC2-mediated Akt S473 phosphorylation that occurs independently of the mTORC1 NFL.

Expression of AMPKα1 in AMPKα1/2 double-knockout cells rescues mTOR S1261 and Akt S473 phosphorylation

As it is a possibility in cultured cells derived from knockout animals, chronic loss of AMPK activity may have unknown effects on mTORC2/Akt signaling through permanent, compensatory adaptations or mutations. To address this concern, we asked whether expression of exogenous HA-AMPKα1 was sufficient to restore AMPK signaling, including mTOR S1261 and Akt S473 phosphorylation, in AMPKα1/2 DKO MEFs. HA-tagged AMPKα1 was stably expressed in AMPKα1/2 DKO MEFs via retroviral transduction by Tsukasa Suzuki (Ken Inoki laboratory, University of Michigan, Ann Arbor, MI). Indeed, HA-AMPKα1 rescued AICAR-stimulated raptor S792 and mTOR S1261 phosphorylation to levels similar to WT, when compared to the parental AMPKα1/2 DKO MEFs (Figure 2-6, compare lanes 2, 4, and 6). The defect in AICAR-stimulated Akt 473 phosphorylation in AMPKα1/2 DKO MEFs was partially rescued upon HA-AMPKα1expression. These experiments demonstrate that the phenotypes exhibited by AMPKα1/2 DKO MEFs in this study are due to lack of AMPK activity and not an unrelated artifact caused by AMPK deletion.

AMPK activation promotes mTOR autophosphorylation within mTORC2 but not mTORC1

The Fingar laboratory has previously shown that the mTOR autophosphorylation site S2481 can be used to mTORC1 and mTORC2 activity within intact cells[108]. I reasoned that if AMPK promotes mTORC2 signaling to Akt, it could also increase mTORC2-assiciated mTOR S2481

autophosphorylation. Because AMPK inhibits mTORC1, it is reasonable to predict that AMPK activation would also inhibit mTORC1-associated S2481 autophosphorylation. To test these ideas, Hugo Acosta-Jaquez serum-starved MEFs, treated them with AICAR to activate AMPK, then immunoprecipitated endogenous rictor and raptor to isolate mTORC2 and mTORC1, respectively. He found that in $Tsc1^{+/+}$ MEFs and $Tsc2^{-/-}$ MEFs stably expressing Tsc2 ($Tsc2^{-/-}$ +Tsc2) mTOR S2481 phosphorylation within mTORC2 increased upon AICAR treatment in a Ku-sensitive manner (Figure 2-7A and B, lanes 1-3). Under the same conditions, mTOR S2481 phosphorylation within mTORC1 decreased, consistent with AMPK suppressing mTORC1. Interestingly, mTOR S2481 autophosphorylation in the whole cell lysate was unchanged by AICAR treatment, which could the result of combined reduction in mTORC1 autophosphorylation with increased mTORC2 autophosphorylation. In agreement with our previous results (Figure 2-2), AMPK activation promoted mTOR S1261 phosphorylation in both mTORC1 and mTORC2. In whole cell lysates, AICAR increased Akt S473 phosphorylation and inhibited S6K1 T389 phosphorylation, consistent with its activation of mTORC2 and inhibition of mTORC1. Neither IRS1 S636/639 phosphorylation nor total IRS1 levels were affected by AICAR treatment in WT MEFs, consistent with the observations in HEK293 and U2OS cells (Figure 2-5B and C).

A previous study reported that Tsc1/2 function is required for growth factor-mediated activation of mTORC2 kinase activity, as measured by mTORC2 in vitro kinase assay using Akt as a substrate[74]. To test whether loss of Tsc1 or

Tsc2 affected AMPK-mediated mTOR S2481autophosphorylation within mTORC2, Hugo Acosta-Jaquez examined phosphorylation status of this site in $Tsc1^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs stably expressing an empty vector ($Tsc2^{-/-}$ +V). He found that Tsc1 is not required for AICAR treatment to induce mTOR S2481 autophosphorylation within mTORC2 (Figure 2-7B, compare lanes 1 and 2 to lanes 4 and 5). $Tsc2^{-/-}$ (+V) MEFs exhibited a higher basal level of mTOR S2481 phosphorylation within mTORC2 than $Tsc2^{-/-}$ (+Tsc2) MEFs (Figure 2-7A, compare lanes 1 and 4). AICAR was still able to further stimulate mTORC2-associated S2481 autophosphorylation in $Tsc2^{-/-}$ (+V) MEFs by 58% (mean of two experiments), indicating that Tsc2 is not absolutely necessary for this effect (Figure 2-7A, compare lanes 4 and 5). As expected, mTORC1-associated S2481 autophosphorylation is not sensitive to AICAR in the absence of Tsc1or Tsc2, consistent with the necessity of Tsc1/2 in AMPK suppression of mTORC1. Tsc1 and Tsc2 *are* required for AMPK to promote Akt S473 phosphorylation in serum-starved MEFs, supporting the previous finding that Tsc1/2 is necessary for mTORC2 to phosphorylate Akt [74]. AICAR induces mTOR S1261 in both WT and Tsc1- or Tsc2-deficient MEFs, suggesting that Tsc1/2 is not required for AMPK to phosphorylate mTOR. It should be noted that the total amounts of some proteins (including raptor and mTOR) are slightly lower in WT MEFs than in $Tsc1^{-/-}$ or $Tsc2^{-/-}$ MEFs and that this is most evident in raptor immunoprecipitates. Though the reason for these differences is unclear, they are consistent with previous observations of the Fingar laboratory[22]. Taken together, these data demonstrate

that AMPK activates cellular mTORC2 (as indicated by S2481 autophosphorylation) in addition to promoting downstream signaling to Akt.

I next considered the possibility that AMPK-activating compounds could promote mTORC2-associated mTOR autophosphorylation independent of AMPK, as AMPK-independent effects have been reported for both AICAR and A769662 [215]. To test this idea, I asked whether AMPK catalytic activity was required for A769662 to promote mTOR S2481 autophosphorylation in HEK293 cells. I expressed exogenous AMPK (HA-AMPKα, myc-AMPKβ1, and myc-AMPKγ1) along with myc-mTOR and compared HA-AMPKα1 and α2 with their kinase-dead (KD) counterparts AMPKα1 K56R and AMPKα2 K45R. A

After transfection, these HEK293 cells were serum-starved and then treated with A769662 for 2 hours to activate AMPK. I found that A769662 increased myc-mTOR S2481 autophosphorylation in cells co-expressing AMPKα1-WT or AMPKα2-WT (Figure 2-8, compares lanes 2 to 3 and 5 to 6). In the presence of AMPKα1-KD and AMPKα2-KD, however, A769662 did not increase myc-mTOR S2481 autophosphorylation (Figure 2-8, compares lanes 2 to 4 and 5 to 7). These data show that AMPK kinase activity is required for A769662 to promote mTOR S2481 autophosphorylation.

Loss of AMPK correlates with increased AICAR-induced apoptosis

The LKB1 tumor suppressor, a critical AMPK T172 kinase, was reported to protect cells from energy stress-induced apoptosis[300]. In that study, AICAR treatment induced Caspase 3 and PARP cleavage (two common readouts of apoptosis) more in cells lacking LKB1 than in wild-type cells[300]. Although AICAR

activates AMPK, it also has pleiotropic effects, including reduction of cellular ATP levels and apoptosis, thus causing a state of stress independent of AMPK [215,216,315]. Considering that LKB1 activates AMPK through phosphorylating AMPKα T172, I reasoned that the loss of AMPK in MEFs may increase sensitivity to energy stress-induced apoptosis in a manner similar to the loss of LKB1. I therefore examined the apoptotic status of WT and AMPKα1/2 DKO MEFs after treatment with AICAR and found that AICAR treatment for 2 h increased PARP cleavage in AMPKα1/2 DKO MEFs but not WT MEFs (Figure 2-9A, compare lanes 1 and 2 to lanes 4 and 5).

I then asked whether mTOR inhibition exacerbates the apoptotic phenotype in AMPK-deficient cells. The addition of Ku enhanced PARP cleavage and this increase was substantially greater in AMPKα1/2 DKO MEFs than in WT MEFs, where the increase was very slight (Figure 2-9A, compare lanes 2 and 3 to lanes 5 and 6). This result suggests that combined loss of AMPK and inhibition of mTOR promotes apoptosis more than inhibition of mTOR alone. Because mTORC2 promotes anti-apoptotic functions through Akt, the possibility that AMPK promotes cell survival through an mTORC2-Akt is intriguing. AICAR increased Akt S473 phosphorylation and phosphorylation of the Akt substrate FoxO3a (T32) in WT but not AMPK-deficient MEFs, consistent with AMPK-dependent activation of mTORC2 and Akt signaling (Figure 2-9A, compare lanes 1 and 2 to lanes 4 and 5). Because inhibition of mTORC2 during AICAR treatment in WT MEFs did not induce apoptosis to the same degree as in

AMPKα1/2 DKO MEFs, AMPK likely has mTORC2-independent pathways that suppress apoptosis.

Thus I found that the loss of AMPK promotes energy stress-induced apoptosis, akin to the loss of LKB1, which was reported previously[300]. Furthermore, I found that the apoptotic sensitivity of AMPKα1/2 DKO MEFs can be reversed by expression of HA-AMPKα1 (Figure 2-9B). AICAR treatment of serum-starved AMPKα1/2 DKO MEFs induced both PARP cleavage and Caspase 3 (Casp3) cleavage (Figure 2-9B, lanes 3 and 4). Stable expression of HA-AMPKα1 was sufficient to reverse the apoptotic sensitivity of AMPKα1/2 DKO MEFs to AICAR, such that they behave like WT MEFs (Figure 2-9B, compare lanes 1 and 2 to lanes 5 and 6). Taken together, these data support a model whereby LKB1-AMPK signaling protects cells against energy stress-induced apoptosis and that may depend, in part, on mTORC2 signaling.

2-4. Discussion

mTOR and its binding partners raptor and rictor are phosphorylated on many sites in intact cells. Recent studies by the Fingar laboratory and others have demonstrated that phosphorylation contributes to the regulation of mTOR complexes [22,23,104,109,117,316]. The Fingar laboratory previously characterized the phosphorylation of mTOR S1261, an event that promotes mTORC1 signaling[22]. An *in vitro* kinome screen was employed to identify the unknown mTOR S1261 kinase. Here I report the identification of AMPKα1 and AMPKα2 as mTOR S1261 kinases. The finding that AMPK phosphorylates mTOR S1261 presents a

conundrum, as AMPK inhibits mTORC1 signaling by phosphorylating Tsc2 and raptor [103,104] yet our previous data indicate that mTOR S1261 phosphorylation promotes mTORC1 signaling [22]. To explain this paradox, I first postulated that AMPK phosphorylates mTOR S1261 in mTORC2 but not mTORC1. The data, however, indicate that AMPK phosphorylates mTOR in both complexes (Figure 2-2). Though AMPK seems to be a major mTOR S1261 kinase in MEFs, it is possible that another kinase phosphorylates mTORC1-associated S1261 to promote mTORC1 signaling in the absence of energy stress or in a different cellular context. Indeed, our kinome screen revealed other kinases in the CaMK/AMPK-like kinase family that could function as physiologically relevant mTOR S1261 kinases. Further studies will be required to determine whether another kinase mediates mTORC1-associated mTOR S1261 phosphorylation to promote mTORC1 signaling.

AMPK activation via AICAR, metformin, and other drugs has been widely reported to enhance insulin sensitivity in tissues that have succumbed to insulin resistance. The molecular mechanisms underlying this response, however, remain poorly understood[183]. Identifying AMPK as the mTOR S1261 kinase led us to ask whether AMPK plays a role in mTORC2 signaling, as mTORC2 is gaining acceptance as an important regulator of glucose homeostasis and insulin sensitivity[10,11,60,281]. In this study, AMPK activation promoted the phosphorylation of Akt on the mTORC2 target site (S473) in serum-deprived conditions through a mechanism that does not rely on suppression of the mTORC1 negative feedback loop (NFL). The evidence against NFL involvement is threefold. First, AMPKα1/2

DKO MEFs cultured under full-serum conditions exhibit similar mTORC1 signaling as WT MEFs yet show reduced Akt P-473 (Figure 2-3A). In addition, AICAR in U2OS cells and A769662 in HEK293 cells failed to suppress S6K1 P-T389 yet these drugs induced Akt S473 phosphorylation (Figure 2-4 and Figure 2-5B and C). Second, rapamycin treatment in serum-starved MEFs, U20S cells, or HEK293 cells affected neither Akt P-S473 nor the ability of AMPK activators to increase Akt P-S473 (Figure 2-5). Third, IRS1 S636/636 phosphorylation and total IRS1 protein levels were unchanged by AMPK activation in serum-starved U2OS or HEK293 cells (Figure 2-5B and C). Therefore I propose that AMPK positively controls mTORC2-mediated substrate phosphorylation in a novel way. mTORC2-regulated pathways (such as Akt) that are otherwise repressed due to lack of growth factors in cultured cells or due to pathological conditions, such as insulin resistance, could in this way be activated through AMPK.

As AMPK phosphorylates mTOR (S1261) and promotes mTORC2 signaling, I hypothesized that AMPK activates mTORC2 through a direct mechanism. mTOR S2481 autophosphorylation is a practical measure of mTORC1- and mTORC2-specific activity in intact cells that does not depend on analysis of a downstream substrate[108]. In the context of mTORC2, this is important because Akt S473 phosphorylation, which is often used as a readout for mTORC2 signaling, can be phosphorylated on this site by other kinases[66–69]. Because AMPK promoted mTORC2-associated mTOR S2481 autophosphorylation and AMPK-mediated Akt phosphorylation was Ku-sensitive, I conclude that AMPK activates mTORC2. Tsc1- and Tsc2-deficient cells were

defective in AMPK-mediated Akt S473 phosphorylation, consistent with mTORC2 requiring Tsc1/2 for phosphorylation of downstream substrates[74]. In contrast, AMPK does not require Tsc1/2 to induce mTORC2-associated S2481autophosphorylation. Tsc1/2, known to co-immunoprecipitate with mTORC2[74], is probably important for some aspect of extrinsic substrate phosphorylation (e.g. docking, recruitment, or positioning) but may not be critical for mTORC2-associated mTOR autophosphorylation, a readout that may reflect on mTOR intrinsic catalytic activity[108]. Though mTOR S1261 phosphorylation closely coincides with AMPK activity and AMPK-mediated mTORC2 signaling, the contribution of mTOR S1261 phosphorylation to the activation of mTORC2 remains unclear and requires further exploration. It is also unknown whether AMPK and mTORC2 can be co-immunoprecipitated, a fact that could shed light on the precise mechanism of mTORC2 activation by AMPK. Notwithstanding these points, our data suggest a novel mechanism of mTORC2 activation by AMPK and that in addition to promoting mTORC2 signaling to Akt, AMPK activation also promotes mTORC2-specific autophosphorylation.

Many studies have suggested that AMPK potentiates insulin signaling in part through cross activation of Akt-dependent pathways[293,299,301,317]. Signaling by LKB1, an AMPK activation loop kinase, was reported to promote Akt S473 phosphorylation and promotes anti-apoptotic functions thought to be mediated by Akt [302,303]. Indeed, Shaw *et al.* found that LKB1$^{-/-}$ MEFs were highly sensitive to energy stress-induced apoptosis[300]. We found that AMPK-deficient MEFs are similarly sensitive, thus supporting a cytoprotective role for the LKB1-AMPK

signaling axis during energy stress. Sestrin1 and 2 are DNA damage-induced proteins that activate AMPK through an unclear mechanism[318,319]. Recently, sestrin2 was found to promote Akt-related cell survival in an AMPK-dependent fashion[250]. These reports along with my observations that AMPK promotes both mTORC2-Akt signaling and cell survival during energy stress, suggests that AMPK may promote cell survival through mTORC2, an intriguing possibility that requires further study.

In addition to promoting Akt HM (S473) phosphorylation, we found that AMPK activation also promoted phosphorylation of the Akt activation loop (T308) and that these phosphorylation events are both inhibited by the mTOR inhibitor Ku. It is believed that mTORC2 functions as the major Akt HM kinase in response to growth factors and that PDK1 is the major Akt activation loop kinase. Combined Akt S473 and T308 phosphorylation maximally activates Akt[138]. The question of interdependence between these two sites, however, remains controversial. Akt T308 phosphorylation should be mTORC2-independent, yet Hug Acosta-Jaquez and I found that Akt T308 phosphorylation induced by AMPK is mTOR-dependent (Ku-sensitive) in MEFs and U2OS cells. Initial characterization of Ku showed that this drug inhibits Akt P-T308 but does not alter PI-3,4,5-P_3 levels or inhibit PDK1[314]. Torin1 and PP-242, two other mTOR catalytic inhibitors that inarguably inhibit Akt S473 phosphorylation were also found to inhibit Akt T308 phosphorylation. PP-242 reduced Akt P-T308, but phosphorylation was insensitive to this drug upon disruption of mTORC2 assembly or mutation of Akt S473 to alanine (S473A)[320]. Torin1 inhibits Akt P-

T308 but at concentrations that also inhibited PI3K[321]. It has also been reported that mutation of Akt S473 to alanine inhibits Akt T308 phosphorylation[322]. In rictor-, Sin1-, and mLst8-null cells, S473 phosphorylation is reduced, yet T308 phosphorylation remains intact[40,41,155]. On the other hand, RNAi-mediated knockdown of rictor impairs both Akt S473 and T308 phosphorylation[61,152]. Furthermore, it has been hypothesized that Akt S473 phosphorylation exposes T308 or creates a docking site for the T308 kinase, PDK1, implying that Akt T308 depends on S473 phosphorylation[138]. From all of this conflicting data I conclude that phosphorylation of Akt S473 and T308 occur both independently and inter-dependently. Further work is necessary to determine how AMPK promotes Akt T308 phosphorylation in an mTOR-dependent manner.

In conclusion, I propose a model whereby AMPK coordinates mTORC1 and mTORC2 signaling to manage periods of acute energy stress. AMPK suppresses mTORC1 as part of a cellular transition from anabolism to catabolism in order to generate ATP. AMPK promotes mTORC2 signaling to enhance insulin/IGF-dependent pathways, such as Akt, and to promote cell survival during stress recovery. I propose that AMPK activates mTORC2 directly in a manner independent of mTORC1 negative feedback signaling. Figure 2-10 illustrates how AMPK as a novel, direct activator of mTORC2 could stimulate growth factor-dependent signaling, which may be a role for AMPK during energy stress and represent the mechanism whereby AMPK facilitates the anti-diabetic benefits of certain drugs, such as metformin and rosiglitazone. Recent studies have implicated mTORC2 in glucose/energy homeostasis, as loss of the mTORC2

component rictor in adipose, liver, and pancreatic tissue caused metabolic pathologies reminiscent of type 2 diabetes[10,11,60,281]. AMPK action potentiates insulin sensitivity, counteracting metabolic disorders through increased glucose uptake, reduced gluconeogenesis, and reduced fatty acid synthesis. Therapeutic approaches that activate AMPK could therefore bypass chronically defective signaling pathways, such as PI3K-Akt, which is suppressed in tissues experiencing insulin resistance. AMPK activity is also up-regulated by hormones such as leptin, adiponectin, and ghrelin, which utilize AMPK signaling to promote fatty acid oxidation in adipose tissue, suppress gluconeogenesis in the liver, and regulate appetite in the hypothalamus[291–293,296,297,323]. If these hormones activate pathways downstream of mTORC2, my model could provide a molecular explanation for these observations. Thus, future work should explore signaling downstream of mTORC2 in the context of AMPK activation by both pharmacological compounds as well as endocrine signals.

2-5. Figures

Figure 2-1. AMPK phosphorylates mTOR on S1261

(A) AMPK phosphorylates GST-mTOR fragment containing S1261 *in vitro*. GST-mTOR (1223-1271) was subject to *in vitro* kinase assay with recombinant AMPKα1/β1/γ1 or AMPKα2/β1/γ1 for 30 min at 37°C. mTOR phosphorylation was monitored with a phospho-mTOR S1261 antibody. BSA was used as a control. The GST-mTOR fragment is depicted below the IVK data. S1261 is highlighted in red. 25T – mTOR HEAT repeat 25T. Below GST-mTOR is the AMPK phosphorylation consensus motif (Φ: hydrophobic residue, β: basic residue, S/T: serine or threonine; x: any residue). **(B)** AMPK-deficient cells have reduced mTOR S1261 phosphorylation. Mouse embryonic fibroblasts (MEFs) from WT or AMPKα1/2 double-knockout (DKO) mice were analyzed by immunoblot for P-mTOR S1261, AMPK signaling (P-AMPKα T172 and P-Acc1 S79) and AMPK levels (pan-AMPKα, AMPKα1, and AMPKα2). **(C)** AMPK phosphorylates full-length mTOR *in vitro*. mTOR immunoprecipitates from AMPKα1/2 DKO MEFs were incubated with AMPKα1/β1/β2 or AMPKα2/β1/γ1 as in *(A).* Where indicated, Compound C (Cpd C; 30μM) was incubated with AMPK for 30 min at 37°C prior to IVK with mTOR immunoprecipitates. **(D and E)** P-mTOR S1261 is inhibited by Compound C. *(D)* WT MEFs *(F)* HEK293 cells were treated with or without Compound C (10μm, 2 h) and analyzed as indicated. **(F)** Immunodepletion of AMPKα1 reduces mTOR S1261 phosphorylation by cell lysates *in vitro.* HEK293 lysates were subject to immunodepletion using no antibody, an unreactive antibody, an AMPKα1 antibody, or an AMPKα2 antibody. GST-mTOR (1223-1271) was added to Immunodepleted lysates and incubated for 30 min at 37°C. Phosphorylation of GST-mTOR (1223-1271) was monitored using a P-mTOR S1261 antibody, and immunodepletion was verified by immunoblot of AMPKα1 and AMPKα2 in the depleted lysate. BSA was used instead of lysate as a control in the IVK.

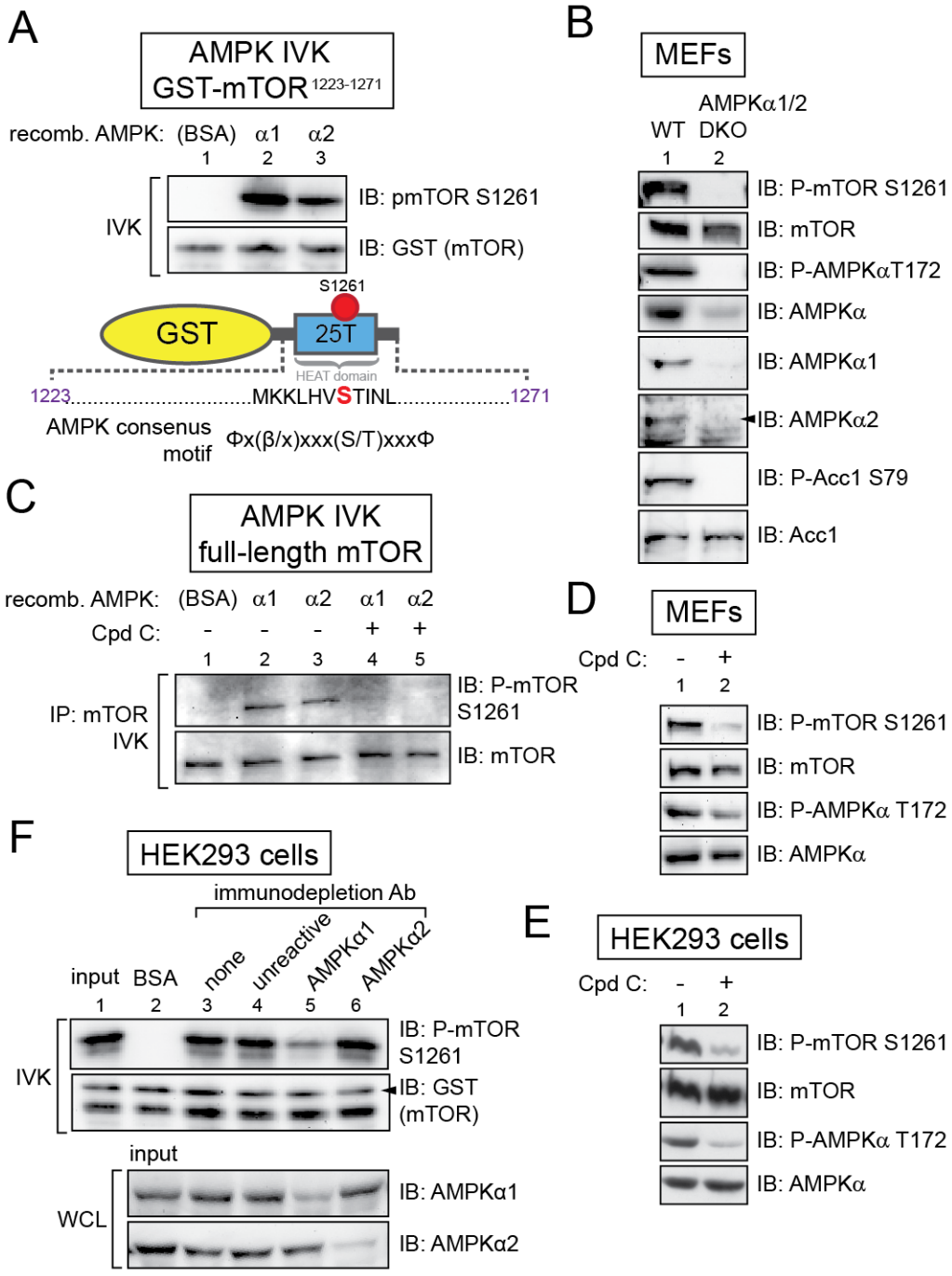

A
AMPK IVK
GST-mTOR 1223-1271
recomb. AMPK: (BSA) α1 α2
1 2 3
IVK
IB: pmTOR S1261
IB: GST (mTOR)
S1261
GST
25T
HEAT domain
1223.....MKKLHVSTINL.....1271
AMPK consenus motif
Φx(β/x)xxx(S/T)xxxΦ
B
MEFs
AMPKα1/2
WT DKO
1 2
IB: P-mTOR S1261
IB: mTOR
IB: P-AMPKαT172
IB: AMPKα
IB: AMPKα1
IB: AMPKα2
IB: P-Acc1 S79
IB: Acc1
C
AMPK IVK
full-length mTOR
recomb. AMPK: (BSA) α1 α2 α1 α2
Cpd C: - - - + +
1 2 3 4 5
IP: mTOR
IVK
IB: P-mTOR S1261
IB: mTOR
D
MEFs
Cpd C: - +
1 2
IB: P-mTOR S1261
IB: mTOR
IB: P-AMPKα T172
IB: AMPKα
E
HEK293 cells
Cpd C: - +
1 2
IB: P-mTOR S1261
IB: mTOR
IB: P-AMPKα T172
IB: AMPKα
F
HEK293 cells
immunodepletion Ab
input BSA none unreactive AMPKα1 AMPKα2
1 2 3 4 5 6
IVK
IB: P-mTOR S1261
IB: GST (mTOR)
input
WCL
IB: AMPKα1
IB: AMPKα2

Figure 2-2. mTOR S1261 is phosphorylated in both mTORC1 and mTORC2.

(A) mTOR S1261 is phosphorylated in WT and AMPKα2 single knockout (SKO) MEFs, but not AMPKα1/2 DKO or AMPKα1 SKO MEFs. MEFs were cultured in DMEM +10% FBS ("steady-state") and lysed in lysis buffer containing 0.3% CHAPS. mTORC2 and mTORC1 were isolated by immunoprecipitation with anti-rictor or anti-raptor antibodies, respectively. Rictor IPs, raptor IPs, and whole cell lysates (WCL) were analyzed by immunoblot using the indicated antibodies. **(B)** as in *(A)*, except cells were cultured in serum-free media (DMEM + 20mM HEPES pH 7.2) for 24 h prior to lysis. **(C)** HEK293 cells were serum-starved as in *(B)* followed by treatment with 2-deoxyglucose (2DG; 50mM, 30 min), AICAR (2.5mM, 2 h), A769662 (10μM, 2 h), or Compound C (Cpd C; 10uM, 2 h). Cells were lysed and rictor and raptor IPs were performed as in *(A)* and *(B)*. The experiment in *(C)* was performed by Hugo Acosta-Jaquez.

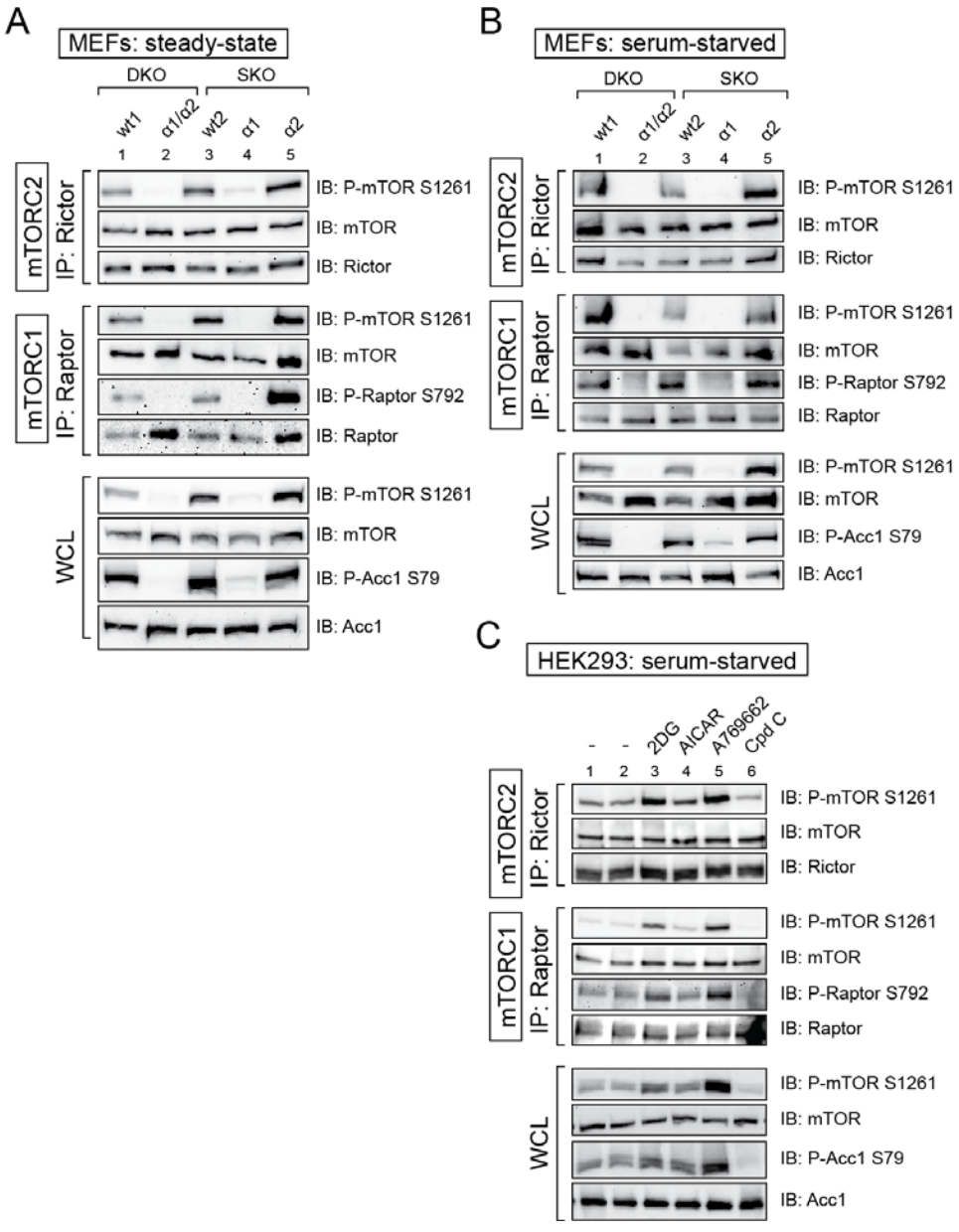
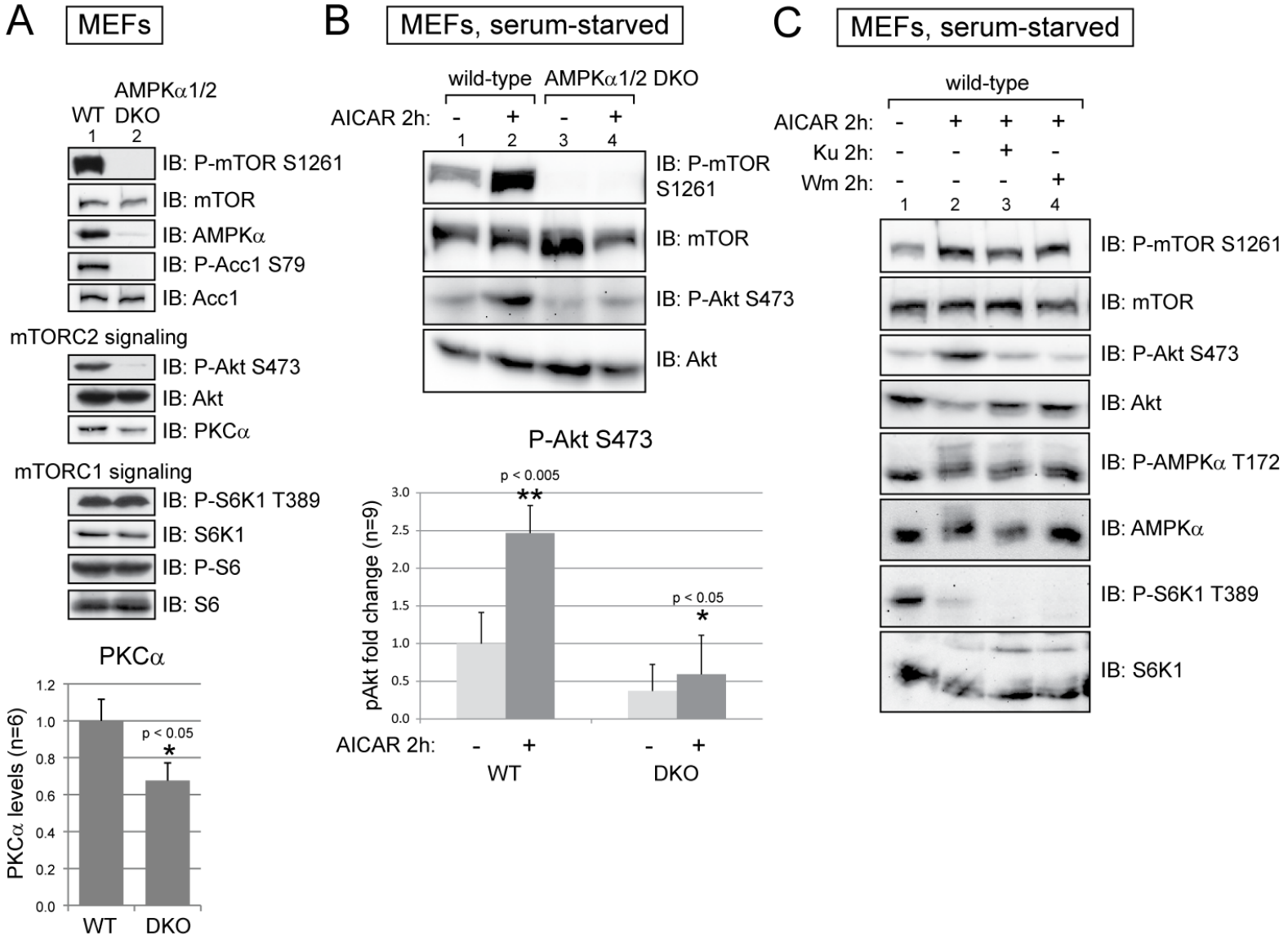
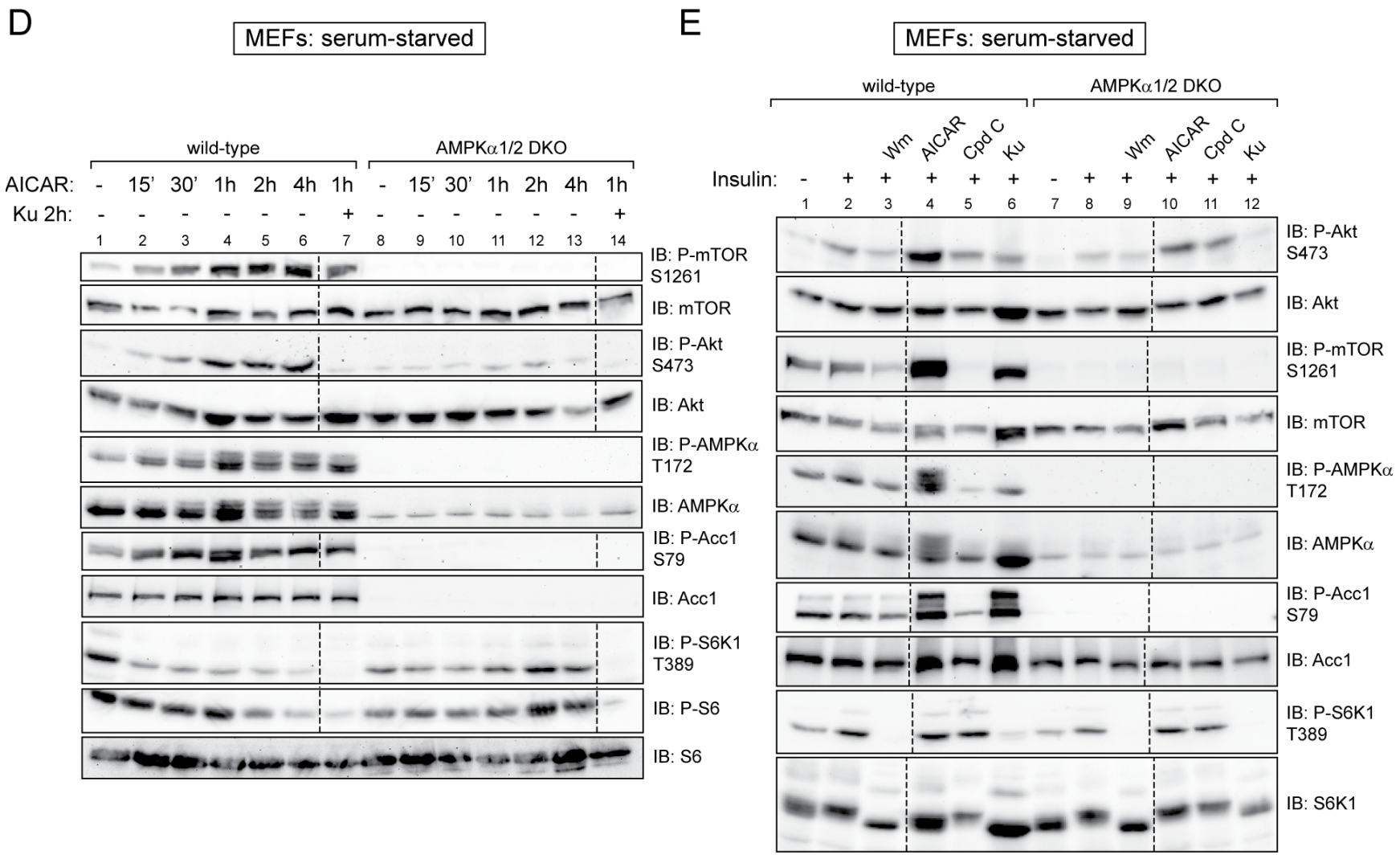

A
MEFs: steady-state
DKO
SKO
wt1
α1/α2
wt2
α1
α2
1
2
3
4
5
mTORC2
IP: Rictor
IB: P-mTOR S1261
IB: mTOR
IB: Rictor
mTORC1
IP: Raptor
IB: P-mTOR S1261
IB: mTOR
IB: P-Raptor S792
IB: Raptor
WCL
IB: P-mTOR S1261
IB: mTOR
IB: P-Acc1 S79
IB: Acc1
B
MEFs: serum-starved
DKO
SKO
wt1
α1/α2
wt2
α1
α2
1
2
3
4
5
mTORC2
IP: Rictor
IB: P-mTOR S1261
IB: mTOR
IB: Rictor
mTORC1
IP: Raptor
IB: P-mTOR S1261
IB: mTOR
IB: P-Raptor S792
IB: Raptor
WCL
IB: P-mTOR S1261
IB: mTOR
IB: P-Acc1 S79
IB: Acc1
C
HEK293: serum-starved
-
-
2DG
AICAR
A769662
Cpd C
1
2
3
4
5
6
mTORC2
IP: Rictor
IB: P-mTOR S1261
IB: mTOR
IB: Rictor
mTORC1
IP: Raptor
IB: P-mTOR S1261
IB: mTOR
IB: P-Raptor S792
IB: Raptor
WCL
IB: P-mTOR S1261
IB: mTOR
IB: P-Acc1 S79
IB: Acc1

Figure 2-3. AMPK promotes mTORC2 signaling and mTOR S1261 phosphorylation in MEFs

(A) AMPK-deficient MEFs have reduced mTORC2 signaling. WT or AMPKα1/2 double-knockout (DKO) MEFs were cultured in DMEM +10% FBS, lysed and analyzed by immunoblot using the indicated antibodies. mTORC2 signaling was determined by Akt S473 phosphorylation and PKCα protein levels. The bar graph below represents total PKCα levels relative to a loading control (Akt) from 6 replicates and normalized to the mean WT value. AMPKα1/2 DKO MEFs show a 32% decrease in PKCα levels relative to WT. **(B)** Activation of AMPK with AICAR promotes mTOR S1261 and Akt S473 phosphorylation in WT but not AMPK-deficient MEFs. WT or AMPKα1/2 DKO MEFs were serum-starved for 20 h, then treated with or without AICAR (2.5mM, 2 h). P-mTOR S1261 and P-Akt S473 were analyzed by immunoblot. The bar graph below represents P-Akt S473/Akt ratios for 9 replicates, normalized to the mean WT (untreated) value. **(C)** AMPK activation of Akt S473 phosphorylation is mTOR- and PI3K-dependent. WT or AMPKα1/2 DKO MEFs were serum-starved for 20 h then treated with AICAR (2.5mM), Ku (1μM), and/or Wm (100nM) for 2 h where indicated. **(D)** Time course of AMPK activation. WT and AMPKα1/2 DKO MEFs were serum-starved for 20 h and treated with AICAR (2.5mM) for the indicated times or a 1 h pre-treatment with Ku (1μM) followed by 1 h AICAR treatment. **(E)** AMPK activation enhances insulin-mediated Akt S473 phosphorylation. WT and AMPKα1/2 DKO MEFs were serum-starved for 20 h and treated as indicated. MEFs were treated for 2 h (total time) with Wm (100nM), AICAR (2.5mM), Cpd C (10μM), or Ku (1μM) and insulin was added (where indicated) for the final 30 min. Error bars are standard deviation. * $p < 0.05$ (two-tailed, paired *t* test); ** - $p < 0.005$ (two-tailed, paired *t* test). *Abbreviations:* Ku: Ku-0063794; Wm: wortmannin; Cpd C: Compound C.


A
MEFs
AMPKα1/2
WT DKO
1 2
IB: P-mTOR S1261
IB: mTOR
IB: AMPKα
IB: P-Acc1 S79
IB: Acc1
mTORC2 signaling
IB: P-Akt S473
IB: Akt
IB: PKCα
mTORC1 signaling
IB: P-S6K1 T389
IB: S6K1
IB: P-S6
IB: S6
PKCα
PKCα levels (n=6)
p < 0.05
*
WT DKO
B
MEFs, serum-starved
wild-type AMPKα1/2 DKO
AICAR 2h: - + - +
1 2 3 4
IB: P-mTOR S1261
IB: mTOR
IB: P-Akt S473
IB: Akt
P-Akt S473
pAkt fold change (n=9)
p < 0.005
**
p < 0.05
*
AICAR 2h: - + - +
WT DKO
C
MEFs, serum-starved
wild-type
AICAR 2h: - + + +
Ku 2h: - - + -
Wm 2h: - - - +
1 2 3 4
IB: P-mTOR S1261
IB: mTOR
IB: P-Akt S473
IB: Akt
IB: P-AMPKα T172
IB: AMPKα
IB: P-S6K1 T389
IB: S6K1


D
MEFs: serum-starved
wild-type
AMPKα1/2 DKO
AICAR: - 15' 30' 1h 2h 4h 1h - 15' 30' 1h 2h 4h 1h
Ku 2h: - - - - - - + - - - - - - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IB: P-mTOR S1261
IB: mTOR
IB: P-Akt S473
IB: Akt
IB: P-AMPKα T172
IB: AMPKα
IB: P-Acc1 S79
IB: Acc1
IB: P-S6K1 T389
IB: P-S6
IB: S6
E
MEFs: serum-starved
wild-type
AMPKα1/2 DKO
Wm AICAR Cpd C Ku
Insulin: - + + + + + - + + + + +
1 2 3 4 5 6 7 8 9 10 11 12
IB: P-Akt S473
IB: Akt
IB: P-mTOR S1261
IB: mTOR
IB: P-AMPKα T172
IB: AMPKα
IB: P-Acc1 S79
IB: Acc1
IB: P-S6K1 T389
IB: S6K1

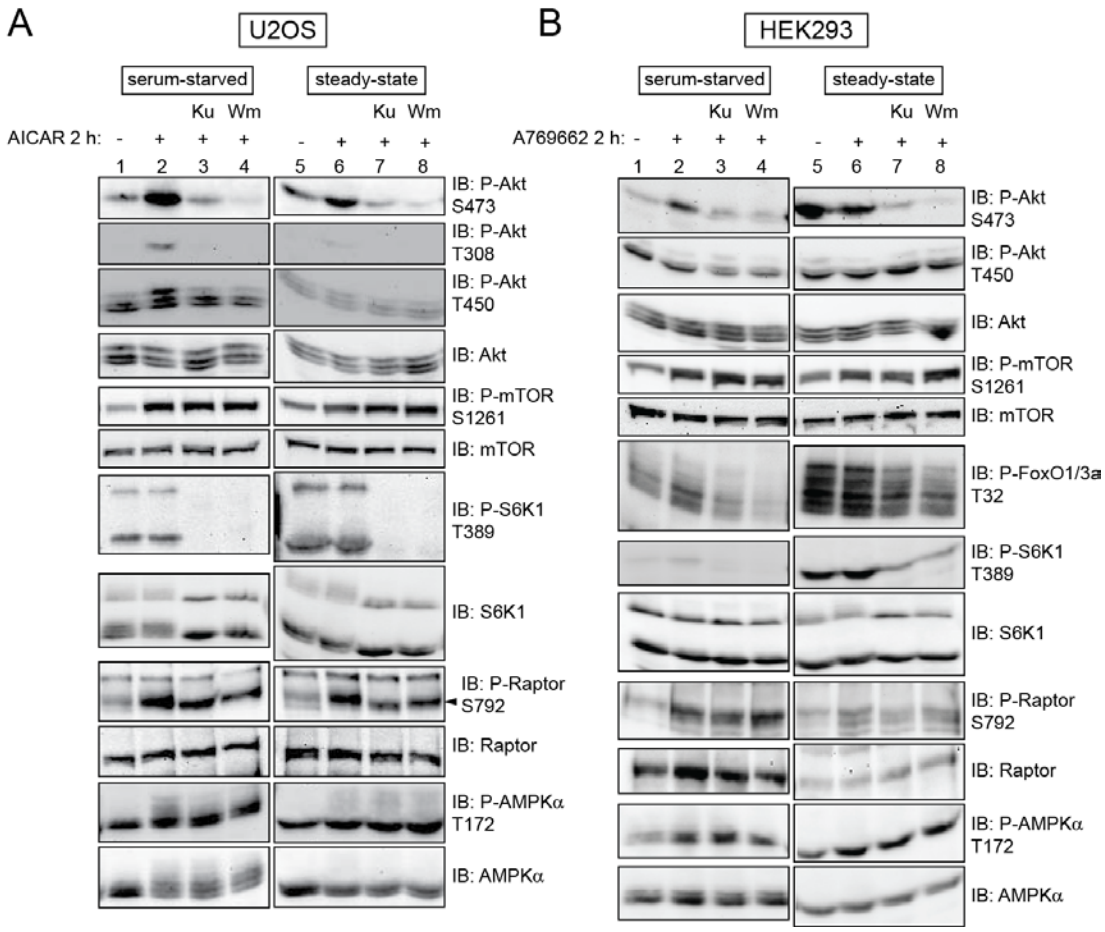
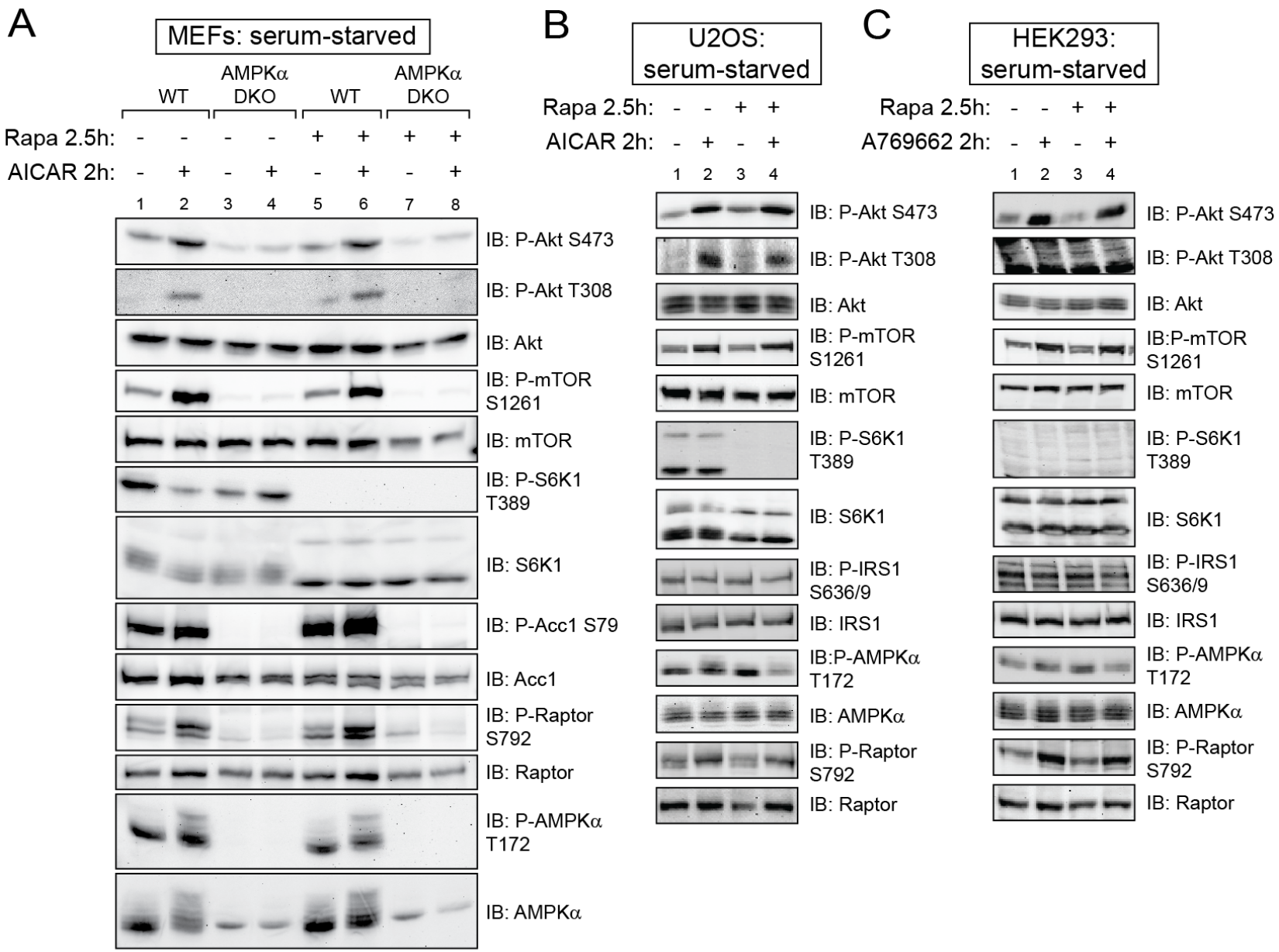


Figure 2-4. AMPK activation promotes mTORC2 signaling to Akt in U2OS and HEK293 cells

(A) AMPK activation with AICAR promotes Akt S473 and T308 phosphorylation in U2OS cells. U2OS cells were serum-starved for 20 h (left panel) or grown in full-serum conditions ("steady-state"; right panel) and treated with AICAR (2.5mM, 2 h), Ku (1mM, 2h), or Wm (100nM, 2h) as indicated. **(B)** AMPK activation with A769662 promotes Akt S473 phosphorylation in serum-starved HEK293 cells. HEK293 cells were serum-starved for 24 h (left panel) or cultured in steady-state conditions (right panel), and treated with A769662 (10μM, 2 h), Ku (1mM, 2h), or Wm (100nM, 2h) as indicated. Abbreviations: Ku: Ku-0063794; WM: wortmannin. The experiments in *(A)* and *(B)* were performed by Hugo Acosta-Jaquez.

Figure 2-5. AMPK activation mediates Akt phosphorylation independently of the mTORC1 negative feedback loop

Rapamycin (Rapa) does not induce Akt S473 or T308 phosphorylation in serum-starved MEFs, U2OS cells, or HEK293 cells and does not alter AMPK-activated Akt phosphorylation. **(A)** WT or AMPKα1/2 DKO MEFs were serum-starved for 20 h and pretreated with rapamycin (20ng/mL) for 30 min where indicated followed by AICAR (2.5mM) for 2 h. **(B)** U2OS cells were treated and analyzed as in *(A)*. **(C)** HEK293 cells were serum-starved for 20 h and pretreated with rapamycin for 30 min where indicated followed by A769662 (10μM) for 2 h. The experiment in *(A)* was performed by me and the experiments in *(B)* and *(C)* were performed by Hugo Acosta-Jaquez.


A
MEFs: serum-starved
WT
AMPKα DKO
WT
AMPKα DKO
Rapa 2.5h: - - - - + + + +
AICAR 2h: - + - + - + - +
1 2 3 4 5 6 7 8
IB: P-Akt S473
IB: P-Akt T308
IB: Akt
IB: P-mTOR S1261
IB: mTOR
IB: P-S6K1 T389
IB: S6K1
IB: P-Acc1 S79
IB: Acc1
IB: P-Raptor S792
IB: Raptor
IB: P-AMPKα T172
IB: AMPKα
B
U2OS: serum-starved
Rapa 2.5h: - - + +
AICAR 2h: - + - +
1 2 3 4
IB: P-Akt S473
IB: P-Akt T308
IB: Akt
IB: P-mTOR S1261
IB: mTOR
IB: P-S6K1 T389
IB: S6K1
IB: P-IRS1 S636/9
IB: IRS1
IB:P-AMPKα T172
IB: AMPKα
IB: P-Raptor S792
IB: Raptor
C
HEK293: serum-starved
Rapa 2.5h: - - + +
A769662 2h: - + - +
1 2 3 4
IB: P-Akt S473
IB: P-Akt T308
IB: Akt
IB:P-mTOR S1261
IB: mTOR
IB: P-S6K1 T389
IB: S6K1
IB: P-IRS1 S636/9
IB: IRS1
IB: P-AMPKα T172
IB: AMPKα
IB: P-Raptor S792
IB: Raptor

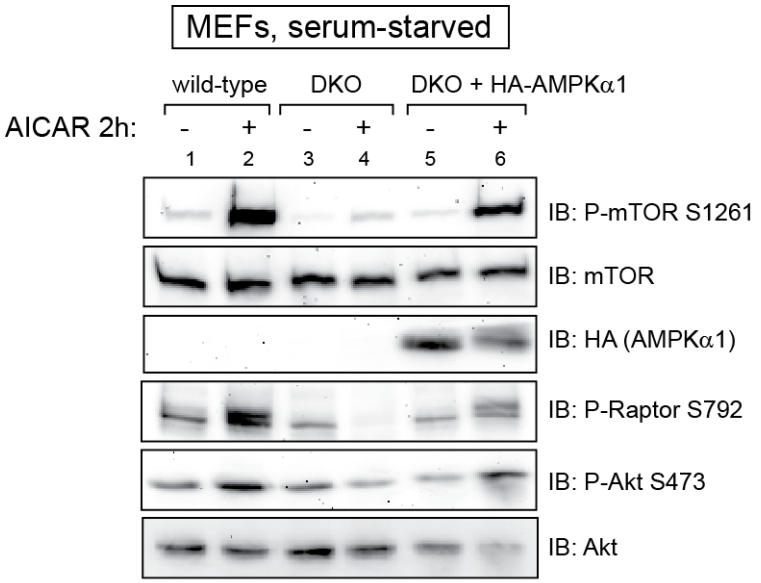
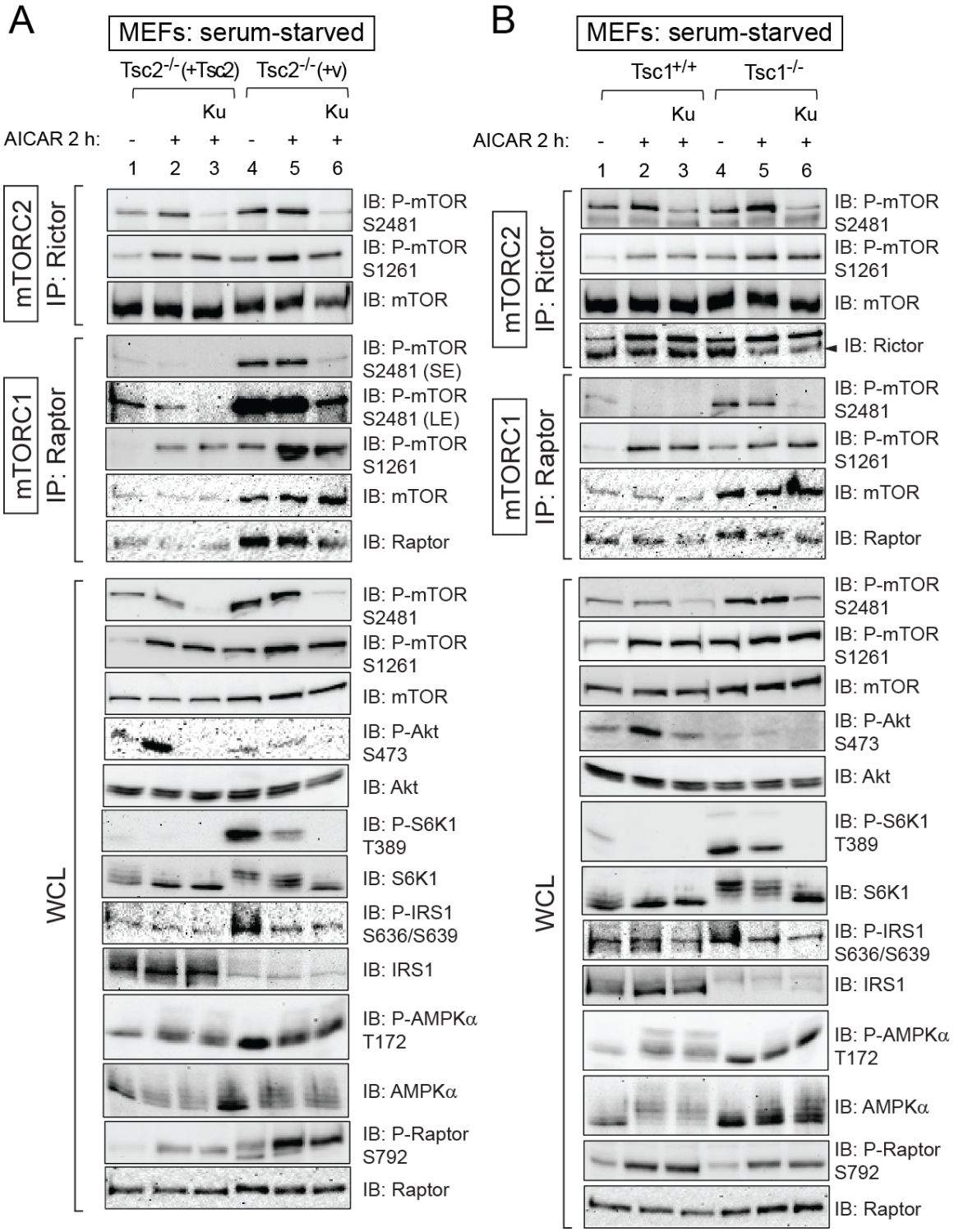


Figure 2-6. Expression of AMPKα1 in AMPKα1/2 double-knockout cells rescues mTOR S1261 and Akt S473 phosphorylation

AMPKα1 rescues mTOR S1261 and Akt S473 phosphorylation in AMPKα1/2 DKO MEFs. HA-AMPKα1 was stably expressed in AMPKα1/2 DKO MEFs. WT AMPKα1/2 DKO, or AMPKα1/2 DKO (+HA-AMPKα1) MEFs were serum-starved for 20 h and treated with or without AICAR (2.5mM) for 2 h. Cells were lysed and analyzed by immunoblot with the indicated antibodies. The AMPKα1/2 DKO MEFs and DKO MEFs expressing HA-AMPKα1 were from Tsukasa Suzuki (Ken Inoki laboratory, University of Michigan, Ann Arbor, MI) and the experiment was performed by me.

Figure 2-7. AMPK promotes mTOR S2481 autophosphorylation within mTORC2 but not mTORC1

For both *(A)* and *(B)*, cells were serum-starved for 20 h followed by treatment with AICAR (2.5 mM) and Ku (1 μM) where indicated. Cells were lysed with a buffer containing 0.3% CHAPS and mTORC2 and mTORC1 were isolated by anti-rictor or anti-raptor immunoprecipitation, respectively. mTOR autophosphorylation was analyzed using an mTOR P-S2481 antibody. **(A)** Rictor and raptor immunoprecipitates and whole cell lysates (WCL) from $Tsc2^{-/-}$ MEFs stably expressing Tsc2 (+Tsc2) and $Tsc2^{-/-}$ MEFs expressing an empty vector (+v) were analyzed by immunoblot with the indicated antibodies. **(B)** As in *(A)*, but with $Tsc1^{-/-}$ and $Tsc1^{+/+}$ MEFs. SE: short exposure; LE: long exposure. The experiments in (A) and (B) were performed by Hugo Acosta-Jaquez.


A
MEFs: serum-starved
Tsc2-/-(+Tsc2)
Tsc2-/-(+v)
Ku
Ku
AICAR 2 h: - + + - + +
1 2 3 4 5 6
mTORC2 IP: Rictor
IB: P-mTOR S2481
IB: P-mTOR S1261
IB: mTOR
mTORC1 IP: Raptor
IB: P-mTOR S2481 (SE)
IB: P-mTOR S2481 (LE)
IB: P-mTOR S1261
IB: mTOR
IB: Raptor
WCL
IB: P-mTOR S2481
IB: P-mTOR S1261
IB: mTOR
IB: P-Akt S473
IB: Akt
IB: P-S6K1 T389
IB: S6K1
IB: P-IRS1 S636/S639
IB: IRS1
IB: P-AMPKα T172
IB: AMPKα
IB: P-Raptor S792
IB: Raptor
B
MEFs: serum-starved
Tsc1+/+
Tsc1-/-
Ku
Ku
AICAR 2 h: - + + - + +
1 2 3 4 5 6
mTORC2 IP: Rictor
IB: P-mTOR S2481
IB: P-mTOR S1261
IB: mTOR
IB: Rictor
mTORC1 IP: Raptor
IB: P-mTOR S2481
IB: P-mTOR S1261
IB: mTOR
IB: Raptor
WCL
IB: P-mTOR S2481
IB: P-mTOR S1261
IB: mTOR
IB: P-Akt S473
IB: Akt
IB: P-S6K1 T389
IB: S6K1
IB: P-IRS1 S636/S639
IB: IRS1
IB: P-AMPKα T172
IB: AMPKα
IB: P-Raptor S792
IB: Raptor

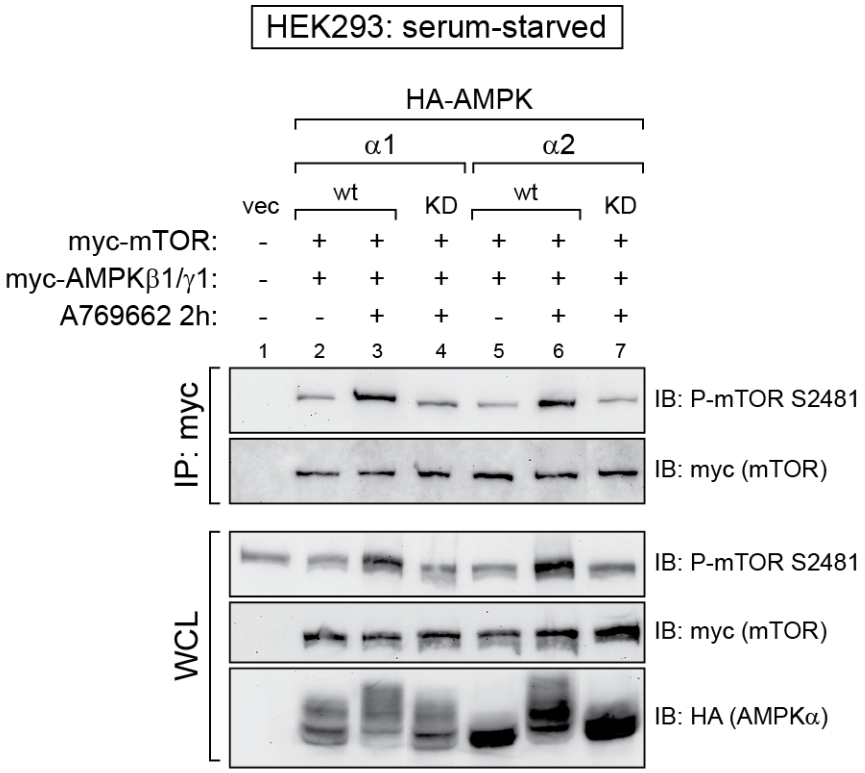


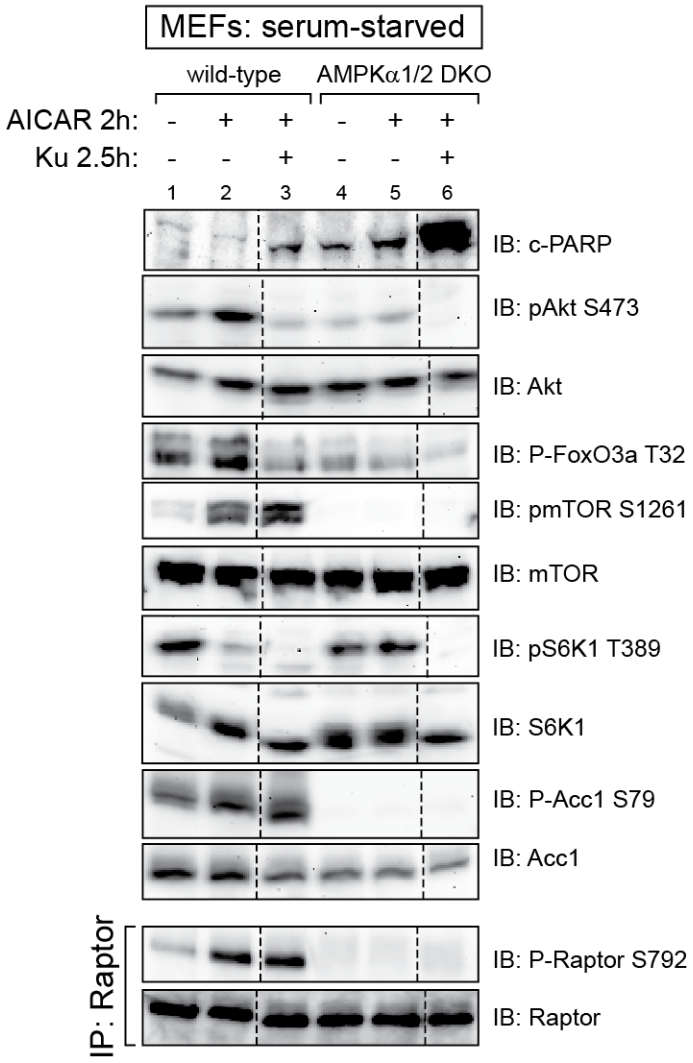
Figure 2-8. Activation of exogenous AMPK promotes mTOR S2481 autophosphorylation

HEK293 cells grown in 10 cm dishes were transfected with HA-AMPKα (α1-wt, α1-KD, α2-wt, or α2-KD), myc-AMPKβ1, myc-AMPKγ1, and myc-mTOR where indicated. 24 h post-transfection, cells were serum-starved for an additional 20 h and treated with A769662 (10μM) for 2 h. Cells were lysed in a buffer containing 0.3% CHAPS, and myc-mTOR was immunoprecipitated with an anti-myc antibody. Autophosphorylation of myc-mTOR was assayed using a P-S2481 antibody in the myc IP. mTOR S2481 autophosphorylation was also monitored in whole cell lysate (WCL). Activation of AMPK was monitored by HA-AMPKα upward mobility shift.

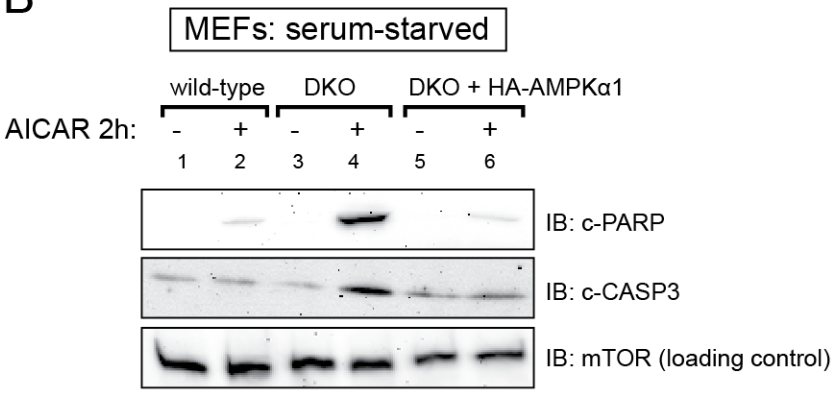
Figure 2-9. Loss of AMPK correlates with increased AICAR-induced apoptosis

(A) Acute AICAR treatment induces apoptosis in AMPKα1/2 DKO MEFs, but not WT MEFs. WT or AMPKα1/2 DKO MEFs were serum-starved for 20 h and treated with AICAR (2.5mM, 2 h), Ku (10μM, 2 h), and Cpd C (10μM, 2 h) where indicated. Apoptosis was monitored by PARP cleavage (c-PARP). Akt signaling was monitored by Akt S473 and FoxO3a T32 phosphorylation. **(B)** HA-AMPKα1 rescues resistance to AICAR-induced apoptosis in AMPKα1/2 DKO MEFs. WT, AMPKα1/2 DKO, or AMPKα1/2 DKO MEFs stably expressing HA-AMPKα1 where serum-starved for 20 h and treated with or without AICAR (2.5mM) for 2 h. Apoptosis was monitored by PARP cleavage (c-PARP) and Caspase 3 cleavage (c-Casp3). mTOR was used as a loading control. AMPKa1/2 DKO MEFs expressing HA-AMPKa1 were from Tsukasa Suzuki (Ken Inoki laboratory, University of Michigan, Ann Arbor, MI) and the experiment was performed by me.

A


MEFs: serum-starved
wild-type
AMPKα1/2 DKO
AICAR 2h: - + + - + +
Ku 2.5h: - - + - - +
1 2 3 4 5 6
IB: c-PARP
IB: pAkt S473
IB: Akt
IB: P-FoxO3a T32
IB: pmTOR S1261
IB: mTOR
IB: pS6K1 T389
IB: S6K1
IB: P-Acc1 S79
IB: Acc1
IP: Raptor
IB: P-Raptor S792
IB: Raptor

B


MEFs: serum-starved
wild-type
DKO
DKO + HA-AMPKα1
AICAR 2h: - + - + - +
1 2 3 4 5 6
IB: c-PARP
IB: c-CASP3
IB: mTOR (loading control)

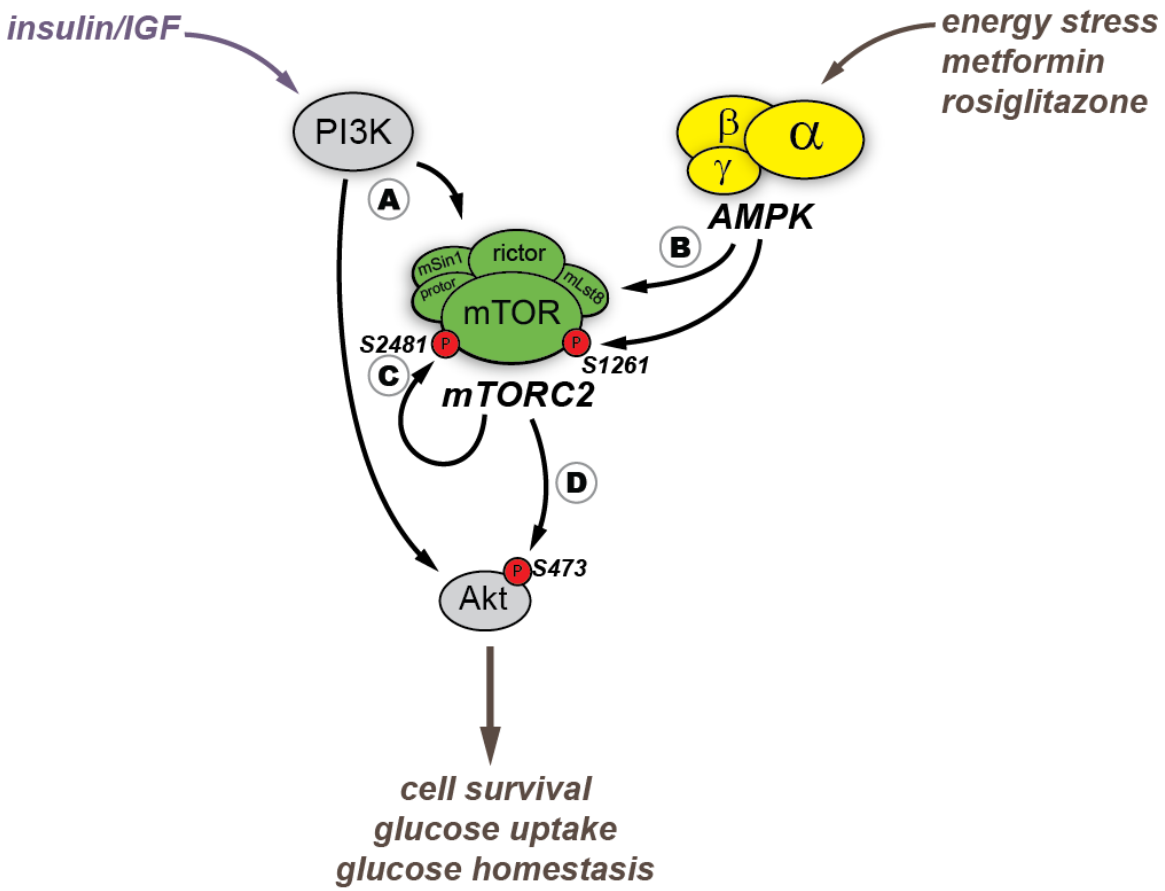


Figure 2-10. Model for how AMPK promotes mTORC2 signaling

(A) In the presence of growth factors, such as insulin and IGF, PI3K signaling leads to phosphorylation and activation of Akt. Akt S473 phosphorylation is mediated by mTORC2, which may depend on PI3K for its activity. **(B)** Energy stress or AMPK-activating drugs promote AMPK signaling. AMPK phosphorylates mTOR on S1261 and activates mTORC2, **(C)** promoting mTORC2-associated mTOR S2481 autophosphorylation and **(D)** Akt S473 phosphorylation. mTORC2-Akt signaling, which promotes metabolic and survival processes, is active in the presence of growth factors, but through AMPK can also be activated in absence of growth factors. During insulin resistance, the insulin-PI3K pathway is depressed. Drugs used to treat diabetes, such as metformin and rosiglitazone, activate AMPK which may enhance insulin signaling by activating mTORC2, ultimately restoring metabolic homeostasis.

CHAPTER 3

Conclusions

3-1. Discussion

The mTOR S1261 kinase: unfinished business

The identification of AMPK as an mTOR kinase was the window through which I viewed AMPK as a regulator of mTORC2. The original screen for mTOR S1261 kinases, which revealed AMPKα1 and AMPKα2 as likely candidates, is an unbiased discovery approach for signal transduction that is gaining popularity. Although the kinome coverage was reasonable (~300 kinases), it depended on the availability of purified, active kinases, thus leaving out predicted, poorly-characterized, or otherwise difficult to prepare kinases, which includes more than 200 kinases. Nevertheless, this screen had other positive hits that have not been thoroughly analyzed. These included several CaMK/AMPK-like kinases and NEK family kinases. The AMPK-like kinases include the MAP/microtubule-affinity protein kinases (MARKs), which are phosphorylated by LKB1[198]. Although my data suggest that AMPK is the dominant mTOR S1261 kinase in mouse fibroblasts, they do not preclude the possibility that one or more other kinases also contribute to mTOR S1261 phosphorylation in other cell types or contexts. I attempted an initial shRNA-based screen in three cell lines using lentiviral

vectors, however several issues prevented a complete analysis of these knockdowns including lack of knockdown confirmation and a lack of a reporter (e.g. bi-cistronic GFP) to confirm shRNA expression (which was merely assumed if cells survived selection). Thus, preliminary knockdown experiments may have resulted in false-negative results. This attempt at analyzing the kinome screen with shRNAs was not without positive results, however. Both AMPKα1and AMPKα2 shRNAs, as well as the AMPK-related MARK4, reduced mTOR S1261 phosphorylation. We know very little about MARK4, which was cloned less than 10 years ago[324], but a recent study found that global MARK4 knockout in mice lead to insulin hypersensitivity, a phenotype similar to S6K1 knockout and suggested a role in metabolic homeostasis[306,325]. Preliminary data from Hugo Acosta-Jaquez and I showed that knockdown of MARK4 reduced S1261 phosphorylation. Hugo also observed reduced S6K1 T389 phosphorylation, suggesting that MARK4 promotes mTORC1 signaling. This is consistent with previous findings from the Fingar laboratory indicating that mTOR S1261 phosphorylation promotes mTORC1 signaling[22]. MARK4 could, therefore, be a kinase that mediates mTORC1-associated mTOR S1261 phosphorylation, a hypothesis that requires further investigation.

The fact that many of the NEK kinases regulate mitotic processes is curious, because mTORC1 is reportedly hyperactive during mitosis, promoting IRES-dependent translation during this cell cycle phase[111]. The mTORC1 component raptor is also phosphorylated by cdc2 and GSK3 during mitosis[111,112]. While one could test the NEK kinases directly, simply asking whether mTOR

S1261 phosphorylation changes during the cell cycle would be a reasonable first step. This could be accomplished by inducing cell cycle arrest and synchronizing cells pharmacologically or isolate cells in difference phases by cell-sorting techniques.

In general, the mTOR S1261 kinome screen candidates should be more carefully analyzed by knockdown-based screening, but with a few improvements: 1) using a viral vector with a reporter (GFP) to assess infection efficiency, 2) a quantitative PCR approach to verify knockdown and uncover false-negatives, followed by immunoblot analysis of actual positives and 3) considering replacement of the viral vector shRNA approach with siRNA transfection, which is both time-efficient and requires no selection scheme. The use of AMPK knockout MEFs in my studies has proved invaluable and while not all of the mTOR S1261 candidate kinases have been knocked out in mice, any available MEFs from knockouts that have been generated would nicely complement RNAi-based screening. While the remaining candidate kinases may not prove to be physiologically relevant mTOR kinases, in the course of screening one could analyze mTORC1 and mTORC2 signaling readouts for serendipitous discovery of novel, indirect mTOR regulators.

mTORC2 regulation: AMPK and beyond

AMPK is an energy-sensing kinase that regulates both metabolism and cell survival. Some of its upstream regulators, such as LKB1 and sestrin1/2, promote Akt signaling[250,300]. While it has not been described previously, it is not surprising that mTORC2, the principal Akt hydrophobic motif kinase, is involved

in this process. The significance of my work and future studies that build off of my work may therefore lie in *how* AMPK regulates mTORC2. AMPK phosphorylates mTOR within mTORC2 and that it promoted mTORC2-associated autophosphorylation. While autophosphorylation does not technically measure mTOR intrinsic catalytic activity, it does represent a practical, correlative readout of mTORC1 or mTORC2 activity in intact cells[108], an advantage over *in vitro* kinase reactions (which are necessarily cell-free).

In Chapter 2, I proposed a model whereby AMPK promotes mTORC2 signaling to Akt, independent of growth factors. In normal, healthy tissues, growth signals are not constantly engaging cells and therefore hormone-dependent survival signals are often suppressed. I hypothesize that AMPK is mobilized by routine metabolic stresses, such as exercise (muscle contraction), to utilize these survival pathways in the absence of growth signals. In pathological states, this suppression may be chronic, as in insulin resistance, and re-activation of these pathways by AMPK may be due to this AMPK-mTORC2 signaling mechanism. Importantly, the action of anti-diabetics such as metformin and rosiglitazone, which activate AMPK, may be explained mechanistically by this AMPK-mTORC2 model.

I found that AMPK phosphorylates mTOR on S1261 and increases mTORC2 -associated S2481 autophosphorylation. Further investigation is necessary to clarify how this works. First, does AMPK promote mTORC2 activity towards its substrate *in vitro*? We expect kinase activity of mTORC2 toward its downstream substrate to reflect on its ability to autophosphorylate, however it

should be verified through *in vitro* kinase assay. AMPK could enhance kinase activity through direct binding, thus one could test whether AMPK is sufficient to promote mTORC2 activity by adding active, recombinant AMPK$\alpha/\beta/\gamma$ to an mTORC2 *in vitro* kinase reaction. Is AMPK interaction with mTORC2 more than just transient? My attempts to see if endogenous AMPKα co-precipitated with raptor, rictor, or mTOR were negative and hence inconclusive. A more rigorous approach is warranted to co-immunoprecipitate AMPK subunits with mTORC2 subunits (both endogenous and exogenous) to find whether AMPK binding is transient, constitutive, or, perhaps, regulated.

A second approach to help understand the activation of mTORC2 by AMPK is to clarify the role of mTOR S1261 phosphorylation. To demonstrate the importance of S1261 in mTORC2 signaling, I have attempted overexpressing both mTOR S1261A and S1261D mutants, however neither affected Akt S473 phosphorylation. Suppressing or enhancing Akt phosphorylation in this manner could require the mTOR S1261A mutant to act dominantly, which it may not. The experiment most likely requires knockdown of endogenous mTOR in addition to expression of mTOR S1261A. Unfortunately, I have been unable to knock down mTOR sufficiently to inhibit mTORC2 signaling to Akt. This issue must be resolved before it can be determined whether AMPK promotes mTORC2 signaling through phosphorylation of mTOR S1261. I have not studied the effect of mTOR S1261A mutant expression on mTORC2 substrates other than Akt. Analysis of mTORC2 signaling to SGK1 and PKCα may prove to be more sensitive to mTOR knockdown than Akt, as other kinases have been shown to

phosphorylate Akt, compensating for chronic loss of mTORC2 within the cell[66,69]. The Fingar laboratory previously reported that mutation of mTOR S1261 to alanine reduced mTORC1-associated mTOR S2481 autophosphorylation but we do not know whether it has a similar effect in mTORC2[22]. We also do not know whether this mutant reduces mTORC1 or mTORC2 phosphorylation of downstream substrates in *in vitro* kinase reactions. Such experiments would support the hypothesis that AMPK regulates mTORC2 through S1261 phosphorylation.

Experiments should also be designed to test the possibility that AMPK does not actually activate mTORC2 through a direct interaction. One possibility is that AMPK activates mTORC2 through Tsc1/2. AMPK phosphorylates and activates Tsc2 (S1345) to suppress mTORC1 signaling and co-immunoprecipitates with Tsc2[103]. Tsc1/2 was recently found to activate mTORC2, despite the fact that it inhibits mTORC1, and Tsc1/2 also co-immunoprecipitated with mTORC2, but not mTORC1[74]. These two studies suggest the possibility that AMPK, Tsc2, and mTORC2 may form a complex that facilitates AMPK-mediated mTOR phosphorylation and activation of mTORC2. Consistent with Huang *et al.*, Tsc1/2 function is necessary for the ability of mTORC2 to phosphorylate Akt, however Tsc1/2 function is not absolutely required for AMPK to activate mTORC2 intrinsic catalytic activity. Therefore, while Tsc1/2 does not play a role in kinase activation by AMPK *per se*, Tsc1/2 may be necessary to promote substrate recruitment, docking, or positioning relative to the kinase. Identifying and characterizing an AMPK-Tsc2-mTORC2

signaling complex would greatly broaden our understanding of mTORC2 regulation.

I found that in growth factor-deprived cells, mTORC1 signaling, already very low, was not responsible for AMPK-mediated Akt phosphorylation. Rapamycin, which inhibits mTORC1 negative feedback signaling to the PI3K/Akt pathway, did not increase Akt phosphorylation nor did it enhance AMPK-mediated Akt phosphorylation. This suggests that AMPK does not act on mTORC2 indirectly through mTORC1 but rather through a novel mechanism. It would be interesting, however, to see if rapamycin treatment inhibited AMPK-induced Akt phosphorylation during insulin treatment. The reasoning is that in the presence of growth factors, AMPK could promote Akt phosphorylation *in part* through suppression of the mTORC1 negative feedback loop. AMPK could employ two mechanisms of promoting mTORC2/Akt signaling, depending on growth factor status: first, direct activation of mTORC2 under growth factor-deprived conditions (which I have demonstrated), and second, a combined direct and indirect activation during growth factor stimulation. Under this second scenario, rapamycin treatment would inhibit the ability of AMPK to promote Akt phosphorylation. Another negative feedback pathway, recently discovered, involves S6K1 phosphorylation of rictor at T1135 in the presence of growth factors, which may inhibit mTORC2 signaling, though its exact role is controversial[116–119]. Perhaps AMPK inhibits mTORC1 in part to suppress this feedback pathway, ensuring maximum activation of mTORC2. The rictor T1135A mutant, which was reported to resist Akt dephosphorylation following insulin

treatment, could sensitize mTORC2 to AMPK signaling. While these alternative pathways should be explored, the significance of our work is that AMPK activates mTORC2 in a novel way, independent of serum growth factors and independent of mTORC1 feedback signaling.

AMPK and mTORC2 both promote survival and metabolic signaling through various targets, many of which regulate transcription. If AMPK directly regulates mTORC2, the sets of target genes would overlap. Literature mining reveals both parallel and paradoxical pathways. For example, AMPK promotes Akt (and possibly SGK1) functions, which included the phosphorylation and inhibition of FoxO transcription factors[250], however AMPK phosphorylates FoxO3a on unique sites in mammals and *C. elegans*, promoting transcription of a subset of its targets[248]. The nature of this apparent opposition is unclear, so in lieu of analyzing the intermediate signaling pathways one could ask: what genes are regulated by both AMPK and mTORC2? Using modern approaches for analyzing genome-wide gene expression (i.e. RNA-Seq), one could easily obtain gene regulation data after activating AMPK with and without mTORC2 (rictor or Sin1 knockdown of the same cell line, or compare cells from wild-type or knockout mice). Many of the target genes can be inferred by comparing expression analyses already published for AMPK and Akt activation, though selectively probing mTORC2 would additionally yield Akt-independent results. Importantly, this could indirectly reveal novel mTORC2 effectors, ultimately broadening our knowledge of mTORC2 functions.

AMPK-mTORC2 *in vivo*

Animal studies are the next logical step in determining the physiological role for both AMPK-mediated mTOR S1261 phosphorylation (S1261) and AMPK-mTORC2 signaling. To begin with, one should examine various healthy, mammalian tissues for mTOR S1261 phosphorylation to formulate a baseline survey of its tissue specificity. Given a strong correlation between AMPK and mTORC2 in glucose homeostasis, some important tissues to examine first would be liver, pancreas, skeletal muscle, adipose tissue, the gut, and the brain (hypothalamus). In addition, examining these primary tissues could also uncover a correlation between S1261 phosphorylation and mTORC2 signaling readouts (e.g. Akt, SGK1, and PKCα phosphorylation and/or stability). Because mTOR S1261 is regulated by AMPK, we would ask whether exercise, which elevates AMPK signaling in some tissues, or treatment of these animals with AMPK activating drugs, such as metformin or AICAR, also increases mTOR S1261 phosphorylation. Next, I would move from healthy animals into models of acquired metabolic syndrome, such as *ob*/*ob* mice or Zucker rats fed high-calorie/high-fat diets. As treatment with AMPK-activating drugs enhances insulin sensitivity and restores metabolic homeostasis in these animals, perhaps this coincides with a rise in mTOR S1261 phosphorylation. In all of these experiments, mTORC2-associated mTOR S2481 autophosphorylation status could be monitored to make the case that mTORC2 catalytic state also correlates with AMPK activation *in vivo*.

Though these proposed studies could demonstrate that AMPK-mediated mTOR S1261 phosphorylation and AMPK-mTORC2 signaling occurs *in vivo*, what physiological function does it serve? A transgenic mouse (mTOR S1261A knock-in, for example), could be generated to test its role in AMPK and mTOR signaling in mammalian physiology. Without first demonstrating in cultured cells that mTOR S1261 phosphorylation contributes to mTORC2 activity and signaling by AMPK, it would be difficult to justify this *in vivo* approach. Given that the mTOR sequence surrounding S1261 is reasonably conserved in invertebrates (though not conserved in fungi or plants), it may prove valuable to first find if it is phosphorylated and physiologically relevant in genetic model systems such as *D. melanogaster*. The position of mTOR S1261 in the fruit fly (TOR T1232) is conserved as a threonine and the AMPK consensus motif is reasonably conserved as well, supporting the possibility that it is phosphorylated. The lack of serine or threonine at the equivalent site in *C. elegans* mTOR (LET-363) suggests that this phosphorylation event is not conserved in all invertebrates. LKB1-AMPK signaling has been implicated in extending lifespan in both *C. elegans* and *D. melanogaster* [255] and Akt signaling correlates with decreased lifespan[326,327]. While it is possible that activation of mTORC2/Akt by AMPK is not a conserved pathway between invertebrates and mammals, it would be interesting to see if AMPK was capable of cross-activating Akt in invertebrate systems.

Does loss of mTORC2 components exacerbate loss of AMPK components *in vivo*? Global knockouts of rictor and Sin1 are embryonic lethal, thus tissue-

specific or conditional knockout approaches have been implemented to study mTORC2, and metabolic defects have been reported for rictor loss in adipose, skeletal muscle, pancreas, and liver[39]. These mice should be revisited in the context of AMPK signaling. Perhaps they respond more poorly to AMPK activating drugs when they develop insulin resistance or hyperglycemia. AMPKα1/2 double knockout mice are embryonic lethal, however AMPKα1 and α2 single knockout mice have very mild metabolic phenotypes. AMPKα1 is ubiquitously expressed, but AMPKα2, which is expressed highest in skeletal muscle. Crossing one of these with a tissue-specific rictor knockout mouse would allow us to test whether the loss of AMPK results in a more severe phenotype. Both Akt and AMPK control glucose uptake, a critical function in skeletal muscle, so global knockout of AMPKα2, which is highly expressed in normal muscle, combined with skeletal muscle-specific knockout of rictor could, in comparison with the parental mice, have a severe glucose uptake defect leading to hyperglycemia and hyperinsulinemia.

Closing Remarks

The studies presented here have shed light on how specific cellular stresses regulate mTORC1 and mTORC2 in mammalian cells. The model for upstream regulation of mTORC2, which is currently not well understood, is now expanded to include direct modification and activation by AMPK. Further work is necessary to determine what consequences AMPK regulation of mTORC2 has in downstream signaling *in vitro*, as how this mechanism translates to primary tissue, organ, and whole-body homeostasis. Pathological consequences of

defects in both mTORC2 and AMPK include poor glucose and lipid metabolism, insulin resistance, and hyperglycemia, all hallmarks of type 2 diabetes, a growing, modern epidemic[60,281,328,329] . As we gain insight into how mTORC2 and AMPK interact at both the molecular and organismal levels, not only will we better understand the mechanism underlying current approaches for treating human metabolic disorders that activate AMPK, but we can also design new therapeutics based on targeting both AMPK and mTORC2. The balancing of metabolic and survival pathways is both complex and still very much a mystery, and I hope the researchers who continue this work meet these challenges with profound thoughtfulness and eagerness.

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