UNIVERSITY OF CALIFORNIA, SAN DIEGO

Identification of Novel Substrates of the Energy-Sensing Kinase, AMP-Activated Protein Kinase, and Regulation of mTOR Signaling During Energy Stress and Mitosis

A Dissertation Submitted in Partial Satisfaction of the Requirements for the Degree Doctor of Philosophy

in

Biology

by

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Dissertation of Dana Meredith Gwinn is approved, and it is acceptable in quality and format for publication on microfilm and electronically:

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this chapter. Chapter Two contains excerpts from material as it appears in Gwinn, D.M., Shackelford, D.S., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., and Shaw, R.J. (2008) AMPK phosphorylation of Raptor mediates a metabolic checkpoint. Molecular Cell 30(2), 214-26. On this publication, I was the primary author. Reuben Shaw directed and supervised this project. David Shackelford assisted with attaining the FACS data, Dan Egan assisted with IP-data, Maria Mihaylova and Debbie Vasquez isolated, treated and analyzed the primary hepatocytes, Annabelle Mery assisted in performing immuno-fluorescence, Ben Turk performed peptide library profiling of AMPK optimal substrate motif.

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ABSTRACT OF THE DISSERTATION

Identification of Novel Substrates of the Energy-Sensing Kinase, AMP-Activated Protein Kinase, and Regulation of mTOR Signaling During Energy Stress and Mitosis

by

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Doctor of Philosphy in Biology

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The ability of cells to sense and respond to changes in cellular nutrient or energy availability is crucial for their survival. AMP-activated protein kinase (AMPK) is a key sensor of cellular energy levels, and is responsible for shutting down energy-costly processes, and turning on processes that will help restore cellular energy levels. The ability of AMPK to inhibit mTORC1 signaling allows

it not only to shut down costly processes like protein synthesis and ribosome biogenesis, but also to allow the energy-restoring process, autophagy to proceed. Additionally, action of AMPK in specialized metabolic tissues like muscle and liver allow it to regulate blood glucose levels, and glucose uptake in the whole organism. Using a bioinformatic and proteomic approach, we have identified the mTORC1 component, raptor, as a novel substrate of AMPK that contributes to its role in downregulation of mTORC1, which is required for engagement of an energy-stress checkpoint. Loss of the ability of AMPK to phosphorylate raptor sensitizes cells to apoptosis during energy poor conditions. We have also investigated the regulation of mTORC1 signaling during mitosis, and its role in altering translation during the cell cycle. Finally, we have identified AMPK phosphorylation sites in the RalGAP complex that may contribute to AMPK regulation of the Ral small GTPases, and the exocyst complex, which has been shown to be important for glucose uptake, insulin secretion and cellular migration. These findings contribute to our understanding of how AMPK functions both in a cell-autonomous fashion in the restoration of cellular energy levels, and cell-non-autonomously in regulation of organismal metabolism.

CHAPTER ONE:

Introduction to mTOR and AMPK signaling: Key Mediators of Cellular and Organismal Growth and Metabolism

Introduction

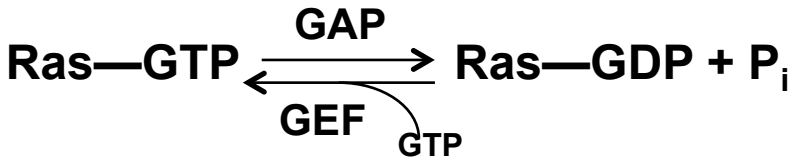
The ability of cells to sense and respond to changes in their intra- and extra-cellular environment is crucial for their growth and survival in dynamic conditions, such as changes in nutrient levels or damage to the cell. In order to detect such changes, cells are dependent on sensor molecules that are capable of being activated under particular environmental conditions. These activated molecules can then "transmit" the signal by modifying another protein, which can then modify another protein, and so on, until an effector molecule is activated, and elicits some change in the cell to help cope with the environmental change. By far the best-studied mechanism of this type of signal transduction is phosphorylation, whereby a kinase catalyzes the addition of a phosphate ion from a molecule of ATP onto a substrate molecule, and this activity is opposed by phosphatases that remove phosphate groups from substrates:

$$\text{Substrate} + \text{ATP} \underset{\text{Phosphatase}}{\overset{\text{Kinase}}{\rightleftharpoons}} \text{Substrate-P}_i + \text{ADP}$$

The mechanisms by which phosphorylation can elicit a change in a substrate molecule are many: the conformation of the protein can be changed, such that catalytic activity might be effected; the localization of the substrate can change based on phosphorylation status; stability of a protein, or its targeting for proteasomal degradation can be regulated by phosphorylation;

and phosphorylation can change binding surfaces such that substrates either bind or dissociate from binding partners.

Another common mechanism of signal transduction is the cycling of small GTP-binding proteins (such as Ras) between an active GTP-bound state and inactive GDP-bound state. The regulation of the GTP-binding is achieved by GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity of the small GTP-binding protein, and thus their inactivation. Opposing the activity of GAPs are guanine nucleotide exchange factors (GEFs) that mediate the switching of GDP for GTP, and thus activation of the small GTP-binding protein.



Many small GTPases contain a CAAX motif at the C-terminus that is lipid modified, and directs the small GTPase to a specific membrane domain of a subcellular compartment. This type of distribution allows small GTPases to regulate their effectors by recruitment to specific subcellular locations, as small GTPases direcly bind their effector molecules (reviewed in Wenneberg et al., 2005).

mTOR signaling

While there are generally a great many signaling molecules within any given cell, there is not a unique sensor, and signaling pathway for every single possible environmental condition. Indeed, many signaling pathways converge at different effector molecules such that the same molecular machinery can be used to control similar cellular processes under diverse conditions. These convergence points, or nodes, are often key effector molecules of the cell. One such node of signaling is the atypical serine/threonine protein kinase, mammalian target of rapamycin (mTOR). In mammalian cells, mTOR exists in one of two complexes; bound to the scaffolding protein, Raptor, GβL (mLST8), and the negative regulatory subunits PRAS40 and DEPTOR, it is referred to as mTOR complex 1 (mTORC1). In complex with GβL, DEPTOR, the scaffolding protein, Rictor, Protor, PRR5, and mSin1, it comprises mTOR complex 2 (mTORC2) (reviewed in Zoncu et al., 2010). TOR was originally identified in yeast as the cellular target of the anti-growth compound, rapamycin; since then, mTORC1 has been found to be a key regulator of protein synthesis, ribosome biogenesis, certain transcriptional programs, metabolism and autophagy, and through regulation of these processes, mTORC1 is tightly linked to cellular growth control. The best characterized substrates of mTORC1 are 4EBP1, S6K and ULK1. Phosphorylation of 4EBP1 by mTORC1 is required for it to release its binding partner, eIF4E, which is then free to promote cap-dependent translation. Phosphorylation of the major mRNA

translation regulating kinase, S6K at its hydrophobic motif by mTORC1 enhances its activity, and thus protein synthesis. ULK1 is the most upstream kinase in the autophagy cascade, and phosphorylation by mTORC1 is thought to inhibit this process (Kim et al., 2011).

The importance of mTORC1 signaling is underscored by the variety of environmental changes, and signaling pathways that modify mTORC1 activity through a variety of mechanisms (Figure 1). mTORC1 is responsive to growth factors, glucose, amino acids, energy stress and hypoxia. mTORC1 activity requires association with the small GTPase, Rheb (Saucedo et al., 2003), which has been suggested to reside in association with a lysosomal compartment (Sancak et al., 2008). Rheb activity is negatively regulated by the tuberin/hamartin (TSC2/TSC1) GAP complex (Inoki et al., 2003; Tee et al., 2003; Zhang et al., 2003), and loss of either component leads to upregulation of mTORC1 signaling. Interestingly, in Drosophila, TCTP has been suggested to have Rheb GEF activity (Hsu et al., 2007), but, this has been called into question (Rehmann et al., 2008), and no GEF has been identified for Rheb in mammalian cells.

Recently, the Rag small GTPases have been identified as key mediators of amino-acid regulation of mTORC1. Unlike most small G-proteins, the Rags act as heterodimers containing RagA or RagB in their GTP-bound forms, and RagC or RagD in their GDP-bound forms (Sancak et al., 2008; Kim et al., 2008). Through the “Ragulator” complex, consisting of MP1, p14 and p18, the

Rags directly bind raptor, and recruit mTORC1 to the Rheb-containing lysosomal compartment, thus contributing to activation of mTORC1 (Sancak et al., 2010). Amino acid deprivation causes mTORC1 to dissociate from these compartments in a Rag-Ragulator dependent way (Sancak et al., 2010). While in yeast, Vam6 has been identified as a GEF for the yeast Rag complex (Binda et al., 2009), no GAPs or GEFs have been identified for the mammalian Rag GTPases.

In addition to regulation by small G-proteins, a number of kinase pathways have been shown to regulate mTOR signaling. A large number of inputs to mTORC1 converge on tuberin (TSC2). Following growth factor stimulation of Akt (Inoki et al., 2002; Potter et al., 2002), Erk (Ma et al., 2005), and RSK (Roux et al., 2004) phosphorylate and inhibit tuberin, allowing Rheb to remain GTP bound, and mTORC1 activity to be high. These signaling pathways to mTORC1 represent important mechanisms by which growth factors can regulate protein synthesis and autophagy.

The energy sensitive LKB1-AMPK pathway (discussed subsequently) also signals to mTORC1 via stimulatory phosphorylation of TSC2 (Inoki et al., 2003; Shaw et al., 2004), resulting in decrease mTORC1 signaling. This axis represents one mechanism by which energy status can be linked to processes downstream of mTORC1. AMPK phosphorylation of TSC2 at S1387 serves as a priming site for phosphorylation of TSC2 by GSK3 at S1383 and S1379 (Inoki et al., 2006). Activation of Wnt signaling inhibits GSK3, and thus TSC2 serves

as an integration point for energy stress signaling via AMPK and Wnt signaling to dictate TSC2 activity and thus downstream mTORC1 signaling.

While phosphorylation of TSC2 appears to be a major hub of signaling input to mTORC1, it has become apparent in recent years that direct phosphorylation of mTORC1 components represents an important mechanism of regulation of mTORC1 activity. The negative regulatory subunit, PRAS40 is phosphorylated by Akt upon growth factor stimulation, causing its dissociation from the complex, and enhanced mTORC1 activity (Sancak et al., 2007; Vander Haar et al., 2007). Additionally, we have shown that AMPK directly phosphorylates raptor during energy stress conditions, inducing association with the phospho-binding protein, 14-3-3, and inhibiting mTORC1 activity (Gwinn et al., 2008). Raptor has also been shown to be directly phosphorylated by the Rsk (Carriere et al., 2008), Erk (Carriere et al., 2011; Langlais et al., 2011), ULK1 (Dunlop et al., 2011), and mTOR itself (Wang et al., 2009; Foster et al., 2010). Thus like TSC2 before it, raptor has emerged as a integration point for kinases mediating different environmental signals.

Intrinsic to mTORC1 signaling is a negative feedback loop, in which S6K phosphorylates a scaffolding protein, IRS1, causing its degradation (Harrington et al., 2004; Shah et al., 2004). By doing so, signals can no longer be transmitted from an activated RTK to PI-3K, shutting down insulin-mediated signaling to mTORC1 via Akt. Another major source of negative feedback on PI3K signaling was recently identified as mTORC1 directly phosphorylating the

Grb10 SH2 adapator protein (Hsu et al., 2011; Yu et al., 2011). This suppression of PI-3K-Akt signaling by mTORC1 is believed to have important therapeutic implications for mTORC1 inhibition as it will simultaneously result in increased activation of PI3K/Akt.

Regulation of translation downstream of mTOR appears to be an important factor in cellular response to nutrient status, but in addition to environmental changes, translation is also regulated in a cell cycle dependent manner, suggesting that mTOR signaling may also be coordinately regulated during cell cycle progression. Specifically, it has been shown to be important that the cell halts cap-dependent translation during mitosis, such that IRES-dependent translation of transcripts important for mitosis can proceed. One suggested mechanism of this alteration of translation is through mitotic up-regulation of 14-3-3σ, which binds and sequesters a number of translation/ initiation factors. In cells lacking 14-3-3σ, the lack of suppression of cap-dependent translation prevents the proper levels of translation of transcripts important for cytokinesis, such as Cdk11, and leads to an increase in bi-nucleate cells. Interestingly, inhibition of mTOR by rapamycin is sufficient to rescue this phenotype (Wilker et al., 2007), demonstrating the importance of regulation of mTOR during mitosis. Accordingly, many components of the mTOR pathway are phosphorylated by the mitotic Cdk, Cdc2 during mitosis, including TSC1 (Astrinidis et al., 2005), raptor (Gwinn et al., 2010; Ramírez-Valle et al., 2010), S6K (Papst et al., 1998; Shah et al., 2003, Hou et al., 2007),

and 4EBP1 (Heesom et al., 2001, Greenberg et al., 2005). Because many of the components of the pathway are regulated during mitosis, it is difficult to determine the importance of regulation of any single component. Mutation of the Cdc2 sites in raptor does not perturb the ability of mTOR signaling to be regulated during mitosis. However, an allele of raptor with mutations in sites of multiple different kinases (including an AMPK site, an mTOR site, and putative GSK3 sites) does show a phenotype typical of mis-regulation of mitotic translation (Ramírez-Valle et al., 2010). This supports the hypothesis that regulation of mTOR signaling during cell cycle progression is more complicated that phosphorylation by a single kinase.

AMPK

The primary sensor of energy stress in all eukaryotic cells is the hetero-trimeric AMP-activated protein kinase (AMPK). AMPK consists of two regulator subunits (β and γ), and a catalytic subunit (α). Like most kinases, AMPK activity requires phosphorylation of its activation loop, which enhances its kinase activity several hundred fold. Biochemical and genetic analyses in worms, flies, and mice have revealed that the serine/threonine kinase LKB1 is the major kinase phosphorylating the activation loop of AMPK under energy stress conditions across metazoans (Hardie, 2007). While LKB1 appears to be the major kinase in most tissues, it has also been shown that CAMKKb is capable of phosphorylating the actiation loop of AMPK in vivo (Towler and

Hardie, 2007). The ability of AMPK to sense the energy status of a cell is dependent on three nucleotide binding CBS domains within the γ subunit. The site 4 nucleotide-binding site is always occupied by AMP, and does not contribute to the regulation of AMPK activity. As intracellular ATP levels fall, and ADP and AMP levels rise, either AMP or ADP bind the second nucleotide binding site, causing a conformational change such that the activation loop phosphorylation is protected from dephosphorylation by a phosphatase, allowing AMPK kinase activity to remain high. The final nucleotide binding site of AMPK can only be occupied by AMP, and when AMP is bound, it causes an allosteric change that enhances AMPK activity 2-5 fold (Xiao et al., 2011).

Upon activation under low ATP conditions, AMPK acts as a metabolic checkpoint in the cell, halting cell growth and suppressing ATP-consuming processes while stimulating ATP-generating processes to restore the initiating loss of ATP (Shaw et al., 2004). In addition to its widespread cell-autonomous role as an energy checkpoint, AMPK also plays key roles in glucose and lipid metabolism in specialized metabolic tissues in mammals and higher eukaryotes such as liver, muscle and adipose tissue (Kahn et al., 2005).

Notably, LKB1, the kinase responsible for phosphorylation of the activation loop of AMPK, is also responsible for phosphorylating the activation loops of the entire family of AMPK related kinases (AMPKRs). This 14-kinase family includes AMPKα1 and α2, the salt-inducible kinases (SIK1, 2, 3), the mammalian Par homologues MARKs 1-4, as well as NUAK1, 2 and BRSK1, 2

and SNRK (Lizcano et al., 2004; Jaleel et al., 2005). Importantly, of all the AMPKRs, only AMPKα1 and AMPKα2 are activated under low ATP conditions, probably due to the fact that only they interact with AMPKγ, which contains the AMP-binding sites (Al-Hakim et al., 2005). However, two other family members, SNARK/Nuak2 and SIK2, have been reported to be activated under low energy conditions (Suzuki et al., 2003; Lefebvre and Rosen, 2005; Du et al., 2008).

Unlike most kinases, LKB1 is not regulated by phosphorylation of its activation loop; instead, its active conformation is achieved through obligatory interactions with the pseudokinase, STRAD, and the scaffolding protein, MO25, which stabilize the activation loop of LKB1 (Zeqiraj et al., 2009). Because LKB1 stability requires its association with STRAD and MO25, and because binding to these proteins holds LKB1 in its active conformation, in most cell types, LKB1 is active. Though there are numerous phosphorylation sites within LKB1 (phosphosite.org) including sites phosphorylated by PKA (Collins et al., 2000), PKC (Song et al., 2008), ATM (Sapkota et al, 2002) and RSK (Sapkota et al., 2001), it is unclear how they affect LKB1 activity.

An important energy-restoring process, autophagy, in which the cell breaks down and recycles proteins and organelle is highly regulated during energy stress conditions (Egan et al., 2011b). As mentioned previously, we have shown that AMPK directly phosphorylates TSC2 as well as the mTORC1 subunit, raptor under conditions of energy stress to downregulate mTORC1

signaling (Inoki et al., 2003; Shaw et al., 2004; Gwinn et al., 2008). This downregulation of mTORC1 signaling lifts the negative regulation of autophagy via mTORC1 inhibitory phosphorylation of ULK1 (Kim et al., 2011). In addition to regulating autophagy through mTORC1 inhibition, AMPK has also recently been shown to directly phosphorylate and promote the activity of the autophagy initiating kinase, ULK1 (Egan et al., 2011a; Kim et al., 2011). Regulation of autophagy represents an important mechanism AMPK employs to restore cellular energy levels.

Akt

Just as AMPK is a key mediator of the cellular response to energy stress, the serine-threonine protein kinase, Akt (PKB) is a key target of insulin/IGF signaling. Upon insulin/IGF binding to the insulin receptor, the receptor undergoes a major conformational change, inducing transphosphorylation of the cytosolic domain, creating binding sites for scaffolding proteins including insulin receptor substrates 1 and 2 (IRS1, IRS2). When bound to the insulin receptor, IRS1 and IRS2 creating binding sites for the regulatory subunit of the lipid kinase, phosphoinositide 3 kinase (PI-3K), and this interaction is responsible for insulin-mediated activation of PI-3K (Manning et al., 2004). Upon activation, PI-3K phosphorylates the membrane lipid phosphoinositide-4-5-biphosphate (PIP_2) to phosphoinositide-3-4-5-triphosphate (PIP_3), which then acts as a signaling molecule, recruiting and activating both PDK1, an upstream kinase of Akt, as well as Akt itself.

Opposing the action of PI-3K is the PIP_3 phosphatase, PTEN (reviewed in Engelman, 2009). Upon activation, Akt plays a key role in cell growth both by stimulation of mTOR signaling, and promoting progression through the cell cycle; through a number of mechanisms including inhibition of apoptosis, and activation of pro-survival pathways, Akt activation exerts protective effects for the cell; and Akt has well documented roles in glucose metabolism and uptake.

Cross-talk between PI-3K and LKB1-AMPK signaling

Given the important roles of AMPK during energy stress and Akt in growth factor signaling, it is not terribly surprising that they regulate a number of the same substrates that are important for cellular responses to nutrient status (Figure 2). As discussed above, AMPK phosphorylates and activates TSC2 during energy stress to downregulate mTORC1 signaling, while Akt phosphorylation on distinct sites of TSC2 following growth factor stimulation inhibits the complex to active mTORC1 signaling. The ability to regulate TSC2 is important for both Akt and AMPK to signal to mTORC1, and thus to properly regulate translation and autophagy according to the nutrient status of the cell.

In addition to cross-talk between Akt and AMPK at TSC2, the tumor suppressor, forkhead box transcription factor, FOXO3a has also been demonstrated to be a substrate of both kinases. Akt phosphorylation of FOXO3a induces association of FOXO3a with 14-3-3, and shuttling from the nucleus to the cytoplasm (Brunet et al., 1999). Conversely, phosphorylation of

the transactivation domain of FOXO3a by AMPK enhances its transcriptional activity (Greer et al., 2007). Regulation of FOXO3a by both Akt and AMPK represent important mechanism of transcriptional changes enacted by the cell in response to growth factor stimulation or energy stress, respectively.

Other substrates shared by Akt and AMPK include the Rab GAPs, AS160/TBC1D4 and TBC1D1, which have been implicated in Glut4 translocation to the plasma membrane (reviewed in Sakamoto et al., 2008). Unlike other substrates of Akt and AMPK discussed, the activity of Akt and AMPK toward AS160 and TBC1D1 is not opposing, rather phosphorylation by either kinase promotes 14-3-3 association, and Glut4 translocation (Chen et al., 2008). In skeletal muscle tissue, this is important for both insulin-stimulated glucose uptake, and for contraction- and hypoxia- induced glucose uptake.

The functional overlap in substrates of Akt and AMPK, such as TSC2 in mTOR signaling, FOXO3a in transcriptional regulation, and TBC1D1 in glucose uptake during growth factor signaling and energy stress suggests that the nodes of cross-talk between these two pathways represent important targets for cellular responses to these environmental conditions.

Common cancer mutations lead to hyperactivation of mTOR signaling

Cancer cells begin to acquire a number of “hallmarks” of cancer as a result of genetic lesions that impair the ability of signaling pathways and effector molecules, such as discussed above, to be switched on or off. For example, cancer cells must not only be able to continue to grow in the absence

of proliferative signals, but must also acquire the ability to ignore growth suppressive signals; they become resistant to cell death programs, achieve replicative immortality, and activate migration and invasion programs to metastasize (reviewed in Hanahan and Weinberg, 2011). Genes whose products are aberrantly active, or overexpressed in cancers are referred to as oncogenes, while those genes inactivated or lost in cancers are tumor suppressors. Much research has been directed both at determining how specific oncogenes or tumor suppressor contribute to acquisition of hallmarks of cancer, and conversely, how specific hallmarks arise.

Interestingly, misregulation of mTOR signaling is a very frequent occurrence in human cancers. Very telling of the contribution of aberrant mTOR signaling to cancer is the observation that it lies downstream of four different genes mutated in familial cancer syndromes: LKB1/STK11 in Peutz-Jeghers Syndrome, PTEN in Cowden's disease, NF1 in neurofibromatosis, and TSC1/TSC2 in tuberous sclerosis complex (reviewed in Shaw and Cantley, 2006). Collectively, these syndromes comprise the hamartoma syndromes that have in common misregulation of mTOR signaling; and are so called as patients present with benign polyps deemed hamartomas. In mouse models of each of these diseases, mTOR signaling is elevated in arising tumors compared to the normal adjacent epithelium (Shaw and Cantley, 2006), and the tumors generally respond to inhibition of mTOR signaling (Podsypanina et al., 2001; Majuber et al., 2004; Jogennessen et al., 2008).

In addition to being mutated in familial cancer syndromes, LKB1, PTEN and NF1 mutations are found in sporadic cancers. LKB1 mutations are also found in 30-40% of sporadically occurring non-small cell lung carcinoma (NSCLC) (Sanchez-Cespedes et al., 2002; Ji et al., 2007), and about 20% of cervical cancers (Wingo et al., 2009). Moreover, genetically engineered mice bearing a conditionally inactivated allele of LKB1 have been deleted in a number of tissues revealing that loss of LKB1 in prostate, skin, uterus, gut, and pancreas is sufficient to initiate hyperplasia and tumorigenesis in some of these tissues (Hezel et al., 2008). Similarly, PTEN is one of the most frequently lost tumor suppressors in a broad range of human cancers including breast, endometrium, thyroid, prostate, leukemias, gliomas, melanomas, lung, liver bladder, and kidney (reviewed in Hollander et al., 2011). Additionally, destabilization of NF1 as a result of hyperactivation of PKC has been shown to be important for tumor growth in sporadic glioblastomas (McGillicuddy et al., 2009). mTOR also falls downstream of some of the most common oncogenes in a wide variety of human cancers, including PI-3K, Akt, EGFR, Raf and Ras.

Therapeutic possibilities

Because of the prevalence of hyperactivation of mTOR signaling in cancers, developing inhibitors for mTOR has been a great focus in therapeutic cancer research. Discouragingly, drugs that mimic rapamycin (rapalogs) have had mediocre clinical outcome (reviewed in Markman et al., 2010). While the

rapalog everolimus has been approved for treatment of renal cell carcinoma, in most cases, rapalogs have been ineffective as cancer therapeutics. This may be due to the effects of inhibition of mTOR on the S6K-IRS1 feedback loop; accordingly, focus is now being placed on dual mTOR/PI-3K inhibitors, where the mTOR inhibition is by kinase inhibition rather than breaking the mTORC1 complex apart as rapamycin does. This will allow for inhibition not only of mTORC1, but also of mTORC2, an upstream activating kinase of Akt.

Therapeutics activating AMPK also hold a potential for cancer, not only for the potential ability to downregulate mTOR signaling, but also for inhibitory effects on lipogenesis. Several compounds exist that are known to activate AMPK, including the biguanides metformin and phenformin that increase the AMP:ATP ratio, and the AMP-mimetic, AICAR. Interestingly, AMPK activation by metformin and AICAR inhibits the growth of tumor cells in culture as well as in xenograft models (Buzzai et al., 2007). In glioblastomas with activating mutations in EGFR, AICAR treatment inhibits tumor cell proliferation better than rapamycin treatment, primarily through inhibition of lipogenesis (Guo et al., 2009). Moreover, metformin treatment suppresses naturally arising tumors in transgenic and carcinogen-treated rodent cancer models (Schneider et al., 2007; Anisimov et al., 2005; Huang et al., 2008). Interestingly, a study comparing the effects of metformin, phenformin and the small molecule Abbott A769662 found that pheforming and A769662 were more potent at suppressing tumors in Pten +/- mice, and this tumor suppressive action correlated with the

ability of these compounds to activate AMPK and suppress mTOR signaling in a wide array of tissues (Huang et al., 2008). The ability of A769662, an allosteric AMPK agonist, to suppress tumors in this study strongly suggests that AMPK is a key target of biguanides in tumor reduction.

Interestingly, the antifolate drug pemexetred was approved by the FDA as an anticancer drug, and was recently shown to activate AMPK, due to an increase in the AMP-like molecule, ZMP, and inhibit mTOR (Racanelli et al., 2009; Rothbart et al., 2010), suggesting that like other AMPK agonists, pemexetred may exhibit clinical efficacy in tumors with aberrant mTOR activity. Notably, pemexetred is used clinically in non-small cell lung carcinoma, 30% of which bear LKB1 mutations, thereby introducing the possibility that pemexetred may work selectively on either LKB1-positive or LKB1-negative tumors. Future studies will help illuminate this exciting possibility.

Importantly, compounds that activate AMPK will not only inhibit tumorigenesis via suppression of mTORC1 and lipogenic targets such as ACC, but perhaps also through alterations in organismal metabolism such as reducing blood glucose and insulin resistance, leading to lowered systemic blood insulin levels as well. Because of its effects on AMPK, and therefore blood glucose levels, metformin is the most widely used type II diabetes drug worldwide, with more than 100 million patients taking the drug. Initial epidemiological studies have shown that type II diabetes patients on metformin compared to other drugs have a statistically significant decrease in tumor

incidence (Evans et al., 2005; Bowker et al., 2006). It will be important to determine if particular tumor types or specific tumor genotypes best predict therapeutic efficacy of metformin. Tumor cells lacking LKB1 are hyper-sensitive to apoptosis in culture following treatment with energy stress inducing agents, presumably through the inability to activate AMPK to restore ATP levels (Shaw et al., 2004, Carretero et al., 2007, Memmott et a l., 2008), suggesting biguanides like metformin and phenformin may have greater efficacy in the absence of LKB1. Indeed, the ability of AMPK to restore ATP levels may results in tumor cell survival in the presence of LKB1.

LKB1-AMPK, mTOR in specialized metabolic tissues

In addition to broad roles in controlling cell growth in all mammalian cell types, mTOR and AMPK play key roles in a number of “professional” metabolic tissues in mammals.

In the hypothalamus, food intake is controlled by neurons in the arcuate nucleus. Hypothalamic AMPK is activated in response to low glucose, endocannibinoids, AgRP, or the gastric hormone ghrelin, all of which are increased during fasting. Conversley, AMPK activity is decreased upon refeeding or administration of insulin or leptin. Consistent with its suppressive effect on AMPK, leptin induces mTORC1 activity in neurons of the arcuate nucleus (Woods et al., 2008). Mice bearing disruptions in AMPK or core

mTORC1 components exhibit a variety of defects in food intake and organismal energy metabolism (Cota et al., 2006; Claret et al., 2007).

Skeletal and cardiac muscles are additional tissues where AMPK and mTOR play key roles in glucose metabolism, hypertrophy, and the response to exercise. In skeletal muscle, AMPK activation has been shown to promote mitochondrial biogenesis at least in part through transcription effects downstream of PGC-1α, and PPARδ (Jager et al., 2007; Narkar et al., 2008). Consistently, mice lacking AMPK function in muscle, either from expression of a dominant-negative AMPK or deletion of LKB1, exhibit loss of mitochondrial mass and a dramatic reduction in voluntary exercise (Mu et al., 2001; Thomson et al., 2007). Resistance exercise in humans has been shown to decrease mTORC1-dependent phosphorylation of 4EBP1 coincident with maximal activation of AMPK (Dreyer et al., 2006). Whether AMPK-deficient mice show elevations in mTOR within subtypes of muscle following exercise and whether mTORC1 plays any role in the metabolic reprogramming of muscle fiber type remains to be examined. The picture in skeletal muscle is likely to be complex, as both AMPK and mTOR have been reported to stimulate PGC-1α-dependent mitochondrial biogenesis in this tissue, albeit via distinct mechanisms (Jager et al., 2007; Cunningham et al., 2007). Indeed, previous observation suggests isoform-specific AMPK activation in individual muscle types (McGee et al., 2008), suggesting a thorough analysis of all fiber types in muscle groups in the individual AMPKα1 and AMPKα2 knockout mice and ultimately a skeletal

muscle tissue-specific deletion of both, will prove necessary to define where and when AMPK is most rate-limiting for mTOR suppression and PGC-1α regulation following specific stimuli. Notably, mTORC1 signaling following insulin or leucine or electrical stimulation is suppressed by AICAR pre-treatment in EDL, gastrocnemius, and extensor digitorum longus muscles, respectively (Deshmukh et al., 2008; Pruznak et al., 2008; Thomson et al., 2008). Importantly, utilizing muscle from knockout mice lacking AMPKα2 or AMPKγ3, it was recently demonstrated that each of these AMPK isoforms are required for AICAR to suppress mTORC1 activity (Deshmukh et al., 2008). Indeed, AMPK activation by metformin or AICAR or by overexpression of activated LKB1 inhibits protein synthesis and hypertrophy in neonatal rat cardiac myocytes coincident with suppression of mTORC1 signaling (Chan et al., 2004; Noga et al., 2007). Consistent with these findings, induction of hypertrophy by angiotensin II is accompanied by inhibition of AMPK and activation of mTORC1 (Stuck et al., 2008), whereas loss of AMPKα1 accelerates over-loading-induced hypertrophy and results in elevated mTORC1 activity (Mounier et al., 2009)). In critical genetic tests of the involvement of AMPK in cardiac hypertrophy, two independent studies found that following isoproterenol (Zarrinpashneh et al., 2008), or transverse aortic constriction (Zhang et al., 2008), increased hypertrophy was observed in AMPKα2-deficient mice, which correlated with dramatic increases in mTORC1 signaling in the AMPKα2-deficient hearts (Zhang et al., 2008). Recently, cardiac myocyte-

specific deletion of LKB1 was shown to cause developmental defects in the atria and ventricles accompanied by increased left ventricular hypertrophy with reduced AMPK and elevated mTORC1 activity (Ikeda et al., 2009). Although much remains to be elucidated in these models and the molecular interplay between AMPK and mTOR signaling in skeletal and cardiac muscle, it is clear that AMPK modulation of mTOR may play a central role in cardiac hypertrophy.

In liver, AMPK plays key roles in glucose and lipid metabolism. Hormones that activate AMPK in liver including glucagon (Kimball et al., 2004) and adiponectin (Wang et al., 2007) have been reported to suppress mTORC1 signaling. Because the effects of AMPK on hepatic gluconeogenesis are mediated by direct phosphroyaltion of transcription factors and coactivators the regulate transcription of gluconeogenic enzymes (Yang et al., 2001; Hong et al., 2003; Koo et al., 2005; Inoue et al., 2006), AMPK regulation of mTOR signaling is unlikely to play a role in AMPK-mediated regulation of gluconeogenesis. In contrast, lipogenesis is controlled in part by mTORC1-dependent signals. One key regulator of lipogenesis is the SREBP-1 transcription factor. SREBP-1 is a sterol-sensing transcription factor that drives lipogenesis not only in liver, but also in a large variety of mammalian cells. Recently, mTORC1 signaling was shown to be required for nuclear accumulation of SREBP-1 and the induction of SREBP-1 target genes (Parstmann et al., 2008). Similar to rapamycin, treatment with AMPK agonists including AICAR and 2DG resulted in suppression of nuclear SREBP-1

accumulation (Parstmann et al., 2008). Similarly, nuclear SREBP-1 is suppressed in the liver of mice treated with metformin (Zhoe et al., 2001). Metformin treatment or overexpression of an activated allele of AMPK was found to be sufficient to reduce triglyceride content in insulin-resistant HepG2 cells (Zang et al., 2004). Mice lacking hepatic AMPK function due to liver-specific LKB1 deletion show elevated SREBP-1 and SREBP-1 target genes resulting in lipid accumulation and hepatic steatosis (Shaw et al., 2004). Metformin treatment of mice leads to robust AMPK-mediated phosphorylation of raptor S792 in murine liver, which is ablated in the LKB1-liver-specific knockout mice further illustrating that this molecular event may be relevant in the context of AMPK-mediated control of lipid metabolism (Gwinn et al., 2008). Beyond hepatic lipogenesis, SREBP-1 has been shown to be critical for cell growth in both Drosophila and mammalian cells (Parstmann et al., 2008) suggesting that it may be a critical target of AMPK and mTOR signaling not only in the context of metabolic disease, but also in tumorigenesis. Consistent with this idea, expression of SREBP-1 trans-criptional target fatty acid synthase (FASN) has been linked to breast cancer proliferation and FASN inhibitors are beginning to be explored clinically as anti-cancer agents (Menendez et al., 2007). It is still unclear how much of the lipid-reducing effects of AMPK are due to direct phosphorylation of lipogenic enzymes such as acetyl-CoA carboxylase (ACC) versus effects of SREBP-1 dependent transcription via AMPK regulation of mTORC1.

Deletion of LKB1 in adult β-cells results in an mTORC1-dependent hypertrophy of β-cells as well as altering their polarity (Fu et al., 2009; Granot et al., 2009). Upon glucose stimulation, LKB1-deleted β-cells secrete more insulin than their wild-type counterparts, and mice with β-cell specific LKB1 knockdown exhibit elevated glucose tolerance, though this effect was not ameliorated by treatment with rapamycin, suggesting that this effect is independent of the hyperactivity of mTORC1 with LKB1 loss (Granot et al., 2009). After 16 weeks on a high fat diet, control mice had deficient glucose tolerance, whereas mice lacking LKB1 in β-cells displayed glucose tolerance similar to mice of a normal diet (Fu et al., 2009; Granot et al., 2009). While the effect on β-cell size due hyperactivation of mTORC1 induced by loss of LKB1 is clear, it is unclear what contribution this makes to the phenotypes of insulin secretion and glucose tolerance in these animals.

Regulation of mTORC1 downstream of AMPK may also help explain the well-documented ability of metformin and other AMPK agonists to act as insulin sensitizers (Towler et al., 2007). One explanation for this effect is that the insulin-independent downregulation of gluconeogenesis by AMPK relieves the amount of insulin required to be made by the pancreas to reduce circulating blood glucose. A cell-autonomous explanation is based on the observation that in conditions of hyperglycemia, hyperlipidemia, and hyperinsulinemia, mTORC1 signaling is chronically hyperactive, engaging a negative feedback loop from S6K to the IRS1/2 scaffolding proteins, and causing their

degradation, such that activated insulin receptor can no longer signal downstream to PI-3K (Harrington et al., 2004; Shah et al., 2004). AMPK downregulation of mTORC1 signaling can actually attenuate the suppression of PI-3K by this negative feedback loop, restoring IRS levels, and Akt activation.

Not only does AMPK-dependent regulation of mTORC1 play an important role in the cellular response to energy stress, it plays an important role in specialized metabolic tissues including muscle and liver. Understanding more about these signaling pathways may give us insight into their complex roles in cell-autonomous responses to growth signals and energy stress, as well as more organismal effects on metabolism.

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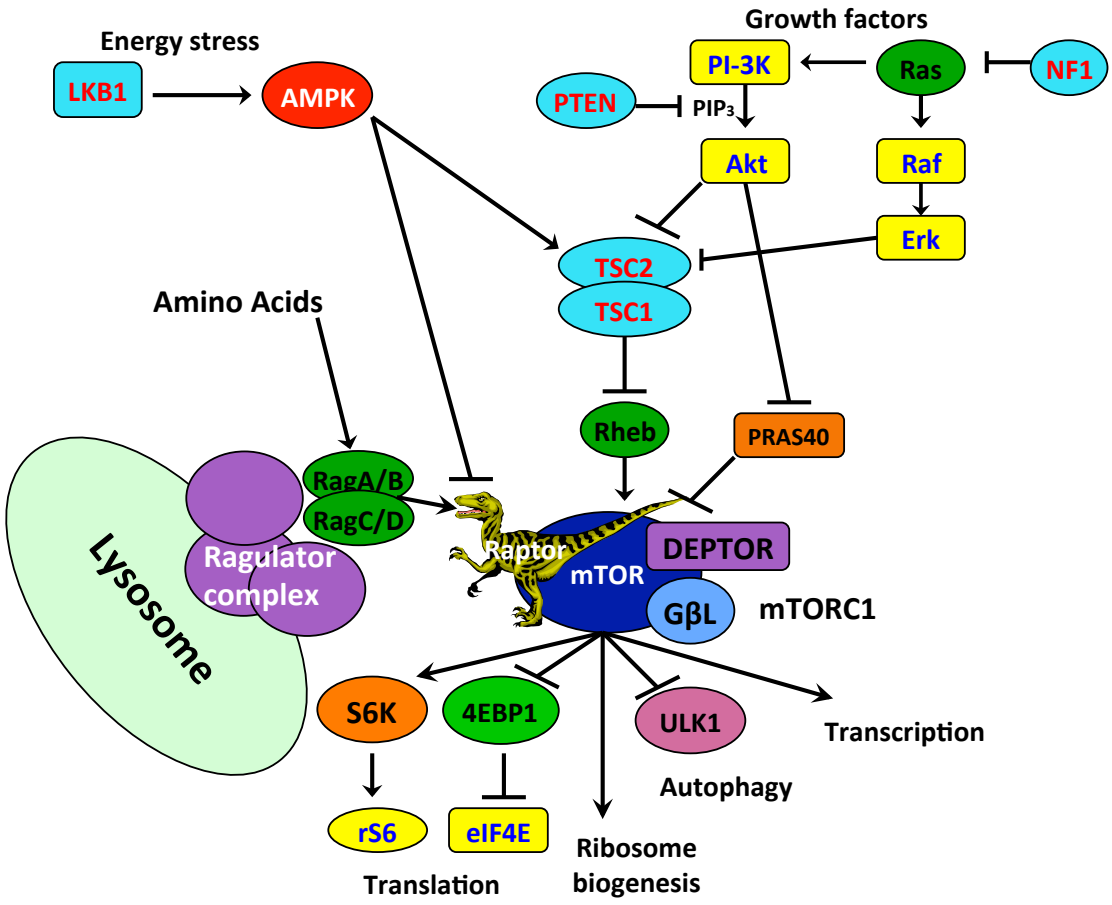
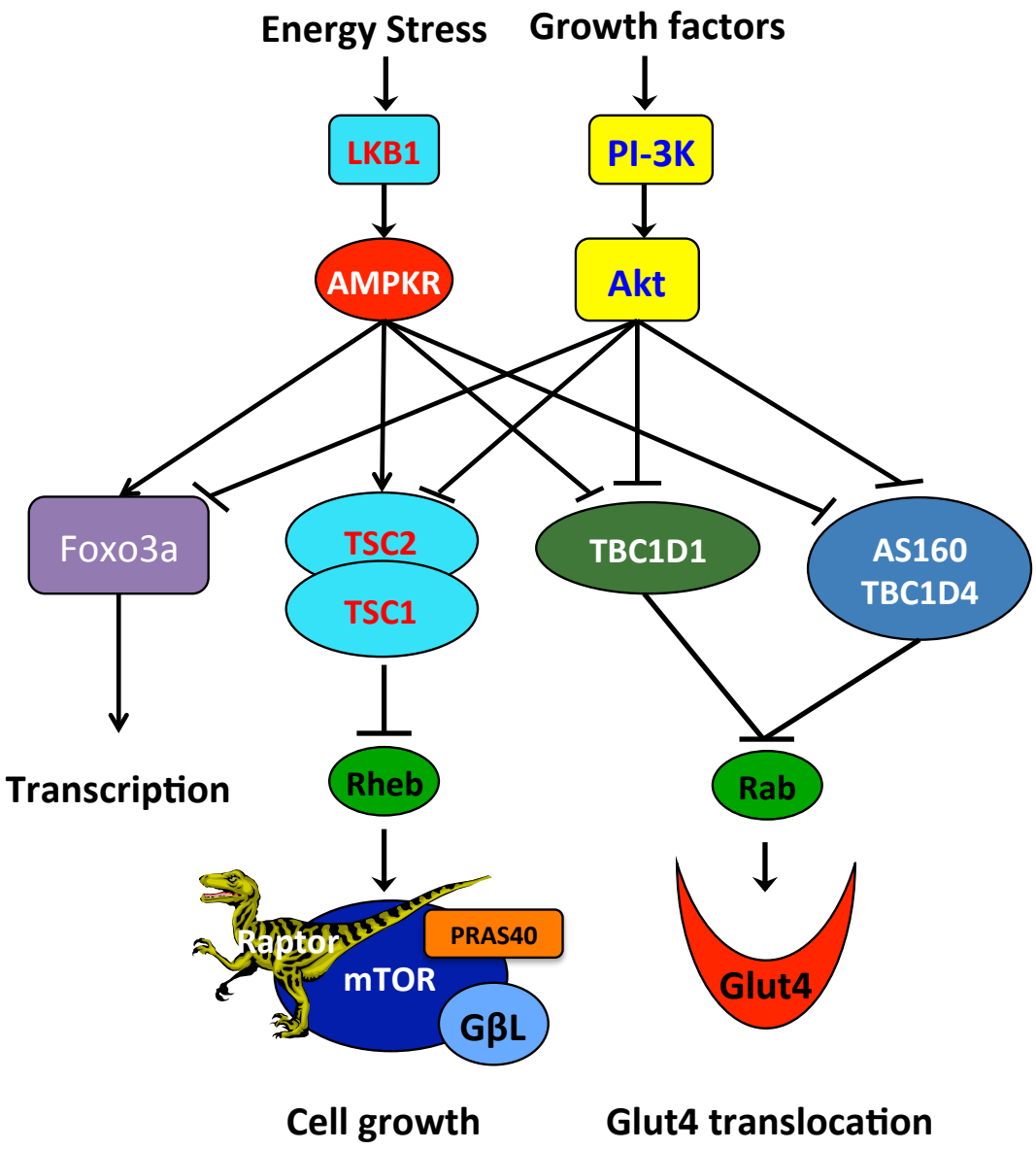


CHAPTER I Figure 1. A model of regulation of mTOR signaling.

The mTORC1 complex receives signals from growth factor signaling by direct phosphorylation of TSC2 and PRAS40 by Akt, and by Erk phosphorylation of TSC2. The LKB1-AMPK pathway inhibits mTORC1 signaling by direct phosphorylation of TSC2 and raptor by AMPK. mTORC1 signaling is sensitive to amino acid withdrawal via regulation of the Rag GTPase and the Ragulator complex. mTORC1 phosphorylates S6K, 4EBP1 and ULK1 to regulate translation and growth control, autophagy, and unknown substrates in regulation of ribosome biogenesis and transcriptional programs.



CHAPTER I Figure 2. Model of Cross-talk between the LKB1-AMPK and PI-3K-Akt pathways.

Both AMPK and Akt phosphorylate Foxo3a to regulate transcriptional programs, TSC2 to regulate mTORC1 signaling, and TBC1D1 and TBC1D4/ AS160 in the regulation of Glut4 trafficking.

CHAPTER TWO:

AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint

Abstract

AMPK is a highly conserved sensor of cellular energy status that is activated under conditions of low intracellular ATP. AMPK responds to energy stress by suppressing cell growth and biosynthetic processes, in part through its inhibition of the rapamycin-sensitive mTOR (mTORC1) pathway. AMPK phosphorylation of the TSC2 tumor suppressor contributes to suppression of mTORC1; however, TSC2-deficient cells remain responsive to energy stress. Using a proteomic and bioinformatics approach, we sought to identify additional substrates of AMPK that mediate its effects on growth control. We report here that AMPK directly phosphorylates the mTOR binding partner raptor on two well-conserved serine residues, and this phosphorylation induces 14-3-3 binding to raptor. The phosphorylation of raptor by AMPK is required for the inhibition of mTORC1 and cellcycle arrest induced by energy stress. These findings uncover a conserved effector of AMPK that mediates its role as a metabolic checkpoint coordinating cell growth with energy status.

Introduction

The AMP-activated protein kinase (AMPK) is a highly conserved heterotrimeric kinase complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits. AMPK is activated under conditions of energy stress, when intracellular ATP levels decline and intracellular AMP increases,

as occurs during nutrient deprivation or hypoxia (Hardie, 2007). Upon energy stress, AMP directly binds to tandem repeats of crystathionine-β-synthase (CBS) domains in the AMPK g subunit. Binding of AMP is thought to prevent dephosphorylation of the critical activation loop threonine in the α subunit (Hardie, 2007). The phosphorylation of the activation loop threonine is absolutely required for AMPK activation. Biochemical and genetic analyses in worms, flies, and mice have revealed that the serine/threonine kinase LKB1 represents the major kinase phosphorylating the AMPK activation loop under conditions of energy stress across metazoans (Apfeld et al., 2004; Shaw et al., 2005; Sakamoto et al., 2005; Mirouse et al., 2007; Lee et al., 2007b).

LKB1 was originally identified as a human tumor suppressor gene mutated in Peutz-Jeghers syndrome (PJS), an autosomal dominant inherited cancer disorder (Hemminki et al., 1998). In addition, LKB1 mutations occur in a large percentage (30%– 40%) of sporadic non-small-cell lung cancers (NSCLC) (San- chez-Cespedes et al., 2002; Ji et al., 2007). PJS shares a number of clinical features with Cowden's Disease, which is caused by inactivating mutations in the PTEN tumor suppressor. This phenotypic overlap suggested that LKB1-dependent signaling might negatively regulate some aspect of PI3-kinase (PI3K) signaling, analogous to PTEN function. However, while classic PI3K/Akt signaling is not elevated in LKB1-deficient cells, mammalian target of rapamycin (mTOR) signaling is uniquely hyperactivated in LKB1-deficient murine embryonic fibroblasts (MEFs) and liver (Corradetti et al.,

2004; Shaw et al., 2004b, 2005). Similarly, mTOR signaling is hyperactivated in hamartomas from LKB1- heterozygous mice (Shaw et al., 2004b) and in LKB1-deficient human lung carcinomas (Carretero et al., 2007).

mTOR is a highly conserved nutrient-responsive regulator of cell growth found in all eukaryotes (Wullschleger et al., 2006). Whereas AMPK is active under nutrient-poor conditions and inactive under nutrient-rich conditions, mTOR is activated in the inverse pattern. In higher eukaryotes, mTOR activation requires positive signals from both nutrients (glucose, amino acids) and growth factors. mTOR, like its budding yeast orthologs, is found in two biochemically and functionally distinct signaling complexes (Wullschleger et al., 2006). The mTORC1 complex is nutrient sensitive, acutely inhibited by rapamycin, and functions as a master regulator of cell growth, angiogenesis, and metabolism (Sabatini, 2006). mTORC1 is composed of four known subunits: mTOR, mLST8/Gβl, PRAS40, and the WD40 repeat-containing subunit raptor (Sabatini, 2006; Sancak et al., 2007; Vander Haar et al., 2007). Raptor acts as a scaffold to recruit downstream substrates such as 4EBP1 and ribosomal S6 kinase (p70 S6K1) to the mTORC1 complex (Nojima et al., 2003; Schalm et al., 2003).

Genetic studies in Drosophila and mammalian cells identified the tuberous sclerosis complex (TSC) tumor suppressors as critical upstream inhibitors of the mTORC1 complex. TSC2 (also known as tuberin) contains a GTPase activating protein (GAP) domain at its carboxyl terminus that in-

activates the Rheb GTPase, which has been shown to associate with and directly activate the mTORC1 complex in vitro (Sancak et al., 2007). Loss of TSC1 or TSC2 therefore leads to hyperactivation of mTORC1.

Phosphorylation of TSC1 and TSC2 serves as an integration point for a wide variety of environmental signals that regulate mTORC1 (Sabatini, 2006). Mitogen-activated kinases including Akt, Erk, and Rsk directly phosphorylate TSC2, leading to its inactivation by an unknown mechanism. In addition, another Akt substrate, PRAS40, was recently shown to bind and inhibit the mTORC1 complex. Upon phosphorylation by Akt, PRAS40 no longer inhibits mTORC1 (Sancak et al., 2007; Vander Haar et al., 2007).

In addition to these growth stimulatory cues that activate mTORC1, the complex is rapidly inactivated by a wide variety of cell stresses, thereby ensuring that cells do not continue to grow under unfavorable conditions. One of the unique aspects of the mTORC1 complex is that unlike many of the aforementioned growth factor activated kinases, it is dependent on nutrient availability for its kinase activity. Withdrawal of glucose, amino acids, or oxygen leads to rapid suppression of mTORC1 activity (Shaw and Cantley, 2006). Upon LKB1- and AMP-dependent activation of AMPK by nutrient loss, AMPK directly phosphorylates the TSC2 tumor suppressor on conserved serine sites distinct from those targeted by other kinases, which constitutes one mechanism through which glucose and oxygen control mTORC1 activation (Inoki et al., 2003; Corradetti et al., 2004; Shaw et al., 2004b; Liu et al., 2006).

We have found that cells lacking TSC2 remain responsive to energy stress, albeit less so than wild-type cells, suggesting that additional AMPK substrates may directly or indirectly modulate mTORC1 activity. Moreover, the relationship between glucose inactivation of AMPK and stimulation of TOR is conserved across all eukaryotes, including several that lack TSC2 orthologs such as *C. elegans* and *S. cerevisiae*. This suggests that either additional mechanisms exist to coordinate the kinase activity of these two master regulators of cell growth and metabolism, or AMPK must target additional conserved components of the pathway. Here, we find that the critical mTOR binding partner raptor is a direct substrate of AMPK, and that phosphorylation of raptor by AMPK is required for suppression of mTORC1 activity by energy stress. Further, we report that raptor phosphorylation is necessary for the full engagement of an AMPK-mediated metabolic checkpoint. These findings have broad implications for the control of cell growth by nutrients in a number of cellular and organismal contexts.

Results

Peptide Library Identification of the Optimal Substrate Motif for AMPK

In an effort to find substrates of AMPK that may mediate its effects on growth and metabolic control, we determined its consensus phosphorylation motif with the aim of identifying proteins that carry optimal phosphorylation sequences. We utilized a positional scanning peptide library (PSPL) technique

in which radiolabeled kinase assays are performed on a spatially arrayed set of peptide mixtures. Each peptide contains one fixed amino acid at a given position relative to a centrally fixed phosphoacceptor (an even mixture of serine or threonine) and degenerate amino acid mixtures at all flanking positions (Hutti et al., 2004). From the relative amount of phosphate incorporated into each peptide mixture, one obtains a quantitative measure of the selectivity for, and against, each individual amino acid residue at each position (Turk et al., 2006). We and our colleagues have previously used this technique to successfully identify optimal substrate motifs for a number of mammalian kinases, including CK2, Erk, PKA, Akt, Pim, Pak, MAP3K, and IKK family kinases (Hutti et al., 2004, 2007; Bullock et al., 2005; Bunkoczi et al., 2007; Rennefahrt et al., 2007).

PSPL profiling revealed that AMPK is a highly selective kinase, strongly preferring basic residues in the -3 and -4 positions relative to the phospho-acceptor site. In addition, hydrophobic residues including leucine and methionine were strongly selected in the +5 position and the +4 position consistent with previous studies of the optimal peptide substrates for AMPK based on mutagenesis and molecular modeling (Scott et al., 2002; Towler and Hardie, 2007). In addition, strong selection for polar residues in the +3 position was noted, with asparagine and aspartate being the most highly selected (Figure 1).

Comparing the optimal motif we identified from the peptide library screen

to all known well-established in vivo substrates of AMPK shows excellent concordance (Figure 2). Each of these substrates contains not only the required basic residue in -3 or -4, and hydrophobic residues in +5 and +4, but they also exhibit strong bias toward the secondary selections for serine and valine in the -2 position and polar residues in the +3 position. The strong selectivity for particular residues in at least four out of the nine flanking residues analyzed makes AMPK one of the most selective mammalian kinases we have examined thus far (out of 60 kinases profiled to date; B.E.T., unpublished data). The high degree of selectivity at multiple residues substantially reduces the odds that any protein will contain serine residues within this sequence context by random chance, especially when examined for evolutionary conservation. This suggests that proteins that do carry this signature sequence are likely to be authentic substrates of AMPK or related kinases. Thus we used our optimal AMPK substrate motifs to mine protein data- bases to search for matching sequences—using bioinformatics tools including Scansite (http://scansite.mit.edu) and Prosite (http://ca.expasy.org/prosite/). We focused our efforts on those candidate substrates bearing optimal AMPK motifs in which the target serine and its critical flanking residues that dictate AMPK-dependent substrate specificity were conserved broadly throughout eukaryotes.

Raptor Is an AMPK Substrate

We first examined potential AMPK substrates that might underlie the ability of AMPK and its upstream kinase LKB1 to regulate cell growth and tumorigenesis. A number of recent studies have revealed that a key effector of AMPK signaling in the control of cell growth is the suppression of the mTORC1 signaling complex. We and others previously reported that the effect of LKB1 and AMPK to regulate mTORC1 is at least in part via direct phosphorylation of the TSC2 tumor suppressor by AMPK (Corradetti et al., 2004; Shaw et al., 2004b). Indeed Ser1387 of human TSC2 con- forms perfectly to the AMPK optimal motif we obtained with our peptide library analysis, and this residue and its flanking sequences are conserved across vertebrates and to Drosophila (Figure 2, data not shown).

However, two pieces of data suggested that TSC2 could not be the only substrate of AMPK to regulate mTORC1 signaling. First, the inverse regulation of TOR and AMPK by glucose levels is found throughout all eukaryotes examined thus far, including C. elegans and S. cerevisiae, although a TSC2 ortholog is not found in either of those species. Second, while performing further experiments to examine the role of TSC2 in regulating energy stress, we found that while TSC2 is needed for rapid suppression of mTORC1 by the AMP-mimetic AICAR and the mitochondrial complex I inhibitor phenformin, mTORC1 is still potently inhibited by both of these AMPK activators in TSC2-/- MEFs (Figure 4). Similar findings have been made by others using glycolytic

inhibitors (e.g., 2-DG) in TSC2-deficient cells (Hahn-Windgassen et al., 2005).

Our bioinformatics analysis revealed that the mTOR binding partner raptor contains two conserved serine sites that match the AMPK consensus motif (serine 722 and serine 792 of human raptor). Importantly, the critical residues flanking raptor Ser792 which were found in the peptide library studies to be important for recognition by AMPK are highly conserved through Drosophila, C. elegans, and Dictyostelium, as well as in both budding and fission yeast (Figure 3). Such a high degree of conservation is rare among phosphorylation sites. For example, of the ten best established AMPK substrates shown in Figure 2, only two of them are conserved across eukaryotes (ACC1 Ser1216 and HMG CoR Ser862). Moreover, half of the known AMPK substrate proteins, including TSC2, have no orthologs in primitive eukaryotes. The striking conservation in the candidate AMPK sites in raptor suggested it could represent an ancestral AMPK target that dictates the responsiveness of TOR to nutrients across eukaryotes.

To test the possibility that raptor is an AMPK substrate, we first examined whether we could detect phosphorylation of raptor in cultured cells using phosphomotif antibodies. These antibodies broadly recognize phosphorylated serine or threonine residues found within a specific sequence motif (Zhang et al., 2002). Interestingly, we found that the ‘‘14-3-3 motif’’ antibody, which was generated against peptides bearing R-X-X-pS or R-X-X-X-pS sequences, recognized raptor in HEK293 cells. Coexpression with wild-

type and kinase-dead LKB1 led to an increase and decrease, respectively, in reactivity of raptor to the antibody (Figure 5). Moreover, cotransfection with a truncated constitutively active allele of AMPKα1 resulted in a dramatic increase in raptor phosphorylation, and activation of endogenous AMPK through the use of the polyphenol compound resveratrol also stimulated acute phosphorylation of raptor (Figure 5).

Tandem mass spectrometry was then used to identify the specific sites of phosphorylation of raptor in cultured cells. Epitope-tagged raptor was cotransfected with mTOR in HEK293T cells. Cells were either untreated, or treated with either resveratrol or phenformin, both of which potently activate AMPK in HEK293T cells. Mass spectrometry (MS) of chymotryptic fragments of raptor from resveratrol- and phenformin-treated cells revealed that the Ser792 site was phosphorylated at high stoichiometry in both samples, with 5 of the 7 peptides identified containing this serine residue being phosphor-ylated with either treatment (Figure 6), unlike the untreated sample that revealed 2 of 9 peptides bearing phosphate at raptor Ser792 (data not shown). The region flanking the candidate Ser722 site was not well repre- sented in our mass spectrometry analysis despite repeated attempts, including digestion with alternative proteases (Figure 6). Notably, during the course of this study, two large-scale analyses of phosphoproteins from rat and mouse liver revealed phosphorylation of endogenous raptor at Ser722, suggesting that it is indeed a bona fide phosphorylation site *in vivo* (Moser and White, 2006; Villen et al.,

2007).

A phosphospecific antibody against the Ser792 site in human raptor was generated, and its specificity was assessed using epitope-tagged wild-type or S792A mutant raptor overexpressed in HEK293T cells, under conditions analogous to those employed for the mass spectrometry (Figure 7). In addition, we examined whether purified AMPK could directly phosphorylate raptor at Ser792 *in vitro*. Active AMPK rapidly and potently induced raptor Ser792 phosphorylation *in vitro* (Figure 7). Similarly, employing non-phosphorylatable mutants, we mapped the sites recognized by the 14-3-3 motif antibody. As expected, we found that reactivity to the phospho-Ser792 antibody was unaffected in the S722A mutant. Surprisingly, the AMPK-induced reactivity of raptor with the 14-3-3 motif antibody was minimally affected in the S792A mutant but was dramatically reduced in the S722A mutant (Figure 8). Reactivity was completely abolished in the S722A/S792A double mutant (henceforth referred to as the ‘‘AA mutant’’). These results suggest that AMPK activation can induce phosphorylation of both Ser722 and Ser792.

To determine whether AMPK is the physiological kinase for phosphorylation of endogenous raptor at Ser792, immortalized wild-type or AMPKα1/α2-deficient MEFs (bearing a targeted disruption of both AMPK α genes) were treated with the AMP-mimetic AICAR, followed by immunoblotting for phospho-Ser792 raptor. As controls, we also examined phosphorylation of two well-established AMPK substrates (ACC1/2 Ser79 and IRS1 Ser789). As

seen in Figure 9, raptor Ser792 is phosphorylated following AICAR treatment in wild-type, but not AMPK null, MEFs, precisely paralleling phospho-ACC and phospho-IRS1, thereby indicating that raptor Ser792 is a bona fide AMPK site in vivo. To further define the physiological conditions under which raptor Ser792 phosphorylation is modulated by AMPK, we examined raptor Ser792 phosphorylation in liver extracts from wild-type or LKB1-liver-specific KO mice fed ad libitum, fasted, or treated with the biguanide diabetes therapeutic metformin. We have previously shown that metformin rapidly activates AMPK in murine liver in a manner completely dependent on LKB1 (Shaw et al., 2005). Here we observed that raptor Ser792 phosphorylation in murine liver was slightly potentiated in fasted mice and was dramatically increased by metformin treatment in a manner completely dependent on intact LKB1 (Figure 10). These results were further extended in isolated primary hepatocytes from wild- type and LKB1-deficient liver (Figure 11). Taken altogether, these results indicate that endogenous raptor Ser792 is phosphorylated in multiple mammalian tissue types in an LKB1- and AMPK-dependent manner following energy stress.

Raptor Phosphorylation Is Required for Inhibition of mTORC1 by AMPK

To examine the requirement for raptor phosphorylation in the regulation of mTORC1 activity by energy stress, we utilized the nonphosphorylatable AA mutant in which both Ser722 and Ser792 were replaced by alanine. To assess the physiological role of raptor Ser722 and Ser792 phosphorylation in

mTORC1 regulation, we replaced the endogenous raptor by creating cell lines stably expressing low levels of human wild-type or AA raptor using retroviral expression, followed by subsequent knock-down of the endo-genous murine raptor utilizing a lentivirally expressed shRNA that does not target the human raptor sequence. In this manner, we replaced endogenous raptor with human wild- type or AA mutant in three murine cell lines: C2C12 myoblasts, TSC2+/+ p53-/- MEFs, and TSC2-/- p53-/- MEFs. In murine raptor lentiviral shRNA infected cultures lacking reconstitution with human raptor, we observed functional suppression of raptor levels and mTORC1 signaling (Figure 12). We then examined the requirement for raptor Ser722 and Ser792 phosphorylation in mTORC1 suppression following AICAR or phenformin treatment in these stable cell lines. Mutation of these sites prevented AMPK agonists from fully suppressing mTORC1, both in cells with normal mTOR signaling (C2C12, TSC2+/+ MEFs) (Figure 13, 14) and in cells lacking TSC2 (Figures 14, 15). Despite the elevated basal levels of mTORC1 activity in TSC2-/- MEFs, AICAR or phenformin treatment potently suppressed mTORC1 signaling, an effect that was almost fully abolished by reconstitution with the AA raptor allele. In AA raptor mutant expressing TSC2-/- MEFs, mTORC1 activity levels were dramatically elevated compared to TSC2+/+ MEFs expressing wild-type raptor at all time points following AICAR treatment (Figure 13). Similar results were seen with phenformin, which activates AMPK via distinct mechanism (AICAR is an AMP mimetic; phenformin is a mitochondrial complex I inhibitor); notably

each may have additional distinct effects on signaling independent of the LKB1/AMPK pathway. Altogether our data demonstrate that raptor phosphorylation on Ser722/Ser792 is required for full mTORC1 suppression by AMPK agonists in all cell types we examined. Furthermore, these findings indicate that TSC2 and raptor represent the major targets of AMPK required for the suppression of mTORC1 in mouse embryonic fibroblasts.

AMPK Phosphorylation of Raptor Induces 14-3-3 Binding

We next considered the mechanism by which AMPK-mediated raptor phosphorylation leads to inactivation of the mTORC1 kinase complex in vivo. We investigated the possibility that phosphorylation of raptor leads to the specific association or disassociation of proteins with the mTORC1 complex. Mass spectrometry was utilized to identify proteins coimmunoprecipitating with overexpressed wild-type raptor following phenformin treatment in HEK293 cells. Among the proteins identified coprecipitating with raptor were two isoforms of 14-3-3 (Figure 19). A common mechanism for phosphorylation-based inactivation of target proteins is through direct phosphorylation-dependent binding to the 14-3-3 family of proteins (Bridges and Moorhead, 2005). As AMPK-mediated phosphorylation of raptor also created an epitope recognized by the 14-3-3 binding motif antibody (Figures 5 and 8), we more closely examined the possibility that phosphorylation of serine 722 and 792 may induce 14-3-3 binding to raptor. Exhaustive peptide library screening and

proteomic analyses have revealed that 14-3-3 proteins generally interact with R-X-X-pS/pT-X-P or R-X-X-X-pS/pT-X-P target sequences. Raptor Ser722 and Ser792 both contain the required upstream arginine residue; however, neither site contains a pro- line residue in the +2 position, although several well-established 14-3-3 binding sites also lack proline at +2 (Cbl, IRS-1, PRAS40). Moreover, both Ser722 and Ser792 in raptor have residues at +1 and +2 that arose as secondary selections in peptide library experiments (Yaffe et al., 1997; Rittinger et al., 1999).

We first examined whether 14-3-3 bound to raptor when coexpressed, in an AICAR- or Ser722/Ser792-dependent manner. Coexpressed GST-14-3-3, but not GST alone, immunoprecipitated with wild-type, but not AA mutant raptor, when cells were subjected to AICAR (Figure 16). In addition, recombinant GST-14-3-3 protein fixed to beads precipitated wild-type, but not AA mutant, raptor in lysates from the MEF stable cell lines treated with AICAR or phenformin (Figure 17). Furthermore, endogenous raptor coprecipitated with recombinant 14-3-3 protein from wild-type, but not LKB1-deficient, MEFs following treatment (Figure 18). Finally, consistent with the original mass spectrometry data (Figure 19), endogenous 14-3-3 ζ and γ isoforms co-immunoprecipitated with wild-type, but not AA mutant, raptor in a phenformin-dependent manner (Figure 20). However, it is worth noting that little specificity has been demonstrated for 14-3-3 isoforms other than 14-3-3 σ, and many of the isoforms can form heterodimers with each other (Gardino et al., 2006;

Wilker et al., 2005). Thus, we expect the 14-3-3 isoforms that bind AMPK-phosphorylated raptor may vary between cell types based on expression levels.

AMPK Phosphorylation of Raptor Regulates mTORC1 IP-Kinase Activity

14-3-3 has been shown to regulate its best studied binding partners through three distinct mechanisms, each involving allosteric conformational changes that (1) induce changes in protein catalytic activity, (2) trigger a disruption of existing protein-protein interactions, or (3) cause changes in subcellular localization. Analyses of crystal structures of 14-3-3 isoforms bound to phosphopeptides suggest that 14-3-3 regulates the activity of many of its binding partners via allosteric stabilization of unfavorable states (‘‘the molecular anvil’’ hypothesis) (Yaffe, 2002). We first examined whether we could detect suppression of mTORC1 IP-kinase activity by immuno-precipitating raptor from AICAR-treated MEFs. Using conditions that were recently reported to reconstitute insulin-dependent stimulation of mTORC1 IP- kinase activity (Sancak et al., 2007), we found that raptor immunoprecipitates from AICAR-treated cells showed a time-and dose-dependent suppression of mTORC1 kinase activity toward purified S6K1 protein that paralleled the inhibition of mTORC1 signaling by AMPK activation in vivo (Figure 15). We subsequently examined the IP-kinase activity of raptor complexes containing the S722A/ S792A double mutant. As seen in Figure 15, mTORC1 complexes containing

AA raptor were refractory to the inhibition of kinase activity seen in mTORC1 complexes containing wild-type raptor. Critically, the amount of mTOR found in association with raptor was not affected by mutation of raptor Ser722/Ser792 or by AMPK activation (Figure 15). These data indicate that immunoprecipitates containing the same amount of complexed mTOR and raptor show differences in mTORC1 kinase activity dependent on Ser722/ Ser792 phosphorylation by AMPK.

We further examined the association of endogenous mTOR and endogenous raptor from wild-type, LKB1-deficient, and AMPKα-deficient MEFs. The amount of raptor and mTOR coimmunoprecipitating was constant in all contexts examined (Figure 21). We also examined whether AICAR induced changes in the amount of endogenous PRAS40 co-immuno-precipitating with raptor. As seen in Figure 15, AICAR treatment induced greater immuno-precipitation of PRAS40 with raptor, which was modestly sup-pressed in cells expressing the nonphosphorylatable raptor. However, the levels of PRAS40 immunoprecipitating with raptor do not strictly correlate with mTORC1 IP-kinase activity or with raptor phosphorylation, suggesting that PRAS40 association is not the key event dictating the impact of raptor phosphorylation on mTORC1 IP kinase activity. AMPK phosphorylation of raptor may lead to changes in the amount of both 14-3-3 and PRAS40 bound, which collectively act to suppress raptor-associated mTOR kinase activity. Finally, the subcellular localization of each of the raptor alleles with and without AMPK activation in the

reconstituted C2C12 myoblasts, TSC2+/+ MEFs, or TSC2-/- MEFs was unchanged (e.g., Figure 22).

AMPK Phosphorylation of Raptor Engages a Metabolic Checkpoint and Prevents Apoptosis

Activation of AMPK by energy stress causes a metabolic checkpoint, in which cells with intact AMPK signaling undergo cell-cycle arrest, while those cells defective for AMPK activation (e.g., LKB1 deficient) or key components of the AMPK pathway (e.g., TSC2 deficient or p53 deficient) continue cycling and subsequently undergo apoptosis (Inoki et al., 2003; Corradetti et al., 2004; Shaw et al., 2004a, 2004b; Jones et al., 2005; Buzzai et al., 2007). A failure to downregulate mTORC1 under conditions of energy stress prefer-entially induces cells to undergo accelerated apoptosis.

We therefore wished to determine whether phosphorylation of raptor by AMPK is required for full activation of this metabolic checkpoint, and whether the inability to phosphorylate raptor would affect the ability of cells to undergo growth arrest or apoptosis following energy stress. To exclude effects of AMPK regulation of TSC2 and p53 in this process, we utilized MEFs lacking both genes that were suppressed for endogenous raptor and reconstituted with human wild-type raptor or AA raptor (as discussed previously—see Figure 4). Importantly, under standard growth conditions these cells grew at comparable rates and dis- played no differences in viability or proliferation. We examined

the response of these cells to several AMPK activating agents, analyzing their DNA content and cell-cycle profile by propidium iodide and flourescence-activated cell sorting (FACS). MEFs lacking TSC2 and p53 but expressing wild-type raptor undergo a significant growth arrest in the G1 and S phases of the cell cycle following treatment with AICAR, depending on the time point examined (Figure 23, 24, and data not shown). This was most readily observed as a decrease in the fraction of cells progressing into G2/M as quantified by DNA content (Figure 23, 24). Cells expressing wild-type raptor undergo a significant arrest (13% in G2/M as compared to 22% in the untreated cells), whereas the cells expressing AA raptor do not. Consistent with engagement of a cell-cycle checkpoint following energy stress, the reduction in the cycling G2/M peak in AICAR- treated cells expressing wild-type raptor was paralleled by a decrease in the levels of the mitotic marker phospho-histone H3 Ser10, as detected by immunoblotting (Figure 24). In parallel cultures ex- pressing the nonphosphorylatable AA mutant raptor, the cells continued cycling, as observed by a complete absence of reduction in the G2/M population and a similar lack of suppression of phospho-histone H3 levels by AICAR (Figures 23, 24). The suppression of mitotic cells was also observed using the phospho-histone H3 Ser10 antibody for immuno-cytochemistry on AICAR- and phenformin-treated cell populations (Figure 24). The percentage of wild-type raptor-expressing cells arresting prior to G2/M and the percentage of AA raptor-expressing cells failing to arrest were concordant in the DNA content

FACS analysis, phospho-histone H3 immuno-cytochemistry, and phospho-histone H3 immunoblotting. By all three assays, AICAR and phenformin led to a similar suppression of mitotic cells in cells expressing wild-type raptor, but not the AA mutant, raptor.

In addition to AMPK phosphorylation dictating cell-cycle arrest, profound effects on apoptosis were observed at later times following energy stress. Previously, in cells lacking LKB1, AMPK, or TSC2 function, inappropriate hyperactivation of mTORC1 was found to promote apoptosis under conditions of energy stress and rapamycin treatment led to suppression of the apoptotic response (Corradetti et al., 2004; Shaw et al., 2004b; Jones et al., 2005; Lee et al., 2007a). TSC2+/+ MEFs expressing human AA raptor underwent a modest increase in apoptosis in response to prolonged (48 hr) treatment with phenformin compared to identical cells expressing human wild-type raptor (48 hr). Strikingly, in cells lacking TSC2 that express the AA raptor mutant, and are thereby severely attenuated in their ability to downregulate mTORC1 following energy stress (see Figure 13), the percentage of cells undergoing apoptosis following phenformin more than doubled when compared to cells lacking TSC2 and expressing the human wild-type raptor (Figure 25).

To ensure that this differential apoptosis was due to signals coming from AMPK signaling and not a gain-of-function effect of the AA mutant, we examined whether the AA raptor mutant sensitized cells to apoptosis in a manner dependent on intact up- stream AMPK signals. To test this hypothesis,

we utilized A549 lung adenocarcinoma cell lines, which bear LKB1 missense mutations and are null for LKB1 protein expression. A549 cell lines stably reconstituted with wild-type or kinase-dead LKB1 were subsequently infected with retroviruses expressing wild-type or AA raptor. Stable cell lines expressing each raptor allele in combination with each LKB1 allele were then treated with phenformin, and as before apoptotic rates were quantified using Annexin V FACS sorting. As seen in Figure 25, wild-type, but not AA mutant, raptor conferred protection from phenformin-induced apoptosis only in cells expressing wild-type LKB1. In cells expressing kinase-dead LKB1 and hence unable to activate AMPK, we observed no difference in the percentage of cells undergoing cell death between those expressing wild-type and those expressing AA mutant raptor. This observation suggests that the survival signal requires both wild-type LKB1 and wild-type raptor, consistent with the maximal suppression of mTORC1 in these cells. Furthermore, the extent of apoptosis observed from overexpressing the AA raptor mutant in cells with wild- type LKB1 (42%) was equivalent to the degree of apoptosis in cells expressing kinase-dead LKB1 (42%–48%). These results indicate, at least in A549 cells, that raptor phosphorylation is a key control point in the response to energy stress, and other targets of LKB1/AMPK signals such as TSC2 or p53 are not sufficient to induce effective growth arrest and prevent apoptosis in these cells. Taken together with the cell-cycle analysis, these data suggest that cells unable to inhibit mTORC1 through LKB1-AMPK-raptor signaling continue to

proliferate inappropriately under energy stress conditions, ultimately leading to increased rates of apoptosis.

Discussion

A fundamental requirement of all cells is that they couple the availability of nutrients to signals emanating from growth factors to drive proliferation only when nutrients are in sufficient abundance to guarantee successful cell division. We show here that the direct phosphorylation of the mTOR binding subunit raptor by AMPK under conditions in which ATP levels are low represents a biochemical mechanism by which eukaryotic cells couple their nutrient status to a central regulator of cell growth and proliferation.

Taken together with previous studies, the findings reported here suggest that energy stress results in LKB-dependent activation of AMPK, which directly phosphorylates both TSC2 and raptor to inhibit mTORC1 activity by a dual-pronged mechanism (Figure 26). It recently has become apparent that Akt-mediated activation of mTORC1 is also controlled via phosphorylation of two substrates by Akt: TSC2 and an mTORC1 inhibitor, PRAS40 (Sancak et al., 2007; Vander Haar et al., 2007). In parallel opposing pathways, AMPK-mediated phosphorylation of raptor induces 14-3-3 binding and inhibition of mTORC1, while Akt-mediated phosphorylation of PRAS40 induces its binding

to 14-3-3 and activation of mTORC1.

We have demonstrated here that the AMPK phosphorylation sites in raptor play a key role in the function of AMPK as a metabolic checkpoint. This metabolic checkpoint is fully analogous to the DNA damage checkpoint, with kinases serving as sensors of the stress (here ATP loss), and then initiating a response to correct the pathological damage from the stress (stimulating creation of ATP) and halting cell-cycle progression while the damage is being corrected. This metabolic checkpoint function of AMPK has been shown to be critical in a variety of cell types under conditions of low glucose, hypoxia, or following acute treatments with mitochondrial inhibitors, glycolytic inhibitors, or AMP-mimetics (Inoki et al., 2003; Corradetti et al., 2004; Shaw et al., 2004a, 2004b; Jones et al., 2005; Liu et al., 2006; Buzzai et al., 2007; Lee et al., 2007a). Inactivation of mTORC1 has previously been demonstrated to be critical for the ability of AMPK to en- force a metabolic checkpoint (Inoki et al., 2003; Shaw et al., 2004b). When mTORC1 cannot be inactivated under energy stress conditions, we show here that cells continue through the cell cycle and ultimately undergo apoptosis.

Recent evidence suggests the AMPK-mediated metabolic checkpoint on cell growth is widely conserved across eukaryotes. Hyperactivation of AMPK suppressed cell proliferation in both Drosophila and Dictyostelium mutants with defective mitochondrial function (Mandal et al., 2005; Bokko et al., 2007). In C. elegans, AMPK (aak-2) and LKB1 (par-4) orthologs are required for the

extended cell-cycle arrest of germ cells in dauer worms (Narbonne and Roy, 2006) as well as the arrest of L1 stage V lineage cells under starvation conditions (Baugh and Sternberg, 2006). In both lineages, AMPK or LKB1 loss causes inappropriate proliferation under nutrient-poor conditions. In addition, AMPK activation is required in C. elegans for lifespan extension by daf-2, heat shock, and glycolytic inhibitors (Apfeld et al., 2004; Schulz et al., 2007). In budding yeast (SNF1) and Arabidopsis (KIN10/11), AMPK orthologs play key roles in regulating growth and lifespan in response to diverse nutrient and environmental stresses (Ashrafi et al., 2000; Baena-Gonzalez et al., 2007; Hong and Carlson, 2007; Thelander et al., 2004). Given these conserved functions for AMPK, it will be interesting to determine whether the predicted AMPK phosphorylation sites in raptor orthologs in lower organisms play a role in these nutrient-dependent controls on cell growth, aging, and stress response.

Taken altogether, our findings indicate that AMPK utilizes multiple targets in mammalian cells to effectively suppress mTORC1 signaling. The integral role that raptor plays in mTORC1 function and the remarkable conservation of the AMPK sites across eukaryotes suggest that raptor phosphorylation by AMPK orthologs may be an ancestral mechanism for coupling cell growth to nutrient status. The phosphorylation of raptor by AMPK could also play a physiological role in other mammalian processes that both AMPK and mTORC1 regulate, including autophagy, angiogenesis, insulin

sensitivity, mitochondrial metabolism, and specific transcriptional responses. In addition, the existence of this direct regulation of mTORC1 by AMPK suggests that widely used diabetes therapeutics such as metformin, which act through AMPK activation, or environmental factors such as exercise and diet that contribute to physiological AMPK activation, may modulate tumorigenesis through this distinct signaling route. The direct phosphorylation and inhibition of raptor function by AMPK also suggest a possible therapeutic window for the use of AMPK agonists to treat tumors arising in patients with tuberous sclerosis complex or tumors exhibiting hyperactivation of mTOR via other genetic lesions.

As the response to a shortage of environmental nutrients and resultant loss in cellular energy represents one of the most fundamental pathological events of all organisms, we anticipate that further investigation of the downstream targets of AMPK will provide great insight into the emerging nexus of cancer, diabetes, and lifespan extension controlled by this ancestral signaling pathway.

Experimental Procedures

DNA Constructs

pBABE-Flag-w.t. LKB1 and Flag-Kinase dead (K78I) LKB1, HA-4E-BP1, and HA-S6K1 were described previously (Shaw et al., 2004a, b). pEBG and pEBG-alpha1 1-312 AMPK were kind gifts of Dr. Lee Witters (Dartmouth Medical School, Hanover, NH). myc-raptor, myc-mTOR, and HA-Gbl constructs originating in Dr. David Sabatini's lab (MIT, Cambridge, MA) and pEBG-14-3-3 zeta constructs originating in Dr. Joseph Avruch's lab (MGH, Boston, MA) were obtained from Addgene.org (Cambridge, MA). Long-range Quikchange mutagenesis was utilized to introduce alanine or aspartate mutations into the myc-raptor construct at serine 722 and 792. The CMV-myc-raptor constructs were then subcloned into FBneo and pBABE-hygro retroviral expression constructs for stable introduction into various cell lines. All raptor cDNAs were sequenced in their entirety to verify no additional mutations were introduced during PCR or mutagenesis steps.

Cell Culture

Littermate-derived LKB1+/+ and -/- MEFs were described previously (Shaw et al, 2004a,b) TSC2-/- p53-/- and control p53 -/- littermate MEFs were obtained from Dr. David Kwiatkowski as previously described (Zhang et al, 2003). SV40 immortalized wild-type and AMPKα1-/-, α2-/- double knockout MEFs were obtained from Keith Laderoute with permission from Benoit Viollet

(Laderoute et al., 2006). C2C12 cells were obtained from ATCC. Cells were cultured and retrovirally infected as previously described (Shaw et al., 2004a). For the MEF experiments in Figure 2A: 1x106 cells of each genotype were plated and the next day serum-starved for 24h. Cells were either lysed as such or their media replaced with fresh DMEM with 10% FBS, DMEM with 10% FBS and 2mM AICAR, DMEM with 10% FBS and 5mM phenformin for 90mins. Phenformin and AICAR stocks are made fresh by diluting each in DMEM at a 200X working concentration. For HEK293T experiments, cells were transfected with Lipofectamine 2000 (Invitrogen) and at 24h, treated with vehicle (EtOH), or 50μM resveratrol (in EtOH) for 30 min, where indicated with 20 min pre-treatment with STO-609 to reduce the high background of AMPK activation in HEK293T cells due to constitutive CAMKK2 activation. HEK293T were treated with 5mM phenformin for 30 min where indicated. For replacement of endogenous murine raptor with human raptor, C2C12 or TSC2+/+, p53-/- MEF or TSC2-/-, p53-/- MEF cell lines were infected with retroviruses expressing human raptor alleles and selected for 5 days in neomycin. Resulting stable cell lines were subsequently infected with either pLKO or pLKO-raptor (TRCN0000077472) that recognizes mouse but not human raptor. Cells were selected in puromycin for 3 days then western lysates were made to verify efficiency of shRNA knockdown. These cells were treated with 5mM phenformin or 2mM AICAR diluted in DMEM as indicated.

Apoptosis Assay

Cells were seeded at a concentration of 1 x 105 cells/mL and treated with AMPK agonists to induce apoptosis. Cells were collected at the appropriate time point, washed once in PBS, once in 1x Annexin V buffer and treated as described by the Annexin V staining protocol (BD Pharmingen, San Diego, CA). Briefly, cells were resuspended in Annexin V buffer to a concentration of 10^6/mL. Cells were then stained with phycoerythrin (PE)-conjugated Annexin V antibody (BD Pharmingen) and 7-amino-actinomycin D (7AAD) and incubated at room temperature for 15 minutes. Annexin V buffer was then added to each sample with gentle mixing. Staining intensities of viable cells were measured by using a FACSCalibur (Becton Dickinson). Flow cytometry data was analyzed using FlowJo 8.6 software (Tree Star Inc.)

Immunocytochemistry and Imaging

MEFs reconstituted with wild type or AA Raptor were plated on glass cover-slips at a density of 3x105 cells per well in 6-well tissue culture plates. Cells were treated with either 2mM AICAR or 2.5mM phenformin 18h after plating. 18h later, cells were fixed in 4% PFA in PBS for 10 minutes and permeabilized in 0.2% Triton in PBS for 10 minutes. The following primary antibodies were used: rabbit anti-phospho-histone H3 ser10 (Millipore, 1:200) and mouse anti-myc epitope (9B11, Cell Signaling Technologies, 1:2000). Secondary antibodies were anti-rabbit Alexa488 and anti-mouse Alexa594

(Molecular Probes, 1:1000). DNA was stained with DAPI and where indicated with rhodamine phalloidin (Molecular Probes) to visualize actin. Coverslips were mounted in FluoromountG (SouthernBiothech). Images were acquired on a Zeiss Axioplan2 epifluorescence microscope coupled to the Openlab software. 3 random fields per condition were acquired using the 20x objective. The total number of cells and the number of phospho-histone H3-positive cells were counted in each field and the 3 fields were added for each condition. The total number of cells counted was 902 for wt Raptor control, 802 for wt Raptor AICAR, 632 for wt Raptor phenformin, 720 for AA Raptor control, 843 for AA Raptor AICAR and 479 for AA Raptor phenformin.

Biochemistry

MEFs were lysed in boiling SDS-lysis buffer (10mM Tris pH7.5, 100mM NaCl, 1% SDS) after the indicated treatments. After trituration, lysates were equilibrated for protein levels using the BCA method (Pierce) and resolved on 6 to 12% SDS-PAGE gels, depending on the experiment. Gels were transferred to PVDF and western blotted according to the antibody manufacturer suggestions. Immunoprecipitations, kinase assays and 7-methyl GTP pulldowns were performed as previously described (Shaw et al., 2004a,b), except for immunoprecipitations to examine raptor-mTOR association were performed in lysis conditions exactly as described (Sancak et al., 2007). To detect endogenous 14-3-3 co-immunoprecipitation, due to migration of 14-3-3

on SDS-PAGE at the same size as the immunoglobulin light chain, myc-tagged raptor immunoprecipitates on beads were eluted for 1 hr at RT in 100 µl of 50 mM Tris pH 7.5, 150 mM NaCl, and 1 mg/ml myc peptide (Covance). Recombinant GST or GST-14-3-3 were produced in E. coli as previously described (Yaffe et al.,1997) then purified on glutathione sepharose and eluted with free glutathione. 10 µg of GST or GST-14-3 was added to cell extracts and incubated with rocking at 4 degrees for 2 hours. Complexes were precipitated with the addition of gluthathione sepharose. In vitro mTORC1 kinase assays on myc-tagged Raptor immunoprecipitates were performed exactly as previously described (Sancak et al., 2007). 150 ng of S6K1 purified from rapamycin treated HEK293 was used per kinase sample. To prepare S6K1 protein for use as a substrate: HEK293T cells in 6 cm plates were transiently transfected with a plasmid encoding N-terminally Flag tagged rat S6K1. 44 hr posttransfection, cells were pretreated with 100 nM rapamycin for 1 hr, washed once with cold PBS, and extracted with lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X100, 1 mM DTT, 1 mM PMSF, 10 µg leupeptin, 2 µg/ml pepstatin A, 10 µg/ml aprotinin). Lysates were cleared (10 min at 16,000 x g, 4º C) and incubated with M2 beads (Sigma, 6.7 µl per plate) for 2 hr at 4 ºC. Beads were washed twice with lysis buffer and twice with wash buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM DTT, 0.01% Igepal CA630, 10% glycerol), and then S6K1 was eluted in wash buffer containing 0.5 mg/ml Flag peptide (Sigma, 15 µl/plate, 1 hr at room temperature). Protein was

quantified by Bradford assay, snap frozen and stored at -80 ºC.

Mice

8 week old littermate LKB1 +/+ or fl/fl FVB males were tail-vein injected with 75ul adenovirus CMV5-cre recombinase (pfu 1x1011; courtesy of the University of Iowa Vector Core). One week after injection mice were fasted for 24h or fed ad libitum Half the fasted mice were injected with 250mg/kg metformin in 0.9% saline solution and the other half were injected with the equivalent amount of saline vehicle. One hour after injection, livers were harvested, flash frozen in liquid nitrogen and tissue extracts were made as previously described (Shaw et. al, 2005). Primary hepatocytes were isolated as previously described (Dentin et al., 2007).

Peptide Library Screening

FLAG-tagged human AMPKα1, rat AMPKβ1, and rat AMPKγ1 were co-expressed by transient transfection in HEK293 cells. Active AMPK was purified from cell lysates by binding to immobilized M2 FLAG antibody (Sigma) and elution with 5 mM free M2 peptide in 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.01% Igepal CA630, 10% glycerol for 2 hr at 4º C. PSPL screening was done essentially as described (Turk et al, 2006). Briefly, a set of 180 peptides with the general sequence Y-A-X-X-X-X-X-S/T-X-X-X-X-A-G-K-K(biotin) was used in which X represents a

roughly equimolar mixture of the 17 amino acids (excluding serine, threonine and cysteine), and S/T indicates an even mix of serine and threonine. For each peptide, one of the nine X positions was substituted with one of the 20 amino acids (20 amino acids x 9 positions = 180 peptides total). Peptides were arrayed in 384-well plates a spatially addressable manner in 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and active AMPK and γ-[^{32}P]-ATP was added to all wells (final [peptide] = 50 µM and [ATP] = 100 µM, 0.025 µCi/µl in each well). After incubating 2 hr at 30 ºC, aliquots of the reactions were spotted onto streptavidin membrane (Promega), which was quenched, washed extensively, dried, and exposed to a phosphor storage screen.

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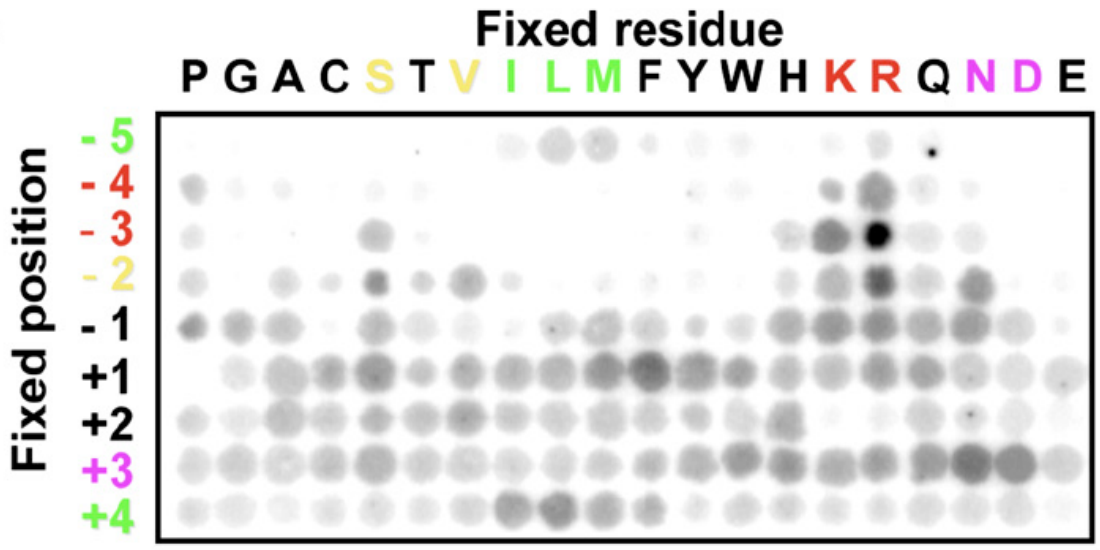
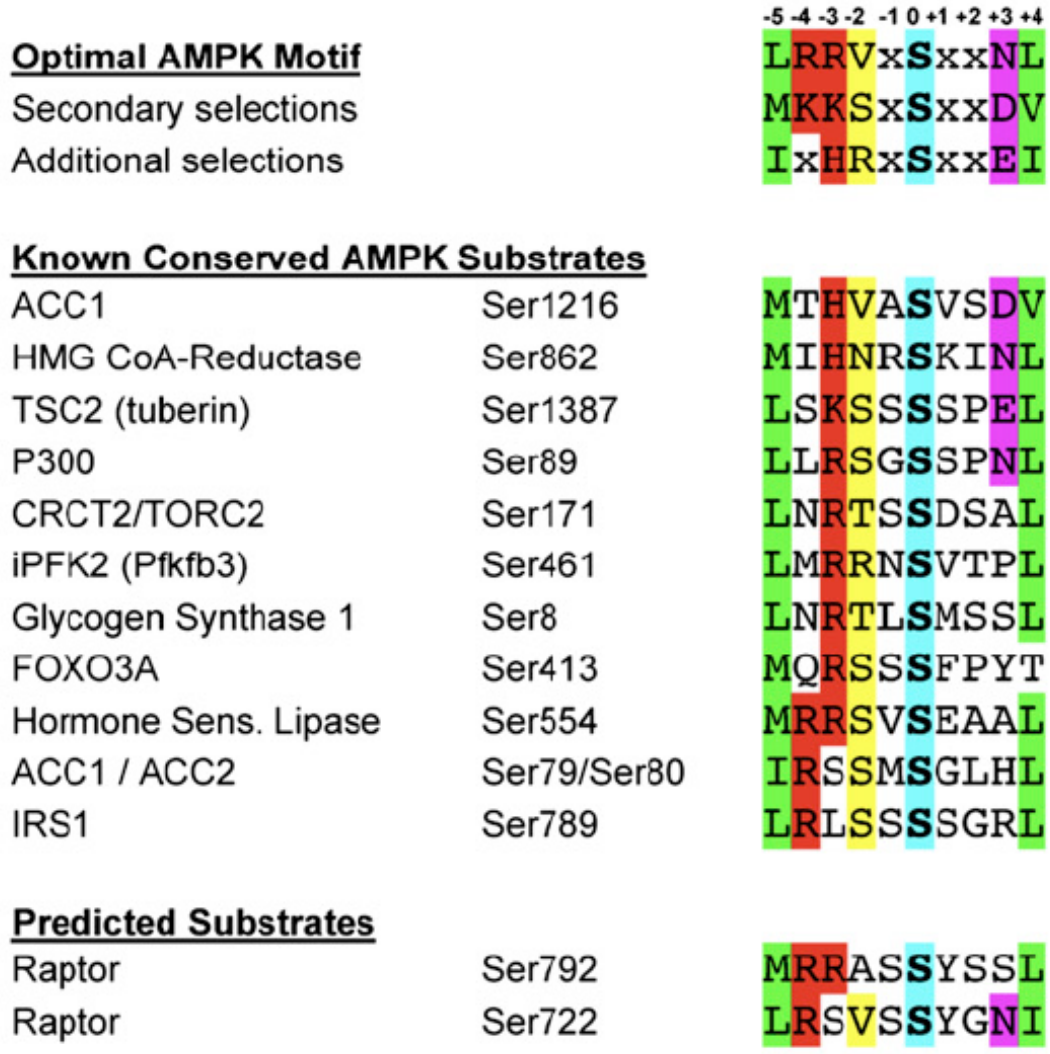
On this publication, I was the primary author. Reuben Shaw directed and supervised the writing and oversaw the project which formed the basis of this chapter.



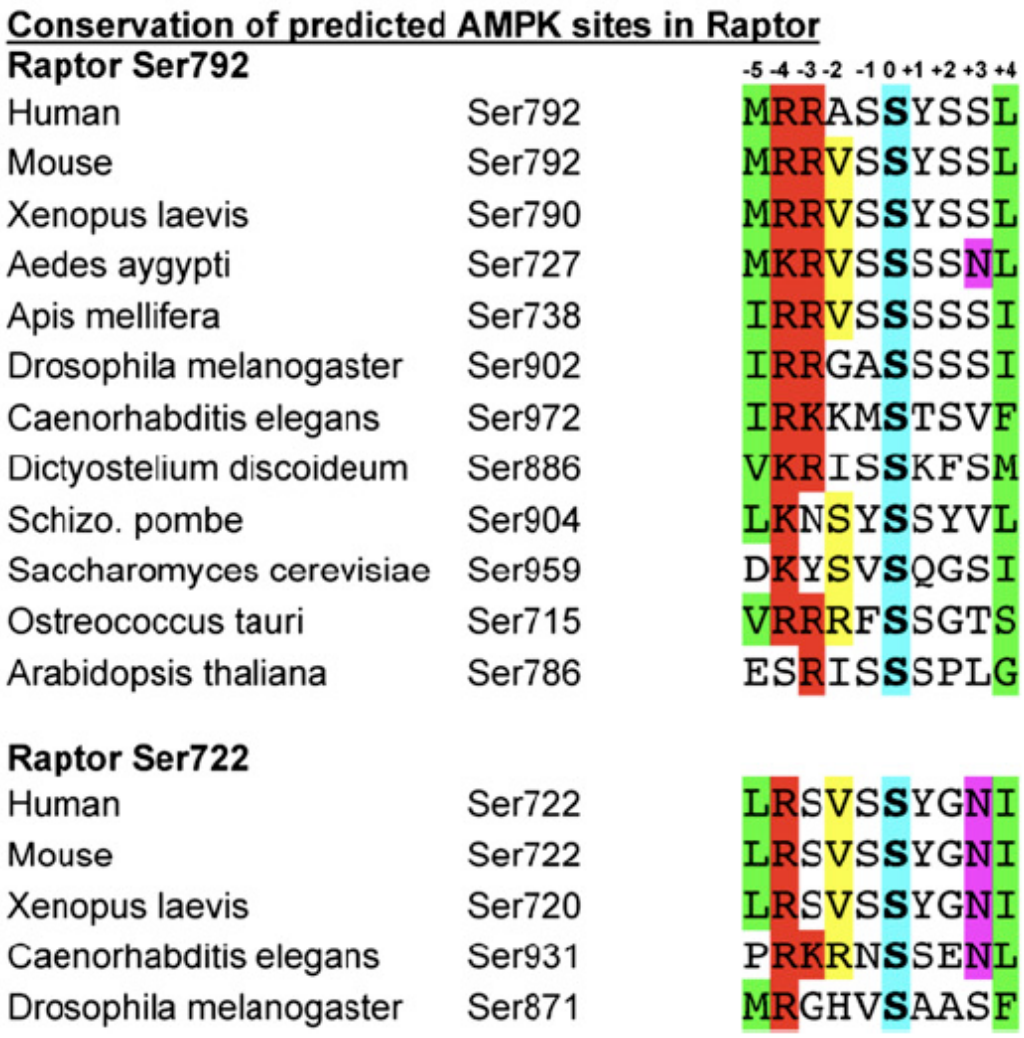
Chapter II Figure 1. Peptide Library Profiling the Optimal Substrate Motif for AMPK

A spatially arrayed PSPL was subjected to in vitro phosphorylation with active AMPKa1 and radiolabeled ATP. Each peptide contained one residue fixed at one of nine positions relative to the centrally fixed phosphoacceptor residue (an equal mix of serine and threonine). Aliquots of each reaction were spotted onto avidin membrane, which was washed, dried and exposed to a phosphor storage screen, providing the array of spots shown in the figure. AMPK displayed strong selectivity at the -5, -4, -3, -2, +3 and +4 positions.



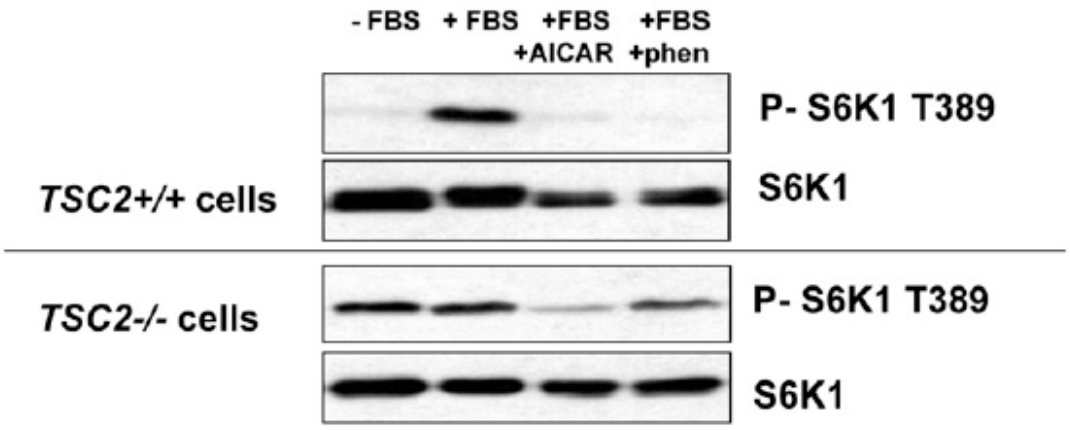
CHAPTER II Figure 2. Comparison of optimal AMPK substrate motif with known and candidate substrates

The optimal and secondary selections taken from triplicate analyses as in Figure 1 are displayed. AMPK phosphorylation sites in the best established in vivo substrates of AMPK conform to the substrate motif derived from the peptide library data. All substrates shown were isolated in bioinformatics searched for proteins containing a conserved AMPK phosphorylation motif. The same search yields two predicted AMPK sites in raptor.

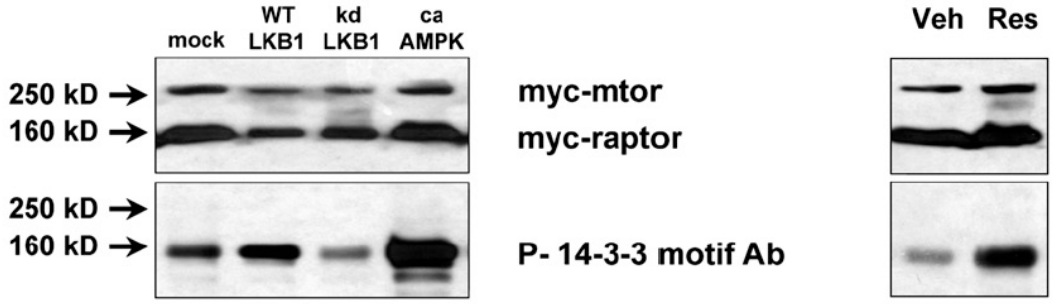


CHAPTER II Figure 3. Evolutionary conservation of predicted AMPK sites in raptor

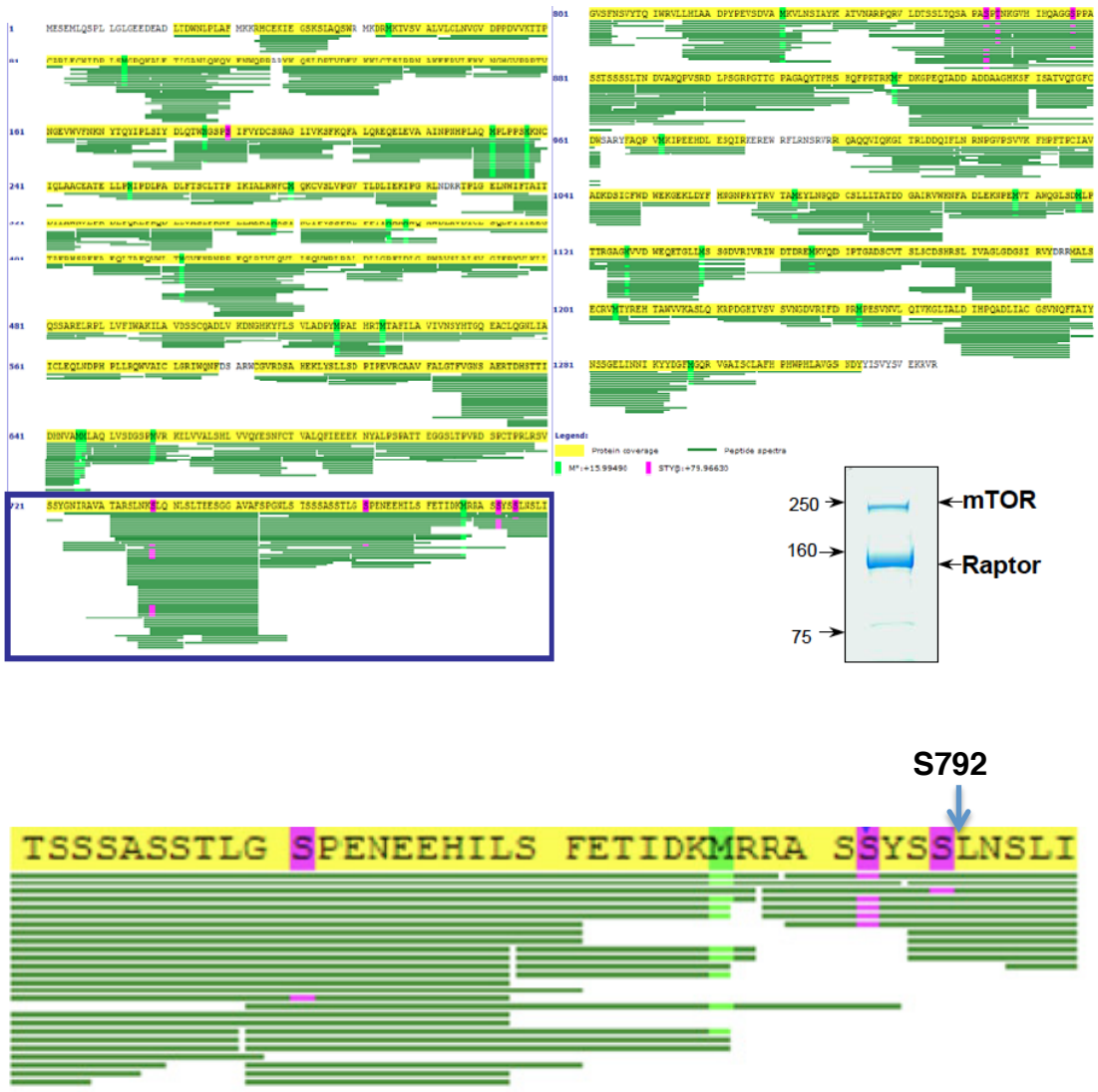
The predicted AMPK sites in raptor are highly conserved across evolution.



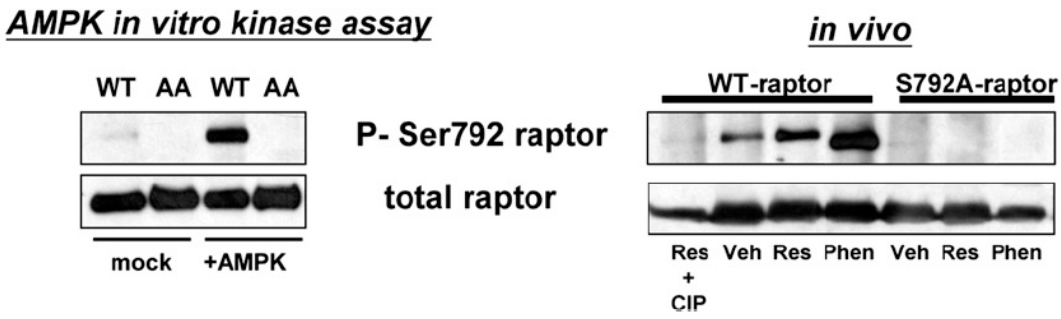
CHAPTER II Figure 4. mTORC1 signaling in TSC2-deficient cells remains responsive to energy stress.
TSC2+/+ or TSC2-/- matched MEFs were serum starved overnight (-FBS) and replaced with fresh media containing 10% fetal bovine serum (+FBS) or serum-containing media with 2 mM AICAR or 5 mM phenformin. Cells were lysed 1 hr after media replacement. Lysates were immuno-blotted for the mTORC1-dependent Thr389 phosphorylation in p70 S6K1 and for total S6K1 protein.



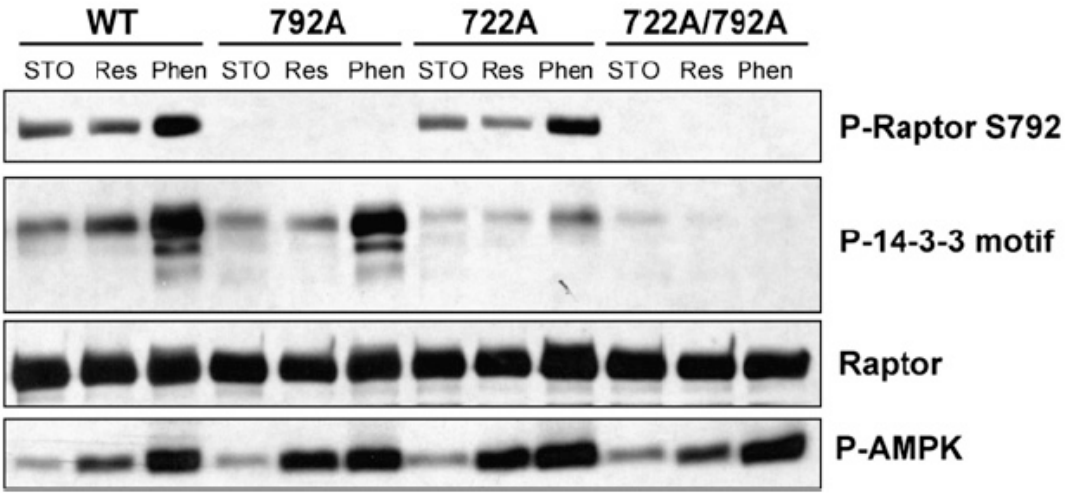
CHAPTER II Figure 5. Overexpressed raptor is phosphorylated in HEK293 cells in an LKB1- and AMPK-dependent manner.
(Left panel) myc-tagged mTOR and myc-tagged raptor were coexpressed in HEK293 cells with empty vector, wild-type LKB1, kinase-dead LKB1, or a constitutively active AMPKα1 allele (1-312 truncation). Raptor phosphor-ylation was detected using the phospho-14-3-3 motif antibody. (Right panel) HEK293 cells expressing mTOR and raptor were treated with 50 mM resveratrol for 30 min and phosphorylation of raptor was detected with the phospho-14-3-3 motif antibody.



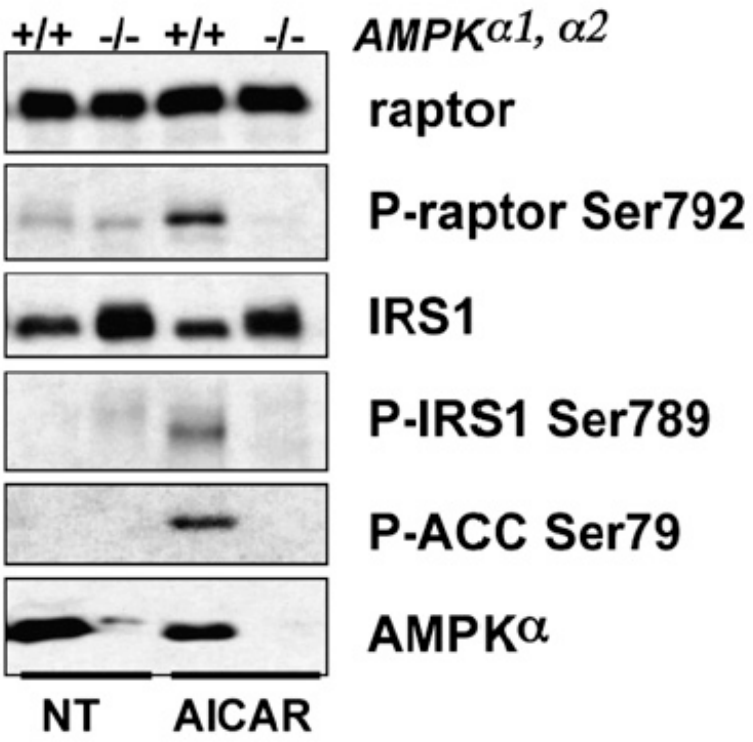
CHAPTER II Figure 6. Raptor is phosphorylated at a high level on Ser792 following resveratrol treatment.
Mass spectrometry was performed on raptor protein purified from resveratrol-treated HEK293 cells. Coomassie-stained raptor protein was isolated from an SDS-polyacrylamide gel (lower right corner) and subjected to chymotryptic digestion prior to analysis by LC-MS/MS. Amino acids 761–800 of human raptor are highlighted here. Each recovered peptide is illustrated by a single green line. Phosphorylated residues are shown in magenta.



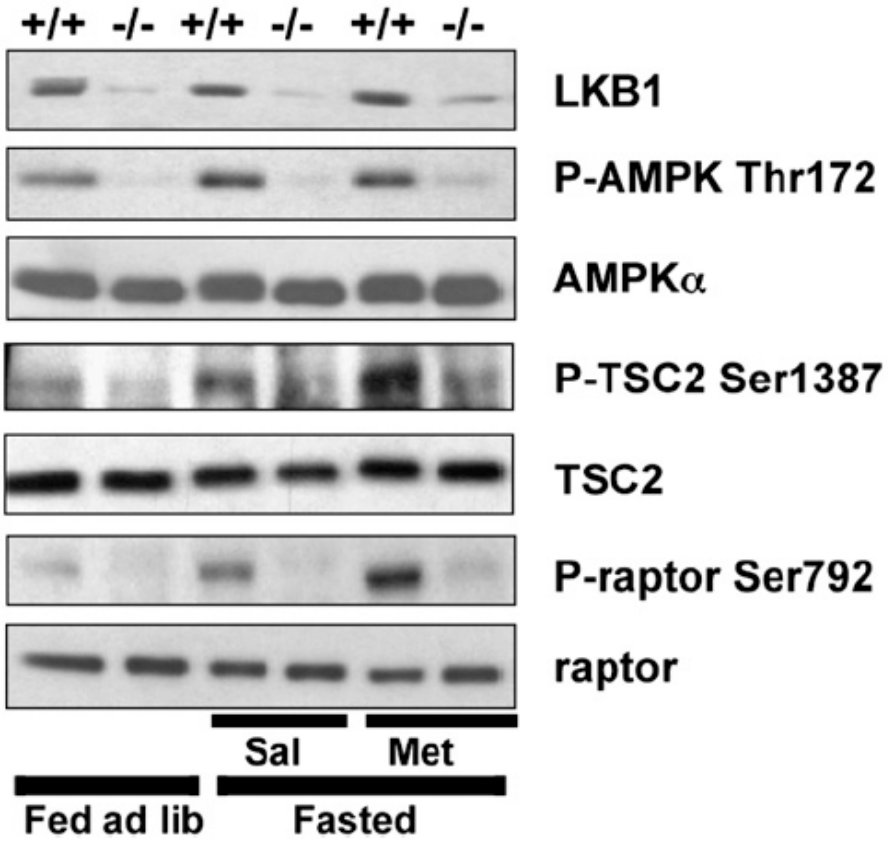
CHAPTER II Figure 7. AMPK directly phosphorylates raptor at Ser 792. A phosphospecific antibody against Ser792 of raptor recognizes raptor phosphorylated in vitro by AMPK (left) as well as wild-type, but not S792A mutant, raptor (right) following treatment with 50 μM resveratrol or 5 mM phenformin in HEK293 cells.



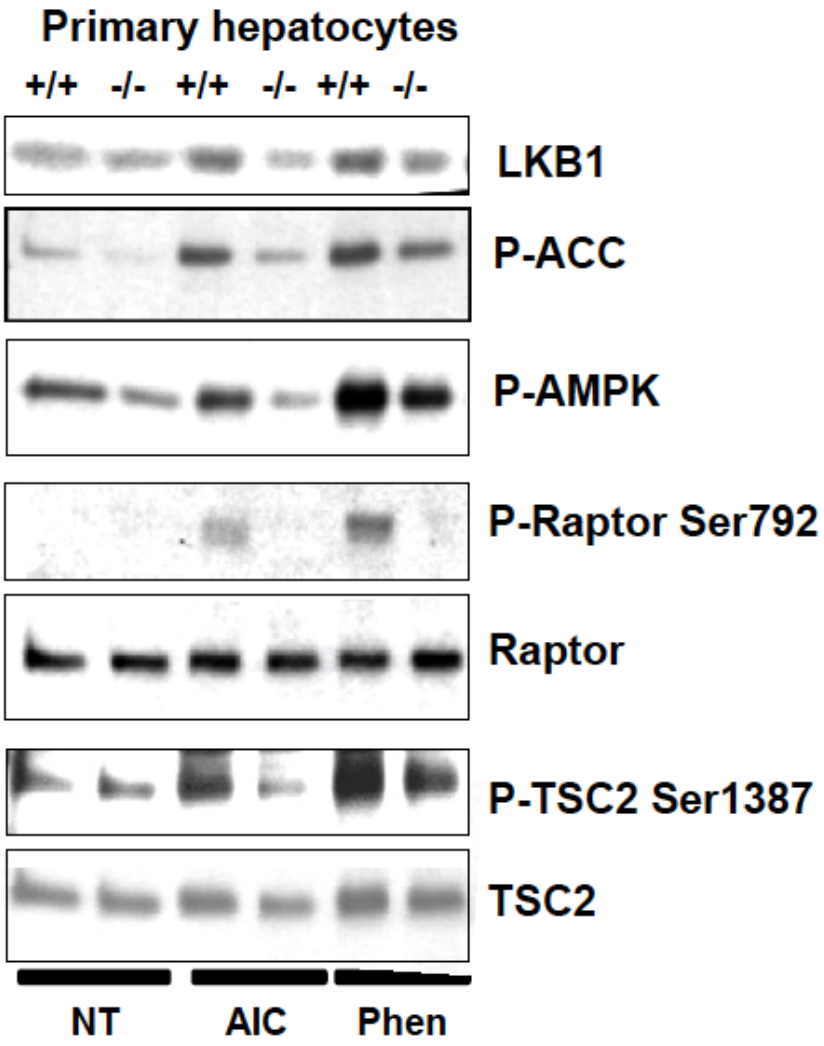
CHAPTER II Figure 8. Both Ser722 and Ser792 are phosphorylated in an AMPK-dependent manner in HEK293 cells.
HEK293 cells were transfected with wild-type, S722A, S792A, or the double mutant S722A/S792A raptor allele and treated as indicated. Raptor was immunoprecipitated and immunoblotted with the phospho-Ser792, phospho-14-3-3 motif, or anti-Myc epitope tag antibody. Phospho-ACC was immuno-blotted from the total cell extracts to illustrate the degree of AMPK activation in the cells.



CHAPTER II Figure 9. Endogenous raptor is phosphorylated by AMPK. Endogenous raptor is phosphorylated at Ser792 in wild-type, but not AMPK-deficient (AMPKα1-/- α2-/-), immortalized MEFs. MEFs were treated with 2 mM AICAR for 1 hr (AICAR) or left untreated (NT), and total cell extracts were immunoblotted with the indicated antibodies.

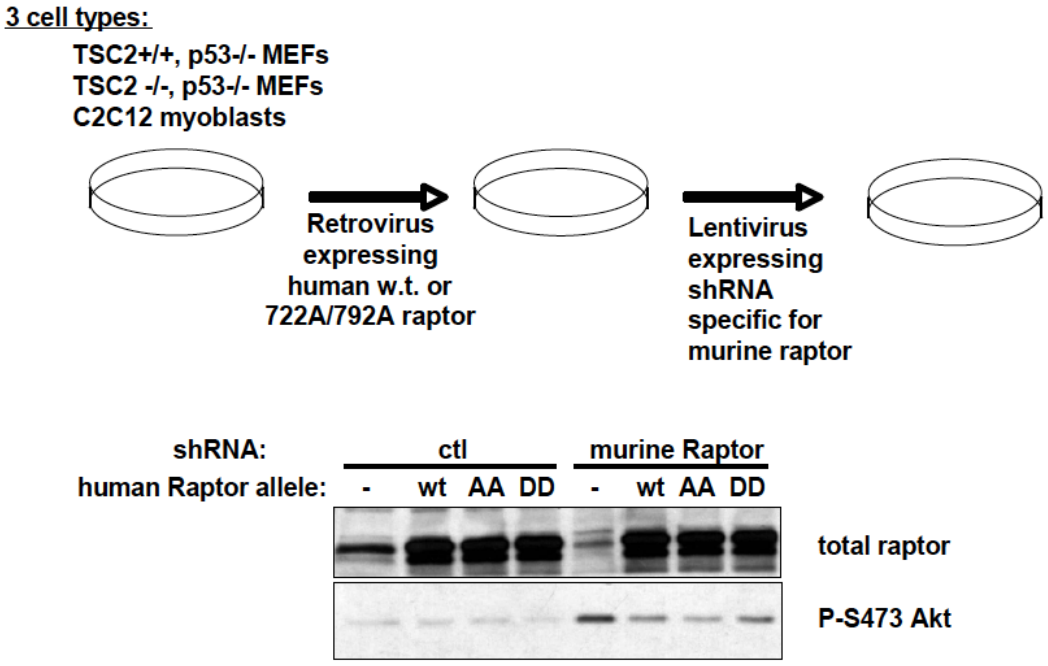


CHAPTER II Figure 10. Endogenous raptor is phosphorylated in an LKB1-dependent manner in murine livers.
Endogenous raptor is phosphorylated at Ser792 in wild-type, but not LKB1-deficient, murine liver following fasting and metformin treatment. Eight-week old mice were either fed ad libidum (ad lib) or fasted 18 hr and treated with either 250 mg/kg metformin in saline (Met) or saline alone (Sal) for 1 hr. Total cell extracts made from harvested livers were immunoblotted with the indicated antibodies.



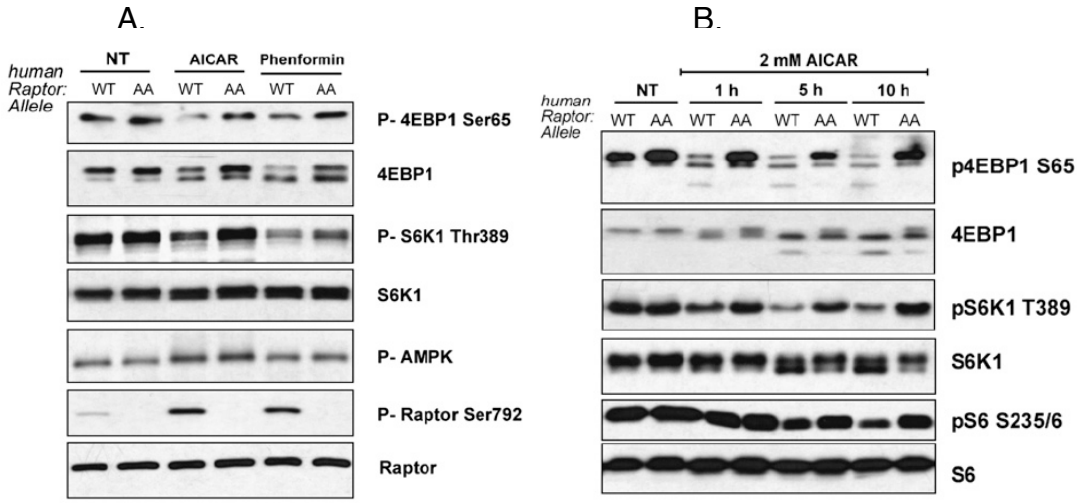
CHAPTER II Figure 11. Raptor Serine 792 Is Phosphorylated in an LKB1-Dependent Manner Following AMPK Agonists in Primary Mouse Hepatocytes

LKB1+/+ or -/- primary hepatocytes were treated with 2mM AICAR or 2mM Phenformin for 1h and total cell extracts were immunoblotted with the indicated antibodies.

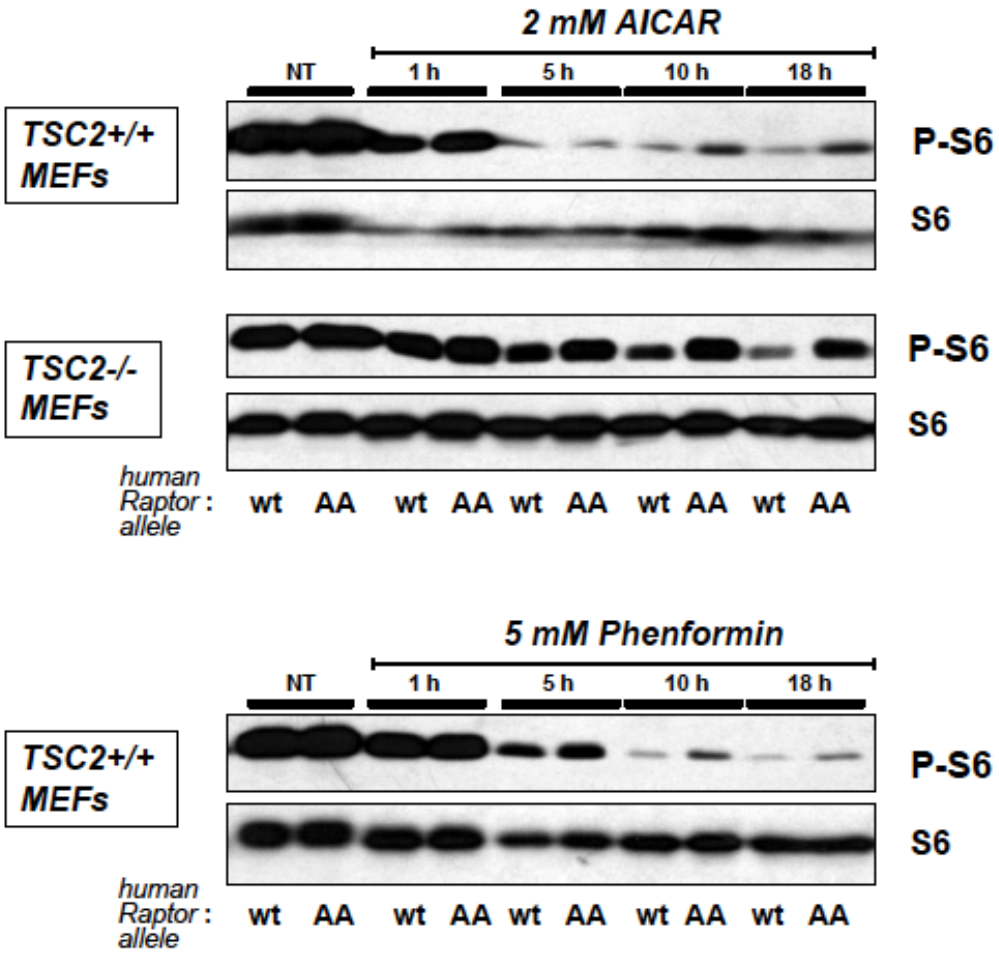


CHAPTER II Figure 12. Strategy for Replacing Endogenous Murine Raptor with Human Raptor cDNAs Expressing Wild-Type or S722A/S792A "AA" Nonphosphorylatable Raptor Alleles

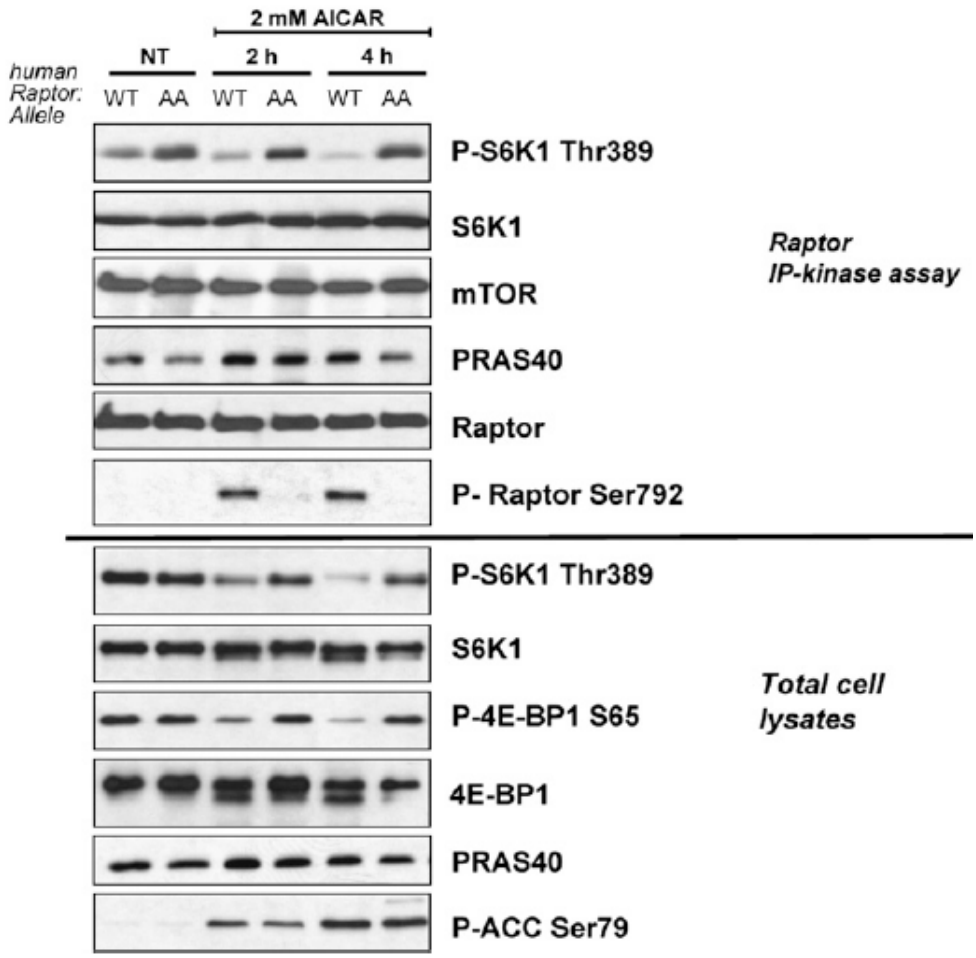
C2C12 myoblasts, TSC2+/+, p53-/- MEFs, and TSC2-/-, p53-/- MEFs were infected with retroviruses expressing wild-type or AA mutant raptor, selected, then infected with lentiviruses expressing an shRNA recognizing murine but not human raptor. These cells were then further selected. Extracts were made from a plate of cells just after selection and immunoblotted with antibodies against raptor and against Akt phospho-Ser473.



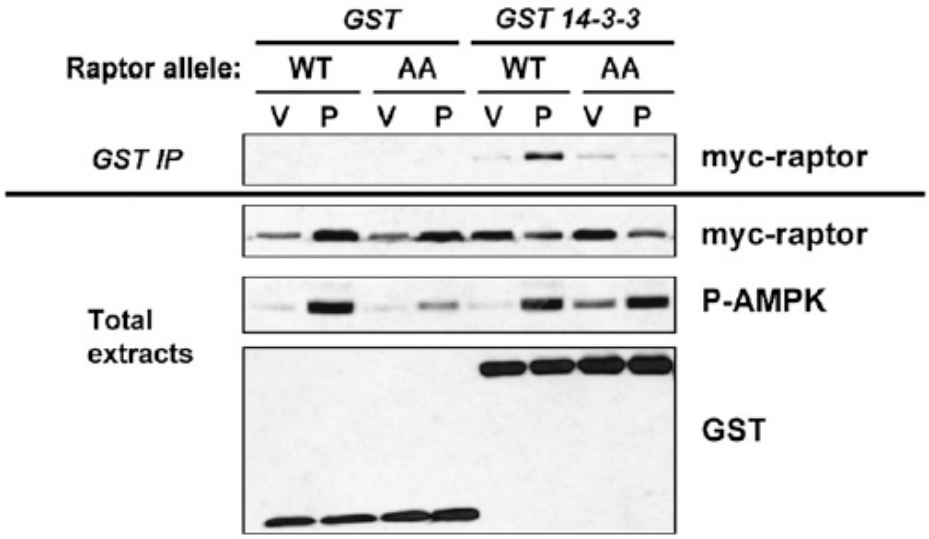
CHAPTER II Figure 13. Phosphorylation of Ser722 and Ser792 Is Required to Inhibit mTORC1 Following Energy Stress in a Variety of Cell Types
(A) C2C12 cells in which endogenous raptor has been knocked down were stably reconstituted with human wild-type or AA raptor (see Figure S3), and were treated with 1 mM AICAR or 1 mM phenformin for 1 hr as indicated. Total cell extracts were immunoblotted with indicated antibodies to examine mTORC1 signaling.
(B) TSC2-/-, p53-/-, raptor knockdown MEFs stably reconstituted with wild-type or AA raptor were treated with 2 mM AICAR as indicated and immunoblotted with indicated antibodies to examine mTORC1 signaling.



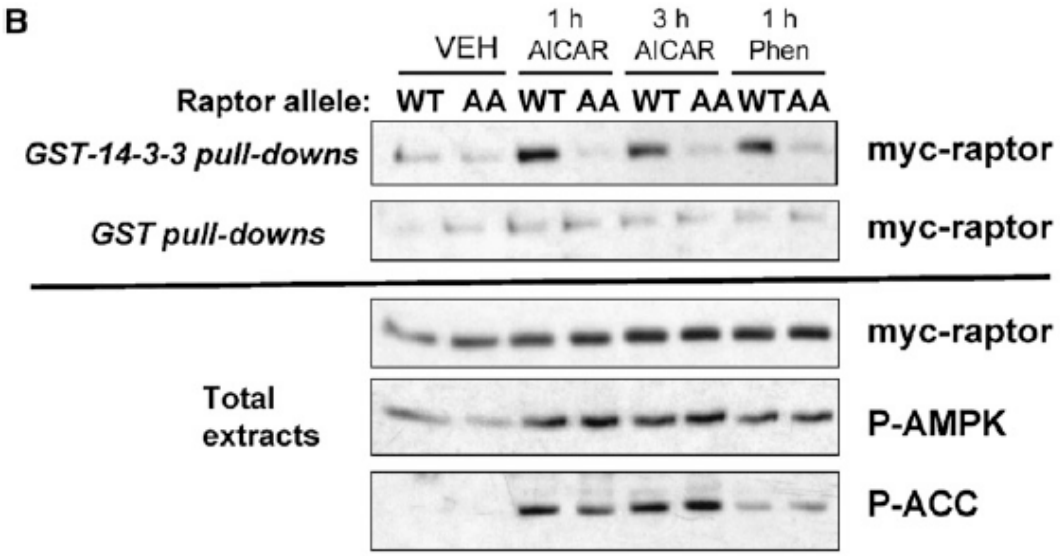
CHAPTER II Figure 14. Phosphorylation of Raptor Ser722 and Ser792 Is Needed to Fully Inhibit mTORC1 Following Energy Stress
Extracts from TSC2+/+, p53-/- MEFs, and TSC2-/-, p53-/- MEFs reconstituted with wild-type or AA mutant raptor treated as indicated were immunoblotted for phospho-S6 or total S6.



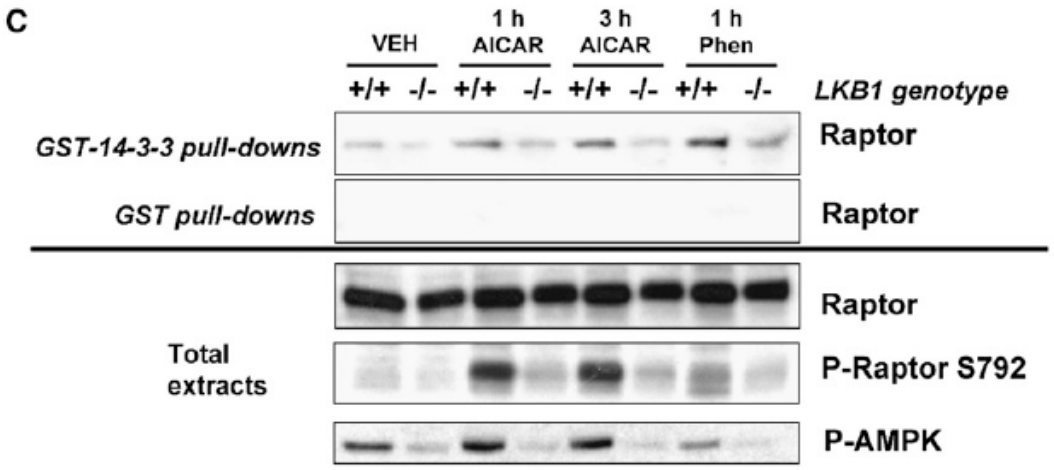
CHAPTER II Figure 15. AMPK regulates the IP-kinase activity of mTORC1.
TSC2-/- p53-/-, raptor knockdown MEFs stably reconstituted with wild-type or AA raptor were treated with 2 mM AICAR as indicated. Raptor was immuno-precipitated in CHAPS buffer and assayed for mTORC1 kinase activity using purified S6K1 as a substrate as previously described (Sancak et al., 2007). (Top) IP-kinase assays were immunoblotted for phosphorylation of purified S6K1 substrate using phospho-Thr389 S6K1 antibody as well as for level of immunoprecipitated raptor, mTOR, and PRAS40. (Bottom) Five percent of the total cell extracts that raptor was immunoprecipitated from were immunoblotted with indicated antibodies.



CHAPTER II Figure 16. AMPK regulates association between exogenously expressed raptor and exogenously expressed 14-3-3. Wild-type, but not AA mutant, raptor complexes with 14-3-3 only under energy stress conditions. HEK293 cells were cotransfected with pEBG or pEBG-14-3-3 with wild-type or AA mutant raptor, and then complexes were precipitated on glutathione beads. Beads or total cell extracts were immunoblotted with the indicated antibodies. Cells were treated with V, vehicle (DMEM) or P, 5 mM phenformin for 1 hr.



CHAPTER II Figure 17. Recombinant GST 14-3-3 proteins precipitate stably expressed raptor in an AMPK-dependent manner.
Wild-type, but not AA mutant, raptor precipitates with recombinant GST-14-3-3 protein in extracts from energy stress treated TSC2-/- MEFs stably reconstituted with human raptor alleles. GST protein pulldowns or total cell extracts were immunoblotted with the indicated antibodies.



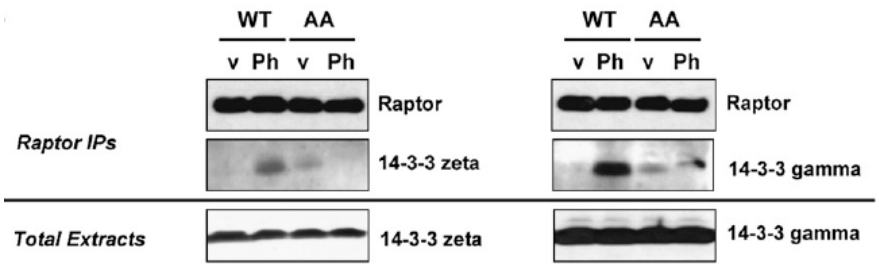
CHAPTER II Figure 18. Endogenous raptor is precipitated by recombinant GST-14-3-3 protein in an LKB1-dependent way. Endogenous raptor binds to immobilized recombinant GST-14-3-3 protein, but not recombinant GST protein, from extracts of cells treated with energy stress in an LKB1-dependent manner. GST protein pull-downs or total cell extracts were immunoblotted with the indicated antibodies.

Protein Name = cDNA FLJ76227, Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide - Homo sapiens (Human)								
Sequence	Charge	Ret. time	Sf	MH+	Xcorr	deltaCn	Sp	TIC
YLAEVAAGDDKK	1289-1291	2 28.27	0.94	1279.4	4.08	0.23	1841	34922
SVTEQGAELSNEER	1328-1329	2 28.82	0.94	1548.5	4.62	0.20	1697	9291

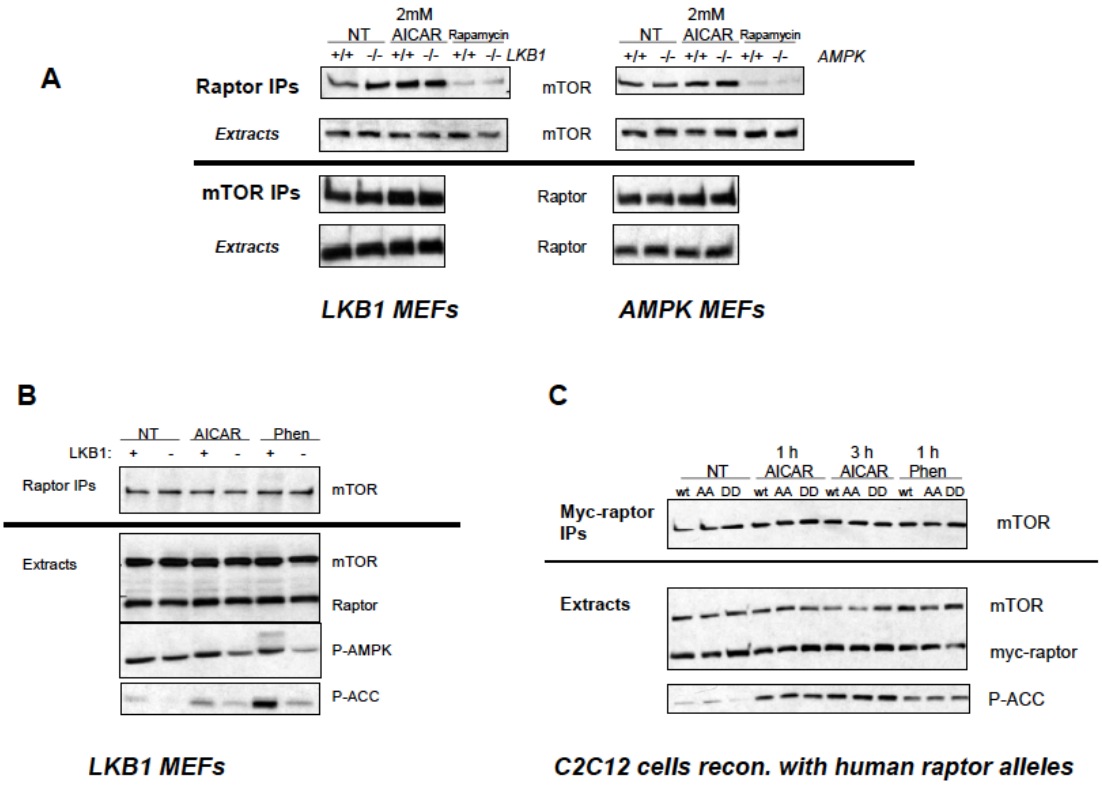
Protein Name = YWHAG protein - Homo sapiens (Human)								
Sequence	Charge	Ret. time	Sf	MH+	Xcorr	deltaCn	Sp	TIC
NVTELNEPLSNEER	1260	2 31.18	0.93	1643.6	4.79	0.39	945	2797
YLAEVATGEK	1134	2 9.35	0.87	1080.3	3.61	0.18	1020	15405
ATVVESSEK	910	2 26.09	0.54	950.8	2.48	0.16	533	5344

CHAPTER II Figure 19. Raptor Immunoprecipitates with Endogenous 14-3-3 Isoforms in HEK293T Cells

Immunoprecipitates of myc-tagged wild-type raptor from 5mM phenformin treated HEK293T cells were digested with with trypsin and subjected to MS/MS. Protein identities were predicted using SeaQuest software. Shown are several peptides derived from 14-3-3 gamma and zeta isoforms.

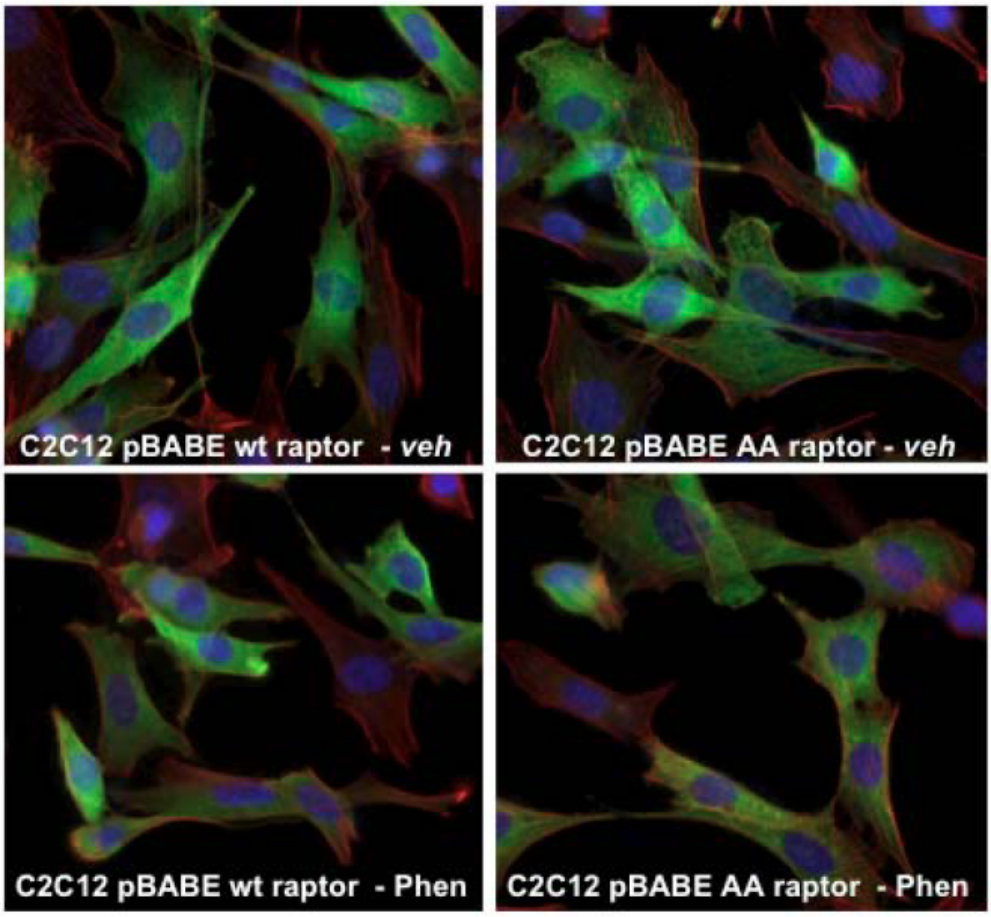


CHAPTER II Figure 20. Endogenous 14-3-3 isoforms are precipitated by stably expressed raptor in an AMPK-dependent manner.
Myc-tagged raptor immunoprecipitates from phenformin (Ph)- or vehicle (v)-treated cells were eluted with myc peptide and immunoblotted for endo-genous 14-3-3 isoforms as indicated.



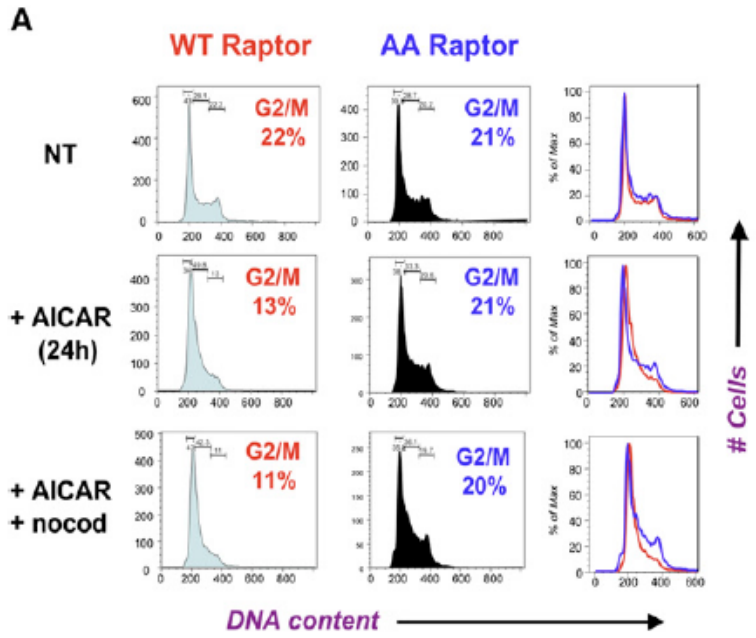
CHAPTER II Figure 21. Endogenous Raptor-mTOR Association Is Not Affected by Treatment with Energy Stress, LKB1 Deficiency or AMPK Deficiency, or Mutation of Raptor Ser722 and Ser792

(A) MEFs of the indicated genotypes (WT, LKB1-/-, or AMPK (α1/α2 double null) were treated with AICAR or rapamycin then lysed. Endogenous raptor or mTOR were immunoprecipitated and immuno-blotted for raptor or mTOR as indicated. As expected, rapamycin treatment resulted in disruption of the mTOR-raptor complex. No effect was observed following AICAR treatment.
(B) MEFs of the indicated LKB1 genotype were treated with AICAR or phenformin and endogenous raptor was immunoprecipitated. Immuno-precipitates or total cells extracts were immunoblotted as indicated.
(C) C2C12 cells stably reconstituted with myc-tagged wt or AA or DD raptor were treated as indicated and immunoprecipitated for myc-raptor. Immunoprecipitates or total cells extracts were immunoblotted as indicated.



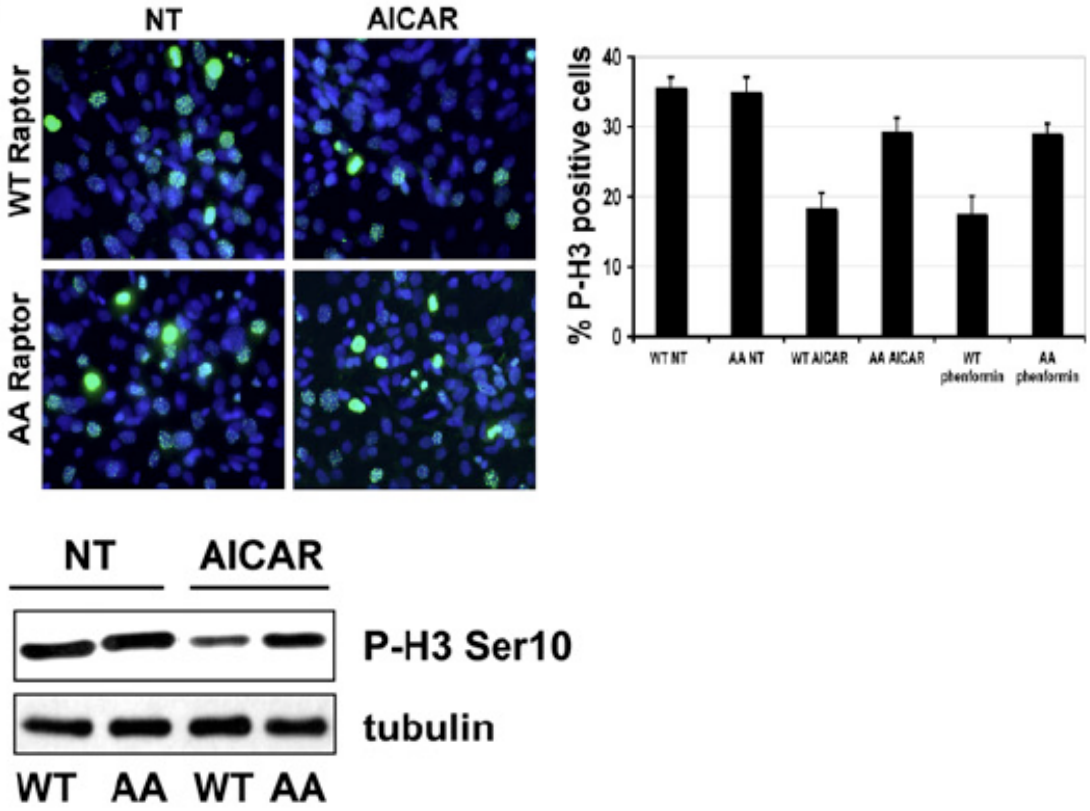
CHAPTER II Figure 22. Localization of Raptor Is Not Affected by Energy Stress or Mutation of Ser722 and Ser792

C2C12 cells stably reconstituted with myc-tagged wt or AA raptor were treated as indicated and immunocytochemistry with anti-myc epitope tag (green) or rhodamine phalloidin to visualize actin (red) or DAPI to visualize DNA (blue) was performed. No effect of raptor localization was observed with mutation of Ser722/Ser792 or treatment with energy stress.



	wt raptor	AA raptor
NT	G1: 43% S: 28% G2: 22%	G1: 40% S: 29% G2: 21%
+AICAR (24h)	G1: 34% S: 50% G2: 13%	G1: 38% S: 34% G2: 20%
+AICAR + noc (24h)	G1: 43% S: 42% G2: 11%	G1: 35% S: 36% G2: 20%

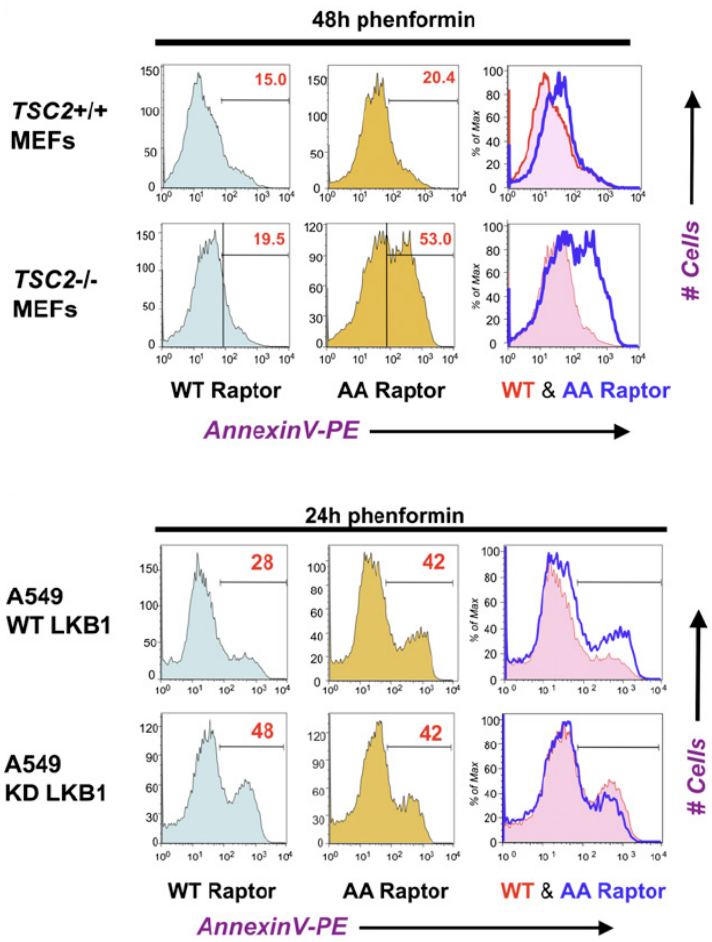
CHAPTER II Figure 23. TSC2-/-, p53-/- MEFs expressing wild-type raptor undergo G1/S arrest following AICAR treatment, while those expressing AA mutant raptor do not.
Cells were left untreated (NT) or treated with 2 mM AICAR, or treated with 2 mM AICAR and 3 hr later exposed to nocodazole (+nocod) to arrest any cycling cells in G2/M. At 24 hr after AICAR treatment, all cells were fixed and analyzed for DNA content using propidium iodide and FACS analysis. The percentage of cells in the G2/M phase of the cell cycle is highlighted in each population. Quantitation is beside.



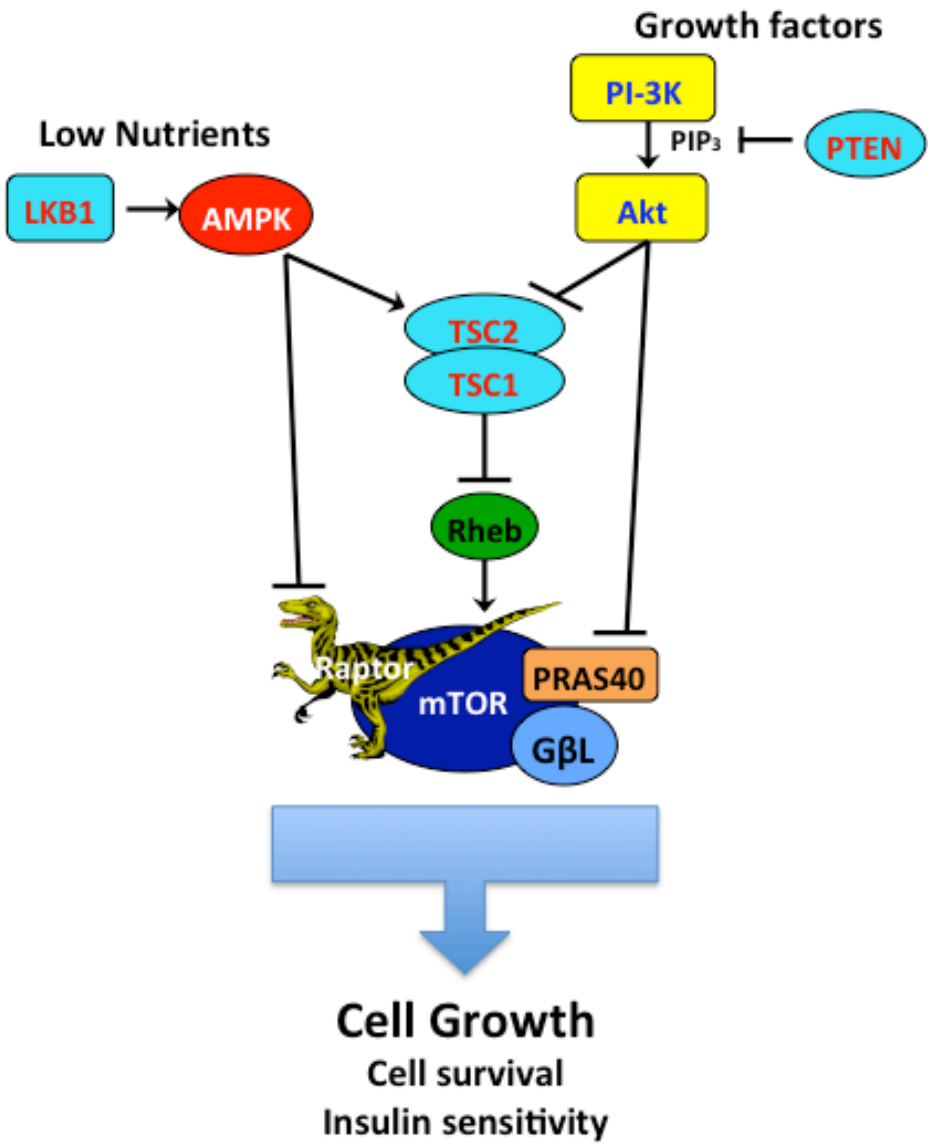
CHAPTER II Figure 24. Cells lacking the ability of AMPK to phosphorylate raptor proceed into M-phase more than cells with wild-type raptor following energy stress.

(Above) Cells were plated on coverslips and the next day left untreated (NT) or treated with 2 mM AICAR and fixed 18 hr later. Cells were processed for phospho-histone H3 Ser10 immunocytochemistry to visualize the cells actively going through mitosis at the time the cells were fixed. DAPI was used as a nuclear counterstain. Histogram quantifies phospho-histone H3 immuno-cytochemistry on indicated cells treated with 5 mM phenformin, or 2 mM AICAR for 18 hr. At least 300 cells were scored for each condition. Error bars indicate standard deviation.

(Below) Cell extracts from parallel plates to the ones analyzed above were immunoblotted for phospho-histone H3 Ser10 as a marker of the percentage of cells in mitosis.



CHAPTER II Figure 25. AMPK phosphorylation of raptor protects cells against apoptosis during energy stress.
(Above) TSC2+/+, p53-/- MEFs or TSC2-/-, p53-/- MEFs expressing AA mutant raptor undergo apoptosis to a greater extent than those expressing WT raptor at later time points following energy stress treatment. Cell populations of indicated genotypes were treated with 5 mM phenformin, and at 48 hr the percentage of cells undergoing apoptosis was quantified using Annexin V staining and FACS analysis. Histograms of cells expressing wild-type raptor (red trace) and AA raptor (blue trace) are overlaid in the rightmost panel. The percentage of apoptotic cells in the Annexin V-positive population is indicated in red at the upper right hand corner of each histogram.
(Below) Upstream AMPK signals from LKB1 are needed for the protective effect of WT raptor on apoptosis following energy stress. A549 human lung adenocarcinoma cells, which are null for LKB1, were stably reconstituted with wild-type LKB1 (WT) or mutant kinase-dead K78I (KD) LKB1-expressing retroviruses. These cells were subsequently stably infected with retroviruses expressing wild-type or AA raptor. Each of the four resulting populations was treated with 5 mM phenformin and analyzed for apoptosis as above.



CHAPTER II Figure 26. Nutrients and Growth Factors Control mTORC1 Activity through Common and Unique Downstream Targets
AMPK and Akt both converge to phosphorylate distinct sites in TSC2. In addition, AMPK directly phosphorylates raptor and Akt directly phosphorylates PRAS40 to regulate the activity of the mTORC1 complex through separate means. Strikingly, both AMPK-mediated suppression of raptor and Akt-mediated suppression of PRAS40 involve the phosphorylation sites in each protein binding to 14-3-3, resulting in the inactivation of those targets. Inherited mutations in LKB1, TSC1. TSC2, and PTEN all result in hamartoma syndromes in humans, indicating that hyperactivation of mTORC1 is a common biochemical mechanism underlying these genetic disorders.

CHAPTER THREE:

Raptor is Phosphorylated by Cdc2 During Mitosis.

Abstract

We have discovered that raptor becomes highly phosphorylated in cells in mitosis. Utilizing tandem mass spectrometry, we identified a number of novel phosphorylation sites in raptor, and using phosphospecific antibodies demonstrated that raptor becomes phosphorylated on phospho-serine/ threonine-proline sites in mitosis. A combination of site-directed mutagenesis in a tagged raptor cDNA and analysis with a series of new phospho-specific antibodies generated against different sites in raptor revealed that Serine 696 and Threonine 706 represent two key sites in raptor phosphorylated in mitosis. We demonstrate that the mitotic cyclin-dependent kinase Cdc2/CDK1 is the kinase responsible for phosphorylating these sites, and its mitotic partner Cyclin B efficiently coimmunoprecipitates with raptor in mitotic cells.

Introduction

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) is a key mediator of the cellular response to nutrient status through its regulation of translation, ribosome biogenesis, mitochondrial metabolism, and autophagy (LaPlante and Sabatini, 2009). mTOR is present in one of two complexes within the cell: mTORC1 is defined by raptor, GβL/mLST8, and negative regulatory subunits PRAS40 and DEPTOR, whereas mTORC2 contains rictor, mSin1, and Protor as well as GβL/mLST8 and DEPTOR

(Peterson et al., 2009).

The best-established substrates of mTORC1 demonstrate the importance of mTOR in translational control. mTOR phosphorylates S6K1 at T389 to enhance S6K1 activity, which amongst other things phosphorylates the S6 subunit of the ribosome to promote translation. mTOR also phosphorylates 4EBP1, causing its dissociation from its binding partner eIF4E, which is then free to associate with the cap-complex to promote cap-dependent translation (Wullschleger et al., 2006).

The activity of mTORC1 is dependent on the small Ras-like GTPase, Rheb, whose GTP-loaded state is regulated by a GTPase-accelerating protein (GAP) complex composed of the TSC1 and TSC2 tumor suppressors. Inputs from a variety of pathways converge on the TSC1/2 complex to regulate mTORC1 signaling (Huang et al., 2008). Following growth factor stimulation, Akt, Erk and Rsk can phosphorylate and inactivate TSC2, leading to activation of mTORC1. Under conditions of low ATP, the energy-sensing kinase AMPK is activated and phosphorylates and activates TSC2, inhibiting mTORC1.

In addition to the hub of signaling at TSC2, phosphorylation of components of mTORC1 have recently been shown to have important regulatory roles in mTOR signaling (Copp et al., 2009, Acosta-Jaquez et al., 2009, Foster et al., 2009, Sancak et al., 2007, Vander Haar et al., 2007, Gwinn et al., 2008, Carriere et al., 2008). PRAS40 is a substrate of both Akt and mTOR, where upon phosphorylation, PRAS40 dissociates from mTORC1,

relieving inhibition of mTORC1 activity following growth factor stimulation. mTOR also phosphorylates the recently identified mTORC1 component DEPTOR, marking it for degradation and further alleviating inhibition of mTORC1 (Peterson et al., 2009). Raptor (regulatory associated protein of TOR) is thought to act as the key mTORC1 scaffolding protein that binds mTOR substrates via the TOR signaling (TOS) motif, facilitating their phosphorylation by mTOR. A handful of recent studies have demonstrated the importance of phosphorylation of raptor on various sites in the regulation of mTOR signaling by pro- and anti-proliferative signals. Phosphorylation by Rsk at S721 as well as by mTOR at S863 have been shown to enhance mTORC1 activity (Carriere et al., 2008), whereas phosphorylation at S722 and S792 by AMPK create 14-3-3 binding sites and inhibit mTORC1 activity (Gwinn et al., 2008). The exact mechanism of augmentation or inhibition of mTOR activity by raptor phosphorylation remains elusive.

We have shown previously that under energy stress conditions, fewer cells proceed into G2/M and that this cell cycle arrest is dependent on AMPK phosphorylation of raptor and inhibition of mTORC1 activity. This suggested that perhaps mTOR signaling might play a role in mitosis, as suppression of mTOR blocks entry into G2/M and inappropriate activation of mTOR signaling drives cells into G2/M. In our investigations into the regulation of mTOR signaling in mitosis, we identified several sites in raptor phosphorylated by Cdc2 that may play a role in mitotic progression.

Results

Raptor Is Phosphorylated on S/T-P Sites in Cells Stalled in Mitosis with Nocodazole

We have shown previously that cells undergoing energy stress arrest in G2/M and that if they lack the ability to downregulate mTORC1 signaling during energy stress, they proceed inappropriately into mitosis (Gwinn et al., 2008). In the course of further examining how mTORC1 signaling is regulated during mitosis, we observed that stalling cells in mitosis through use of the microtubule destabilizing drug nocodazole caused a shift in the mobility of raptor on SDSPAGE, and this was reversed by in vitro phosphatase treatment of the immunoprecipitates (Figure 1a). Similar results were also seen with the microtubule stabilizing drug taxol, which also promotes mitotic arrest (Figure 1b). Phosphorylation-induced mobility shifts are often indicative of phosphorylation on serine/threonine-proline residues, and indeed we see an increase in immunoreactivity of an anti-phospho-threonine-proline antibody on raptor immunoprecipitates isolated from arrested cells compared to asynchronous cells (Figure 1b).

Mass Spectrometry Analysis of Raptor Reveals Several Novel In Vivo Phosphorylation Sites

In attempts to identify the residues of raptor responsible for the nocodazole-induced bandshift, microcapillary liquid-chromatography/tandem

mass spectrometry (LC/MS/MS) was performed on raptor immunoprecipitated from cells with or without nocodazole treatment. We identified several novel phosphorylation sites from this analysis, including Ser696, Thr706, Ser738, Ser771 and Ser877 (Figure 2a, Table 1). Of these sites, Ser696, Thr706, Ser771, and Ser863, Ser877 are S*-P sites, all of which are evolutionarily conserved through vertebrates (Figure 2b).

Interestingly, mapping our phosphorylation sites along with all those from phospho-proteomic databases including PhosphoSite-Plus (www.phosphosite.org; Hornbeck et al., 2004) reveals that phosphorylation sites within raptor cluster to two regions located between the HEAT repeat region and the WD-repeat containing C-terminal of raptor (Figure 2c).

Raptor Is Phosphorylated on Ser696 and T706 during Mitosis

Next we examined whether mutation of any of the identified serine/ threonine-proline sites to alanine would alter the mobility shift induced in raptor upon nocodazole or taxol treatment. From this analysis, we discovered that an allele of raptor with mutation of Ser696 and Thr706 to alanine, showed reduced band-shifting (Figure 3). Mutation of the adjacent serine-proline residue, Ser711, to alanine in combination with 696/706 was found to further collapse the bandshift, suggesting that S711 is also phosphorylated in cell blocked in mitosis. Mutation of other reported S/T*-P sites in raptor did not collapse the bandshift (Fig. 3, data not shown).

To directly determine the residues of raptor up-regulated following nocodazole treatment, phospho-specific-antibodies against Ser696, Thr706, Ser877, and Ser863 were generated and verified as recognizing only wild-type myc-tagged raptor immunoprecipitated from HEK293T cells, but not raptor mutated at the specified phospho-acceptor residue (Figure 4).

Treatment with nocodazole to block cells in mitosis greatly increased phosphorylation of raptor on Ser696 and Thr706 (Figure 4) but notably, not any of the other S/T*-P sites tested (Figure 4).

Cdc2 Is the Raptor Ser696, Thr706 Kinase

Having identified several residues of raptor phosphorylated in cells blocked in mitosis, we next sought to identify the upstream kinase for Ser696 and Thr706. Knowing the upstream kinase was active following nocodazole treatment, and was proline-directed, we decided to examine the mitotic CDK family member Cdc2.

First we tested whether recombinant Cdc2-cyclin B kinase complexes were capable of in vitro phosphorylation of raptor immunoprecipitated from hydroxyurea treated HEK293T cells (and hence derived from cells where Cdc2 would be inactive) (Figure 5). Indeed Cdc2/cyclin B induced robust phosphorylation of raptor in vitro on Thr706 and Ser696.

To examine whether Phospho-Thr706 can be detected during natural mitotic progression and is not simply due to kinases activated by microtubule

stress, we synchronized A549 cells using double thymidine block and endogenous raptor was immunoprecipitated at various timepoints following thymidine release and immunoblotted with the phospho-raptor Thr706 antibody (Figure 6). Mitotic entry peaked at 8 to 10 hours following thymidine release in these cells as demarcated by increased mitotic markers phospho-histone H3 and phospho-Plk1, coinciding exactly with maximal Thr706 phosphorylation on endogenous raptor (Fig 6). Importantly, endogenous raptor phosphorylation was observed during mitosis in the synchronized cells similar to that observed following nocadozole treatment when all of the cells are arrested in mitosis. We further examined cdc2 involvement through acute treatment of nocodazole arrested cells with the cdc2 inhibitor roscovitine. Roscovitine resulted in inhibition of endogenous phospho-raptor Thr706 (Fig. 6, lanes 9–10).

Finally, we examined whether we could detect and in vivo association between raptor and the cdc2/cyclin B kinase complex. Utilizing HeLa cells stably expressing low levels of tagged raptor, raptor immunoprecipitates from cycling or taxol-arrested cells revealed the presence of endogenous cyclin B (Fig 7).

Cdc2 Phosphorylation of Raptor on Ser696, Thr706 Does Not Impact mTOR1 Complex Formation

mTORC1 is a multi-protein complex whose function requires proper association of all components (Guertin and Sabatini, 2007). To test whether Cdc2 phosphorylation of raptor might change the association of various

components of mTORC1, epitope tagged cDNAs of the components of mTORC1 were expressed in HEK293E cells with or without nocodazole. No changes in the amount of HA-GβL or Flag-PRAS40 that co-immunoprecipitated with myc-tagged raptor were observed with either nocodazole treatment or raptor allele (Figure 8). However, in whole cell lysates taken from the same cells, endogenous 4EBP1 phosphorylation increased, which occurs regardless of raptor allele.

It has been demonstrated that raptor acts as a scaffolding protein between mTOR and its substrates (Schalm and Blenis, 2002, Schalm et al., 2003), so we tested whether Cdc2 phosphorylation of raptor changes the association of known mTOR substrates S6K and 4EBP1. We observed no significant change in the amount of Flag-tagged S6K or 4EBP1 immunoprecipitated with myc-raptor in HEK293T cells treated with or without nocodazole regardless of raptor allele (Figure 9), and the same is true of the reciprocal immunoprecipitations.

To confirm these results in a more physiological system, cell lines stably over-expressing a low level of myc-tagged raptor alleles with stable knock-down of endogenous raptor with short hairpin RNA that targets the 39 UTR of raptor (Sarbassov et al., 2005) were generated in HeLa cells. No changes in the ability of endogenous mTORC1 components to co-immunoprecipitate with myc-raptor were observed with either nocodazole treatment or different raptor alleles (Figure 10). Taken together, these data suggest that phosphorylation of

raptor by Cdc2 does not significantly change the composition of mTORC1 nor the ability of mTOR to signal to downstream substrates S6K or 4EBP1 during mitosis.

Discussion

How signaling pathways coupled to nutrient uptake and expenditure couple to the cell cycle machinery and proliferation control has been an area of increasing investigation. The mTORC1 signaling pathway is a critical integrator of environmental inputs into protein translation and cell growth. However, the precise role of mTORC1 signaling in mitotic progression remains enigmatic (Wang and Proud, 2009).

Our previous studies indicated the presence of G2/M metabolic check-point enforced by AMPK in a manner dependent on its ability to phosphorylate raptor and suppress mTORC1 activity. Cells expressing non-phosphorylatable alleles of raptor continued to progress through mitosis unabated unlike those expressing wild-type raptor, and ultimately displayed increased rates of apoptosis. Consistent with an AMPK/mTORC1 dependent checkpoint, AMPKα2 and its upstream kinase LKB1 were isolated in an RNAi screen for modulators of G2/M in mammalian cells (Moffat et al., 2006). Furthermore, increased phosphorylation at the AMPKα2 activation loop was observed in a proteomic study for kinases activated during G2/M (Daub et al., 2008) and

more recently, activated AMPK has been proposed to reside at the mitotic spindle (Vazquez-Martin et al., 2009), hinting at both spatial and temporal regulation of AMPK in mitosis which may restrict or target its regulation of mTOR to specific locations or phases of mitosis.

Previous studies also suggest that mTOR signaling plays a positive role in the progression through mitosis in a variety of species. In budding yeast, a temperature sensitive allele of raptor or rapamycin-treatment of cells both induce mitotic delay and a prolonged G2 (Nakashima et al., 2008). In contrast, in fission yeast rapamycin induces early mitotic onset in synchronized cultures (Peterson and Nurse, 2007), though in both yeasts, TOR activity has been tied to the control of Polo kinase activation. Further work is needed in each of these biological settings to further dissect the role of TOR in mitotic control.

We demonstrate here that the mitotic kinase cdc2 directly phosphorylates raptor during mitosis, though we have been unable to demonstrate the contribution those phosphorylations play to overall mitotic progression or mTORC1 signaling during mitosis in the tumor cell settings we have examined thus far. Importantly, our data suggest there may be additional Cdc2 sites beyond Ser696, Thr706, and Ser711 in raptor and until these sites are fully identified, the phenotype of a fully cdc2 nonphosphorylatable raptor remains unknown. Nonetheless, the cdc2 sites in raptor may be more critical for growth control in nontumorigenic settings, which is an area requiring further investigation.

One additional complicating factor is these analyses is the fact that cdc2 has been reported to directly phosphorylate both S6K1 (Papst et al., 1998, Shah et al., 2003, Hou et al., 2007) and 4EBP1 directly (Heesom et al., 2001, Greenberg et al., 2005). Indeed the well-characterized Ser65 and Ser7 phosphorylation sites in 4EBP1 have been proposed to be sites of phosphorylation by cdc2, events that are dependent on mTORC1 activity. Additionally, cdc2-dependent phosphorylation of eEF2K was shown to be suppressed by amino acid deprivation and increased in cells lacking TSC2, conditions that respectively serve to inhibit and stimulate mTORC1 signaling, leading to the suggesting that mTORC1 activity may serve to contribute to cdc2-dependent regulation of eEF2K (Smith and Proud, 2008). The possibility exists therefore that both cdc2 and mTORC1 kinase complexes serve to inter-regulate one another depending on the precise timing and localization of each during different stages of mitosis. The fact that several components of the mTORC1 pathway are targeted by cdc2 may result in no single one of them being critical in isolation as a cdc2 target whose phosphorylation is absolutely required for mitotic progression.

A complication of much of the previous literature studying the effect of mTOR on G2/M progression utilizing rapamycin is that recent finding from several labs that rapamycin does not fully inhibit mTORC1 kinase activity. Kinase inhibitors directed at mTOR itself yield changes in mTORC1 signaling and growth arrest phenotypes more similar to RNAi for raptor (Feldman et al.,

2009, Thoreen et al., 2009, Garcia-Martinez et al., 2009). Importantly, these effect of mTOR kinase inhibitors were demonstrated to be independent of the mTORC2 complex and its function (Feldman et al., 2009, Thoreen et al., 2009). These findings are also consistent with a variability of rapamycin in inhibiting S6K1 signaling but not 4EBP1 phosphorylation universally in mammalian cells, unlike RNAi or genetic deletion of raptor or mTOR (Thoreen et al., 2009, Choo et al., 2008). The inability of rapamycin to suppress 4EBP1 phosphorylation indicates that previous studies in mammalian cells studying effects of rapamycin on mitosis were not accounting for the full role of mTORC1. Future studies using these new direct mTOR kinase inhibitors will be needed to fully dissect its requirement in different stages of mitotic progression.

Additional tools including phospho-specific antibodies which can work for immunolocalization may better reveal where the population of cdc2-phosphor-ylated raptor and 4EBP1 are during the different stages of mitosis. Understanding how AMPK activity and mTOR activity are controlled spatially and temporally during mitosis will undoubtedly lead to fundamental insights into how nutrients control cell division as well as to how protein translation is coupled to timely cell cycle exit during differentiation or stem cell renewal. A deeper understanding of how mTORC1 controls cell cycle progression is essential for use of targeted mTOR inhibitors in the treatment of cancer and many other mTOR related pathologies (Guertin and Sabatini, 2009).

Experimental Procedures

Antibodies and Plasmids

Myc-raptor, AU1-mTOR, HA-GβL and Flag-PRAS40 originated in Dr. David Sabatini's Lab (MIT, Cambridge, MA) and were obtained from Addgene.org (Cambridge,MA). HA-tagged 4EBP1 and S6K1 were obtained from Dr. John Blenis (Harvard Medical School, Boston,MA). Myc-raptor was subcloned into pENTR3C (Invitrogen), and serine to alanine point mutations were made using QuikChange II XL (Stratagene). Mutant alleles were then put into an FBneoDEST vector by LR reaction (Invitrogen). All constructs were fully sequence verified. Phospho-Plk1 T210 was from BD Pharmingen (#558400).

Phospho-raptor (S696, T706, S863, S877) and Phospho-histone H3 antibodies were obtained from Millipore. Anti-raptor used for endogenous raptor immunoprecipitations was from Invitrogen (#42-4000). The 9E10 anti-myc antibody was used for immunoprecipitations (Roche). Antibodies against raptor (#2280), mTOR (#2983), GβL (#3274), PRAS40 (#2610), phospho-S6K T389 (#9234), pAurora A, B, C (T288/T232/T198) (#2914), Cyclin B1 (#4138), phospho-4EBP1 (T37/46) (#2855), phospho-4EBP1 (S65) (#9451), myc-tag (#2272), phospho-threonine-proline (#2321), and GST-tag (#2622) were from Cell Signaling Technologies.

Cell Culture

HeLa, A549 and HEK293T cells were grown in DMEM with 10% FBS at 37°C with 5% CO_2. Cells were transfected with Lipofectamine 2000 (Invitrogen) as per manufacturer's instruction for 32–36 hours. Nocodazole (1 μg/mL) (SIGMA) or taxol (1 μM) (Cell Signaling Technologies) treatment was administered for 16–18 hours prior to lysis (usually 16 h post-transfection). Replacement of endogenous raptor with myc-tagged raptor was achieved by infecting HeLa cells with a retrovirus expressing myc-wt or myc-S696/T706/S711AAA raptor in the FBneo vector, and selection in neomycin. These stables were subsequently infected with a lentivirus expressing a short-hairpin RNA that targets the 39 UTR of human raptor in the pLKO vector and selected in puromycin and distributed by Addgene. A549 cells were synchronized in G1/S by double thymidine block as follows: 2 mM thymidine was added to the media for 14–16 hours, plates were washed twice with PBS, then complete thymidine-free media was added. Eight to ten hrs later, 2 mM thymidine was added again for 14–16 hrs, cells were washed twice with PBS, then released into thymidine-free media. 50 μM roscovitine was administered for 6 hrs following 16 hrs nocodazole treatment in A549 cells. Torin1 (50 nM) (Dr. D. Sabatini, MIT) was added for 1 h.

Biochemistry

For immunoprecipitations, cells were washed with ice cold PBS and

collected in lysis buffer 1 (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10 nM Calyculin A, and EDTA-free complete protease inhibitor tablets (Roche) as per manufacturer's directions) for experiments in Figures 1, 2, 3, 4a and 4b or lysis buffer 2 (40 mM HEPES pH 7.5, 150 mM NaCl, 0.3% CHAPS, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 10 nM Calyculin A and EDTA-free complete protease inhibitor tablets) for Figures 4c and 5. Lysates were incubated on ice for 15 min after lysis, then spun at 13,200 rpm at 4°C for 15 minutes. The supernatants were collected and normalized for protein levels by BCA assay (Pierce). Whole cell lysates were incubated with antibodies for 1.5 hrs with constant rocking at 4°C, then protein-A or –G Sepharose beads (Invitrogen) were added for 1 hr. Immunoprecipitates were washed three times with lysis buffer, and sample buffer was added to 1X final, and samples were boiled at 95°C for 5 min. HeLa cells were lysed in boiling SDS-lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1% SDS) and equilibrated by BCA assay. Samples were resolved on 8–12% SDS-PAGE gels, transferred to PVDF and immunoblotted according to the antibody manufacturer's instructions.

Phosphatase Treatment

Anti-myc immunoprecipitations were performed on cell lysates from HEK293T cells transiently transfected with myc-raptor treated with or without

nocodazole. After washing the beads twice in lysis buffer 1, they were washed twice in CIAP buffer (50 mM Tris pH 8.5, 100 μM EDTA) then incubated with 5 μL calf-intestinal alkaline phosphatase (CIAP) (NEB) with constant agitation at 37°C for 30 min.

In Vitro Kinase Assays

Anti-myc immunoprecipitations from HEK293T cells transiently transfected with myc-raptor for 16 hours followed by 12 hrs of 2 μM hydroxyurea treatment were washed three times with lysis buffer 1, then three times with kinase buffer (25 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 5 mM β-glycerophosphate, 2 mM DTT). Immunoprecipitates were then incubated with 20 μL kinase reaction mix (kinase buffer, 10 μM ATP) with or without 175 ng recombinant Cdc2/cyclin B (Cell Signaling Technologies #7518) at 30°C for 30 min with constant shaking. Reaction was quenched by addition of sample buffer to 1X and boiling at 95° for 5 min.

LC/MS/MSTandem Mass Spectrometry

For all mass spectrometry (MS) experiments, myc-Raptor immunoprecipitates were separated using SDS-PAGE, the gel was stained with Coomassie blue, and the myc-Raptor band was excised. Samples were subjected to reduction with dithiothreitol, alkylation with iodoacetamide, and in-gel digestion with trypsin or chymotrypsin overnight at pH 8.3, followed by

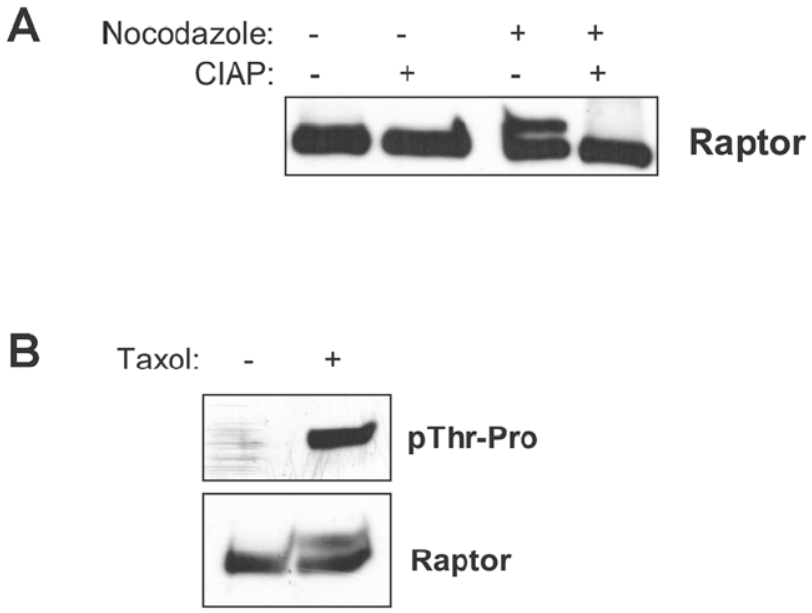
reversed-phase microcapillary/tandem mass spectrometry (LC/MS/MS). LC/MS/MS was performed using an Easy-nLC nanoflow HPLC (Proxeon Biosciences) with a self-packed 75 μm id x 15 cm C18 column connected to a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) in the data-dependent acquisition and positive ion mode at 300 nL/min. MS/MS spectra collected via collision induced dissociation in the ion trap were searched against the concatenated target and decoy (reversed) single entry Raptor and full Swiss-Prot protein databases using Sequest (Proteomics Browser Software, Thermo Scientific) with differential modifications for Ser/Thr/Tyr phosphorylation (+79.97) and the sample processing artifacts Met oxidation (+15.99), deamidation of Asn and Gln (+0.984) and Cys alkylation (+57.02). Phosphorylated and unphosphorylated peptide sequences were identified if they initially passed the following Sequest scoring thresholds against the target database: 1+ ions, Xcorr ≥2.0 Sf ≥0.4, P≥5; 2+ ions, Xcorr ≥2.0, Sf ≥0.4, P≥5; 3+ ions, Xcorr ≥2.60, Sf ≥0.4, P≥5 against the target protein database. Passing MS/MS spectra were manually inspected to be sure that all b- and y- fragment ions aligned with the assigned sequence and modification sites. Determination of the exact sites of phosphorylation was aided using FuzzyIons and GraphMod and phosphorylation site maps were created using ProteinReport software (Proteomics Browser Software suite, Thermo Scientific). False discovery rates (FDR) of peptide hits (phosphorylated and unphosphorylated) were estimated below 1.5% based on reversed database hits.

Acknowledgements

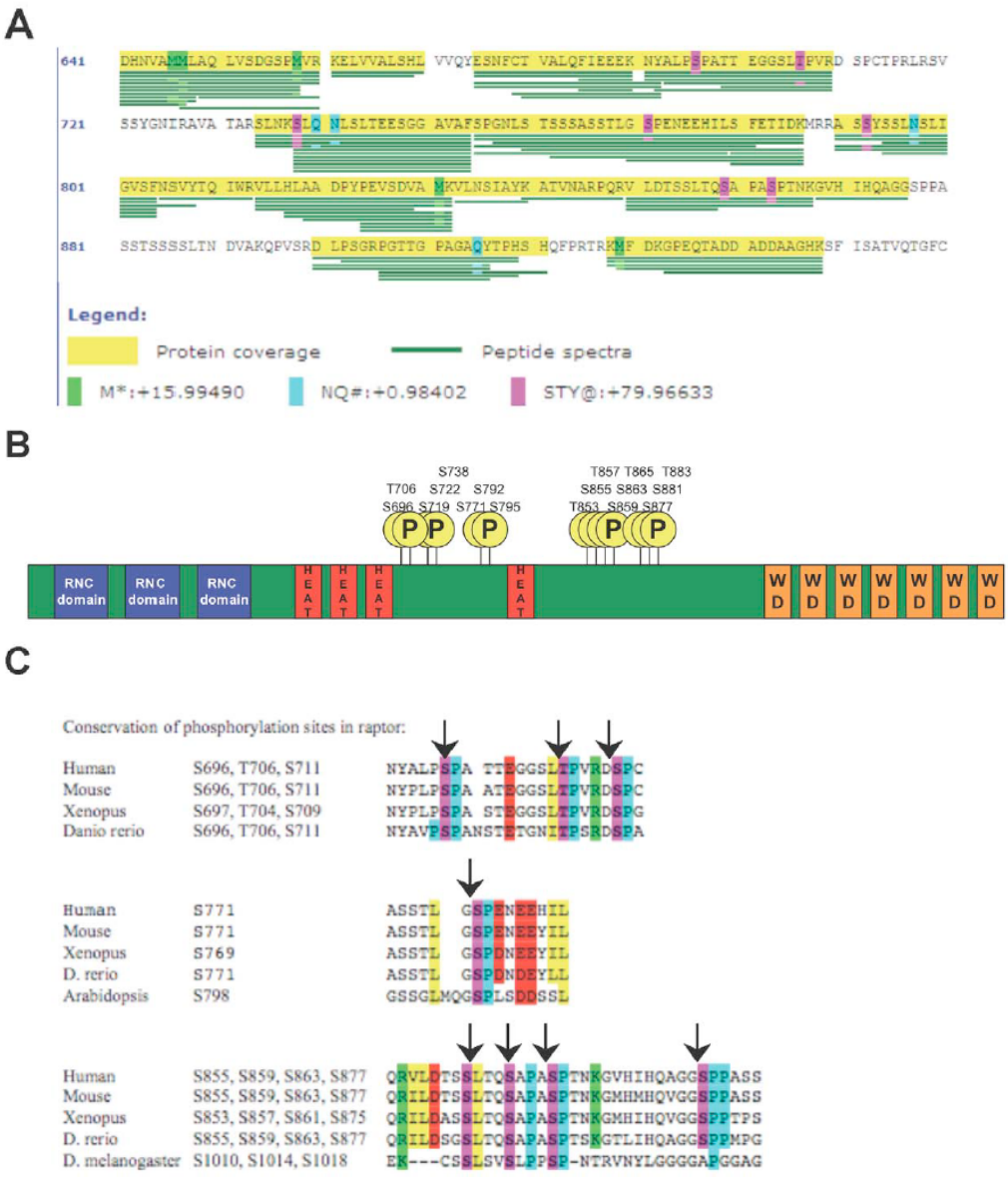
Chapter 3 contains excerpts from material as it appears in:

Gwinn, D.M., Asara, J.M., Shaw, R.J. (2010). Raptor is phosphorylated by cdc2 during mitosis. PloS One 5:e9197.

On this publication, I was the primary author. Reuben Shaw directed and supervised the writing.



CHAPTER III Figure 1. Figure 1. Raptor is phosphorylated on S/T*-P sites in cells treated with nocodazole.
(A) Raptor undergoes a mobility shift on SDS-PAGE following nocadazole which is collapsed by phosphatase treatment. Myc-tagged raptor was expressed in HEK293T cells and nocadazole treated for 16 h. Where indicated, immunoprecipitates were treated with or without calf-intestinal alkaline phosphatase (CIP) and then resolved in SDS-PAGE, and subjected to anti-myc immunoblotting.
(B) Raptor is recognized by a phospho-threonine proline antibody in mitotic arrested cells. HEK293T cells transiently expressing myc-tagged raptor were treated for 16 h with taxol and immunoprecipitates were immunoblotted with an antibody that recognizes phospho-threonine followed by proline.

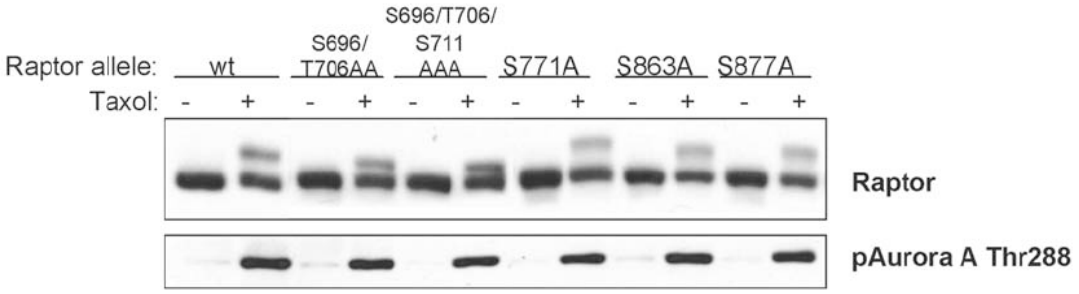


CHAPTER III Figure 2. Mass spectrometry analysis of raptor reveals several novel phosphorylation sites.

(A) Phosphorylation sites in raptor after nocodazole treatment as detected by LC/MS/MS. The presence of a phosphate moiety is indicated by a magenta colored box. Note that four serine-or threonine sites followed by a proline were detected in this analysis. Sites of oxidation (green) and deamidation (blue) represent in vitro artifacts of the mass spectrometry experiment.

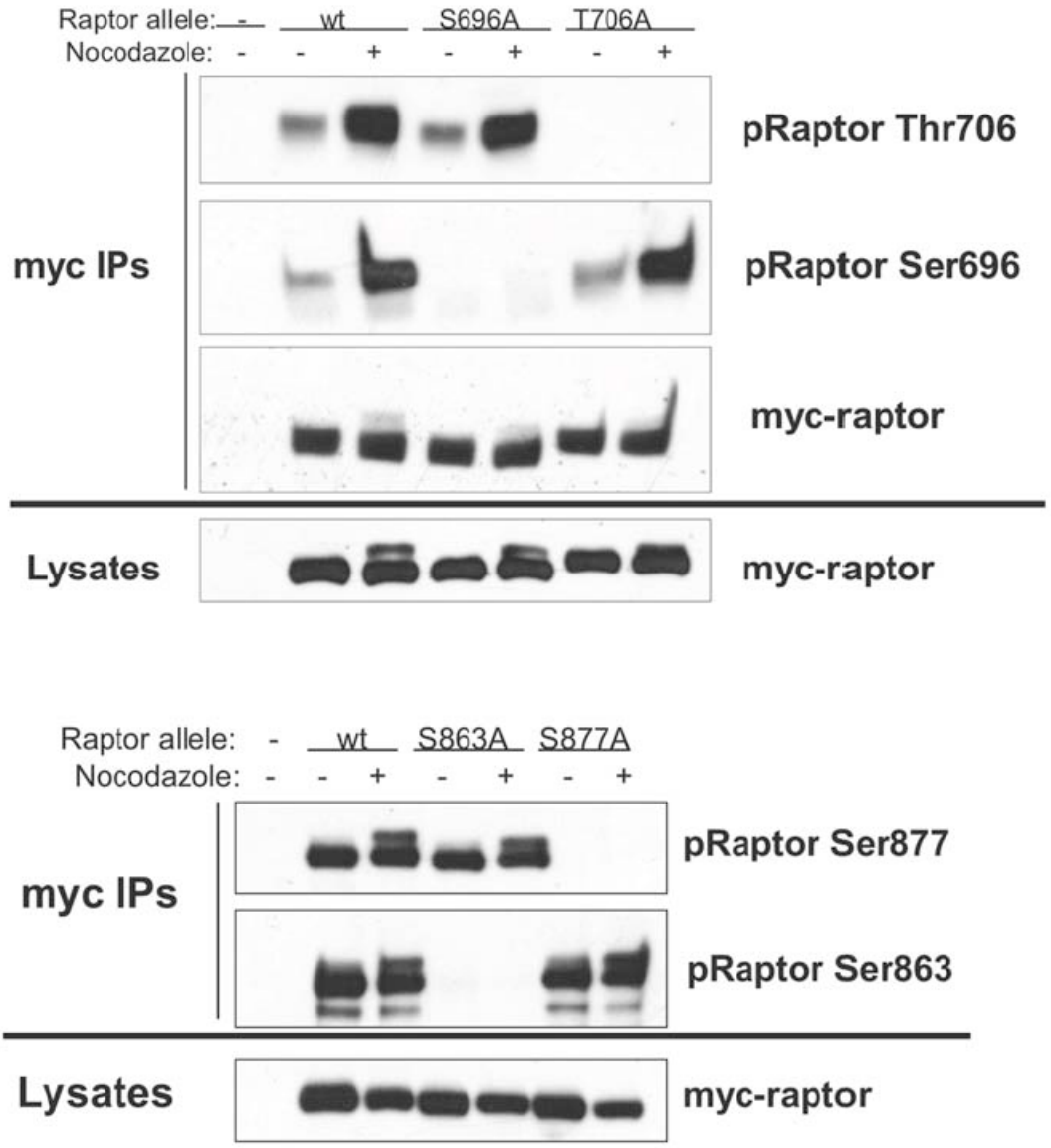
(B). Schematic of human raptor domain structure with all known phosphorylation sites found in this and previous studies. Note that most phosphorylation sites cluster in two regions of the protein.

(C) Conservation of the indicated phosphorylation sites.

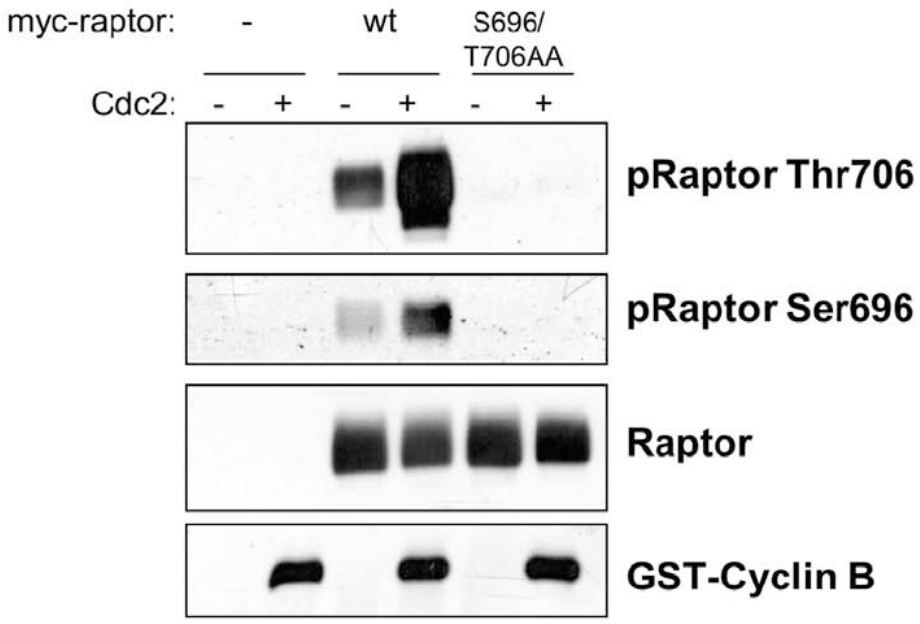


CHAPTER III Figure 3. Mutation of Ser696, Thr706 and Thr711 collapse mitotic bandshift of raptor.

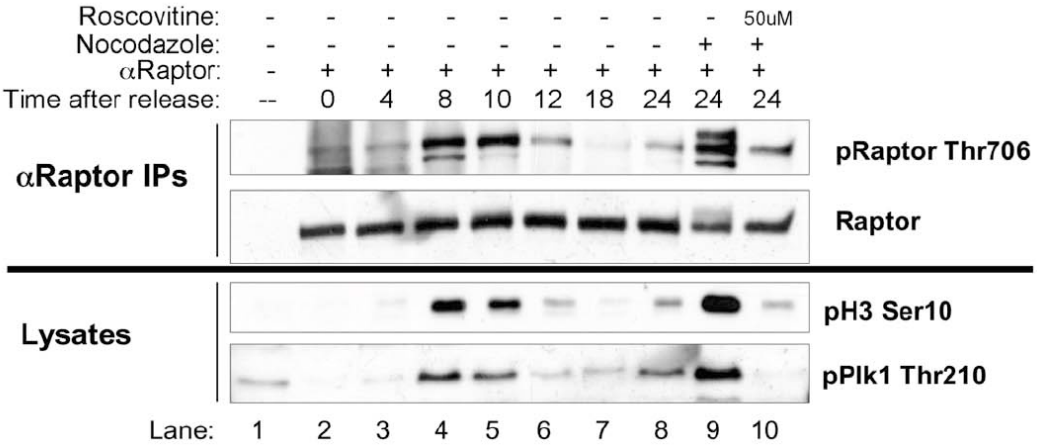
The mitotic induced bandshift is collapsed by mutation of Ser696, Thr706, and Thr711. Indicated serine/threonine-to-alanine non-phosphorylatable raptor mutants were expressed in HEK293T cells treated with taxol as in Figure 1.



CHAPTER III Figure 4. Raptor is phosphorylated at Ser696 and Thr706 during mitosis.
Wild-type or non-phosphorylatable raptor alleles were immuno-precipitated from nocadazol treated HEK293T cells and then immuno-blotted with indicated site-specific phospho-raptor antibodies. Note specificity of each antisera and that Ser696 and Thr706 (above), but not Ser863 or Ser877 are increased by nocadazole treatment (below).

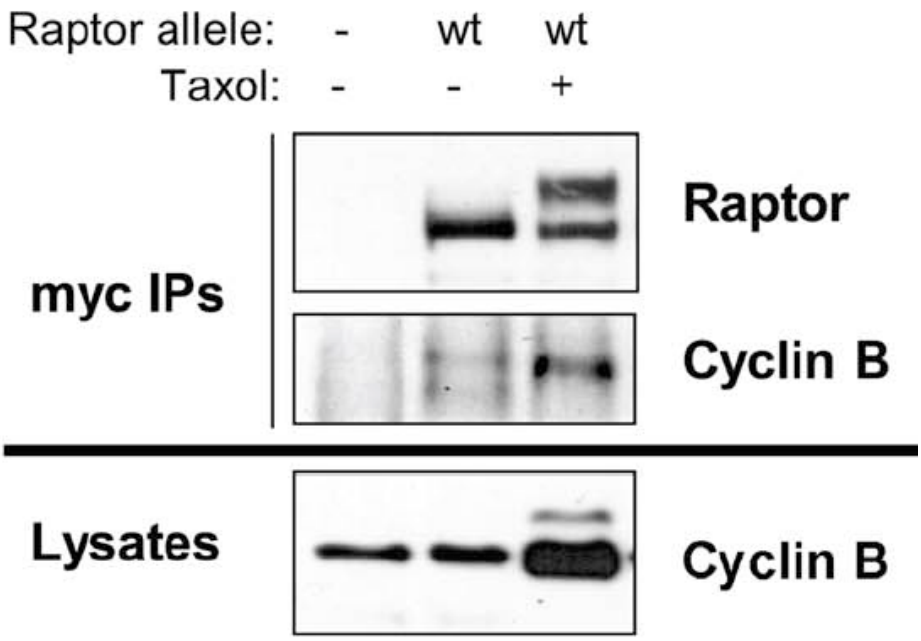


CHAPTER III Figure 5. Cdc2 directly phosphorylates Raptor *in vitro*. Purified cdc2 can directly phosphorylate raptor on Thr706 and Ser696 *in vitro*. Myc-raptor (wild-type or S696/T706AA) was immunoprecipitated from hydroxylurea treated HEK293T cells. Immunoprecipitates were incubated with or without active recombinant Cdc2/cyclin B and immunoblotted with phospho-raptor Ser696, Thr706 or total raptor.

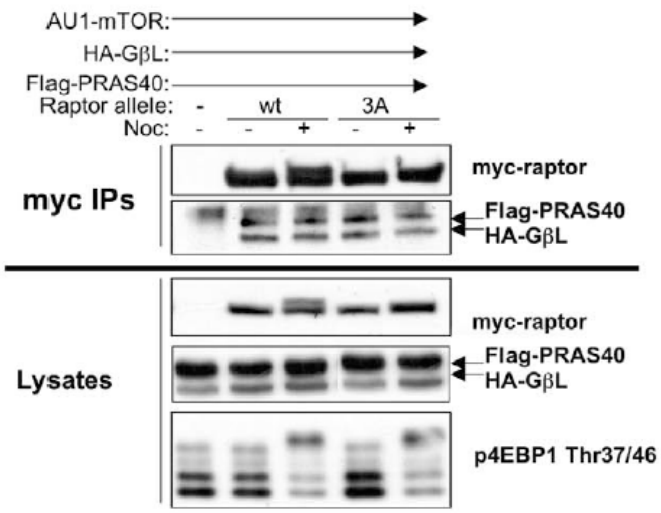


CHAPTER III Figure 6. Endogenous raptor is phosphorylated at Thr706 in a Cdc2-dependent fashion.

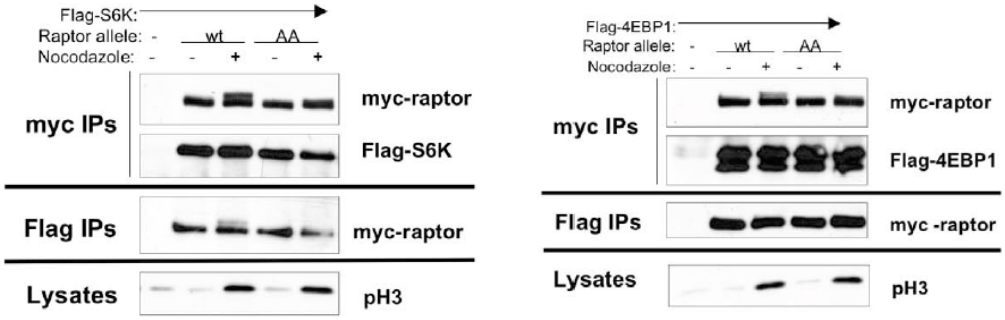
Endogenous raptor is phosphorylated on Thr706 in synchronized cells undergoing mitosis, and this phosphorylation is blocked by the CDK inhibitor roscovitine. A549 cells were synchronized by double thymidine block and endogenous raptor was immunoprecipitated at the indicated times after release with an anti-raptor antibody and immunoblotted with phospho-raptor Thr706. Whole cell lysates taken from the same cells were immunoblotted for mitotic markers phospho-histone H3 Ser10 and phospho-Plk1 Thr210.



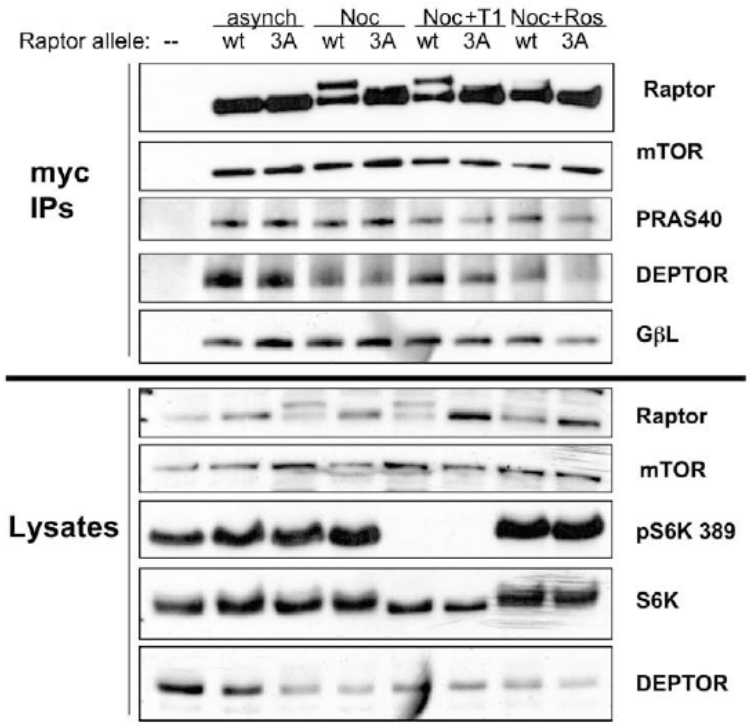
CHAPTER III Figure 7. Raptor interacts with Cyclin B.
Raptor immunoprecipitates with endogenous cyclin B. HeLa cells stably expressing myc-wt raptor with stable knockdown of endogenous raptor treated with or without taxol for 16 hrs. myc-tagged raptor was immunoprecipitated and immunoblotted for Cyclin B.



CHAPTER III Figure 8. Cdc2 phosphorylation of raptor does not effect association of PRAS40 or GbL with mTORC1.
HEK293T cells were transiently transfected with myc-raptor (wild-type or Ser696/Thr706/Ser711AAA: ‘‘3A’’), AU1-mTOR, HA-GbL and Flag-PRAS40 for 16 hours, followed by 16 hours of nocodazole treatment following addition of fresh media to plates. Cells were lysed and myc-raptor was immunoprecipitated using an antibody against the myc-tag. Immunoprecipitates were resolved by SDS-PAGE and probed with antibodies against the Flag- and HA- tags.



CHAPTER III Figure 9. Raptor phosphorylation by Cdc2 does not effect association of substrates with raptor.
HEK293T cells were transiently transfected with myc-raptor (wild-type or 3A) and Flag-S6K or Flag-4EBP1. 16 hours later, media was changed and nocodazole was added for 16 hours. Cells were lysed and lysated were split in two; myc-raptor was immunoprecipitated with an antibody against the myc-tag, and Flag-S6K or 4EBP1 were immunoprecipitated with an antibody against the Flag-tag. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with indicated antibodies.



CHAPTER III Figure 10. Cdc2 phosphorylation of raptor does not change mTORC1 complexes or signaling.
HeLa cells stably expressing myc-raptor (wt or 3A) with stable knockdown of endogenous raptor were treated with nocodazole for 16 hours, then Torin1 or roscovitine were added for 4 hours. Cells were lysed and myc-raptor was immunoprecipitated with an antibody against the myc-tag. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

CHAPTER III Table 1. Phosphorylation sites identified by mass spectrometry in raptor with predicted kinases for each site.
Phosphorylation sites gathered from various publications and mass spectrometry databases (phosphosite.org) are listed. Kinase predictions were made with NetPhorest (netphorest.info).

	Amino Acid at Position:																Conserved in:								
Site	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7		Ms	Xl	Dr	Dm	Ce	Sc		**Predicted kinase:**	**References:**
S696	E	K	N	Y	A	L	P	S	P	A	T	T	E	G	G		x	x	x	?				Cdc2, CDK, MAPK	2, 6
T706	T	T	E	G	G	S	L	T	P	V	R	D	S	P	C		x	x	x					Cdc2, CDK, MAPK	2, 6
S711	S	L	T	P	V	R	D	S	P	C	T	P	R	L	R		x	x	x					Cdc2, CDK, MAPK	
S719	P	C	T	P	R	L	R	S	V	S	S	Y	G	N	I		x	x	x					DMPK, PKB	1, 3
S721	T	P	R	L	R	S	V	S	S	Y	G	N	I	R	A		x	x	x	x	x			Rsk, p70S6K	1, 3
S722	P	R	L	R	S	V	S	S	Y	G	N	I	R	A	V		x	x	x					AMPK	1, 3, 4
Y723	R	L	R	S	V	S	S	Y	G	N	I	R	A	V	A		x	x	x					InsR	1
S738	T	A	R	S	L	N	K	S	L	Q	N	L	S	L	T		x	x	x	?				PKC, NEK1	6
S771	S	A	S	S	T	L	G	S	P	E	N	E	E	H	I		x	x	x	x	x	x		p38, JNK	6
S792	D	K	M	R	R	A	S	S	Y	S	S	L	N	S	L		x	x	x					AMPK	1, 4, 6
S795	R	R	A	S	S	Y	S	S	L	N	S	L	I	G	V			x	x					NEK1, PAKB	6
T853	R	P	Q	R	V	L	D	T	S	S	L	T	Q	S	A		x	x						GSK3, DMPK	1
S855	Q	R	V	L	D	T	S	S	L	T	Q	S	A	P	A		x	x	x	x				NEK1, GSK3	1
T857	V	L	D	T	S	S	L	T	Q	S	A	P	A	S	P		x	x	x					ATM/ATR, NEK1	1
S859	D	T	S	S	L	T	Q	S	A	P	A	S	P	T	N		x	x	x	x				NEK1, GSK3	1, 5, 6
S863	L	T	Q	S	A	P	A	S	P	T	N	K	G	V	H		x	x	x	x				mTOR	1, 2, 5, 6
T865	Q	S	A	P	A	S	P	T	N	K	G	V	H	I	H		x	x	x					PKC, GSK3	1
S877	H	I	H	Q	A	G	G	S	P	P	A	S	S	T	S		x	x	x	x				CDK2/3, p38	1, 3
S881	A	G	G	S	P	P	A	S	S	T	S	S	S	S	L		x	x						RCK, MAPKAPK	1
T883	G	S	P	P	A	S	S	T	S	S	S	S	L	T	N		x	x						NEK1, PAKB	1
S886	P	A	S	S	T	S	S	S	S	L	T	N	D	V	A			x	x					NEK1, ACTR	1
T889	S	T	S	S	S	S	L	T	N	D	V	A	K	Q	P		x	x	x					CK1, DMPK	1

CHAPTER FOUR:

The RalGAP complex acts as a signal integration point for Akt, AMPK and PKD signaling in the regulation of the Ral small GTPases

Introduction

The ability of cell to sense and respond to changes in cellular energy status is largely reliant on the evolutionarily conserved AMP-activated protein kinase (AMPK). AMPK exists as a hetero-trimer, with one catalytic (α), and two regulatory (β, γ) subunits (Hardie, 2007). Direct binding of AMP or ADP to the γ-regulatory subunit of AMPK promotes a conformational change such that the phosphorylation site on the activation loop, which is critical for AMPK kinase activity, is protected from dephosphorylation by PP2Cα, or other phosphatases (Xiao et al, 2011). This mode of regulation allows AMPK to act as an energy gauge in the cell. Active AMPK suppresses energy costly processes such as fatty acid catabolism, glucose storage, protein synthesis, and in the liver inhibits gluconeogenesis. Energy restoring processes such as fatty acid oxidation, increased mitochondrial number and autophagy are also stimulated by AMPK (reviewed in Shackelford and Shaw, 2009).

The tumor suppressor, LKB1 is the major upstream kinase for AMPK, and the entire family of AMPK-related kinases (AMPKRs), and is responsible for phosphorylation of their activation loops, which is absolutely required for their kinase activity (Shackelford and Shaw, 2009). While LKB1 was originally identified as the causally mutated gene in the familial cancer predisposition syndrome, Peutz-Jeghers Syndrome (Hemminki et al., 1998), it has also been found to be mutated in 30-40% of sporadically occurring non-small cell lung carcinoma (NSCLC) (Sanchez-Cespedes et al., 2002; Ji et al., 2007) and about

20% of endometrial cancers (Wingo et al., 2009). In addition to AMPK, LKB1 phosphorylates and activates a family of 12 AMPK-related kinases including the MARK, SIK, NUAK, and SAD subfamilies. Interestingly, the optimal substrate sequence that AMPK and its 12 related kinases prefer to phosphorylate is highly similar, as revealed by arrayed positional scanning peptide library analysis (Turk et al., 2006; Turk and Shaw, unpublished data). The predicted similar substrate sequence specificities for these kinases fits with the findings from several studies that the same protein in one cell type or one condition can serve as a substrate for AMPK, while under other conditions for its family members the MARK or SIK kinases (Dentin et al., 2007; Shaw et al., 2005; Jenkins et al., 2000; Thornton et al., 2011).

Another kinase family not regulated by LKB1, but also sharing an overlapping substrate specificity with AMPK is the protein kinase D (PKD) family of kinases, which consists of three members: PKD1, PKD2 and PKD3. Each PKD contains multiple cysteine-rich domains that function to bind diacylglycerol (DAG), an auto-inhibitory pleckstrin homology (PH) domain, and a C-terminal kinase domain (reviewed in Van Lint et al., 2002). Activation of PKD occurs downstream of DAG-responsive protein kinase C (PKC) family members, which are responsible for phosphorylation of two conserved serine residues in the activation loop of PKD (Van Lint et al., 2002). This occurs downstream of G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) that stimulate phospholipase C (PLC) (Van Lint et al., 2002). In

addition, direct binding of the Gβγ subunit of GPCRs to the PH domain, may contribute to activation of PKD by relieving the auto-inhibitory effect of the PH domain (Van Lint et al., 2002). Changes in localization can also contribute to proper regulation of PKD: the binding of DAGs to the PH domain of PKD recruits PKD to the trans-Golgi network (Maeda et al., 2001; Baron and Malhotra, 2002), and it has also been shown to shuttle in and out of the nucleus.

Upon activation, PKD contributes to trafficking of proteins from the Golgi to the plasma membrane (Van Lint et al., 2002), modulating the ERK and JNK pathways (Brändlin et al., 2002; Wang et al., 2002; Ziegler et al., 2011), promoting cellular survival by inducing the NF-κB and p38 MAPK pathways (Song et al., 2009), cell motility (Prigozhina et al., 2004; Jaggi et al., 2005; Fu and Rubin, 2011), migration (Eiseler et al., 2007) and invasiveness (Bowden et al., 1999; Riol-Blanco et al., 2004) and enhancing sensitivity to apoptosis under certain conditions (Van Lint et al., 2002).

Much research has been directed at determining a role for PKD in cancer, and interestingly, PKD is misregulated in a number of cancers. However, in some cancers (pancreatic, basal cell carcinoma) PKD is upregulated, while in other cancers (breast, gastric), PKD is decreased (LaValle et al., 2010). Interestingly, in pancreatic cancer, where PKD activity is high, inhibition of PKD suppresses growth of pancreatic cancer cell lines, and

xenograft models (Harikumar et al., 2010). Further studies are thus needed to sort out the roles of different PKD family members in different tumor types.

The Ras-related small GTPases, RalA and RalB have been studied most in the context of Ras-induced tumorigenesis, as the RalGDS family of RalGEFs have been shown to be effectors of the Ras oncogene (Hofer et al., 1994, Kikuchi et al., 1994, and Spaargaren et al., 1994), and in certain settings, activation of Ral is important for growth of Ras-transformed cell lines, as well as xenograft tumor growth (Lim et al., 2005). Mice lacking RalGDS develop far fewer Ras-induced tumors than their wild-type counterparts, suggesting that Ral activation may contribute to the oncogenecity of Ras (González-García, et al., 2005). In addition to regulation by Ras, the inhibitory N-terminus of RalGDS has also been suggested to be a substrate of PKC, which allows dissociation from the catalytic domain and enhanced activity of RalGDS toward Ral (Rusanescu et al., 2001); however, bona fide phosphorylation sites have not been reported within this region of the protein. RalGDS, and its family members RGL1 and RGL2 have also been found to have activating mutations in sporadic cancers, suggesting that their misregulation may contribute to tumorigenesis in a non-Ras transformed context as well (Bodemann and White, 2008). Indeed, RalA has also been shown to be an important substrate of the tumor suppressor PP2A Aβ phosphatase (Sablina et al., 2007), which normally acts as a negative regulator of RalA, and loses function in a variety of cancers.

While GEFs for Ral have been studied for some time, a GAP for the Rals has only recently been identified (Shirakawa et al., 2009). The RalGAP complex is an obligate heterodimer, containing one of two catalytic subunits (α), and a scaffolding subunit (β) (Shirakawa et al., 2009). GAP activity of the RalGAP complex shows specificity toward RalA and RalB in vitro compared with Ras, Rap, Rheb, Rho, Rab and Ran members of the Ras super-family (Shirakawa et al., 2009). It is widely expressed in tissues surveyed (Gridley et al., 2006; Shirakawa et al., 2009), with some variability in the relative levels of RalGAPα1 v α2 (Shirakawa et al., 2009). Expression levels and mutational analysis of the RalGAPs in tumors have not been examined, though they are speculated to function as tumor suppressors (Chen et al., 2011c), based in part on the analogous domain structure of the RalGAP complex to the TSC (Tuberous Sclerosis Complex) tumor suppressor complex. Indeed the two RalGAPα genes are the most homologous genes to the TSC2 tumor suppressor in mammals. Though only sharing about 8% sequence identity, they share the same domain structure as TSC2, with their GAP domains located at the very C-terminus. Like TSC1/2, RalGAPs have been proposed to act as negative regulators of mTORC1 signaling, though how Ral signaling ties into mTOR signaling is completely unknown at this time.

Like most small GTPases, RalA and RalB are active in their GTP-bound state, and directly bind their effectors. Through binding RalBP1, a RhoGAP domain-containing protein, Ral activity may regulate Cdc42 and Rac1, though

this has yet to be demonstrated *in vivo* (Jullien-Flores et al., 1995). Ral has also been implicated in endocytosis, as RalBP1 has also been suggested to coordinate endocytosis of clathrin-coated vesicles (Boissel et al., 2007). Ral may regulate density-dependent growth, via its relief of the transcriptional repressive effects of the Y-box transcription factor, ZONAB, Ral promotes transcription of ZONAB targets, including ErbB2 (Frankel et al., 2004).

Perhaps the best-studied effectors of Ral are Sec5 and Exo84, components of the hetero-octomeric exocyst complex (Camonis and White, 2005). Ral is involved in formation of the exocyst holocomplex from sub-complexes, which directs cargo-containing vesicles to the correct plasma-membrane location (Moskalenko et al., 2001, Moskalenko et al., 2003). Via binding to the exocyst complex, Ral has been suggested to regulate polarized exocystosis, filopodia formation (Sugihara et al., 2002), cell migration (Rossé et al., 2006), growth factor signaling via integrin-mediated exocytosis of membrane rafts (Balasubra-manian et al., 2010), activation of the pro-survival kinase TBK1 (Chien et al., 2006), and initiation of autophagy (Bodemann et al., 2010).

One important role of the exocyst complex is to target Glut4 to the plasma membrane following insulin stimulation (Inoue et al., 2003) in a Ral activation dependent manner (Chen et al., 2007). In addition to its role in activating the exocyst complex, RalA also coordinates the motor protein Myo1c to promote Glut4 translocation (Chen et al., 2007).

Interestingly, it was shown that RalGAPα2 is a direct substrate of Akt, providing a mechanism for enhancing Ral activity following insulin stimulation to promote Glut4 targeting to the plasma membrane (Chen et al., 2011b). Akt activation also promotes Glut4 translocation by phosphorylating the Rab GAP proteins TBC1D1 and AS160/TBC1D4 (Chen et al., 2008), suggesting that Akt regulates multiple steps in Glut4 trafficking to induce insulin-dependent glucose uptake.

We now present a model in which the RalGAP complex acts as a signal integration point for multiple signaling pathways. We present here evidence that AMPK and PKD directly phosphorylate the α catalytic subunit of the RalGAP complex to induce 14-3-3 association. Activation of either pathway stimulates Ral activity, indicating that these phosphorylation events play an inhibitory role in the RalGAP complex. Regulation of Ral activity by PKD and AMPK implicate these pathways in regulation of the exocyst complex and Ral-dependent growth control.

Results

The RalGAP complex proteins contain candidate AMPKR/PKD phosphorylation sites

We have previously identified the optimal substrate motif, or sequence of amino acids surrounding the phospho-acceptor residue, for AMPK (Gwinn et al., 2008), and subsequently found that this motif is identical for all members of the AMPK-related kinases (Shaw and Turk, unpublished data). While the AMPKRs have a high degree of specificity compared to many kinases profiled (Turk et al., 2006), several other kinases subfamilies share some elements of the AMPKR motif, though not requiring as many selectivities as the AMPKRs (Figure 1). The best known kinases which select for some elements of the motifs that AMPKRs select for are the PKD and MK families. Thus any sequence that is specific enough to be an AMPKR substrate will by definition contain within that sequence the basic element selected for by PKD. Therefore peptide sequence alone cannot distinguish between a potential PKD substrate from an AMPK substrate; and it is quite possible that these kinases have the ability to phosphorylate the same residues within substrates, as is well-established for the Class II HDACs (Vega et al., 2004; Berdeaux et al., 2007; Mihaylova et al., 2011).

As a part of a larger ongoing effort in our laboratory, we screened the mammalian genome for proteins bearing conserved AMPKR motifs which were conserved back through *Drosophila* or *C. elegans* (Gwinn et al., 2008; Egan et

al., 2011a; Mihaylova et al., 2011). From this analysis, we discovered two candidate AMPKR phosphorylation sites matching the optimal substrate consensus within each of the catalytic subunits of the RalGAP complex (RalGAPα1 and RalGAPα2). These sites appear well-conserved, as at least one site is conserved in *Xenopus*, *Drosophila* and the echinoderm *S. purpuratus*, and both candidate phosphorylation sites are conserved in higher vertebrates including the zebrafish (*Danio rario*), and mouse (*M. musculus*) (Figure Xb).

Interestingly, a PFAM tree of RapGAP-domain containing proteins reveals that RalGAPα2 is the most closely related protein to the Rheb-GAP, tuberin (data not shown). Just as tuberin, both the RalGAP catalytic subunits contain RapGAP domains in their C-termini (Figure 2), with no other discernible domains. Just as tuberin has shown to be a substrate of Akt (Potter et al., 2002; Inoki et al., 2002; Dan et al., 2002), RalGAPα2 was originally identified in a screen for Akt substrates (as AS250, Akt substrate of 250 kDa), and was recently reported to be a direct substrate of Akt (Chen et al., 2011b). Tuberin is a known substrate of AMPK (Inoki et al., 2003; Corradetti et al., 2004; Shaw et al., 2004b; Liu et al., 2006), and we now find candidate AMPKR phosphorylation sites in RalGAPα1 and α2. These similarities suggest that as the tuberin/hamartin complex, the RalGAP complex may be responsive to signals from both the PI-3K-Akt pathway and the AMPKRs.

The previous bioinformatics searches only reveal whether a protein has the specified peptide sequence, but not whether that sequence is a bona fide *in vivo* phosphorylation site. To confirm that the candidate AMPKR/PKD sites can actually be phosphorylated *in vivo*, we performed mass spectrometry on chymotryptic fragments of RalGAPα2 immunoprecipitated from HEK293T cells overexpressing Flag-wt RalGAPα2, as well as mining mass spectrometry databases where phosphorylation sites identified in mass spectrometry studies have been deposited (phosphosite.org). This analysis revealed not only that both candidate sites within RalGAPα1 and RalGAPα2 are bona fide in vivo phosphorylation sites, but also that the RalGAPs are highly phosphorylated proteins; we were able to identify 30 phosphorylation sites within RalGAPα2 (Table 1). This degree of potential regulation suggests that, like tuberin/hamartin complex, the RalGAP complex may be a major integration point for signals from multiple pathways. Supporting this hypothesis, phosphoproteomic analysis of oncogenic receptor tyrosine kinase (RTK) pathways reveals that RalGAPα2 lies downstream of the RSK-MAPK, PI-3K-Akt and mTOR-S6K signaling pathways (Moritz et al., 2010).

RalGAPα2 Ser820 is phosphorylated by AMPK and PKD

To be able to characterize the candidate AMPKR/PKD phosphorylation sites in the RalGAP complex, phospho-motif antibodies were tested for capability to recognize RalGAPα2 site-specifically. Of about 15 antibodies

tested (including the 14-3-3 binding motif antibody and various AMPK-motif antibodies), only the PKD motif antibody (made to recognize peptides of the sequence L-X-R-X-X-pS/pT) specifically recognized Ser 766 and Ser820 of RalGAPα2 immunoprecipitated from HEK293T cells. Basal phosphorylation of RalGAPα2 is low, but upon stimulation of AMPK by phenformin treatment, or constitutively AMPK expression recognition of RalGAPα2 by the PKD motif antibody is enhanced (Figure 3a). This increase in reactivity is partially ablated by mutation of either Ser766 or Ser820 to alanine, and completely ablated when both candidate sites are mutated (Figure 3a), revealing that activation of AMPK can induce phosphorylation of RalGAPα2 at Ser766 and Ser820.

Because of the similarity between the optimal substrate motifs of AMPK and PKD, and because of the documented ability of these two kinases to phosphorylate the same residues in the same substrates (Vega et al., 2004; Berdeaux et al., 2007; Mihaylova et al., 2011), we decided to test whether these sites can also be phosphorylated in a PKD-dependent way. Expression of a constitutively active PKD allele (S738/742EE) increases immunoreactivity of the PKD motif antibody with RalGAPα2 compared to expression of kinase dead (KD) PKD, and as with AMPK, this increase is sensitive to mutation of Ser766 or Ser820, and ablated by mutation of both (Figure 3b).

To ensure that these phosphorylation events are the result of direct phosphorylation of RalGAPα2 by AMPK or PKD, we tested the ability of these kinases to phosphorylate RalGAPα2 *in vitro*. RalGAPα2 was immuno-

precipitated from HEK293T cells overexpressing epitope tagged RalGAPα2, and incubated with active recombinant AMPK or PKD. The cells for the IP to be incubated with AMPK were treated with fresh full serum medium for 30 minutes prior to lysis, while those for the IP to be incubated with PKD were treated with full serum medium with the PKC inhibitor, Gö6976, to ensure that the activity of PKC (and thus PKD) would be low basally. Addition of active AMPK (Figure 4a), or active PKD (Figure 4b) induced phosphorylation of RalGAPα2 at S766/S820, but not when S766/S820 are mutated to alanines. This demonstrates that both AMPK and PKD are capable of directly phosphorylating RalGAPα2 at S766/S820.

Phosphorylation of RalGAPα2 by Akt induces 14-3-3 association

One common mechanism of action for phosphorylation sites for some basophilic kinases is to induce association of the phospho-binding protein, 14-3-3 with the substrate. Analysis of 14-3-3 binding suggests that an important feature of the 14-3-3 binding peptides is that they bend after the phosphosite to exit the 14-3-3 binding pocket (Yaffe et al, 1997). This suggests that proline may be the ideal +2 residue for 14-3-3 binding, but many other residues can be tolerated, and only about half of the reported 14-3-3 binding proteins contain a proline in the +2 position (Johnson et al, 2010).

Our analysis of phosphorylation sites within the RalGAP complex revealed multiple candidate phosphorylation sites that might induce 14-3-3 binding, including one site in RalGAPβ (Ser359) that matches the optimal motif for Akt, and contains a proline at +2 (see Table 1). While Akt has been shown to phosphorylate RalGAPα2 (Chen et al., 2011b), the ability of Akt to induce association with 14-3-3 with the complex has not been assessed, and the candidate Akt site in RalGAPβ has not been characterized. We decided to test whether 14-3-3 associates with the RalGAP complex in an Akt dependent way for two reasons: first, it would represent yet another similarity between the RalGAP complex and the tuberin/hamartin complex, which associates with 14-3-3 upon Akt phosphorylation (Nellist et al., 2002; Li et al., 2002), and because Akt-dependent 14-3-3 association might mask 14-3-3 dependent regulation by other kinases, suggesting that we might try to assess the AMPKR/PKD phosphorylation sites in the context of Akt-inhibition. We found that bacterially made GST-14-3-3ζ protein, but not GST itself can precipitate wt-RalGAPα2 and wt-RalGAPβ, which is diminished by inhibition of Akt with the PI-3K inhibitor LY294002 (Figure 5). Surprisingly, this was not dependent of the perfect 14-3-3 consensus site in RalGAPβ, as mutation of Ser359 to alanine did not effect 14-3-3 association, but rather was due to Akt phosphorylation of RalGAPα2, as mutation of the Akt-sites in RalGAPα2, Thr715 and Ser844 to alanines ablated 14-3-3 association (Figure 5). These sites do not contain proline in the +2, but other residues at +2 can be tolerated for 14-3-3

association (Johnson et al., 2010). Just as Akt phosphorylation of tuberin promotes 14-3-3 binding, similar regulation is seen here with Akt phosphorylation of RalGAPα2 inducing 14-3-3 association.

PKD phosphorylation of RalGAPα2 at Ser766 and Ser820 induces 14-3-3 association

The possibility that phosphorylation of RalGAPα2 at sites distinct from the Akt sites might promote 14-3-3 association is also reminiscent of tuberin, and the RabGAPs TBC1D1 and TBC1D4/AS160 in which phosphorylation by AMPK on residues distinct from those phosphorylated by Akt promotes 14-3-3 binding (Nellist et al., 2003; Li et al., 2002; Pehmøller et al., 2009; Chen et al., 2008). It is quite common for PKD phosphorylation to induce 14-3-3 association with its substrates, including the Class II HDACs (Vega et al., 2004; Dequiedt et al., 2005), the transcription factor SNAIL (Du et al., 2010), and the phosphatase Slingshot (SSH1L) (Eiseler et al., 2009). Given that Akt phosphorylation of RalGAPα2 induces 14-3-3 association, the ability of PKD to induce 14-3-3 association with RalGAPα2 was tested in the context of PI-3K inhibition. Overexpression of wt and constitutively active PKD, but not kinase dead PKD induce 14-3-3 association with RalGAPα2; mutation of Ser820 to alanine dampens this effect, whereas mutation of both Ser766 and Ser820 to alanines ablates the ability of PKD to induce 14-3-3 association with RalGAPα2

(Figure 6). This indicates that Ser766 is important for PKD-induced 14-3-3 association with RalGAPα2.

Activation of endogenous PKD by phorbol-ester mediated PKC activation was sufficient to induce 14-3-3 association with RalGAPα2 and RalGAPβ (Figure 7). Knockdown of PKD1 and PKD2 partially reduced TPA-induced 14-3-3 association, whereas knockdown of both PKD1 and PKD2 ablated the ability of TPA to induce 14-3-3 association with the RalGAP complex (Figure 7). These data demonstrate that activation of either endogenous PKD1 or PKD2 downstream of PKC can induce phorbol-ester mediated 14-3-3 association with the RalGAP complex.

Activation of AMPK or PKD enhances Ral activity

To assess any changes in Ral activity caused by activation of the AMPK or PKD pathways, we pulled down GTP-bound Ral with the effector domain of RalBP1 immobilized on beads. Consistent with an inhibitory effect of AMPK phosphorylation of the RalGAP complex, stimulation of AMPK activity with the small molecular compound A769662 (A76), an allosteric activator of AMPK, increases Ral activity (Figure 8a, b). Cells with stable shRNA mediated knockdown of AMPKα1/α2 have a diminished capacity to enhance RalA activity following AMPK activation by A76 (Figure 8a). While RalA is still responsive to AMPK activation in this setting, some residual AMPK protein remains, and we hypothesize that more complete knockdown of AMPK would

completely ablate the ability of AMPK agonists to activate RalA. Similarly, stimulation of endogenous PKD by phorbol-ester mediated activation of PKC also induces RalA activation (Figure 8b), again indicating that the phosphorylation of RalGAPα2 at Ser766 and Ser820 inhibits the GAP activity of the complex, allowing for increases in GTP-bound Ral.

The potential inhibition of GAP activity by AMPK is in contrast to the role of AMPK phosphorylation in stimulating the GAP activity of TSC2, but is similar to the role of AMPK in regulating the RabGAPs TBC1D1 and AS160/TBC1D4 (Pehmøller et al., 2009; Chen et al., 2008). The induction of 14-3-3 binding by both phosphorylation of RalGAPα2 at Thr715 and Ser844 by Akt, and by phosphorylation of RalGAPα2 at Ser766 and Ser820 by AMPK or PKD suggests that 14-3-3 binding plays an inhibitory role in the RalGAP complex, similar to Akt- and AMPK-dependent 14-3-3 binding to TBC1D1 and AS160.

Discussion

Here, we present a model (Figure 9) where the RalGAP complex acts as a signal integration point for multiple pathways converging on regulation of the Ral small GTPases. This is analogous to the TSC1/2 Rheb GAP complex in regulation of mTOR signaling. Similar to TSC2, RalGAPα2 is a substrate of

both Akt and AMPK, phosphorylation by either inducing association with 14-3-3. Based on the large number of phosphorylation sites present in RalGAPα2, it is plausible that many other signaling pathways regulate Ral activity via the RalGAP complex.

We show here that PKD phosphorylates the same residues within RalGAPα2 as AMPK. This is reminiscent of regulation of HDAC5/7 by AMPKRs and PKD, where these kinases phosphorylate the same residues to promote nuclear exclusion of the HDACs (Vega et al., 2004; Berdeaux et al., 2007; Mihaylova et al., 2011). These observations taken into consideration with the overlapping optimal substrate motifs of PKD and the AMPKRs suggests that the ability of these kinases to phosphorylate the same sites within substrates may be a common theme in these two signaling pathways. It would be interesting to test whether known substrates of PKD can be phosphorylated by AMPK and vice versa.

The regulation of the RalGAP complex by AMPK and PKD ties these two signaling pathways to the processes regulated downstream of the Ral small GTPases including certain transcriptional programs, endocytosis, and vesicular trafficking via exocyst complex. And, the observation that RalBP1 along with components of the exocyst complex contain additional reported phosphorylation sites matching the AMPKR and PKD consensus substrate motifs suggest that regulation of the Ral pathway may be an important function of these kinases. Notably, PKC has previously been implicated in exocyst

function by phosphorylating the RalA-binding domain of Sec5, causing its dissociation from RalA, allowing for cycling of exocytic vesicles without changing the activation state or RalA (Chen et al., 2011a), suggesting that activation of PKC may regulate multiple steps of exocystosis.

Regulation of glucose uptake is process regulated by both Akt and AMPK, and the RabGAPs TBC1D1 and TBC1D4/AS160 have been shown to be an important substrate of both kinases in mediating this process. Akt phosphorylation of TBC1D1/4 mediates glucose uptake in response to insulin, while AMPK phosphorylation of TBC1D1/4 is important for hypoxia-, and contraction- induced glucose uptake in muscle cells (Sakamoto et al., 2008). Interestingly, Ral activation (Chen et al., 2007) and the exocyst complex (Inoue et al., 2003) were shown to be required for GLUT4 to be targeted to the plasma membrane following insulin stimulation, and the phosphorylation of RalGAPα2 by Akt has been shown to be important for insulin-mediated regulation of Ral activity and GLUT4 translocation (Chen et al., 2011b). It has also been shown that PKD can stimulate contraction-induced glucose uptake independent of AMPK, though no mechanism has been determined for the action of PKD in glucose uptake (Luiken et al., 2008). Our data presented here suggest that, like Akt and AMPK regulation of TBC1D1, both kinases regulate Ral GTPase activity to coordinate GLUT4 translocation to the plasma membrane, and also represents a mechanism by which PKD may function in glucose uptake. The convergence of AMPK and PKD on the RalGAP complex, with the potential

regulation of glucose uptake downstream would make the known glucose-transport regulating AMPK substrate, TBC1D1 or AS160 attractive candidate substrates to test for the ability of to be phosphorylated by PKD. Additionally, it would be interesting to investigate the possibility of other steps of Glut4 translocation regulated by both Akt and AMPK. In addition to regulating steps of exocytosis, AMPK- and PKD-dependent regulation of the Ral GTPases also links them to regulation of endocytosis by RalBP1 (Boissel et al., 2007). Suggesting that they may play a larger role in the recycling process of Glut4, and other clathrin-coated pit dependent endocytosis.

Both the exocyst complex (Tsuboi et al., 2005) and RalA (Lopez et al., 2008) have been shown to be important in the insulin exocytosis pathway in pancreatic beta cells. Notably, it has been demonstrated that PKD plays a role in insulin secretion as well (Sumara et al., 2009), and while PKD probably regulates this process at multiple steps, including its well-documented role in fission of transport carriers from the trans Golgi network (Bossard et al., 2007; Liljedahl et al., 2001), the regulation of Ral and exocyst activity via phosphorylation of the RalGAP complex provides an additional mechanism by which PKD may contribute to insulin exocytosis. The potential regulation of insulin secretion by PKD phosphorylation of the RalGAPs implicates this pathway in diabetic conditions. It will be important to discern the relative contribution of the LKB1-AMPK pathway versus PKD in the context of insulin secretion as well.

Delivery of components to the basolateral membrane in polarized cells is also dependent on RalA regulation of the exocyst (Moskalenko et al., 2002). Loss of the ability of RalA to regulate the exocyst results in accumulation of basolateral membrane components including the epidermal growth factor receptor (EGFR) and gp58 at the apical membrane (Moskalenko et al., 2002). Given the role of AMPK and PKD in regulation of RalA activity, these pathways may play a role in establishment and maintenance of proper basolateral membranes.

Because loss of LKB1 would result in decreased Ral activity, and hyperactive Ral activity is implicated in cancer (Lim et al., 2005; González-García, et al., 2005; Sablina et al., 2007), this would suggest that regulation of the Rals does not contribute to LKB1-tumor suppressor function. However, in certain tumor settings, PKD activity is enhanced (LaValle et al., 2010; Harikumar et al., 2010), so it would be very interesting to investigate whether Ral activity is aberrant in these conditions, and whether it contributes to PKD-induced tumorigenesis.

Taken together, our data presented here suggest that regulation of the Ral small GTPases by AMPK and PKD represent important downstream functions of these pathways for multiple biological processes. It will be important in the future to determine the relative contribution of each of these pathways in specific tissues to modulation of Ral activity, and how important regulation of Ral is for these biological processes.

Experimental Procedures

Plasmids

RalGAPα2 cDNA was purchased from OriGene, and subcloned into the pENTR3C vector (Invitrogen). Serine to alanine point mutations were made by PCR with the Phusion polymerase (NEB). RalGAPα2 was put into a pcDNA3-Flag DEST vector by LR reaction (Invitrogen). All ligation and PCR reactions for RalGAPα2 were transformed into CopyCutter e. coli (EpiCentre) due to toxicity of cDNA. Correct constructs were then transformed into DH10β cells (NEB).

Cell Culture and drug treatmens

HEK293, HEK293T and U2OS cells were grown in DMEM supplemented with 10% FBS in 10% CO_2. Cells were transfected with Lipofectamine 2000 (Invitrogen) as per manufacturer's instruction for 32–36 hrs. HEK293T cells were treated with 5 mM phenformin (SIGMA) for 1 hour where indicated. TPA (12-O-Tetra-decanoyl-phorbol-13-Acetate) (Cell Signaling Technologies) was administered at 200 nM for 30-45 min.

Immunoprecipitations

Cells were washed with ice cold PBS, then lysed on ice with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 2.5 mM $Na_4P_2O_7$ mM, 1 mM β-glycerophosphate, 10 nM

Calyculan A, and EDTA-free complete protease inhibitor tablets (Roche) as per manufacturer's directions). Lysates were incubated on ice for 15 min. after lysis, then spun at 13,200 rpm at 4°C for 15 min. The supernatants were collected and normalized for protein levels by BCA assay (Pierce). Whole cell lysates were incubated with antibodies for 1.5 hours with constant rocking at 4°C, then protein-A or –G sepharose beads (Invitrogen) were added for 1 hr. Immunoprecipitates were washed three times with lysis buffer, and sample buffer was added to 1X final, and samples were boiled at 95°C for 5 min.

In Vitro Kinase Assays

Anti-Flag immunoprecipitations from HEK293T cells transiently transfected with Flag-RalGAPα2 and myc-RalGAPβ for 24 hrs. Cells to be tested with PKD were treated with Gö6976 for 1 hr Flag-RalGAPα2 was immunoprecipitated (as above), while cells to be tested with AMPK were treated with full-serum medium for 1 hr, and lysed as above, and Flag-RalGAPα2 was immunoprecipitated. Then immunoprecipitates were washed three times with immunoprecipitation lysis buffer (as above), then three times with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycero-phosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$). Immunoprecipitates were then incubated with 20 μL kinase reaction mix (kinase buffer, 10 μM ATP) with or without 250 ng recombinant PKD (Millipore) or AMPK (Millipore) at 30°C for 30 min. with constant shaking. Reaction was quenched by addition of sample buffer to 1X

and boiling at 95° for 5 min.

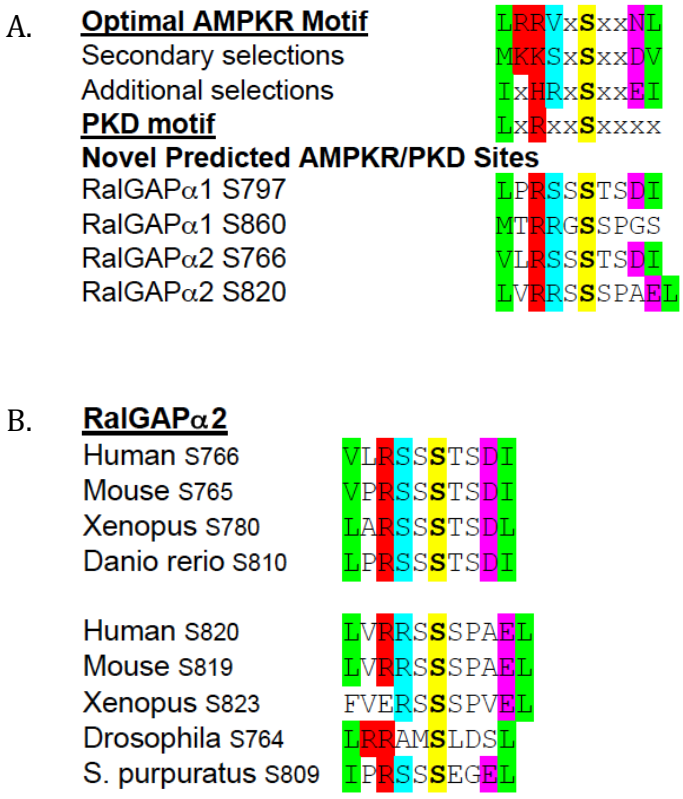
GST Pulls

Recombinant GST or GST-14-3-3ζ were produced in E. coli as previously described (Yaffe et al., 1997) then purified on glutathione sepharose and eluted with free glutathione. Cells were lysed in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 2.5 mM $Na_4P_2O_7$ mM, 1 mM b-glycerophosphate, 10 nM Calyculan A, and EDTA-free complete protease inhibitor tablets (Roche) as per manufacturer's directions). Lysates were incubated on ice for 15 min after lysis, then spun at 13,200 rpm at 4°C for 15 min. The supernatants were collected and normalized for protein levels by BCA assay (Pierce). Whole cell lysates were incubated with 10 μg GST or GST-14-3-3 with rocking at 4°C for 1 hr, followed by addition of glutathione Sepharose (GE Healthcare) to immobilize GST.

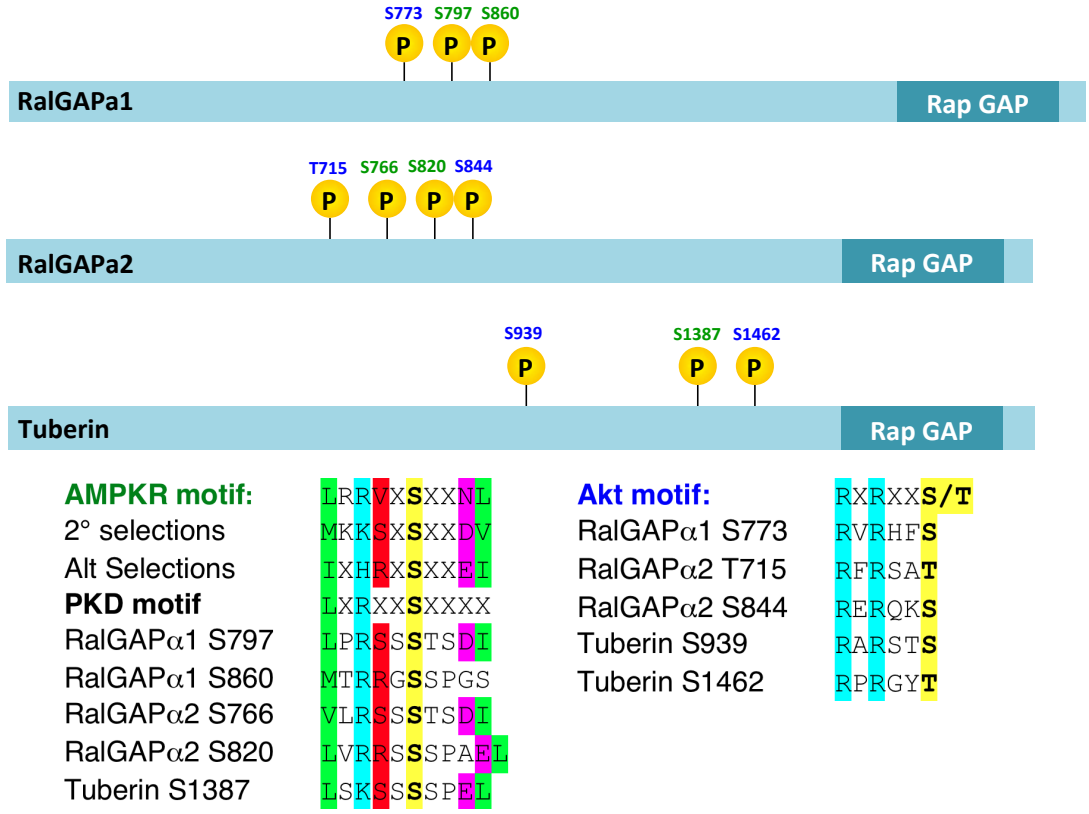
Ral activity assays

Cells were washed in ice cold PBS, then lysed in Ral activity lysis buffer (250 mM Tris-HCl, pH 7.5, 1 M NaCl, 5% NP-40, 50 mM $MgCl_2$, 2.5 mM dithiothreitol with 10 nM Calyculan A, and EDTA-free complete protease inhibitor tablets (Roche) as per manufacturer's directions), then spun at 13,200 rpm at 4°C for 10 min. Extracts of whole cell lysates were taken, then the

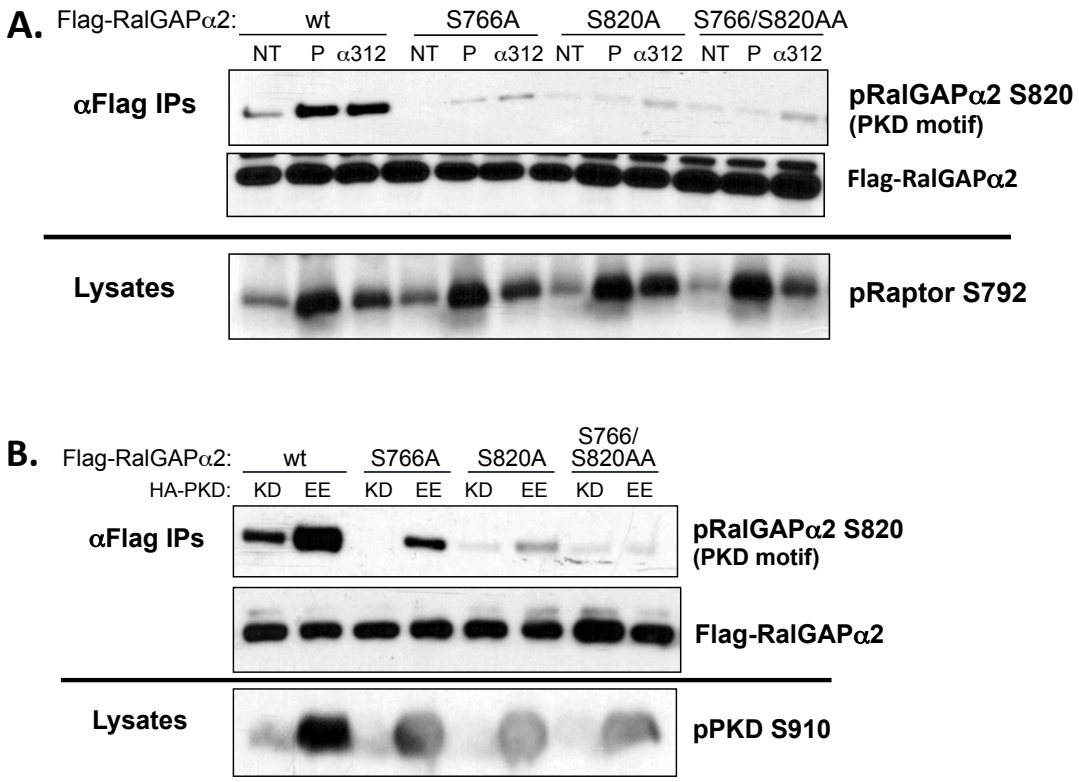
lysates were incubated with RalBP1 agarose (Millipore) for 30 minutes, and washed 3X with Ral activity lysis buffer. Extracts were used to measure protein quantity; lysates were equilibrated to similar protein concentrations, while RalBP1 pull downs were loaded differentially based on protein quantity.



CHAPTER IV Figure 1. RalGAPα1 and RalGAPα2 are candidate substrates of the AMPKRs and PKD.
(A) The optimal substrate motif (and secondary selections) for AMPK and the AMPK related kinases (AMPKRs) overlap with the optimal substrate motif of protein kinase D (PKD). Each catalytic subunit of the RalGAP comples contains two candidate AMPKR or PKD phosphorylation sites.
(B) Candidate AMPKR/PKD phosphorylation sites in RalGAPα2 are well-conserved evolutionarily.

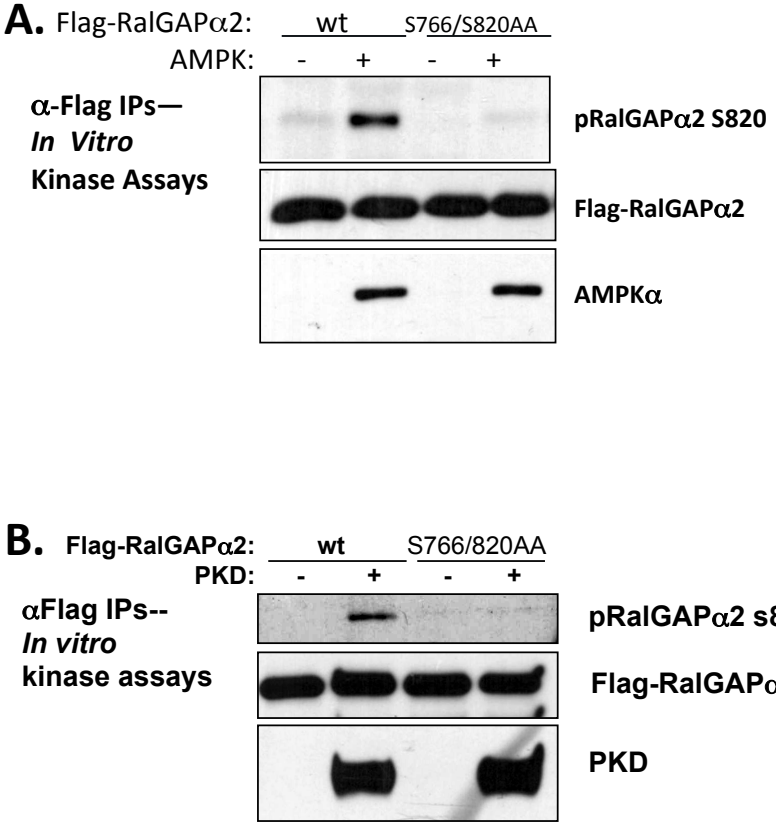


CHAPTER IV Figure 2. Candidate AMPKR/PKD and Akt phosphorylation sites within the RalGAPs compared with tuberin.
Alignment of the domain structures reveals similarities between the catalytic RalGAP subunits and tuberin. Each contain candidate or known phosphorylation sites for the AMPKRs/PKD and Akt.

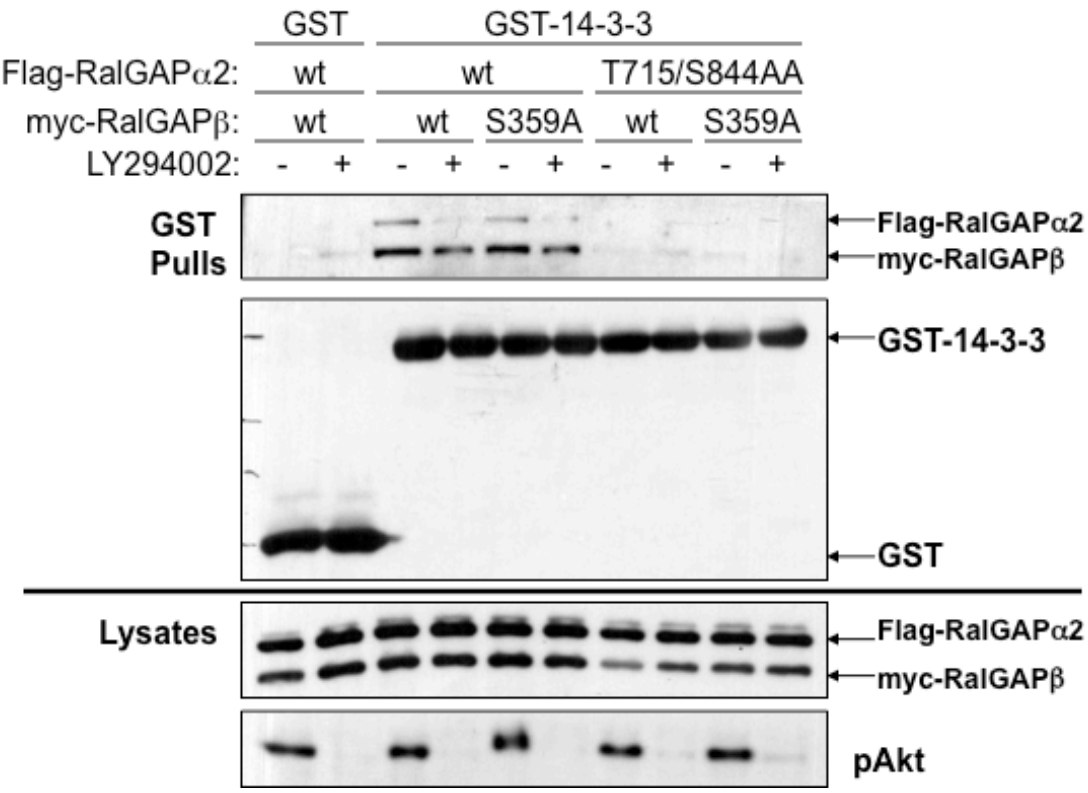


CHAPTER IV Figure 3. RalGAPα2 is phosphorylated in an AMPK and PKD-dependent manner.

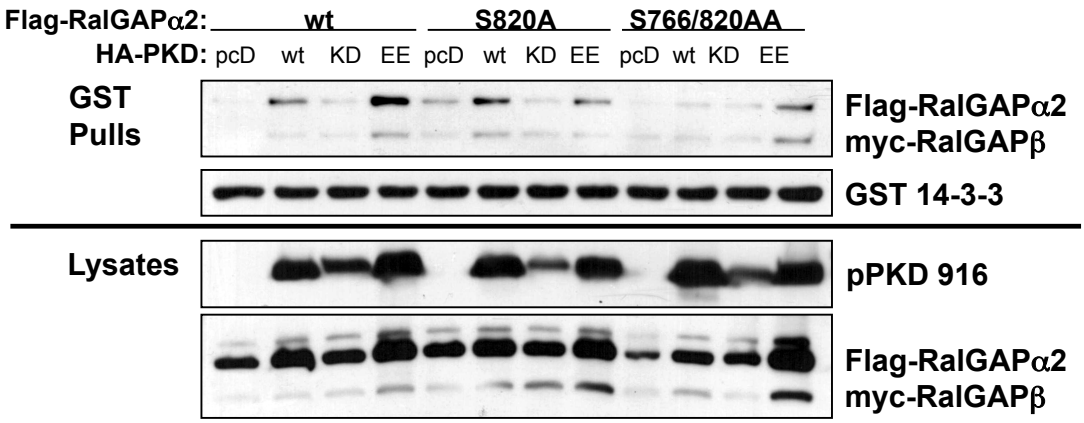
Flag-tagged wt-, S766A-, S820A or S766/S820AA RalGAPα2 and wt-RalGAPβ were co-transfected in HEK293T cells with (A) empty vector (pcD) or constitutively active AMPK (α312), (B) kinase dead (KD), or constitutively active (EE) PKD. Where indicated, cells were treated with 5 mM phenformin for 1 hr. RalGAPα2 was immunoprecipitated with α-Flag and Westerns were blotted with indicated antibodies.



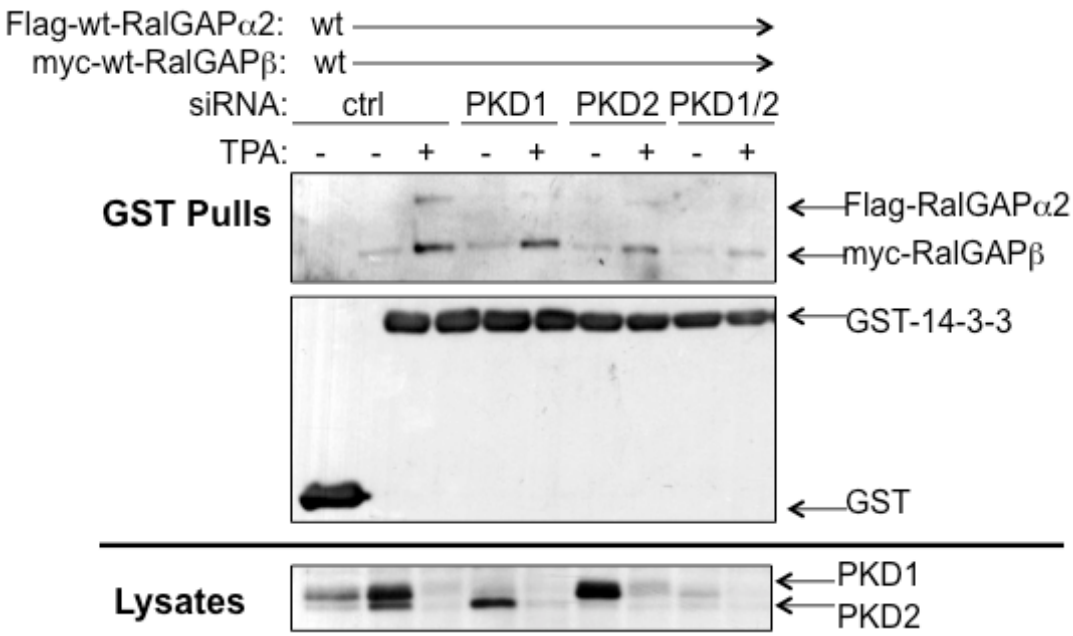
CHAPTER IV Figure 4. AMPK and PKD directly phosphorylate RalGAPα2 at Ser766 and Ser820. Active recombinant AMPK (A) or PKD (B) were incubated with Flag immunoprecipitates from HEK293T cells expressing empty vector (pcD), Flag-wt- or S766/S820AA RalGAPα2 myc-wt RalGAPβ treated with the full serum (A) or the PKC inhibitor, Gö6976 (B). Phosphorylation was detected by Western with PKD motif antibody.



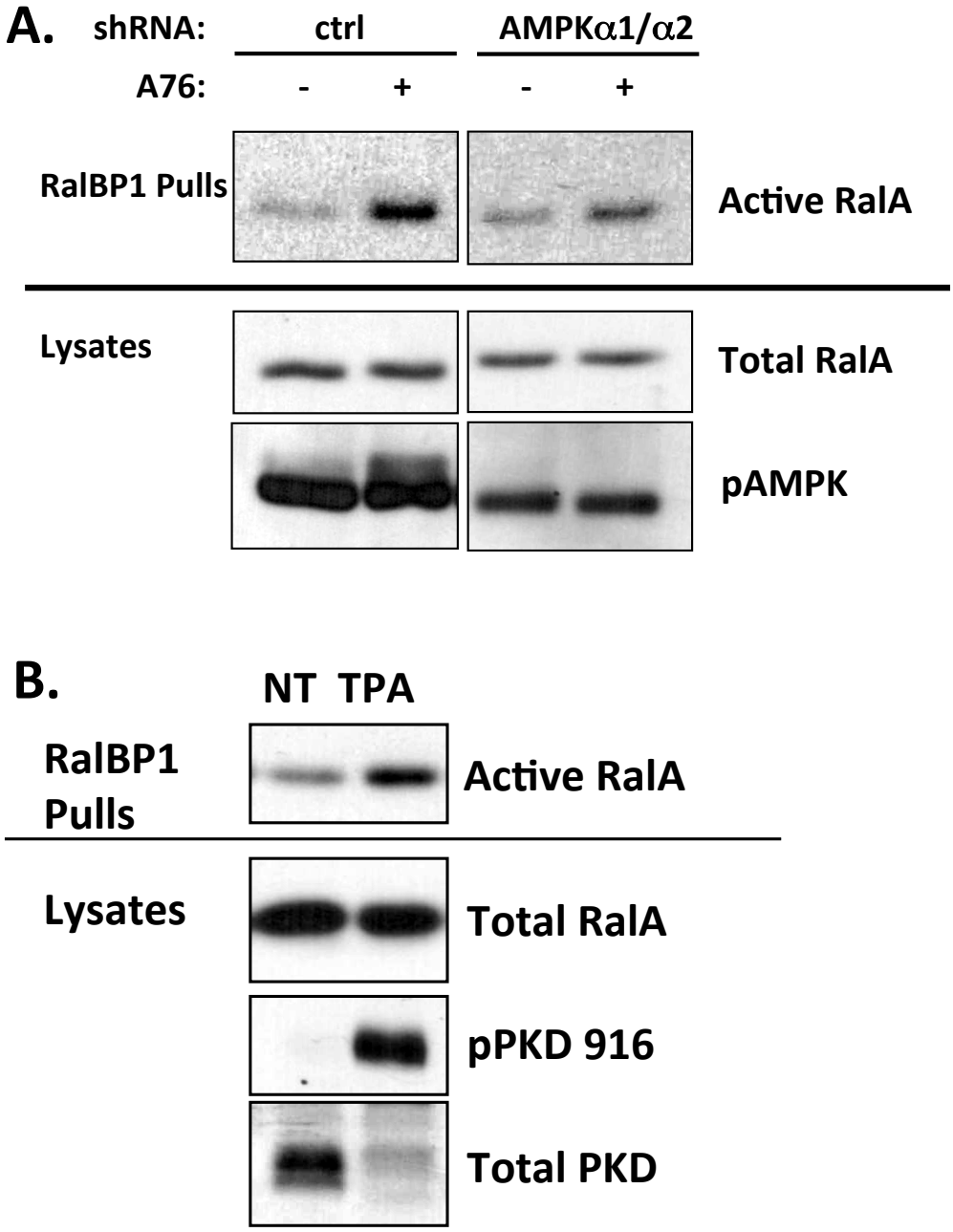
CHAPTER IV Figure 5. Akt phosphorylation of RalGAPα2 induces 14-3-3 association. Flag-RalGAPα2 and myc-RalGAPβ were precipitated from HEK293T cells expressing Flag-wt or T715/S844AA-RalGAPα2 or myc-wt or S359A RalGAPβ treated with LY294002 where indicated with bacterially expressed GST or GST-14-3-3 protein immobilized on GSH beads.



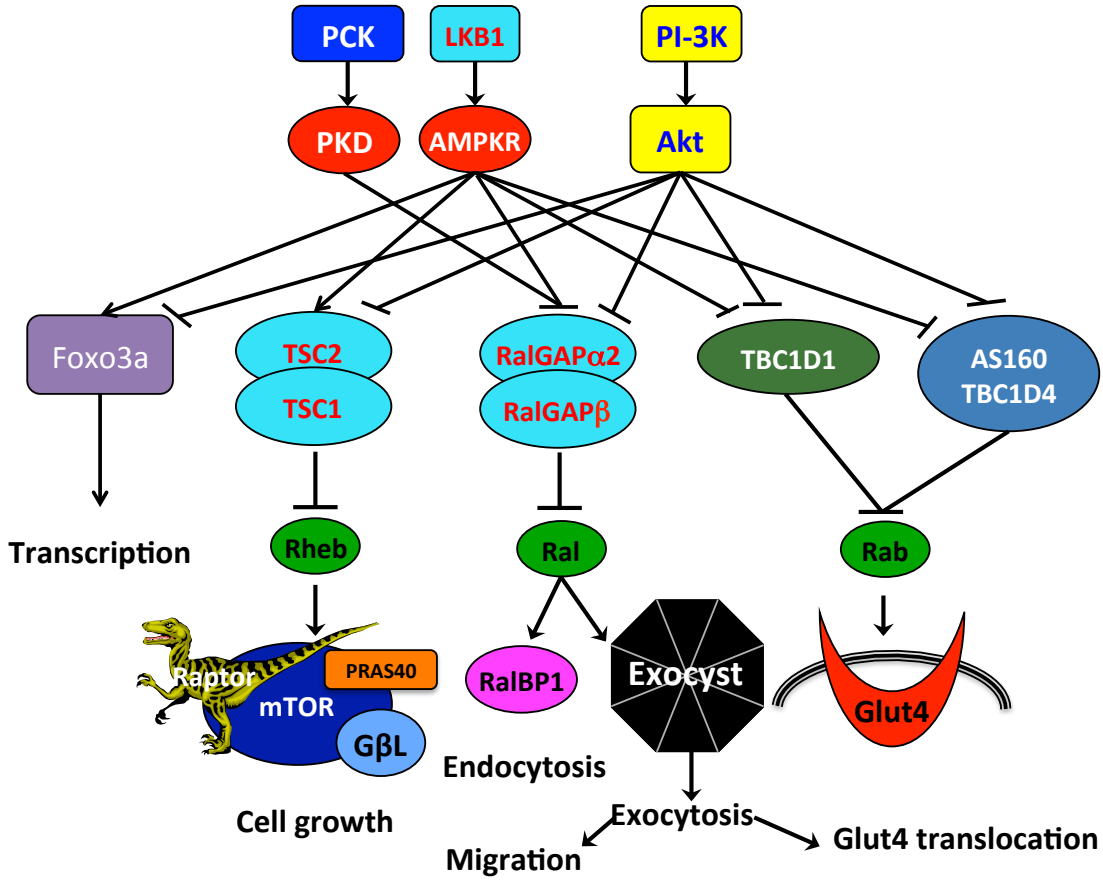
CHAPTER IV Figure 6. Expression of active alleles of PKD induce 14-3-3 association with the RalGAP complex. Flag-RalGAPα2 and myc-RalGAPβ were precipitated from HEK293T cells expressing Flag-wt-, S820A-, or S766/S820AA RalGAPα2 and myc-wt-RalGAPβ with bacterially expressed recombinant GST-14-3-3 protein immobilized on GSH beads.



CHAPTER IV Figure 7. Activation of endogenous PKD induces 14-3-3 association with the RalGAP complex. Bacterially expressed recombinant GST or GST-14-3-3 immobilized on GSH beads was used to precipitate Flag-RalGAPα2 and myc-RalGAPβ from U2OS cells with control or siRNA mediated knockdown of PKD1 PKD2, or both. All cells were treated with LY294002, and TPA where indicated.



CHAPTER IV Figure 8. Activation of endogenous AMPK or PKD stimulates Ral activity.
(A) U2OS cells with stable lentiviral expression of control or shRNA against AMPKa1/a2 were treated with fresh media or fresh media with the allosteric AMPK agonist, Abbott A769662 (A76), then subjected to RalBP1 pulldowns to detect active RalA. (B) HEK293 cells were treated where indicated with the phorbol ester, TPA, and subjected to RalBP1 pulldowns.



CHAPTER IV Figure 9. Updated Model of Cross-Talk Between the PI-3K-Akt and LKB1-AMPK Pathways.
The energy sensor, AMPK and the growth factor stimulated kinase, Akt share in common many substrates including Foxo3a in the regulation of transcriptional programs, TSC2 in mTORC1 signaling, TBC1D1/TBC1D4 in regulation of Glut4 translocation, and now RalGAPa2 in the regulation of the Ral small GTPases, which may represent novel mechanisms for AMPK-dependent Glut4 translocation, and endocytosis. Additionally, PKD can phosphorylate the same residues within RalGAPa2, providing a mechanism whereby PKD can regulated Glut4 translocation, and insulin secretion.

CHAPTER IV Table 1. Phosphorylation sites in the RalGAP complex proteins.

Mass spectrometry data was compiled from databases (phosphosite.org) as well as our own mass spectrometry on RalGAPα2 and RalGAPβ.
*only from our own MS data.

RalGAPα1	
S379	TLTEREP**s**SSsLCsI
S381	TEREPsSSsLCsIDE
S382	EREPsSS**s**LCsIDEE
S385	PsSSsLC**s**IDEEHLT
S683	NLYSLDL**s**DLPLDKL
S711	GHEFQKV**s**VDKSFSR
S721	KSFSRGW**s**RDQPGQA
S740	RSATTTG**s**PGTEKAR
T754	RSIVRQK**t**VDIDDAQ
S773	STRVRHF**s**QSEETGN
S775	RVRHFSQ**s**EETGNEV
S778	HFSQSEE**t**GNEVFGA
S795	EEQPLPR**s**sstsDIL
S796	EQPLPRS**s**STSDILE
S797	QPLPRSS**s**TSDILEP
T798	PLPRSSS**t**SDILEPF
S799	LPRSSST**s**DILEPFT
S860	STMTRRG**s**SPGSLEI
S861	TMTRRGS**s**PGSLEIP
S864	RRGSSPG**s**LEIPKDL
S1000	HSPLGSR**S**QTPSPST
T1002	PLGSRSQ**T**PSPSTLN
S1004	GSRSQTP**S**PSTLNID
S1006	RSQTPSP**S**TLNIDHM
S1478	KEPEPLH**s**PDSERSS
S1801	RELRNLD**s**RQCREtH
T1807	DsRQCRE**t**HKIAVFY

RalGAPα2	
Y290	TRDNENI**y**STKIPYM
Y296	IYSTKIP**y**MAARVVF
S373	TLSDRRL**s**NSSLCSI
S375	SDRRLSN**S**SLCSIEE
S376	DRRLSNS**s**LCSIEEE
S379	LSNSSLC**s**IEEEHRM
S461*	DAEKLGF**s**ETDSKEA
S480	SGHKRSS**s**WGRTYSF
S486	SSWGRTY**s**FTSAMSR
S694	KGTTVGR**s**FsLSWRS
S696	TTVGRSF**s**LSWRSHP
S713	TEPMRFR**s**AtTSGAP
T715	PMRFRSA**t**TSGAPGV
S764*	QQQQVLR**s**SsTSDIP
S766	QQVLRSS**s**TSDIPEP
S776*	DIPEPLC**s**DSSQGQK
S819	ITILVRR**s**ssPAELD
S820	TILVRRS**s**SPAELDL
S821	ILVRRSS**s**PAELDLK
S844	KCRERQK**s**EstNsDT
S846	RERQKsE**s**tNsDTTL
T847	ERQKsEs**t**NsDTTLG
S849	QKsEstN**s**DTTLGCT
S893*	ERHWLQL**s**PTDASNL
S980*	AISLDNQ**s**SPSPPVL
S981*	ISLDNSS**s**PSPPVLI
S1349*	SEPVQYH**s**SAELGNL
S1451*	TEGPVGG**s**PVGSLSD
S1493*	APNGRNP**s**FLISSWR
S1593*	VTSQGQP**s**PVERPRG

RalGAPβ	
S357	LGISRPR**s**DsAPPtP
S359	ISRPRsD**s**APPtPVN
T363	RsDsAPP**t**PVNRLSM
T379	PQSAAVS**t**TPPHNRR
T400*	KATMKTS**t**VSTAHAS
S417	QHQTSST**s**PLssPNQ
S420	TSSTsPL**s**sPNQTSS
S421	SSTsPLs**s**PNQTSSE
S470	NGINRDS**s**MTAITTQ
S496*	QMSDTMV**s**NPMFDAS
Y537	GEEILPA**y**LSRFYML
S720	KSHSRTN**s**GISSASG
S723	SRTNsGI**s**SASGGST
S726	NsGISSA**s**GGSTEPt
S729	ISSASGG**s**TEPttPD
T730	SSASGGS**t**EPttPDS
T733	SGGSTEP**t**tPDSERP
T734	GGSTEPt**t**PDSERPA
S892*	DKEPNPA**s**MRVKDAA
S1300*	PGILKQP**s**LTLELFP

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