



Novel modes of mTORC1 regulation by amino acids

Citation

Buel, Gwen. 2016. Novel modes of mTORC1 regulation by amino acids. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.

Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:33840703>

Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

Novel modes of mTORC1 regulation by amino acids

A dissertation presented

by

Gwen Buel

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

August 2016

© 2016 Gwen Buel

All rights reserved.

Novel modes of mTORC1 regulation by amino acids

Abstract

In order to maintain homeostasis, cells must be able to sense, interpret, and coordinate responses to environmental factors including energy status, amino acid availability, and the presence of growth factors. One important pathway that coordinates these signals and dictates the appropriate response is the mechanistic target of Rapamycin complex 1 (mTORC1) pathway. In conditions that favor growth, mTORC1 activates pathways involved in protein, lipid, and nucleotide synthesis, and inhibits breakdown of biomolecules through lysosomal degradative pathways. Mutations in the pathways that regulate mTORC1 lead to constitutively activated mTORC1, which contributes to disease states such as cancer. My thesis attempts to elucidate the pathways involved in coordinating amino acid availability with mTORC1 activity so we may better understand how healthy cells maintain homeostasis given a constantly changing environment, and how diseased cells with mutations in these pathways will respond to various treatments. My thesis will show that cells respond to withdrawal of individual amino acids in a manner very distinct from the response to a lack of all amino acids. Cells sense the removal of amino acids and downregulate mTORC1 activity. I discovered that withdrawal of the individual amino acids arginine or leucine allows for mTORC1 reactivation for a period of several hours. This reactivation occurs after a brief decrease in mTORC1 activity, and is not observed following withdrawal of total amino acids. I demonstrate that this response requires an upstream activator of mTORC1, Akt, and is not due to amino acids being regenerated via autophagy or other means. I also observe an increase in Akt activity during the time frame of mTORC1 recovery, suggesting that longer term amino acid deprivation stimulates mTORC1 activity through activation of Akt. My thesis

also describes a phenomenon in which proteins involved in mTORC1 regulation alter their location in response to amino acids, and I summarize my work to investigate the mechanism and functions of this movement. I discuss the implications of these findings, and propose alternate interpretations to the current models of mTORC1 activation downstream of amino acids.

Table of Contents

List of Figures	vii
Acknowledgements	ix
Chapter 1 General introduction	1
Discovery of mTOR: The Mechanistic Target of Rapamycin	2
mTOR structure and function.....	3
mTORC1 regulation overview	7
mTORC1 regulation by amino acids	9
The TSC complex: a point of integration of growth factor and amino acid pathways?	13
Other mechanisms of mTORC1 regulation	14
mTORC1-mediated feedback loops.....	15
Processes regulated by mTORC1.....	17
Chapter 2 mTORC1 reactivation follows prolonged deprivation of leucine or arginine	23
Introduction.....	24
Results	26
mTORC1 reactivation follows prolonged deprivation of leucine or arginine.	26
mTORC1 reactivation is observed in many, but not all cell types.....	28
The mTORC1 reactivation is independent of regeneration of amino acids from autophagy/lysosomal degradation or the proteasome	29
The mTORC1 recovery response requires PI3K/Akt activity	31
Akt activity increases during the mTORC1 recovery response in some, but not all cell lines.....	34
The mTORC1 recovery response is independent of mTORC2.....	36
The recovery response is partially mediated by the TSC complex.....	37
The increase in Akt signaling upon prolonged leucine or arginine deprivation is transient	39
Discussion	40
Chapter 3 Rag movement from lysosomes following amino acid stimulation	46
Introduction.....	47
Results	48
RagA moves away from the lysosome following amino acid stimulation in an mTOR-independent fashion	48
Rag localization is governed by GDP/GTP nucleotide binding	51
The Rag proteins enter the nucleus following amino acid stimulation	56

N-terminal modification/lipidation results in dysfunctional RagA protein.....	58
Discussion	62
Chapter 4 Discussion and future directions.....	66
General Discussion	67
The mTORC1 recovery response	67
Rag movement from lysosomes.....	71
Chapter 5 Materials and methods	74
Cell culture and transfections	75
Amino acid deprivations and stimulations.....	75
Antibodies and reagents	76
Cell Lysis and western blotting.....	77
Immunoprecipitations.....	78
Microscopy	78
Generation of Rag fusion proteins	80
Generation of stable cell lines.....	82
Metabolite extraction	82
SUnSET Translation Assay	83
Nuclei isolation for flow cytometry.....	83
Appendix A Supplemental figures.....	84
References.....	89

List of Figures

Figure 1.1 The mTOR complexes	6
Figure 1.2 Regulation of mTORC1.....	9
Figure 1.3 mTORC1 regulation by amino acids.....	13
Figure 1.4 mTORC1-mediated feedback loops	16
Figure 2.1 mTORC1 reactivation follows prolonged deprivation of leucine or arginine.....	27
Figure 2.2 mTORC1 recovery occurs in many but not all cell lines.....	28
Figure 2.3 The mTORC1 recovery response is independent of canonical autophagy or proteasomal degradation of proteins	30
Figure 2.4 The mTORC1 recovery response requires PI3K/Akt activity.....	32
Figure 2.5 Prolonged arginine or leucine deprivation leads to increased PI3K/Akt signaling.	35
Figure 2.6 The mTORC1 recovery response is independent of mTORC2	36
Figure 2.7 mTORC1 reactivation is partially mediated through the TSC complex	37
Figure 2.8 SGK1 plays a role in mTORC1 activation, and inhibition of both Akt and SGK1 robustly inhibits mTORC1 signaling and recovery response	39
Figure 2.9 The increase in Akt signaling upon prolonged leucine or arginine deprivation is transient	41
Figure 2.10 Loss of the Rag GTPases prevents mTORC1 responsiveness/recovery following arginine or leucine withdrawal.....	44
Figure 3.1 RagA moves away from the lysosome following amino acid stimulation	49
Figure 3.2 Rag movement away from the lysosome is independent of mTOR activity	50
Figure 3.3 Rag localization is governed by GTP/GDP binding.....	52
Figure 3.4 RagA enters the nucleus following amino acid stimulation	55
Figure 3.5 RagA entry into the nucleus does not appear to be necessary for mTORC1 activation	57
Figure 3.6 N-terminal modification/lipidation of RagA results in dysfunctional protein.....	59
Figure 3.7 RagA forced localization with the Rheb CAAX motif inhibits activation of mTORC1 to its full extent, but partially rescues mTORC1 activity in amino acid starve conditions	62
Figure A 1 Rapamycin induces a large nuclei phenotype under arginine deprivation conditions	85
Figure A 2 Amino acid levels following arginine removal.....	85
Figure A 3 Amino acid levels following leucine removal	86
Figure A 4 mTORC1 recovery cannot be blocked through additional deprivation of glutamine	87

Figure A 5 Rags appear to be shifting from lysosomes to another endo-membrane structure88

Acknowledgements

I would like to thank everyone who has been a source of support throughout my years in graduate school. In particular, I would like to thank my adviser, Dr. John Blenis, for providing me with valuable advice, space, and resources to conduct research in his lab. I would also like to thank the members of my Dissertation Advisory Committee, Dr. Brendan Manning, Dr. Nika Danial, and Dr. Victor Hsu, for all the advice and encouragement on my projects. Importantly, I want to recognize all the students and post-docs from the Blenis lab with whom I have interacted, who have taught me most of the things I have learned in graduate school, and who have been a source of moral support on a daily basis. I am also indebted to the folks at the Nikon Imaging Center and the Image and Data Analysis Core at Harvard Medical School for helping to teach and advise on all the imaging data I have collected. I would also like to extend my thanks to the community at Weill Cornell who welcomed me as a visiting student when the Blenis lab made the jump to New York City.

I want to recognize my mentor from my undergraduate studies, Dr. Bryan Ballif, for continuing to be a source of advice and moral support throughout my years in graduate school.

I would like to thank my family, for never questioning my abilities or my progress, and for always being supportive despite having their own constant barrage of challenges.

I would like to sincerely thank my classmates, particularly those who were supportive through challenging times and never lost faith in me as a person or a scientist, even when I lost faith in both.

Lastly, I would like to thank my partner in crime for being endlessly supportive and helping me to see the sunlight in every day. You inspire me to be a better person, and your optimism has helped guide me through countless challenges.

To anyone who I have not mentioned explicitly but who has positively influenced me in any way during my time in graduate school, please know that I am deeply indebted to your kindness, thoughtfulness, and generosity.

Chapter 1

General introduction

Introduction

A central hallmark of all cells is the ability to sense their environment and adapt in order to survive in a constantly changing environment. For most organisms, part of this adaptation requires being able to quickly transition between anabolic and catabolic states depending on the availability of nutrients. This allows a cell to avoid death during periods of nutrient depletion by reducing consumption, as well as allowing a cell to proliferate by increasing consumption when nutrients are abundant and growth is beneficial to the organism. One of the main mechanisms by which many organisms mediate this transition relies on a kinase complex called mTORC1, or the mechanistic target of Rapamycin complex 1. mTORC1 is able to sense various aspects of the cellular environment, and accordingly adjust the metabolism of the cell to suit the environment and needs of the organism. My thesis investigates the mechanisms involved in regulation of mTORC1 by the environment, specifically by amino acid levels.

Discovery of mTOR: The Mechanistic Target of Rapamycin

The 289 kDa protein now known as mTOR, or the mechanistic target of Rapamycin, was named after a natural product isolated in the 1970's from a species of bacteria from the island of Rapa Nui (Vezina et al., 1975). The compound, which its discoverers' named Rapamycin after the island of its discovery, was originally found to have little anti-bacterial activity, but displayed strong inhibition of growth of the yeast *Candida albicans* through an unknown mechanism. Rapamycin, which was later found to additionally have an inhibitory effect on immune responses in rats (Martel et al., 1977), was shown through initial characterization to inhibit synthesis of nucleic acids, proteins, and lipids, while

promoting degradation of macromolecules (Singh et al., 1979). Genetic studies in *saccharomyces cerevisiae* were the first to identify the molecular targets responsible for the action of Rapamycin; it was found that Rapamycin forms a complex with a protein now known as FKBP12 (Koltin et al., 1991), and that the Rapamycin-FKBP12 complex in yeast binds two homologues of a protein named TOR (target of Rapamycin) (Heitman et al., 1991). Mammalian cells were later discovered to possess only one ortholog of the TOR genes, which was initially named the mammalian target of Rapamycin (mTOR) (Sabers et al., 1995), in addition to other names given by groups who also discovered the protein around the same time: RAFT1 (Sabatini et al., 1994), FRAP (Brown et al., 1994), SEP (Chen et al., 1994), and RAPT1 (Chiu et al., 1994). In recent years, attempts to streamline nomenclature have led to mTOR having an alternative definition of mechanistic target of Rapamycin (Hall, 2013), although both terms have remained prevalent in the field.

mTOR structure and function

Following the discovery of mTOR, it was found that mTOR has homology to phosphatidylinositol kinases, but it was ultimately shown that mTOR is itself a protein kinase (Brown et al., 1995) with serine/threonine kinase activity (Brunn et al., 1997; Burnett et al., 1998). Subsequent work led to the discovery that mTOR in cells forms a complex with a protein termed Raptor (regulatory associated protein of mTOR) (Hara et al., 2002; Kim et al., 2002), and in a separate complex, mTOR associates with rapamycin-insensitive companion of mTOR (Rictor) (Sarbasov et al., 2004). The Raptor-containing mTOR complex has ultimately become known as mTOR complex 1 (mTORC1), and the Rictor-containing mTOR complex has become known as mTORC2 (Figure 1.1). Both mTORC1 and mTORC1 contain the

protein mLST8 (mammalian lethal with SEC13 protein 8), also known as GβL (Kim et al., 2003), but the two mTOR complexes have distinct sets of substrates.

The earliest identified substrates of mTORC1, the eIF-4E binding proteins (4E-BP1 and 4E-BP2) and p70 S6 Kinase 1 (S6K1) (Brunn et al., 1997; Burnett et al., 1998), are regulators of protein translation, demonstrating a mechanism for the observed role of mTOR in regulation of protein synthesis. In addition to its regulation of protein synthesis, mTORC1 has also been shown to promote synthesis of other biomolecules including lipids (Porstmann et al., 2008; Yecies et al., 2011) and nucleotides (Ben-Sahra et al., 2013, 2016), and to inhibit cellular degradation of proteins and other material via autophagy (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). mTORC1 exerts these effects both through regulation of transcription of metabolic genes (Düvel et al., 2010), and through direct post-translational phosphorylation of proteins (Ben-Sahra et al., 2013; Brunn et al., 1997; Burnett et al., 1998).

In contrast to mTORC1, mTORC2 has been studied less well extensively, but it has been shown that mTORC2 plays roles in cell survival and proliferation through regulation of protein kinase B (PKB, also referred to as Akt) (Sarbasov et al., 2005), and in cytoskeletal reorganization through regulation of protein kinase Cα (PKCα) (Jacinto et al., 2004; Loewith et al., 2002; Sarbasov et al., 2004). Although Rapamycin is not thought to bind to mTOR while it is bound to Rictor, prolonged treatment with rapamycin has been shown to inhibit mTORC2 assembly, seemingly by binding to newly synthesized mTOR before it can bind and complex with Rictor (Sarbasov et al., 2006). Additional proteins found in the mTORC2 complex but not mTORC1 include mSIN1 (mammalian stress-activated MAP Kinase-interacting protein 1) (Frias et al., 2006; Yang et al., 2006a) and PROTOR1/2 (protein observed with Rictor) (Pearce et al., 2007). PROTOR1/2 activates mTORC2 through incompletely understood mechanisms,

while mSIN1 contains a pleckstrin homology (PH) domain and recruits the complex to the cell membrane following activation of the PI3K pathway (Liu et al., 2015).

The structural architecture of the mTORC1 complexes has been hard to elucidate due to the large size of mTOR and the difficulty in crystalizing full-length mTOR bound to its complex components. However, advancements in the field of cryo-electron microscopy have led to structures of the mTOR complexes that have allowed for a better understanding of the overall construction of the mTOR complexes. Both mTORC1 and mTORC2 form dimeric complexes, leading to complexes in the megadalton range (Wullschleger et al., 2005; Yip et al., 2010). The mTOR dimer forms something that resembles a doughnut, with mLST8 perched by the active site, helping to form a cleft, with the mTOR active site at the base of the cleft (Gaubitz et al., 2015; Yang et al., 2013; Yip et al., 2010). In mTORC1, Raptor binds mTOR at the junctions of the two mTOR subunits, seemingly stabilizing the dimer, and additionally making the active site cleft smaller (Aylett et al., 2015). Deletion of sections of mTOR, allowing for a more open active site, increase the activity of mTOR in vitro, supporting a model in which mTOR specificity is dependent on restriction of substrates to the active site (Yang et al., 2013). In agreement with this model, a domain of Raptor is able to recruit substrates containing a TOR signaling (TOS) motif to the catalytic site of mTORC1, helping to overcome the physical restriction of the catalytic site (Aylett et al., 2015; Schalm and Blenis, 2002).

Based on the structural data, it appears that the Rapamycin-FKBP12 complex inhibits mTORC1 by binding to mTOR and further blocking access to the active site (Yang et al., 2013). However, Rapamycin does not have equally strong inhibition towards all mTORC1 substrates, and why this is the case is not fully understood (Choo et al., 2008). The domain of

mTOR that is bound by Rapamycin-FKBP12 is thought to be blocked by Rictor, explaining the insensitivity of mTORC2 to Rapamycin (Gaubitz et al., 2015).

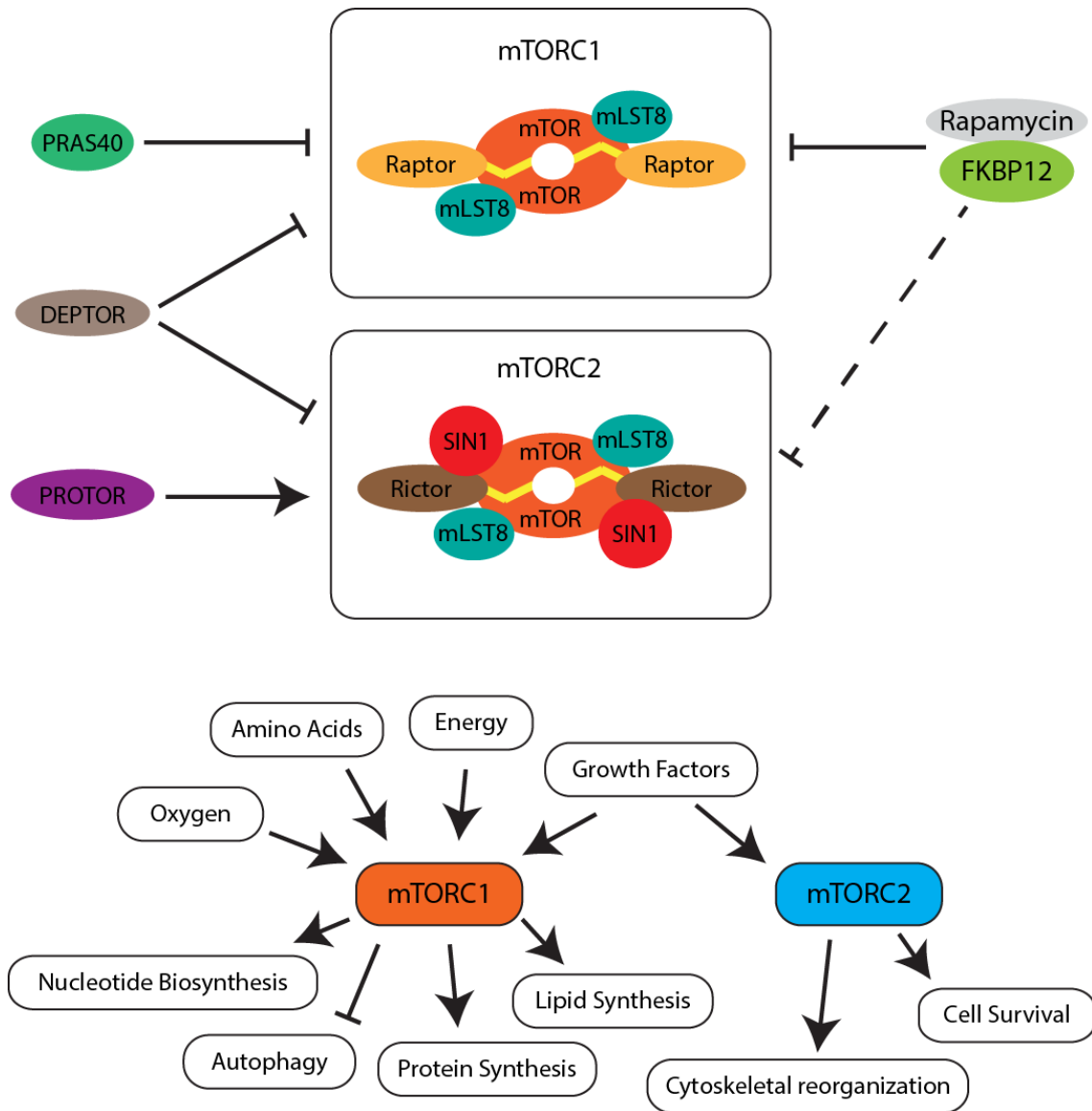


Figure 1.1 The mTOR complexes. Both mTORC1 and mTORC2 form dimers of mTOR which contain mLST8. mTORC1 additionally contains Raptor, while mTORC2 contains Rictor and SIN1. Endogenous proteins that interact with the complexes and regulate the actions of mTOR include PRAS40, DEPTOR, and PROTOR. PRAS40 inhibits mTORC1 but not mTORC2, PROTOR activates mTORC2 but not mTORC1, and DEPTOR inhibits both complexes. The Rapamycin FKBP12 complex inhibits mTORC1 following treatment with Rapamycin, while effects of rapamycin on mTORC2 are only observed following longer term treatment. Growth factors regulate both mTORC1 and mTORC2, while levels of oxygen, amino acids, and energy levels additionally regulate mTORC1. mTORC2 regulates cell survival and cytoskeletal reorganization. mTORC1 regulates cell growth processes including nucleotide biosynthesis, autophagy, protein synthesis, and lipid synthesis.

mTORC1 regulation overview

mTORC1 is regulated by a number of environmental signals, namely growth factors, energy status, oxygen, and amino acids (Figure 1.2). The integration of these signals by mTORC1 is important for cellular functioning, given the prominent role the protein complex plays in regulation of the growth-favoring processes described above. There is a large body of research showing that improper regulation of mTORC1 can cause cells to overconsume under conditions of limiting nutrients, leading to metabolic catastrophe and cellular death (Choo et al., 2010; Efeyan et al., 2013; Teleman et al., 2005). However, hyperactivation of mTORC1 is often seen in cancers, giving cancerous cells a growth advantage, allowing them to “steal” nutrients from the rest of the body in order to produce the nucleotides, lipids, and proteins necessary to fuel cell division (Cargnello et al., 2015). Thus, intricate regulation of mTORC1 in accordance with the cellular environment and the growth requirements of the organism is essential for cellular functioning and organismal survival.

Central to the regulation of mTORC1 is the tuberous sclerosis complex (TSC) complex, which is a protein complex named after a disease of the same name (Henske et al., 2016). The TSC complex consists of TSC1 (also called hamartin), TSC2 (also called tuberlin), and TBC1D7 (Dibble et al., 2012), which together act as a GTPase activating protein (GAP) for the small GTPase Rheb (Ras homolog enriched in brain) (Inoki et al., 2003a; Tee et al., 2003). Rheb binds to mTOR, and while in the GTP-bound form, Rheb activates the kinase activity of mTORC1 (Long et al., 2005) but not mTORC2 (Yang et al., 2006b). Since the TSC2 complex stimulates Rheb to hydrolyze GTP to GDP, the TSC complex acts as a negative regulator of mTORC1. However, the TSC complex can be inactivated by phosphorylation of several residues catalyzed by Akt (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). As a

part of the Phosphoinositide 3 Kinase (PI3K)/Akt pathway, Akt is regulated downstream of growth factor stimulation (Burgering and Coffey, 1995; Franke et al., 1995), and in this way, growth factors such as Insulin and Insulin-like growth factor 1 (IGF-1) can stimulate mTORC1 activity through inactivation of the TSC complex. Additional data suggests that serum- and glucocorticoid-induced protein kinase (SGK1), a kinase highly related to Akt and also regulated by growth factor stimulation, can also phosphorylate TSC2 on the sites phosphorylated by Akt (Castel et al., 2016). In addition to Akt and SGK1, growth factor stimulated pathways result in activation of extracellular regulated kinases 1 and 2 (ERK1/2) and ribosomal S6 Kinase (RSK), which are also able to phosphorylate and inhibit the TSC complex (Ma et al., 2005; Roux et al., 2004).

In addition to being regulated by growth factors, the TSC complex can also be regulated by energy status. In response to low energy, exhibited by low ATP to ADP ratio, AMP kinase (AMPK) becomes activated, and phosphorylates TSC2 at T1227 (Inoki et al., 2003b). In contrast to the phosphorylation of TSC2 by Akt, the phosphorylation site modified by AMPK promotes the activity of the TSC complex, thereby decreasing activation of mTORC1. In addition to the input AMPK gives to mTORC1 through the TSC complex, low energy also leads to AMPK phosphorylation of Raptor, which also leads to inhibition of mTORC1 (Gwinn et al., 2008).

Oxygen is an essential nutrient necessary for much of cellular metabolism, and as such, cells have also evolved mechanisms to signal the availability of oxygen to mTORC1 (Arsham et al., 2003). Under conditions of modest, short-term hypoxia, AMPK is activated and can inhibit mTORC1 through its effects on the TSC complex (Liu et al., 2006). Additionally, low oxygen levels can activate the hypoxia-inducible factor family of transcription factors (HIFs).

One HIF-1 target gene, REDD1 (Shoshani et al., 2002), can inhibit mTORC1 through activation of the TSC complex (Brugarolas et al., 2004; Deyoung et al., 2008; Sofer et al., 2005).

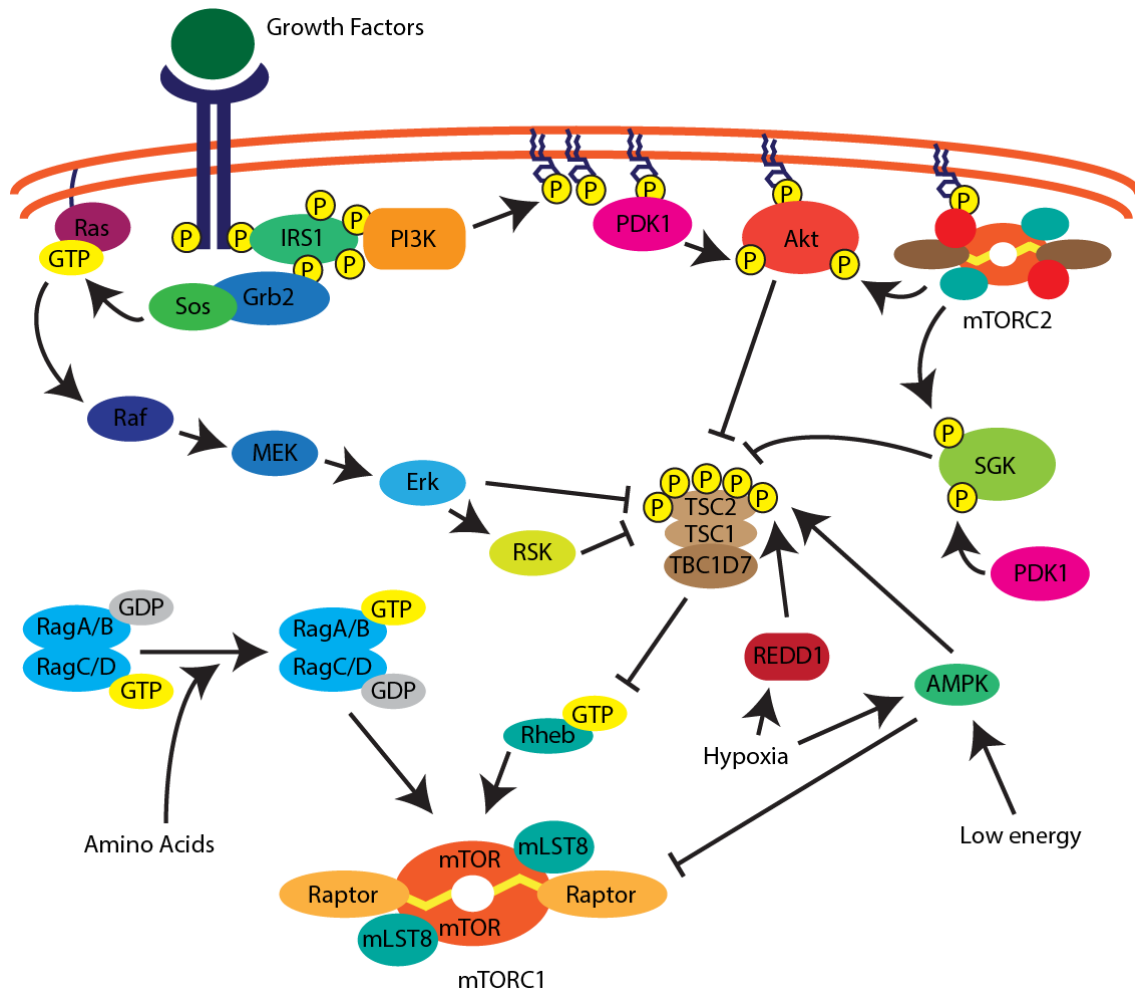


Figure 1.2 Regulation of mTORC1. mTORC1 is regulated by growth factors, amino acids, energy status, and hypoxia. Upon binding of growth factors to growth factor receptors like the insulin receptor, the PI3K and MAPK pathways become activated. This leads to phosphorylation of TSC2 by Akt, Erk, RSK, and SGK at sites that inhibit the ability of the TSC complex to act as a GAP for Rheb. Low energy leads to activation of AMPK, which also phosphorylates TSC2, but at a site that activates the ability of the complex to act as a GAP for Rheb. Acute hypoxia also activates AMPK, and it also leads to transcription of REDD1, which activates the TSC complex. Lastly, amino acids lead to mTORC1 activation through the Rag GTPases.

mTORC1 regulation by amino acids

In contrast to the mechanisms of regulation described above, the mechanisms of mTORC1 regulation by amino acids are largely independent of the TSC complex. Although

it had been clear by the 1990's that amino acids are necessary for mTORC1 activation (Blommaart et al., 1995; Hara et al., 1998; Luiken et al., 1994), the mechanisms of this regulation remained elusive for the next decade. A breakthrough in the field came when it was discovered that amino acids allow for recruitment of mTORC1 to the surface of lysosomes (Kim et al., 2008; Sancak et al., 2008). It was found that this recruitment is mediated by a family of GTPases called the Rag GTPases, which themselves localize to the lysosome due to interactions with a pentameric complex called Ragulator (Sancak et al., 2010). In mammals there are four members of the Rag GTPase family: RagA, RagB, RagC, and RagD. RagA and RagB are orthologs of the yeast protein Gtr1, while RagC and RagD are orthologs of the yeast protein Gtr2 (Bun-Ya et al., 1992; Hirose et al., 1998; Schürmann et al., 1995; Sekiguchi et al., 2001). The Rag proteins form obligate heterodimers of one Gtr1-like protein (RagA or B) and one Gtr2-like protein (RagC or D) (Nakashima et al., 1999; Sekiguchi et al., 2001).

The fact that the Rag proteins form dimers is unusual, given that most GTPases are monomeric. Additionally, the Rag proteins are also unique in that they are not lipidated to allow for their localization to membranes. Instead, for their localization to lysosomal membranes, the Rags require the Ragulator complex, which also serves as a guanine nucleotide exchange factor (GEF) for RagA and RagB (Bar-Peled et al., 2012).

As with all GTPases, the Rags cycle between GTP-bound and GDP-bound states. The switch from GDP to GTP is mediated by GEFs, which stimulate release of GDP and allow for GTP, which is found in much higher concentrations in the cell, to take the place of GDP. The switch from GTP to GDP, however, is mediated by the intrinsic enzymatic activity of the GTPase, which is stimulated by GTPase activating proteins (GAPs). In many cases, the GTP-bound form of a GTPase is considered the "active" form, while the GDP-bound form is the "inactive" form, but this is not always the case. In the case of the Rag proteins, GTP-bound

RagA/B is “active” in its ability to stimulate mTORC1 activity, while the GDP-bound form of RagC/D is “active” in its ability to stimulate mTORC1 activity (Sancak et al., 2008).

The relative contributions to mTORC1 activation by the Rag dimer components is a bit unclear, although there is data to suggest that the nucleotide binding state of RagC/D is more important for mTORC1 binding (and recruitment of mTORC1 to lysosomes) than that of RagA/B (Tsun et al., 2013). However, expression of a dominant active form of RagA/B (constitutively bound to GTP) has been shown to make the mTORC1 pathway completely insensitive to amino acid deprivation (Efeyan et al., 2013; Kim et al., 2008; Sancak et al., 2008), suggesting that RagA/B may regulate an important but less well understood process involved in mTORC1 regulation. Additionally, it is not yet understood why mammals contain four Rag proteins, while yeast only contain the two Gtr proteins; it is likely that there are tissue specific enrichments for RagA versus RagB, and RagC versus RagD, but this has yet to be studied in detail.

As mentioned above, the Ragulator complex serves as a GEF for RagA/B. The Ragulator complex was originally identified to have three components: p18 (LAMTOR1), p14 (LAMTOR2), and MP1 (LAMTOR3) (Sancak et al., 2010). However, two more components of the Ragulator complex were later identified, C7orf59 (LAMTOR4) and HBXIP (LAMTOR5), which were found to be essential for the GEF function of the complex (Bar-Peled et al., 2012).

Following the discovery of the Ragulator as a RagA/B GEF, it was discovered that a trimeric complex now termed GATOR1 (GAP activity toward Rags 1) consisting of the proteins DEPDC5, Nprl2, and Nprl3, functions as a GAP for RagA/B (Bar-Peled et al., 2013). In this regard, the Ragulator complex positively regulates mTORC1 through RagA/B, and the GATOR1 complex negatively regulates mTORC1 through RagA/B. The RagC/D portion of the dimer, however, is not affected by the known GEF/GAP proteins that regulate RagA/B. Rather,

it has been shown that the GAP for RagC/D is Folliculin in complex with its binding partners FNIP1/2 (FLCN-FNIP) (Petit et al., 2013; Tsun et al., 2013), and a GEF for RagC/D has yet to be identified.

The discovery of the GEFs and GAPs involved in the regulation of the Rags was illuminating, but it still did not explain how amino acids are being sensed by the cell, and how this sensing is then regulating the actions of the GEFs and GAPs. There is a body of work suggesting that the amino acid content within the lysosome is important in the regulation of the Rags by amino acids; specifically, the lysosomal amino acid transporter SLC38A9 can sense intralysosomal arginine levels, and together with the lysosomal v-ATPase, is able to promote the Ragulator GEF activity towards RagA/B in amino acid replete conditions (Wang et al., 2015; Zoncu et al., 2011).

In contrast to the lysosomal amino acid sensing pathway that has been proposed, additional proteins involved in sensing cytosolic amino acids have also been uncovered. Specifically, the Sestrin family of proteins have been found to bind directly to leucine (Saxton et al., 2016a; Wolfson et al., 2016), and the CASTOR (cellular arginine sensor for mTOR) proteins have been found to bind directly to arginine (Chantranupong et al., 2016; Saxton et al., 2016b). Both the Sestrins and the CASTOR proteins exert their actions on a complex called GATOR2, which is a negative regulator of GATOR1 (Bar-Peled et al., 2013). In the absence of leucine or arginine, respectively, Sestrins and CASTOR proteins bind to GATOR2, preventing it from inhibiting the GAP activity of GATOR1. In the presence of amino acids, however, binding of Sestrins and CASTOR to their respective amino acids prevents them from binding GATOR2 (Chantranupong et al., 2016; Wolfson et al., 2016). This allows GATOR2 to inhibit GATOR1, allowing for increased levels of GTP-bound RagA/B and therefore increased mTORC1 recruitment and activation (Figure 1.3).

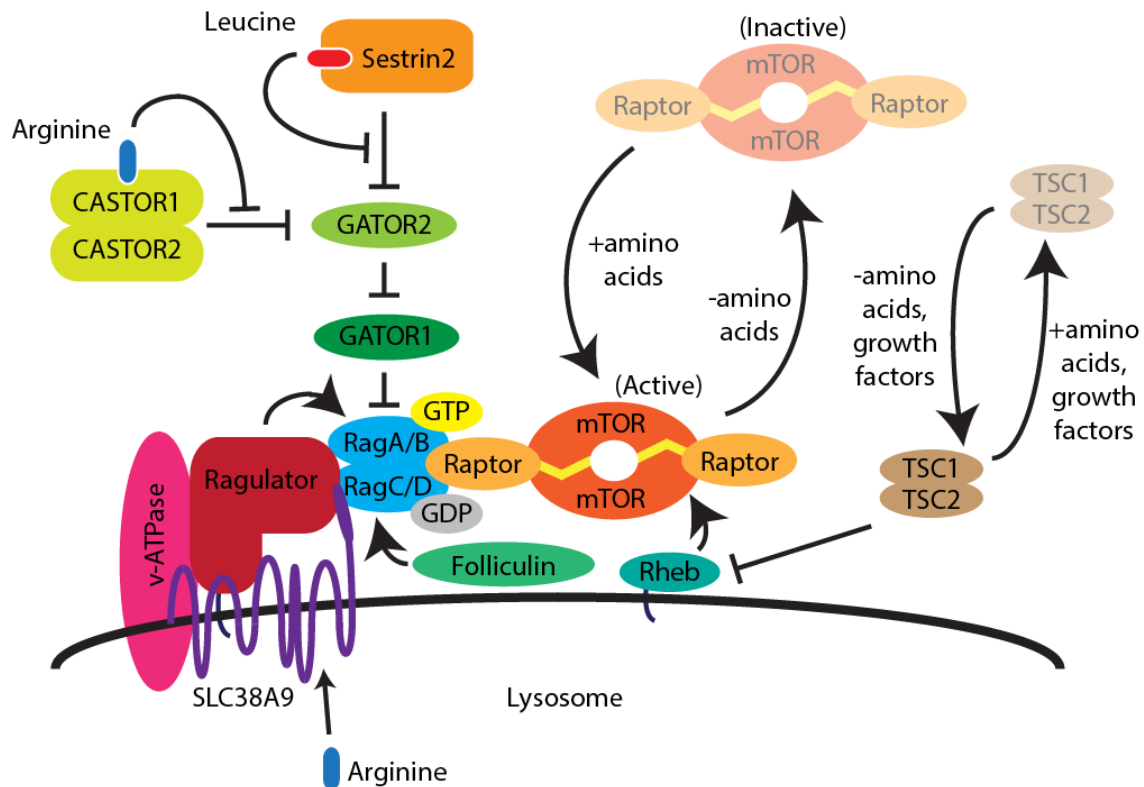


Figure 1.3 mTORC1 regulation by amino acids. mTORC1 is recruited to the lysosomal membrane by the Rag GTPases which localize to the lysosome via interactions with the Ragulator complex. RagA/B are positively regulated by the Ragulator complex, which serves as a GEF for RagA/B. GATOR1 acts as a GAP for RagA/B, negatively regulating the ability of RagA/B to activate mTORC1. GATOR1 is negatively regulated by GATOR2, which is negatively regulated by CASTOR1/2 and Sestrin1/2/3. Upon leucine binding to Sestrin1/2/3 or arginine binding to Castor1/2, the interactions between these proteins and GATOR2 are disrupted, leading to inhibition of GATOR1 and activation of RAGA/B. Folliculin serves as a GAP for RagC/D, positively regulating the ability of RagC/D to recruit mTORC1 to the lysosome. Once mTORC1 is at the lysosome, Rheb is able to activate mTORC1 through direct contacts. The TSC complex is a GAP for Rheb, and localization of the TSC complex to the lysosome is regulated by a host of factors including amino acids and growth factors, and potentially other stresses as well.

The TSC complex: a point of integration of growth factor and amino acid pathways?

To summarize the methods of regulation described above, it may be easiest to classify the factors that regulate mTORC1 into two classes: those that directly regulate the activity of mTORC1 through the TSC complex/Rheb, and those that regulate the activity of mTORC1 indirectly by affecting its intracellular location/proximity to Rheb. In this

classification, growth factors, oxygen, and energy status would be in the first class, and amino acids would be in the second class. However, studies looking at the localization of the TSC complex have blurred the lines of these initially distinct classifications. It has been established that the TSC complex can be recruited to lysosomal membranes in order to inhibit Rheb, but some data shows that growth factor-stimulated Akt phosphorylation of the TSC complex is needed to inhibit this recruitment (Menon et al., 2014), while other data demonstrate that amino acid deprivation allows for TSC complex recruitment (Demetriades et al., 2014). Newer data has suggested that both growth factor and amino acid inputs regulate the localization of the TSC complex, and additionally, other cellular stresses such as hypoxia, osmostress, energetic stress, and pH stress may be able to regulate this process as well (Demetriades et al., 2016). These data suggest that mTORC1 localization via the Rags is only one way by which amino acids can regulate mTORC1 activity, and that TSC complex localization may also play an important role in amino acid regulation of mTORC1. Since multiple factors seem to be able to regulate the localization of the TSC complex, it seems that the TSC complex may serve as a point of intersection of regulatory factors.

Other mechanisms of mTORC1 regulation

Other mechanisms of regulation of mTORC1 activity involve the proteins PRAS40 (proline-rich Akt substrate 40 kDa), DEPTOR (DEP domain containing mTOR interacting protein), and PA (phosphatidic acid). PRAS40 is a Raptor interacting protein that potently inhibits mTORC1 kinase activity in vitro by blocking substrate binding. Phosphorylation of PRAS40 by Akt causes dissociation of PRAS40 from mTORC1, thus promoting mTORC1 activity (Vander Haar et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Wang et al., 2007). PRAS40 can also be phosphorylated by mTORC1 itself, which would

further promote mTORC1 activation once it is activated via other mechanisms (Oshiro et al., 2007).

DEPTOR is an mTOR-interacting protein that also has inhibitory effects towards both mTORC1 and mTORC2 (Peterson et al., 2009). DEPTOR is regulated by its expression levels, with its proteasomal degradation being promoted through ubiquitination by the ubiquitin ligase SCF^{βTrCP}. Phosphorylation of DEPTOR by mTOR, CK1α, RSK1, S6K1, allow for its degron sequence to be recognized by SCF^{βTrCP}, ultimately leading to its degradation and release of inhibition of mTOR (Duan et al., 2011; Gao et al., 2011; Zhao et al., 2011).

PA activates mTORC1 through mechanisms that have not been well defined (Fang et al., 2001). PA is a lipid second messenger that can be incorporated into cellular membranes from exogenous sources, or can be synthesized from lysophosphatidic acid (LPA), diacylglycerol (DG), or phosphatidylcholine (PC), though it seems that the route of synthesis most relevant is from PC through the enzyme phospholipase D (PLD). It has been proposed that PA acts through stabilization of the mTOR complex (Toschi et al., 2009), however, more recent data suggests that PA acts by displacing DEPTOR from mTORC1 in a manner more acute than the methods of DEPTOR degradation described above (Yoon et al., 2015).

mTORC1-mediated feedback loops

Feedback loops have evolved in countless biological pathways to effectively regulate processes. While positive feedback loops produce unstable systems, negative feedback loops produce stable systems that resist change. In the context of mTORC1 activation, unchecked activation could potentially lead to overconsumption. Perhaps as a mechanism to avoid this situation, cells have evolved several negative feedback loops that become activated following mTORC1 activation. The key proteins involved in these feedback

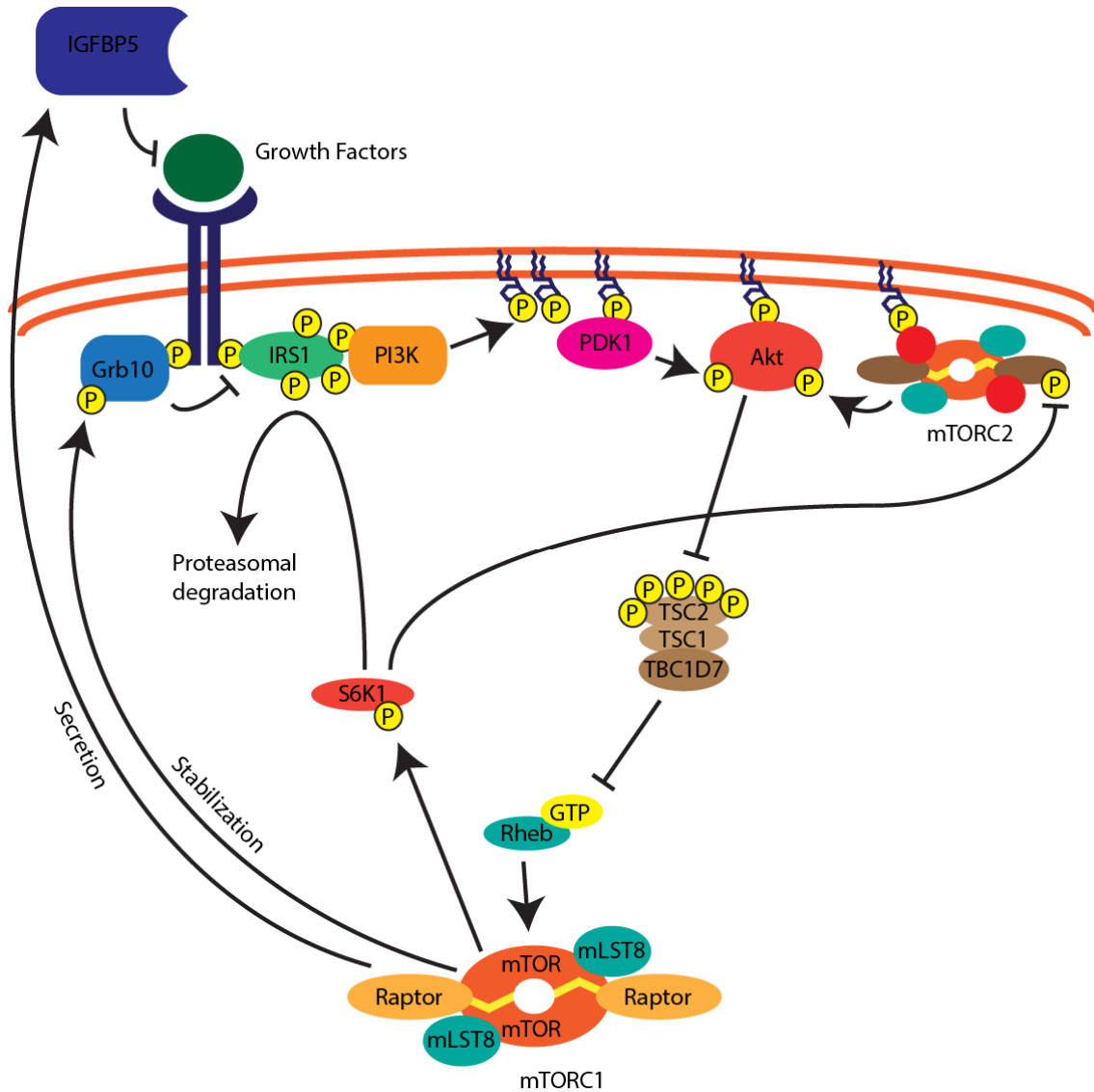


Figure 1.4 mTORC1-mediated feedback loops. Active mTORC1 phosphorylates and activates target proteins, including S6K1 and Grb10. Phosphorylation of Grb10 by mTORC1 leads to stabilization of Grb10, which acts to inhibit signaling downstream of the insulin receptor. S6K1 phosphorylation of IRS1 leads to proteasomal degradation of IRS1, also leading to downregulation of growth factor signaling. S6K1 also phosphorylates Rictor, leading to inhibition of the ability of mTORC2 to phosphorylate and activate Akt. mTORC1 activation also leads to upregulation and secretion of IGFBP5, which is thought to sequester IGF1. All of these mechanisms lead to feedback inhibition of mTORC1.

loops are insulin receptor substrate 1 (IRS1), growth factor receptor-bound protein 10 (Grb10), Rictor, and IGF binding protein 5 (IGFBP5) (Figure 1.4). IRS1 binds to the intracellular portions of activated insulin receptors and IGF-1 receptors, and is tyrosine phosphorylated at multiple residues by these receptors, creating binding sites for proteins such as p85 regulatory

subunit of phosphatidylinositol 3 Kinase (PI3K). This allows for downstream activation of Akt, which then leads to mTORC1 activation, as described above. mTORC1 has been shown to regulate phosphorylation of IRS1 via S6K, and phosphorylation of these sites promote degradation of IRS1 (Haruta et al., 2000; Um et al., 2004). It has also been proposed that S6K1 suppresses IRS1 at the mRNA level (Harrington et al., 2004).

Grb10 is another insulin receptor associated protein, although its actions toward the insulin receptor are inhibitory. It has been shown that mTORC1 directly phosphorylates Grb10 and stabilizes the protein, thus leading to decreased insulin receptor signaling upon mTORC1 activation (Hsu et al., 2011; Yu et al., 2011).

A key step in the activation of Akt is phosphorylation of Akt by mTORC2. It was found that S6K1 can phosphorylate Rictor and that this phosphorylation inhibits the ability of mTORC2 to phosphorylate and activate Akt (Dibble et al., 2009).

In addition to these mechanisms of feedback downstream of receptor activation, another mechanism of mTORC1-mediated negative feedback is through the secreted protein IGFBP5. mTORC1 promotes expression of IGFBP5, which is secreted and is thought to sequester IGF1, blocking signaling through IGFR while mTORC1 is active (Ding et al., 2016).

Processes regulated by mTORC1

The best characterized cellular process regulated by mTORC1 is protein synthesis. As mentioned above, mTORC1 directly phosphorylates S6K and 4E-BP1, which both help mediate the effects of mTORC1 on protein translation. 4E-BP1 is an inhibitor of the translation initiation factor eIF4E, binding it and preventing binding of the scaffold protein eIF4G. Phosphorylation of 4E-BP1 by mTORC1 prevents binding of 4E-BP to eIF4E, allowing

recruitment of eIF4G and eIF4A to the 5' end of mRNA (Brunn et al., 1997). This allows for subsequent recruitment of the initiating Met-tRNA and additional factors comprising the 48S pre-initiation complex, allowing for ribosome scanning and translation initiation (reviewed in Ma and Blenis, 2009).

mTORC1 can phosphorylate both S6K1 and S6K2 (Burnett et al., 1998), though S6K1 has been studied more extensively than S6K2. mTORC1 phosphorylates S6K at T389 in the hydrophobic motif, and allows for docking of PDK1 (phosphoinositide-dependent kinase 1), which phosphorylates S6K at T229 in the catalytic domain of S6K (for review, see Pearce et al., 2010). One of the main mechanisms by which mTORC1 and S6K promote translation is through increased biogenesis of ribosomes. One model proposed this to be mediated by S6K1 phosphorylation of ribosomal protein S6 (RPS6), which promotes translation of mRNAs with an oligopyrimidine tract at their transcriptional start site, termed a 5'TOP. mRNAs encoding ribosomal proteins have been shown to contain 5'TOPs, and thus mTORC1/S6K activation was proposed to promote expression of ribosomal proteins through this mechanism (Jefferies et al., 1997). However, later work showed that the translation of 5'TOP mRNAs is independent of S6K and RPS6 (Tang et al., 2001), bringing into question how mTORC1 promotes translation of ribosomal proteins. mTORC1 also promotes ribosomal biogenesis through increased synthesis of ribosomal RNA (rRNA) and transfer (tRNA). mTORC1 achieves this through activation of the transcription factor tripartite motif-containing protein-24 (TIF-1A), which promotes transcription of rRNA via RNA Pol I (Mayer et al., 2004). mTORC1 also phosphorylates and inhibits Maf-1, which is a repressor of RNA Pol III, and this leads to mTORC1 promotion of transcription of tRNA and 5S rRNA (Kantidakis et al., 2010; Shor et al., 2010). In addition to mTORC1-regulated ribosome biogenesis, S6K can also promote translation directly through interactions with a number of translation initiation factors. S6K

and mTORC1 both associate with eIF3, which acts as a scaffold to allow for efficient regulation of the translation machinery in response to stimuli (Holz et al., 2005). For example, S6K1 can phosphorylate eIF4B at S422, which allows for eIF4B to promote the helicase activity of eIF4A and for recruitment of the translation pre-initiation complex (Raught et al., 2004). S6K1 can also phosphorylate PDCD4 (programmed cell death 4) at S67, causing it to be recognized by the ubiquitin ligase β -TrCP and ultimately be degraded by the proteasome (Dorrello et al., 2006). PDCD4 binds eIF4A, preventing it from being recruited by eIF4G to the translation initiation complex, so S6K1-mediated degradation of PDCD4 is another mechanism to allow for activation of translation by eIF4A. Lastly, S6K has also been shown to promote efficient translation of spliced mRNAs in the pioneer round of translation in a SKAR (S6K1 Aly/REF-like substrate)-dependent manner (Ma et al., 2008).

In addition to regulation of protein synthesis, mTORC1 plays a role in regulation of lipid synthesis. mTORC1 achieves this goal primarily through regulation of the sterol regulatory element-binding protein 1/2 (SREBP1/2) transcription factors (Düvel et al., 2010; Li et al., 2010; Porstmann et al., 2008; Wang et al., 2011). While inactive SREBP1/2 localize to the endoplasmic reticulum (ER), insulin stimulation or sterol depletion leads to transport of SREBP1/2 to the Golgi in COPII-dependent vesicles, where it is proteolytically processed, allowing an active form of SREBP1/2 to enter the nucleus and promote expression of genes involved in fatty acid and cholesterol synthesis. The mechanisms by which mTORC1 regulate SREBP1/2 have been somewhat difficult to elucidate but it appears that it is at least partially dependent on S6K1 (Düvel et al., 2010; Li et al., 2011; Wang et al., 2011). mTORC1 can also directly phosphorylate lipin-1, a phosphatidic acid phosphatase which inhibits SREBP1/2 nuclear localization and transcriptional activity. Phosphorylation of lipin-1 by mTORC1 prevents lipin-1 from localizing to the nucleus and inhibiting SREBP1/2, and this serves as a

mechanism by which mTORC1 can promote SREBP1/2 function (Huffman et al., 2002; Peterson et al., 2011). Another mechanism by which mTORC1 can regulate SREBP1 is through CREB regulated transcription coactivator 2 (CRTC2). CRTC2 interferes with a component of the COPII complex, thus inhibiting transport of SREBP1 to the Golgi for processing to its active form. mTORC1 phosphorylates CRTC2 and inhibits it from acting on the COPII complex, allowing SREBP1 processing to proceed (Han et al., 2015).

Another branch of mTORC1 regulation is that of nucleotide biosynthesis. It has been shown that mTORC1 positively regulates the synthesis of pyrimidines through S6K1-mediated phosphorylation of CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase) at S1859. CAD is the enzyme that catalyzes the first three steps of de novo pyrimidine synthesis, and phosphorylation at S1859 allows for increased flux through the pyrimidine synthetic pathway (Ben-Sahra et al., 2013; Robitaille et al., 2013). Additionally, mTORC1 can promote the synthesis of purines through upregulation of the enzyme methylenetetrahydrofolate dehydrogenase 2 (MTHFD2). mTORC1 was shown to upregulate many enzymes necessary for purine synthesis, but in particular, mTORC1 promoted the stability and transcriptional activity of ATF4 toward its target gene MTHFD2 (Ben-Sahra et al., 2016). MTHFD2 is an enzyme in the mitochondrial tetrahydrofolate cycle, and is essential for forming a pool of formate for use in purine biosynthesis.

In combination with its promotion of protein translation, lipid synthesis, and nucleotide synthesis, mTORC1 promotes consumption of glucose in order to produce the adenosine triphosphate (ATP) required for the energy to drive these processes, and to produce the carbon necessary for synthesis of biomolecules. The main mechanism by which mTORC1 promotes this metabolic shift is through transcriptional and translational upregulation of the transcription factor hypoxia inducible factor 1 α (HIF1 α), which promotes

transcription of many glycolytic genes (Brugarolas et al., 2004; Düvel et al., 2010; Hudson et al., 2002; Laughner et al., 2001).

In addition to promoting consumption and synthesis of biomolecules, mTORC1 also inhibits autophagic recycling of cellular proteins and organelles. Autophagy is necessary for clearing out damaged organelles, but is also induced in response to nutrient starvation as an adaptation response. Following nutrient depletion or mTORC1 inhibition, autophagosomes engulf cellular material, and fuse with lysosomes to allow for degradation and recycling of cellular building blocks. The main mechanism mTORC1 employs to mediate this inhibition is through direct phosphorylation of ULK1/Atg13/FIP200 (unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa), which is a kinase complex involved in the initiation of autophagy (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). In addition to the ULK complex, mTORC1 has also been shown to regulate death-associated protein 1 (DAP1), which is a suppressor of autophagy (Koren et al., 2010). An additional study has identified WIPI2, an ortholog of a yeast protein involved in early autophagosome formation, as a potential mTORC1 effector, suggesting mTORC1 can inhibit autophagy through multiple mechanisms (Hsu et al., 2011).

Another mechanism mTORC1 employs to inhibit degradation of cellular material is through negative regulation of lysosomal biogenesis. mTORC1 mediated phosphorylation of the transcription factor EB (TFEB) leads to its exclusion from the nucleus; inhibition of mTORC1 through nutrient starvation or other means leads to accumulation of TFEB in the nucleus and transcription of genes involved in lysosomal function (Peña-Llopis et al., 2011; Settembre et al., 2011, 2012; Yu et al., 2010).

In addition to autophagy, another cellular machinery involved in cellular degradation is the ubiquitin-proteasome pathway. Two groups have published data to

suggest that mTORC1 inhibits breakdown of proteins through proteasomal degradation (Rousseau and Bertolotti, 2016; Zhao et al., 2015), however, a third group published data to suggest that mTORC1 can promote the expression of genes coding for proteasomal subunits, enhancing the capacity for protein degradation through the proteasome pathway (Zhang et al., 2014). The model put forth by the Bertolotti group proposes mTORC1 as an inhibitor of Erk5, which promotes upregulation of proteasome subunits and regulatory particle assembly chaperone proteins (RACs) (Rousseau and Bertolotti, 2016). The group supporting a model of mTORC1 enhancing proteasome expression shows this upregulation to be dependent on the transcription factor nuclear factor erythroid-derived 2-related factor 1 (NRF1), which seems to be regulated by mTORC1 through SREBP1 (Zhang et al., 2014). The inconsistencies between the models may be due to differences in the extent of mTORC1 perturbation. In conditions of acute mTORC1 inhibition, as was studied by Rousseau and Bertolotti, and Zhao et al., upregulation of proteasomal degradation would increase pools of free amino acids, allowing for translation of proteins necessary for survival, and for reactivation of mTORC1 under times of nutrient or energy stress. In contrast, in situations of prolonged mTORC1 activation, as in the cells studied by Zhang et al., mTORC1-driven upregulation of proteasomes may serve to increase the pool of cellular free amino acids to allow for the increased translation also promoted by mTORC1. It may also help the cell deal with increased levels of misfolded and/or damaged proteins that may result from increased levels of protein synthesis.

Chapter 2

mTORC1 reactivation follows prolonged deprivation of leucine or arginine

Introduction

Growth and function of mammalian cells depends largely on the availability of nutrients in the cellular environment. In order to appropriately function in the face of a changing nutritional environment, cells depend on the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), a highly evolutionary conserved threonine/serine kinase that promotes growth-favoring processes such as protein translation, lipid synthesis, and nucleotide biosynthesis under conditions of nutrient sufficiency (Laplante and Sabatini, 2012). Among the upstream cues that regulate mTORC1 activity, growth factors and amino acids have long been known to be crucial for mTORC1 activity (for review, see Kim et al., 2013b). Growth factor stimulation leads to activation of Phosphatidylinositol-4-5-bisphosphate 3-kinase (PI3K)/Akt signaling, which inactivates the Tuberous Sclerosis Complex (TSC) complex through its component TSC2, leading to activation of the small GTPase Rheb, a key regulator of mTORC1 (Long et al., 2005; Manning et al., 2002; Tee et al., 2003). On the contrary, amino acids have been shown to be communicated through a TSC2/Rheb independent mechanism, instead depending on the family of Rag small GTPases, which exists as heterodimers of RagA or RagB, and RagC or RagD (Nakashima et al., 1999; Sekiguchi et al., 2001). When the Rag-GTPases are active, they recruit mTOR in close proximity to Rheb at the lysosome, which leads to mTORC1 activation when growth factors are present (Sancak et al., 2008, 2010). Among the 20 proteinogenic amino acids, arginine and leucine are known to be key regulators of mTORC1 activity, as deprivation of these amino acids lead to rapid inhibition of Rag-GTPases and mTORC1 activity (Hara et al., 1998). Recently, it was shown that cytosolic leucine and arginine are communicated to the Rag-GTPases by the Sestrin and Castor proteins, respectively, through the Gator1 and Gator2 complexes (Chantranupong et al., 2016; Peng et al., 2014; Saxton et al., 2016a, 2016b; Wolfson et al., 2016).

The majority of studies to date studying amino acid regulation of mTORC1 have focused on short-term mTORC1 regulation and thus, the effects of prolonged deprivation remain poorly characterized. Several studies have observed what appears to be a reactivation of mTORC1 activity upon prolonged deprivation of individual amino acids, but the molecular details and extent of this reactivation remains poorly understood (Chen et al., 2014; liboshi et al., 1999; Ye et al., 2015; Yu et al., 2010). Some reports have suggested that this mTORC1 reactivation is due to regeneration of free amino acids through autophagy, but often these studies have looked at glutamine starvation in combination with serum starvation (Chen et al., 2014; Yu et al., 2010), which would more strongly induce autophagy due to numerous growth factor-regulated signaling pathways that regulate autophagy (He and Kilonsky, 2009). The complexity in regulation may explain why other studies have shown that deprivation of individual amino acids is not a strong enough stimulus to induce autophagy in certain contexts (Sheen et al., 2011).

In this study, I address how prolonged arginine or leucine deprivation affects mTORC1 activity. I demonstrate that short-term leucine or arginine deprivation leads to rapid inactivation of mTORC1 activity, but that prolonged starvation leads to mTORC1 reactivation, reaching activation levels similar to those observed during amino acid sufficient conditions. I find that this reactivation is independent of regeneration of amino acids by autophagy, the lysosome, or the proteasome. Instead, the reactivation is accompanied by increased activation of PI3K-Akt signaling, which is necessary for the reactivation of mTORC1 activity to occur. Our results suggest crosstalk between different arms of mTORC1 regulation, allowing for increased signaling through one arm when input is decreased through another. Given the interest in specific targeting of mTORC1 in diseases such as cancer, compensatory signaling mechanisms such as these have important therapeutic implications.

Results

mTORC1 reactivation follows prolonged deprivation of leucine or arginine.

mTORC1 activity is known to be tightly regulated by the cellular availability of leucine and arginine (Hara et al., 1998), but how prolonged deprivation of these amino acids affects mTORC1 activity remains poorly characterized. In some studies investigating mTORC1 activity upon prolonged leucine starvation, it has been observed that mTORC1 becomes reactivated, but the extent of this reactivation, as well as the molecular mechanism for this recovery response, are still poorly characterized (Chen et al., 2014; liboshi et al., 1999; Yu et al., 2010). To address this further, I first deprived mouse embryonic fibroblasts (MEFs) of leucine or arginine for 45 minutes, and assessed mTORC1 activity by looking at phosphorylation of the mTORC1 substrates S6K1, 4E-BP1, ULK1, and Grb10. In agreement with previous studies (Hara et al., 1998), mTORC1 activity rapidly decreased in cells that had been short-term deprived of arginine or leucine as no phosphorylation of mTORC1 downstream targets could be observed after 45 minutes (Figure 2.1A). However, upon longer times of deprivation of leucine or arginine, mTORC1 was found to be reactivated, reaching activation levels similar to those observed under amino acid sufficient conditions after 60-90 minutes of deprivation. Interestingly, reactivated mTORC1 levels remained comparable to levels found during amino acid sufficient conditions for as long as 12 hours after leucine or arginine deprivation, whereas after 24 hours of starvation, no mTORC1 activity could be detected (Figure 2.1B). The kinetics of mTORC1 reactivation were nearly identical between the arginine and leucine deprivation conditions (Figure 2.1B), but removal of all 20 proteinogenic amino acids from the growth media did not allow for a recovery response similar to that observed for arginine or leucine deprivation (Figure 2.1C).

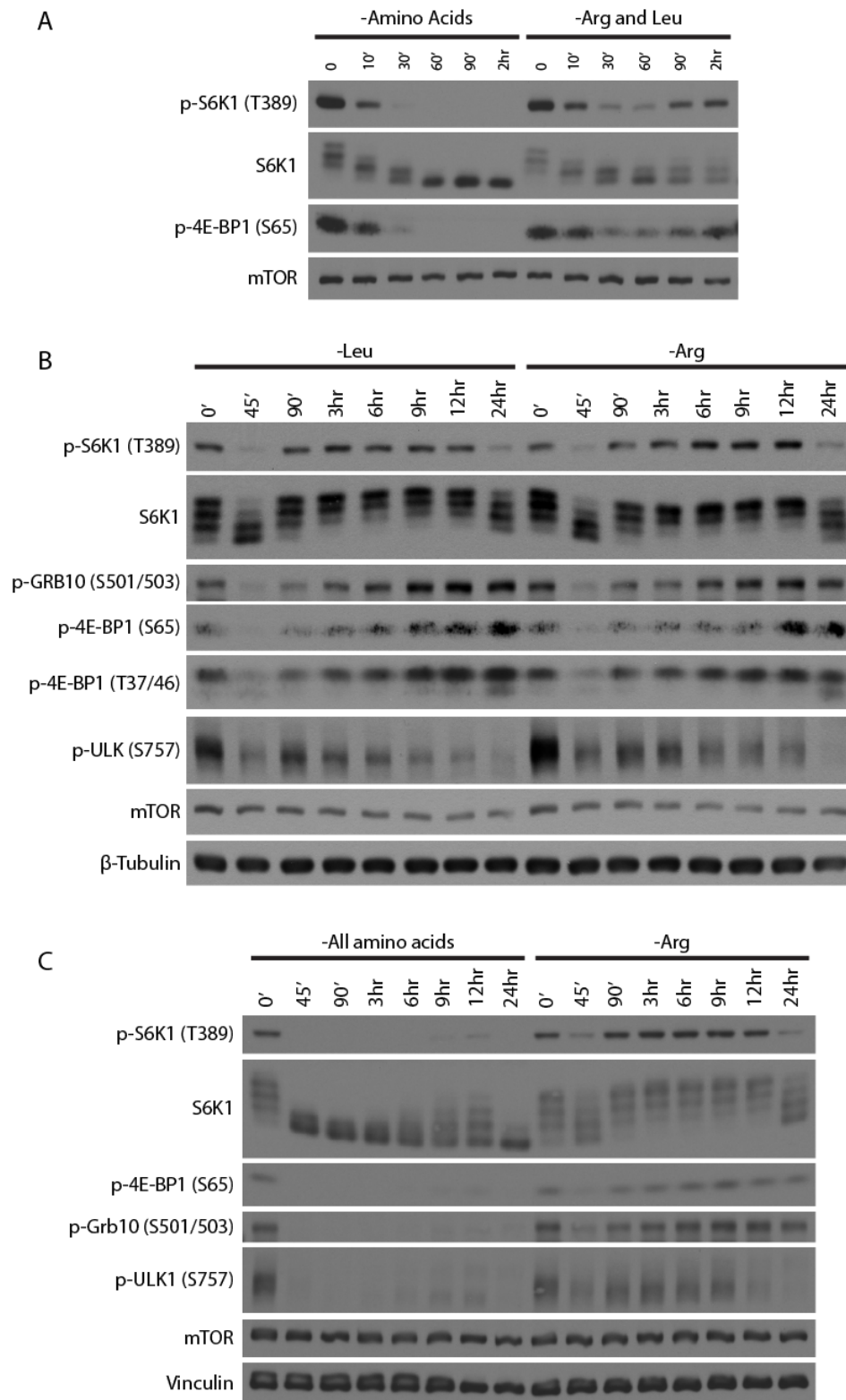


Figure 2.1 mTORC1 reactivation follows prolonged deprivation of leucine or arginine. (A-C) MEFs were deprived of either all amino acids (A,C), both arginine and leucine (A) or arginine or leucine alone (B-C) for the indicated times.

mTORC1 reactivation is observed in many, but not all cell types

We further explored the extent of this reactivation by looking at the reactivation response in other cell lines. I found that the osteosarcoma cell line, U2OS, displayed clear reactivation similar to that observed in the MEFs (Figure 2.2A) as did 293A cells (Figure 2.10). However, in 293T cells, the recovery response was negligible (Figure 2.2B). The absence of an mTORC1 recovery response in 293T cells is significant, given that much of the work done to characterize amino acid sensing and signaling to mTORC1 has been worked out in 293T cells (Bar-Peled et al., 2012; Sancak et al., 2008, 2010). Taken together, our data show that prolonged leucine or arginine deprivation leads to a potent but transient reactivation of mTORC1 activity in multiple cell types.

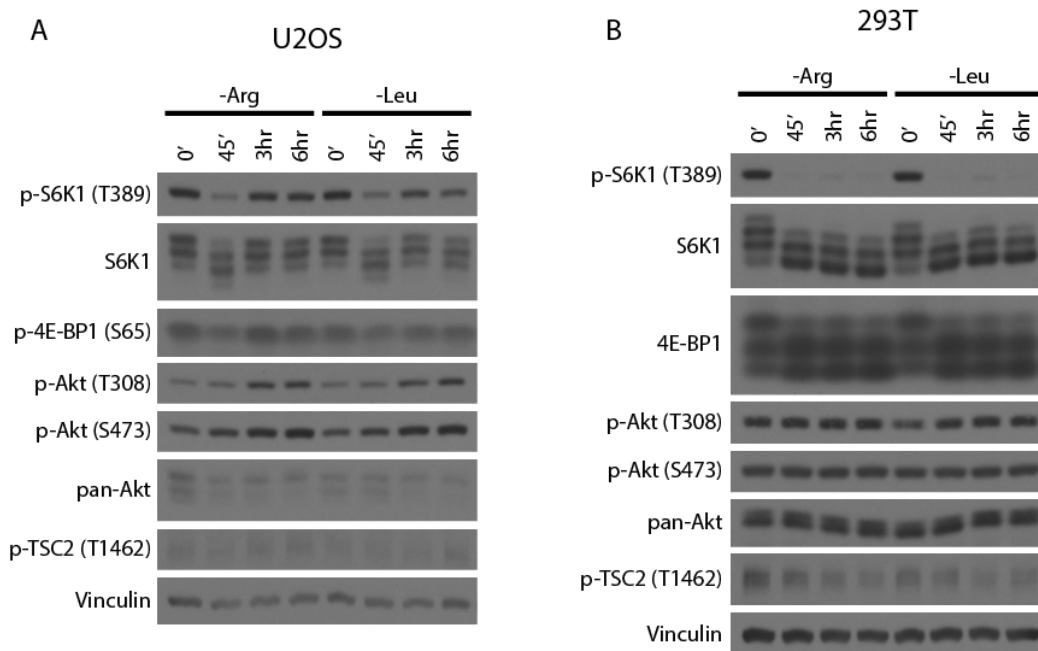


Figure 2.2 mTORC1 recovery occurs in many but not all cell lines. (A-B) U2OS cells (A) or 293T cells (B) were deprived of arginine or leucine for the indicated times.

The mTORC1 reactivation is independent of regeneration of amino acids from autophagy/lysosomal degradation or the proteasome

Under conditions of limiting nutrients, cells can adapt by inducing autophagy to degrade proteins and other cytosolic macromolecules. Release of amino acids due to autophagy has been proposed to co-occur with mTORC1 reactivation (Yu et al., 2010), but it remains unclear if the mTORC1 reactivation is dependent on autophagy per se, or if mTORC1 reactivates in an autophagy-independent manner. To explore this, I utilized MEF cells that lack ATG5, and deprived these cells of leucine and arginine. Interestingly, the mTORC1 reactivation was observed also in the ATG5 autophagy-deficient MEFs (Figure 2.3A), demonstrating that the recovery response is not due to induction of ATG5-mediated autophagy. To further investigate if the reactivation response was due to regeneration of amino acids through either lysosomal or proteasomal degradation of proteins, I treated cells with the lysosomal inhibitor chloroquine, or the proteasomal inhibitor MG132 for 6 hours, in combination with arginine deprivation. Neither inhibition of the lysosome or the proteasome interfered with the mTORC1 reactivation (Figure 2.3B,C), demonstrating that the mTORC1 recovery is not due to amino acids being regenerated through these protein degradation machineries. Moreover, prolonged arginine deprivation did not lead to any increased intracellular levels of arginine during the time course of reactivation, as measured by metabolic profiling (Figure 2.3D), strongly arguing against regeneration of arginine as the cause of the mTORC1 recovery. In sum, these data show that the mTORC1 reactivation observed upon leucine or arginine deprivation is independent of regeneration of these amino acids.

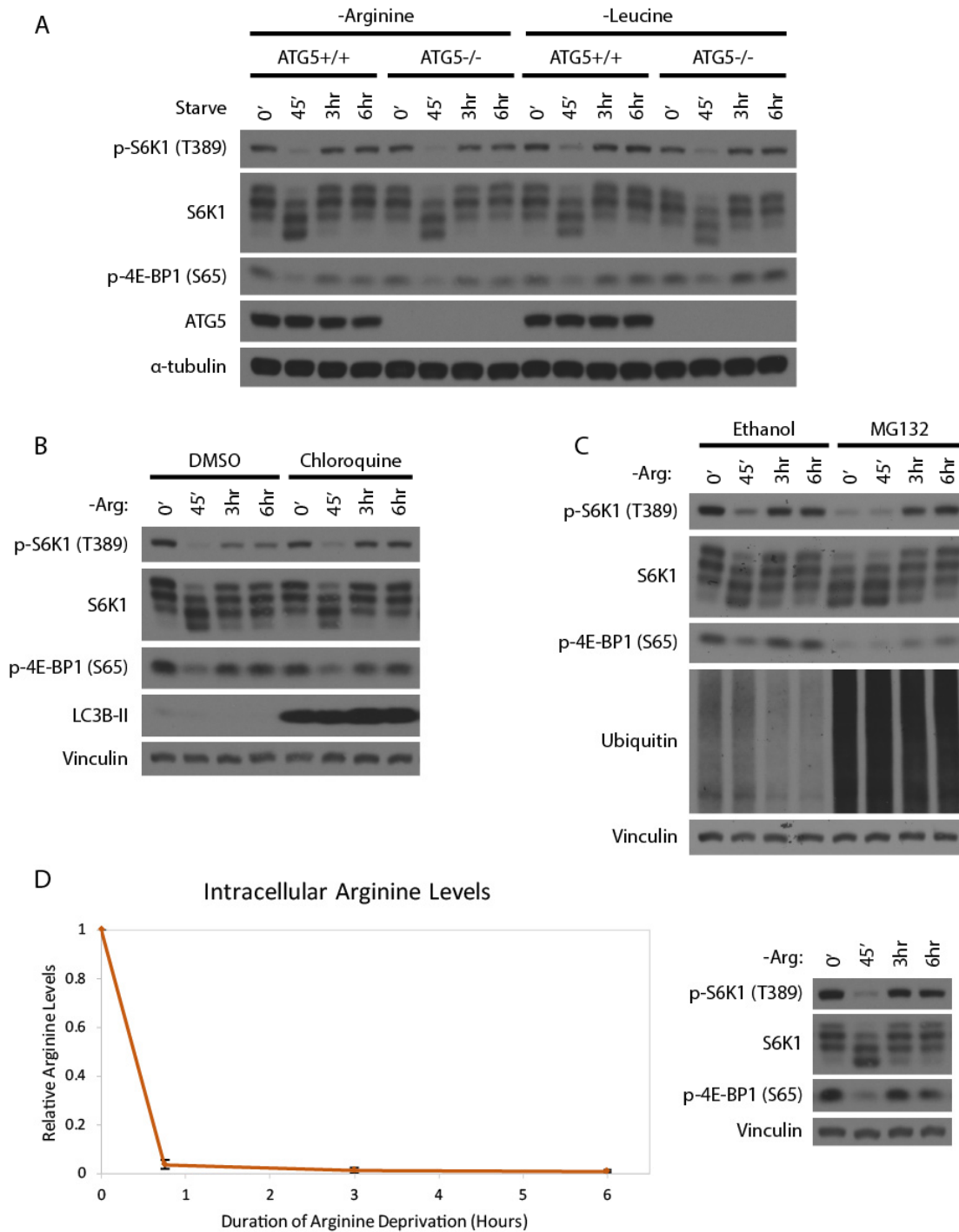


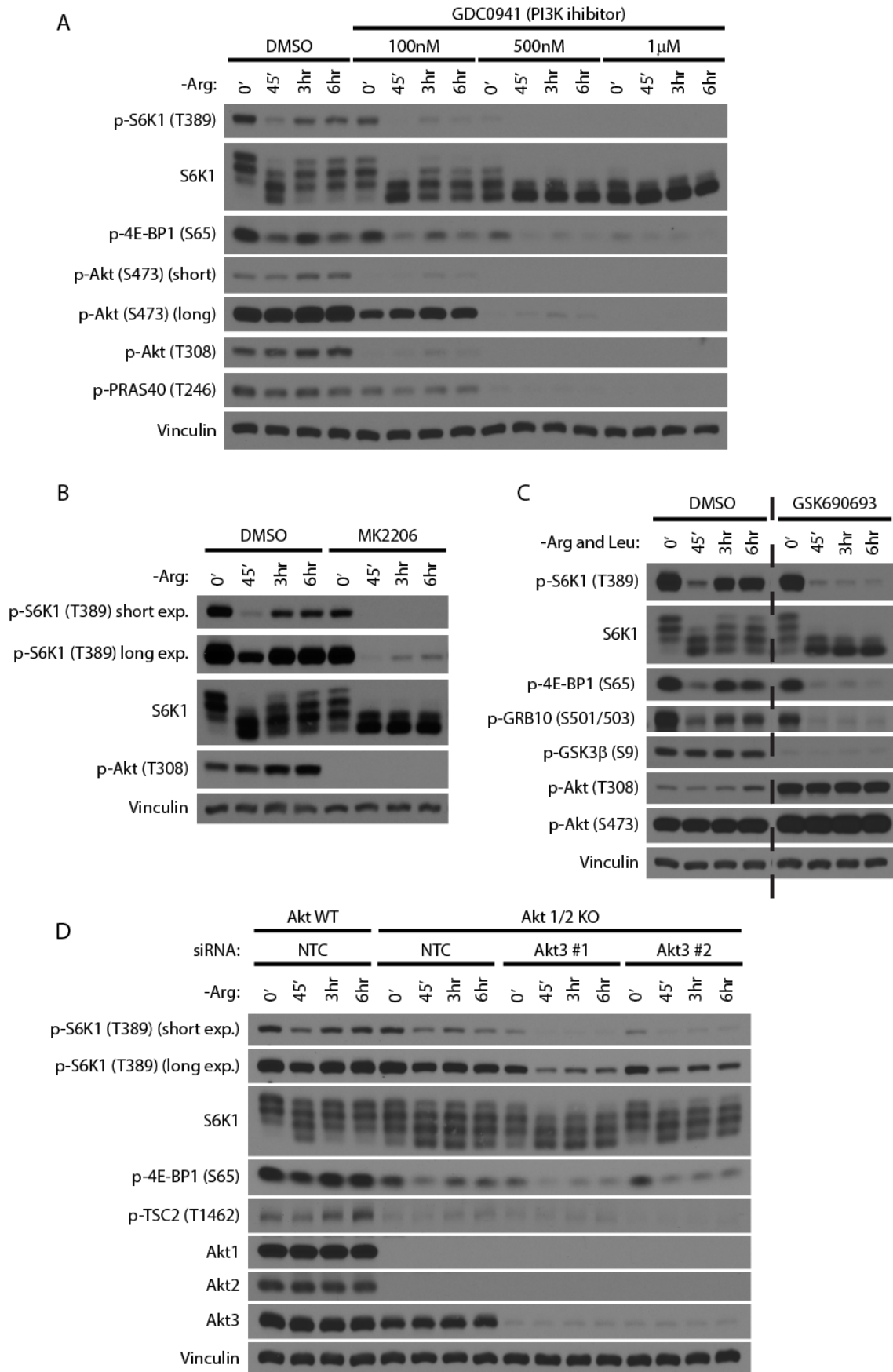
Figure 2.3 The mTORC1 recovery response is independent of canonical autophagy or proteasomal degradation of proteins. (A) Autophagy deficient ATG5^{-/-} MEFs or littermate controls were deprived of arginine or leucine for the indicated times. **(B-C)** MEFs were deprived of arginine in the presence of 100 μM of the lysosomal inhibitor chloroquine **(A)** or 20 μM of the proteasomal inhibitor MG132 **(B)** as indicated. All drug treatments in (B) and (C) were for a total of 6 hours. **(D)** WT MEFs were deprived of arginine for 0 minutes, 45 minutes, 3 hr, or 6 hr, and intracellular metabolite levels were analyzed via mass spectrometry. The graph on the left shows arginine levels with standard deviations averaged from 4 separate experiments (left panel). Western blots of duplicate plates from a representative experiment are shown in the right panel.

The mTORC1 recovery response requires PI3K/Akt activity

Given that the mTORC1 reactivation was found to be independent of regeneration of amino acids, I started to interrogate other signaling components that are known to be involved in mTORC1 activation, to explore their role in the mTORC1 recovery response. This approach included looking at PI3K-Akt signaling, which is a major activator of mTORC1, through inactivation of the mTORC1-inhibitors TSC2 and PRAS40 (Dibble and Cantley, 2015). Interestingly, depriving cells of arginine in combination with pharmacological inhibition of PI3K severely blunted the mTORC1 recovery response (Figure 2.4A), suggesting that activation of the PI3K pathway is needed for the reactivation to occur. To investigate if this was due to PI3K signaling through Akt, I treated cells with two inhibitors of Akt, the PH-domain inhibitor MK2206, and the ATP-competitive inhibitor GSK690693, to investigate if Akt is necessary for the mTORC1 recovery response. As with PI3K inhibition, pharmacological inhibition of Akt significantly blunted the mTORC1 reactivation, further suggesting an important role for PI3K/Akt signaling in the mTORC1 reactivation response. To confirm this, I knocked-down Akt3 in Akt1/Akt2 double knock out (DKO) MEFs, to generate cells nearly completely deficient in Akt, and deprived these cells of leucine or arginine as previously described. Indeed, Akt deficient cells mirrored the pharmacological inhibition of PI3K/Akt signaling inhibitors, as these cells also exhibited a severely blunted mTORC1 recovery response (Figure 2.4D). Taken together, these data show that PI3K/Akt signaling is needed for the mTORC1 reactivation to occur.

Figure 2.4 The mTORC1 recovery response requires PI3K/Akt activity. (A) WT MEFs treated with the PI3K inhibitor GDC0941 at the indicated concentrations in combination with deprivation of arginine for the indicated times. (B-C) MEFs were deprived of arginine (B) or Arginine and Leucine (C) and treated with 2 μ M MK2206 (B) or 10 μ M GSK690693 (C) for up to 6 hours, as indicated. (D) Akt1-2 double KO MEFs treated with two different siRNAs against Akt3 were deprived of arginine for the indicated times.

Figure 2.4 (Continued)



Akt activity increases during the mTORC1 recovery response in some, but not all cell lines

Given that PI3K/AKT signaling was needed for the recovery response to be observed, I also investigated the activation levels of Akt during mTORC1 reactivation. Interestingly, I noted that Akt activation was robustly increased upon prolonged arginine and leucine deprivation, as judged by levels of phospho-Akt at residues T308 and S473, which both correlate with Akt activity (Alessi et al., 1996) (Figure 2.5A). Our initial thought was that this increase in Akt activity was a direct consequence of the inhibition of mTORC1, due to the relief of a well described mTORC1-mediated feedback loop (Dibble et al., 2009; Haruta et al., 2000; Hsu et al., 2011; Yu et al., 2011). However, pharmacological inhibition of mTORC1 with rapamycin did not inhibit Akt activation upon prolonged arginine deficiency, demonstrating that the observed Akt activation is not due to the mTORC1 inhibition per se, but rather connected with the amino acid deficiency (Figure 2.5B). To investigate if the increase in Akt is due to a general activation of growth factor receptor pathways, I probed lysates that had been starved of arginine and leucine with an anti-phosphotyrosine antibody, since a large part of growth factor signaling is mediated through tyrosine phosphorylation (Kawase et al., 1995). Remarkably, I did not find any evidence of a general increase in growth factor signaling activity, as overall phosphotyrosine levels robustly decreased during the arginine and leucine deprivation time course (Figure 2.5C). This data argues against a model in which amino acid deprivation upregulates growth factor signaling generally, and instead, may suggest that amino acid deprivation is activating the PI3K-Akt pathway more specifically. Future investigations will be needed to thoroughly address this topic.

The mTORC1 recovery response is independent of mTORC2

Based on the data from Figure 2.5C suggesting that growth factor receptor signaling is not globally upregulated during the mTORC1 recovery response, I was curious if there was a more specific upregulation happening. Akt is activated through phosphorylation of two key residues: T308 by PDK1 (phosphoinositide-dependent kinase 1), and S473 by mTORC2. To test if Akt phosphorylation by mTORC2 is responsible for the mTORC1 recovery, I obtained Rictor KO MEFs and deprived these cells of arginine or leucine. These cells showed no phosphorylation of Akt at S473, but displayed robust mTORC1 recovery (Figure 2.6), arguing that mTORC2, and phosphorylation of Akt at S473 is not necessary for the mTORC1 recovery response.

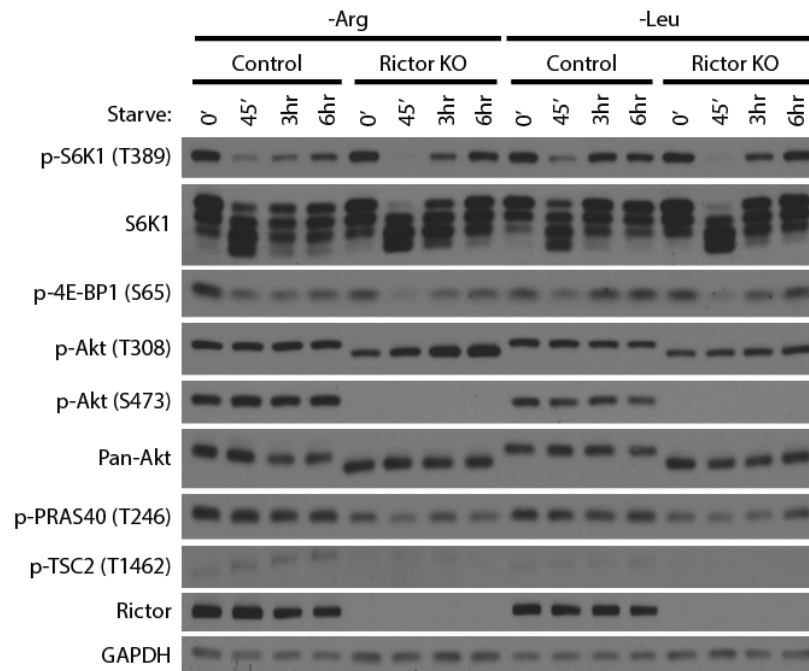


Figure 2.6 The mTORC1 recovery response is independent of mTORC2. Rictor KO MEFs were deprived of arginine or leucine for the indicated times and cell lysates were probed with the indicated antibodies.

The recovery response is partially mediated by the TSC complex

To gain further understanding of how the recovery response might be occurring, I decided to test the role of the TSC complex in the response. One approach would be to test the recovery response in a setting without a functional TSC complex. However, this approach

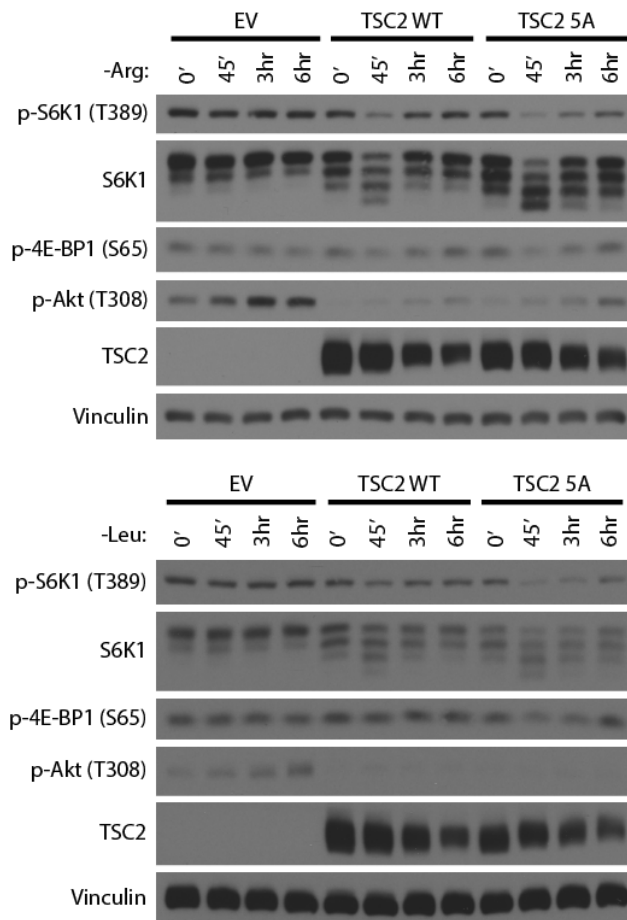


Figure 2.7 mTORC1 reactivation is partially mediated through the TSC complex. TSC2^{-/-} cells were infected with empty vector, TSC2 WT, or a version of TSC2 in which the Akt phosphorylation sites were mutated to alanines (TSC2 5A). Cells were deprived of arginine or leucine for the indicated times. Note, this experiment was only performed one time.

proved difficult; cells lacking the TSC component TSC2 have such hyperactive levels of mTORC1 through Rheb activation, that these cells are largely unresponsive to single amino acid deprivation (Figure 2.7, left four lanes). To overcome this issue, I obtained a construct of TSC2 in which the residues that are phosphorylated by Akt were mutated to unphosphorylatable alanines (TSC2 5A). The protein encoded by this construct is fully

functional in its GAP activity toward Rheb, but it cannot be inhibited by Akt (Menon et al., 2014). Two of my colleagues, Julie Han and Dr. Ana Gomes, virally expressed this construct or a WT TSC2 construct in TSC2^{-/-} MEFs. Re-expression of TSC2 in TSC2 KO MEFs slightly lowered the mTORC1 activity, and restored the responsiveness of mTORC1 activity to arginine and leucine (Figure 2.7, middle four lanes, both panels). Expression of the TSC2 5A construct also lowered the mTORC1 activation levels of the TSC2^{-/-} MEFs, and additionally lowered the phosphorylation levels of mTORC1 targets during the recovery response (Figure 2.7, right four lanes, both panels). The result from this experiment shows that the recovery response seems to be partially, but not completely mediated by TSC2 phosphorylation by Akt.

Based on data suggesting that a kinase related to Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), is able to phosphorylate TSC2 at the sites phosphorylated by Akt (Castel et al., 2016), I decided to investigate the role of SGK in the recovery response. There are three isoforms of SGK, and the compound 14g has selectivity toward SGK1 (Halland et al., 2015). I used this compound alone and in combination with the Akt inhibitor MK2206, and found that alone, the inhibitor had less of an effect on mTORC1 recovery than the Akt inhibitor, however, the two inhibitors together displayed potent inhibition of mTORC1 recovery (Figure 2.8). It is clear that there is some redundancy between the Akt and SGK proteins in regulation of mTORC1, however, the combination of inhibitors robustly inhibited mTORC1 signaling in both the growing condition and the recovery conditions, so it is unclear whether SGK plays a role specific in the recovery phase or simply acts as an mTORC1 activator in all conditions.

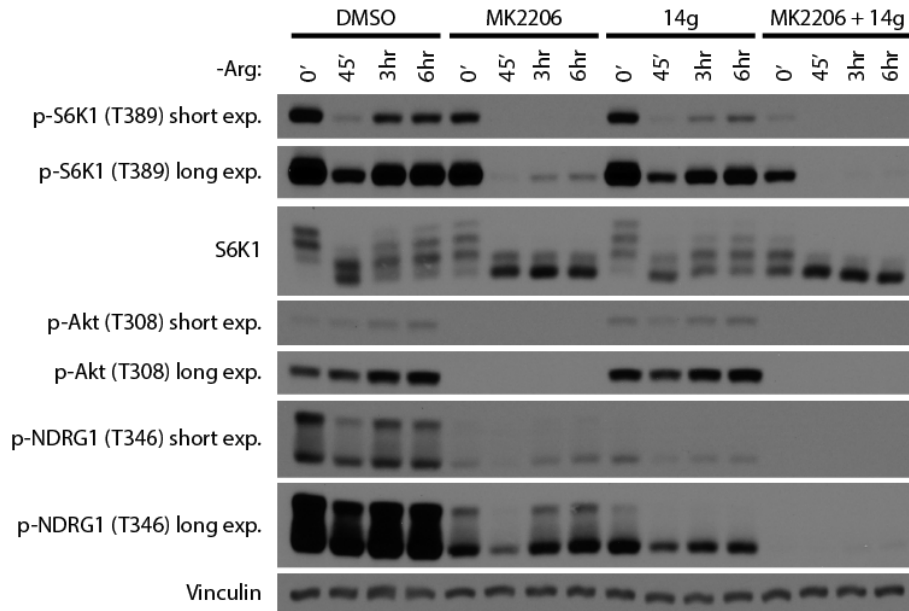


Figure 2.8 SGK1 plays a role in mTORC1 activation, and inhibition of both Akt and SGK1 robustly inhibits mTORC1 signaling and recovery response. MEFs were deprived of arginine in the presence of the Akt inhibitor MK2206 (2 μ M) and/or the SGK1 inhibitor 14g (10 μ M). Note, the experimental conditions containing 14g were only performed one time, and a portion of this gel was shown in Figure 2.4.

The increase in Akt signaling upon prolonged leucine or arginine deprivation is transient

To get a more complete view of the mTORC1 recovery response, I deprived cells of arginine and leucine for a longer period of time. I found that the mTORC1 recovery remained strong through 12 hours, and slowly diminished, reaching a minima around 36 hours (Figure 2.9A). Akt activation mirrored that of mTORC1, but the peak Akt activation and timing of the decrease was slightly quicker than that of mTORC1 (Figure 2.9A).

mTORC1 regulates many cell growth processes, with protein translation being strongly promoted by mTORC1 (Laplante and Sabatini, 2012). I was curious, given the data showing that arginine levels remain low to undetectable during mTORC1 recovery, if protein translation is able to occur during mTORC1 recovery. To address this question, I employed a

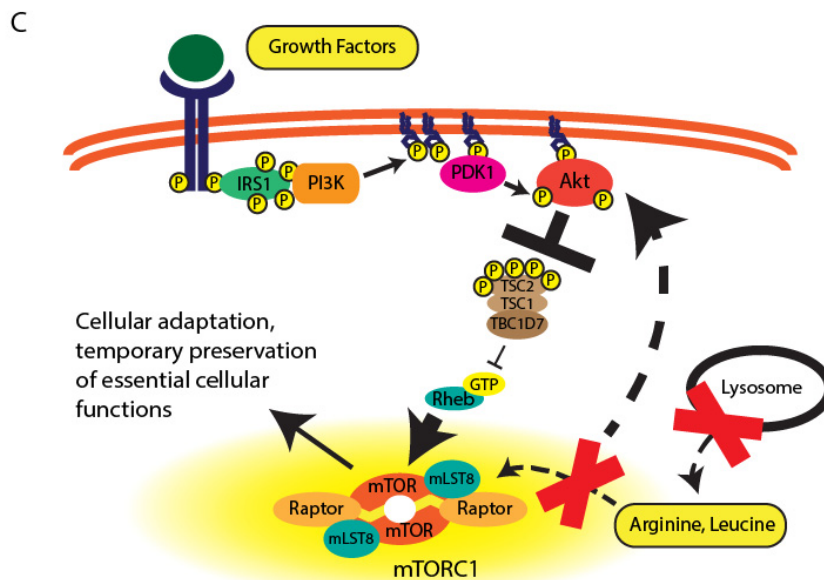
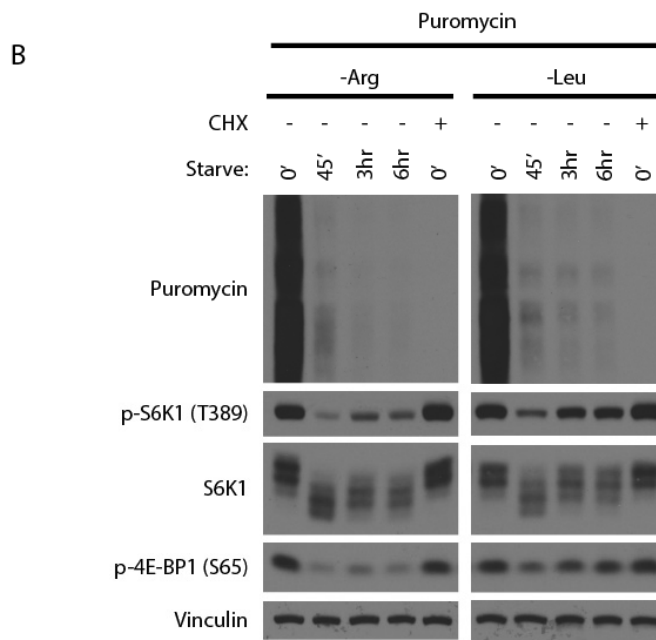
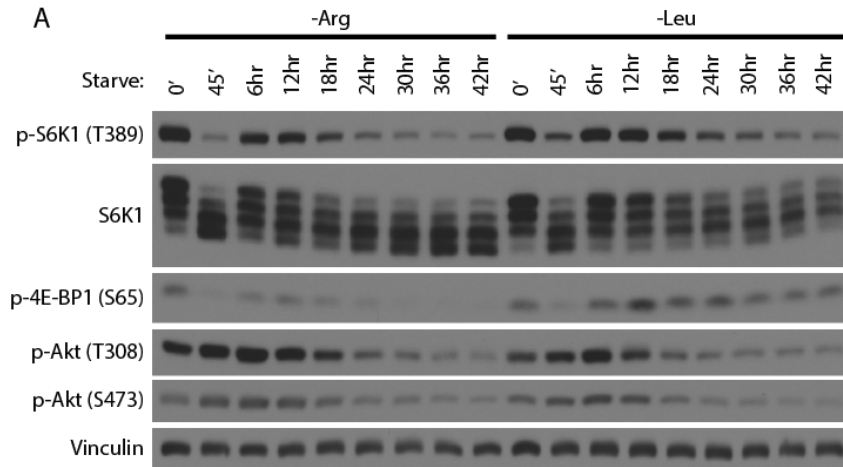
method termed surface sensing of translation (SUnSET) to measure relative rates of global protein translation (Dadehbeigi and Dickson, 2013; Schmidt et al., 2009). This method takes advantage of the fact that puromycin, as a protein synthesis inhibitor, acts by itself incorporating into the end of nascent peptides. Considering that the rate of puromycin incorporation into peptides is proportional to the rate of protein translation, the relative levels of puromycin incorporation can be used as a readout for relative levels of translation. As can be observed in Figure 2.9B, puromycin incorporation into peptides was greatly diminished by 45 minutes of deprivation of either arginine or leucine, and rates did not increase during the timeframe of mTORC1 reactivation. These results indicate that despite the activation of mTORC1 in this context, protein translation remains globally low, presumably due to lack of charged arginine or leucine tRNAs.

Discussion

Taken together, our results demonstrate a novel mechanism of mTORC1 regulation by which Akt-dependent mTORC1 activation occurs following prolonged deprivation of individual amino acids. I demonstrate that this reactivation is independent of amino acid levels, and that the phenomenon is dependent on signaling that can be blocked by disruption of Akt. While mTORC1 activity is known to promote numerous cell growth processes including protein, lipid, and nucleotide synthesis, our data show that at least one arm of this regulation, that of protein synthesis, is not able to resume during the mTORC1 recovery phase. It is not surprising that protein translation remains stalled, since peptides cannot be synthesized without the presence of all proteinogenic amino acids. Further characterization will be necessary to determine if the other effectors of mTORC1 signaling correlate with mTORC1 activity during the recovery phase or not.

Figure 2.9 The increase in Akt signaling upon prolonged leucine or arginine deprivation is transient. (A-B) WT MEFs were deprived of arginine and leucine for the indicated times. In **B**, 10 µg/mL puromycin was added to the media 10 minutes prior to harvest to visualize cellular rates of translation. 25 µM cycloheximide was added to the media 45 minutes prior to harvest, as indicated. The puromycin blot shows approximately from 35-50 kDa. **(C)** Graphical summary of findings.

Figure 2.9 (Continued)



There are various “arms” of regulation of mTORC1, the two best characterized of which can loosely be labeled the growth factor arm and the amino acid arm. The growth factor arm largely relies on the Tuberous Sclerosis Complex (TSC) complex, which serves as a GTPase activating protein (GAP) for Rheb (Inoki et al., 2003b; Tee et al., 2003), an essential activator of mTORC1 (Long et al., 2005), that localizes to endomembranes, including lysosomes. It has been shown that the TSC complex can be recruited to lysosomal membranes in order to inhibit Rheb. However, to date, there is some controversy over what stimuli regulate the localization of the TSC complex. Some data argues that the localization of the TSC complex is regulated by growth factor-stimulated Akt phosphorylation of the TSC complex (Menon et al., 2014), while other data argues that TSC complex localization is regulated by amino acids (Demetriades et al., 2014). Newer data suggests that both models may be true, with TSC localization being regulated by a number of cellular stresses (Demetriades et al., 2016). If both models do indeed hold true, then it seems that the TSC complex could be a point of intersection of the two regulatory arms.

Based on the observed increase in Akt phosphorylation levels following amino acid deprivation and the inability of rapamycin to block this increase (Figure 2.4), it seems that amino acid deprivation is able to stimulate Akt activity independent of mTORC1. This suggests a model in which lack of input through one arm of mTORC1 regulation can be compensated for via upregulation of another arm (Figure 2.9C). This is in agreement with what I find in cells lacking amino acid input through the Rag GTPases; these cells have highly upregulated levels of Akt activation during all conditions tested (Figure 2.10). Additionally, the U2OS cells which also display mTORC1 recovery also display robust Akt activation following amino acid deprivation (Figure 2.2A), whereas the 293T cells which lack mTORC1 recovery show little change in Akt activation, suggesting they lack compensation through this

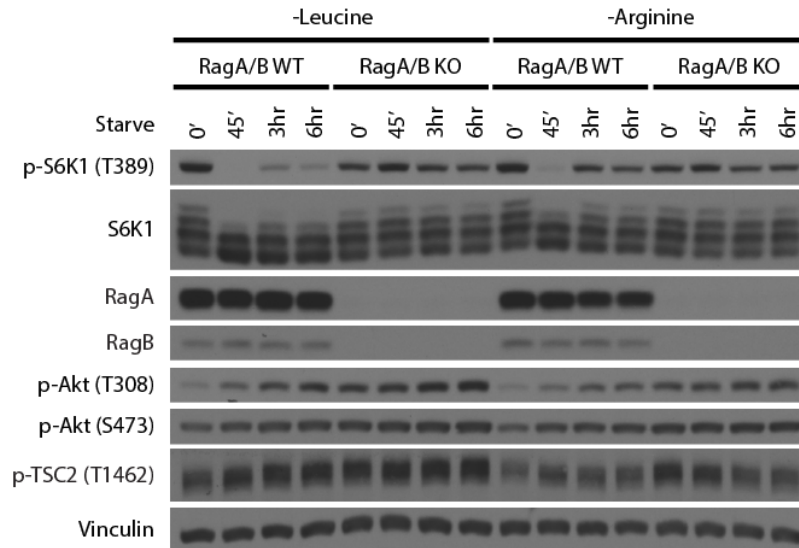


Figure 2.10 Loss of the Rag GTPases prevents mTORC1 responsiveness/recovery following arginine or leucine withdrawal. 293A cells with RagA and RagB knocked out via CRISPR/Cas9 were deprived of arginine or leucine for the indicated times.

mechanism. If indeed, the localization of the TSC complex can be regulated both by the growth factor pathway and the amino acid pathway, then it is possible that this is the point of convergence that is able to modulate mTORC1 activity through a balance of inputs. This compensatory balancing of inputs, however, appears to only be sufficient to overcome deprivation of one or a few amino acids, and does not appear to be able to overcome the strong negative input to mTORC1 resulting from total amino acid deprivation (Figure 2.1C).

Further work will be necessary to understand the functional role of the mTORC1 recovery response. I postulate that the recovery response is an adaptation, allowing the cell to maintain essential cellular functions in the expectation that levels of the lacking amino acid will be restored in the following hours. In addition to the role mTORC1 recovery plays in normal cells, the recovery response is important to take into account for therapeutic interventions, especially those involving specific amino acid depletions such as has been proposed for numerous cancer types (Feun et al., 2008; Huang et al., 2012; Kelly et al., 2012).

Inadvertent activation of mTORC1 and/or Akt in these contexts could have unwanted effects toward cancer cells that should be considered in these treatments.

Chapter 3

Rag movement from lysosomes following amino acid stimulation

Introduction

The activity of mammalian target of rapamycin complex 1 (mTORC1) is regulated by numerous environmental factors, including growth factors, glucose, oxygen, and amino acids. While many of these factors exert their effects by regulating the guanine nucleotide binding state of Rheb (Manning et al., 2002; Tee et al., 2003), an essential activator of mTORC1 (Long et al., 2005), amino acids function in mTORC1 regulation largely through the guanine nucleotide binding states of the Rag GTPases. The Rag GTPases localize to the lysosomal surface, and in the presence of amino acids, the Rag GTPases recruit mTORC1 to the lysosome via interactions with the raptor component of mTORC1. At the lysosome, mTORC1 can interact with Rheb, allowing for activation of mTORC1 under favorable environmental conditions (Bar-Peled et al., 2012; Sancak et al., 2008, 2010).

The Rag GTPases are unusual for two main reasons. Firstly, while most GTPases are monomeric, the Rag GTPases form heterodimers of one Gtr1-like protein (RagA or RagB), and one Gtr2-like protein (RagC or RagD). The GDP/GTP binding of the two components of the heterodimer are regulated independently of each other, suggesting the possibility that the two components can serve distinct functions. Secondly, the Rag GTPases are not prenylated to allow for their localization to membranes as most GTPases are. Instead, the Rag proteins rely on interactions with the Ragulator complex to remain localized to the lysosome, suggesting that constitutive localization of the Rags through lipidation has been evolutionarily selected against. There is data to suggest that interactions between the Rags and the Ragulator proteins weaken following amino acid stimulation (Bar-Peled et al., 2012), but it has not been reported that the Rag proteins can move away from the Ragulator complex, and the current model maintains that the Rag proteins are constitutively localized to the lysosomal surface. In contrast to the current model, I find that the Rag proteins move

away from the lysosomal surface following amino acid stimulation. This finding is counterintuitive given that the Rags are essential for mTORC1 recruitment to the lysosomal membrane during amino acid replete conditions (Sancak et al., 2008), and suggests that the Rags may serve another unidentified function in amino acid sensing and mTORC1 signaling.

Results

RagA moves away from the lysosome following amino acid stimulation in an mTOR-independent fashion

Based on published data suggesting a regulated interaction between the Rag proteins and the regulator complex (Bar-Peled et al., 2012), I decided to investigate whether the Rag proteins can move from the lysosomal surface to other cellular compartments. To do this, I performed immunofluorescence (IF), co-staining mouse embryonic fibroblasts (MEFs) with antibodies to endogenous RagA and LAMP1, as a marker of lysosomes. I starved the cells of amino acids and stimulated with amino acids for various time points. As a control, I also stained cells separately with mTOR and LAMP1. As expected, mTOR was largely diffuse throughout the cytosol in amino acid starvation conditions, but a large amount of mTOR was found co-localized with LAMP1 in the amino acid stimulation conditions (Figure 3.1 A and C). Interestingly, the RagA staining pattern followed the opposite trend, with RagA becoming more diffuse throughout the cytosol following amino acid stimulation (Figure 3.1 B and D). The changes I observe in localization of mTOR and RagA correlate well with the change in activation of mTORC1 as assessed by phosphorylation levels of the mTORC1 target protein S6K (Figure 3.1 E).

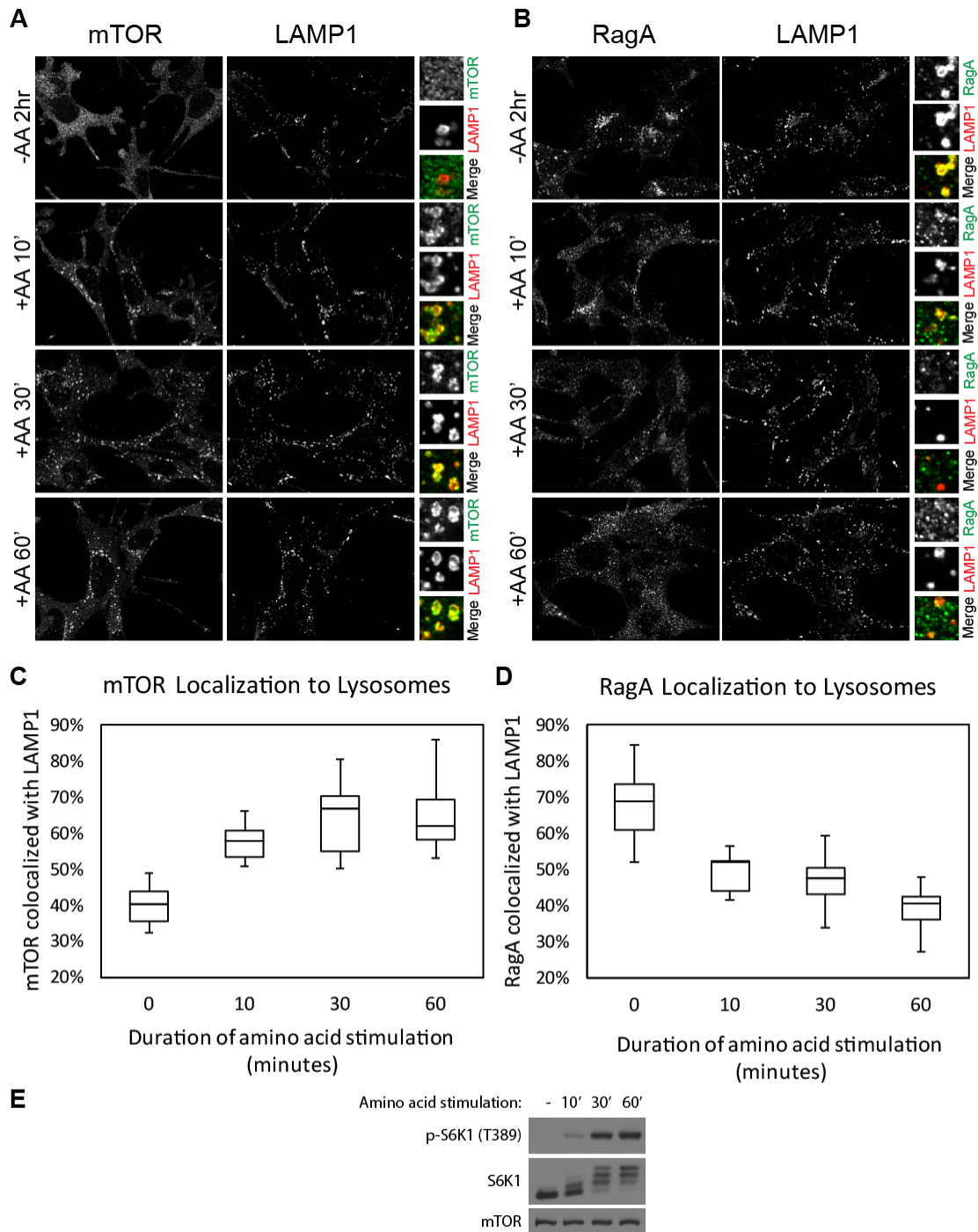


Figure 3.1 RagA moves away from the lysosome following amino acid stimulation. (A-B) MEFs were deprived of amino acids and stimulated with amino acids for either 10, 30, or 60 minutes, and stained with the indicated antibodies. **(C-D)** Box plots of the colocalization of the indicated antibodies with the lysosomal marker, LAMP1. Data quantified in these plots are from Z-stacks of the entire cell volume, analyzed to calculate the percent volume of RagA signal above threshold colocalized with LAMP1 signal. Data for each timepoint represents an average of a minimum of 12 distinct fields of view. Each plot is from a representative experiment. **(E)** Western blot of MEF cell lysates, showing the phosphorylation levels of the mTORC1 target protein S6K1 at the times indicated.

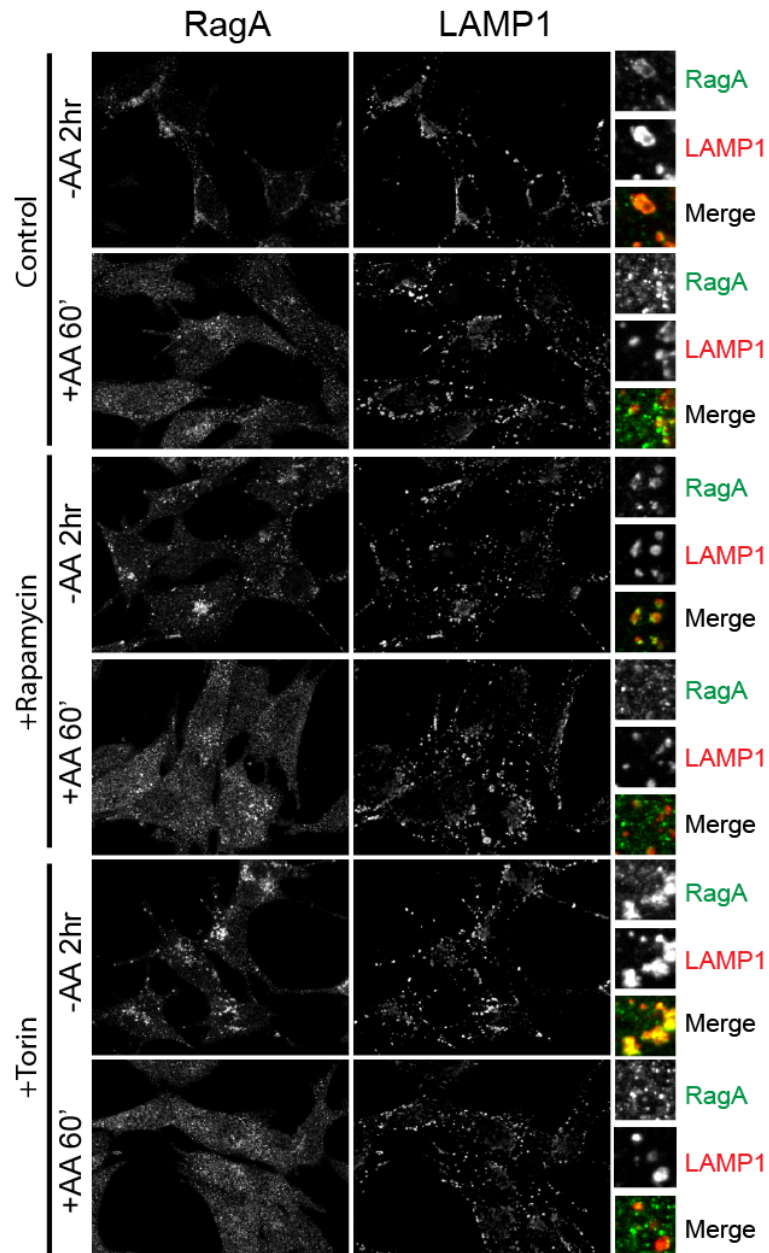


Figure 3.2 Rag movement away from the lysosome is independent of mTOR activity. MEFs were starved and stimulated of amino acids in the presence of the ATP-competitive mTOR inhibitor Torin (250 nM) or the mTORC1-specific inhibitor Rapamycin (20 nM). Cells were fixed and stained for RagA and the lysosomal marker LAMP1.

I next questioned whether the change in RagA localization is a consequence of mTOR activation, or if it occurs independently of the mTOR activation state. To address this, I looked at the localization of RagA in starved and stimulated cells in the presence of the ATP-competitive mTOR inhibitor Torin, the mTORC1-specific inhibitor Rapamycin. In the presence of either of these inhibitors, I observe the diffusion of RagA following amino acid stimulation, demonstrating that the change in localization of RagA occurs independently of mTOR activity (Figure 3.2)

Rag localization is governed by GDP/GTP nucleotide binding

Amino acids are known to regulate the Rag-mTORC1 interaction by modulating the GDP/GTP binding status of the Rag proteins. Thus, I was curious if the changes in RagA localization I was observing were due to changes in the guanine nucleotide bound to the Rag protein. To address this, I took advantage of mutants that lock the GTPase in either a GTP-bound state or a nucleotide-free state that mimics the GDP-bound state. A colleague of mine, Dr. Sang Gyun Kim, stably expressed these mutants in MEFs and stained the cells for mTOR localization and Rag localization. Unsurprisingly, the cells expressing nucleotide-free RagB (RagB TN) displayed diffuse staining of mTOR irrespective of the presence of amino acids, due to the inability of this mutant to bind mTORC1. In contrast, the cells expressing RagB constitutively bound to GTP (RagB QL) displayed enrichment of mTORC1 at lysosomes irrespective of the presence of amino acids. The staining pattern of RagB, however, displayed the opposite trend as mTOR: the RagB TN was largely lysosomal, and the RagB QL was largely diffuse (Figure 3.3). These results suggest that the guanine nucleotide binding of RagA/B regulates the localization of the Rag proteins.

Figure 3.3 Rag localization is governed by GTP/GDP binding. MEFs stably expressing FLAG-tagged RagB (C), or RagB containing the Q99L mutation rendering RagB constitutively GTP-bound (B), or RagB containing the T54N mutation rendering RagB unable to bind nucleotides (A) were starved of amino acids for 90 minutes (-AA) or starved of amino acids for 60 minutes followed by 30 minutes of amino acid stimulation (+AA). Cells were stained with FLAG and LAMP1 antibodies and imaged via confocal microscopy.

Figure 3.3 (Continued)

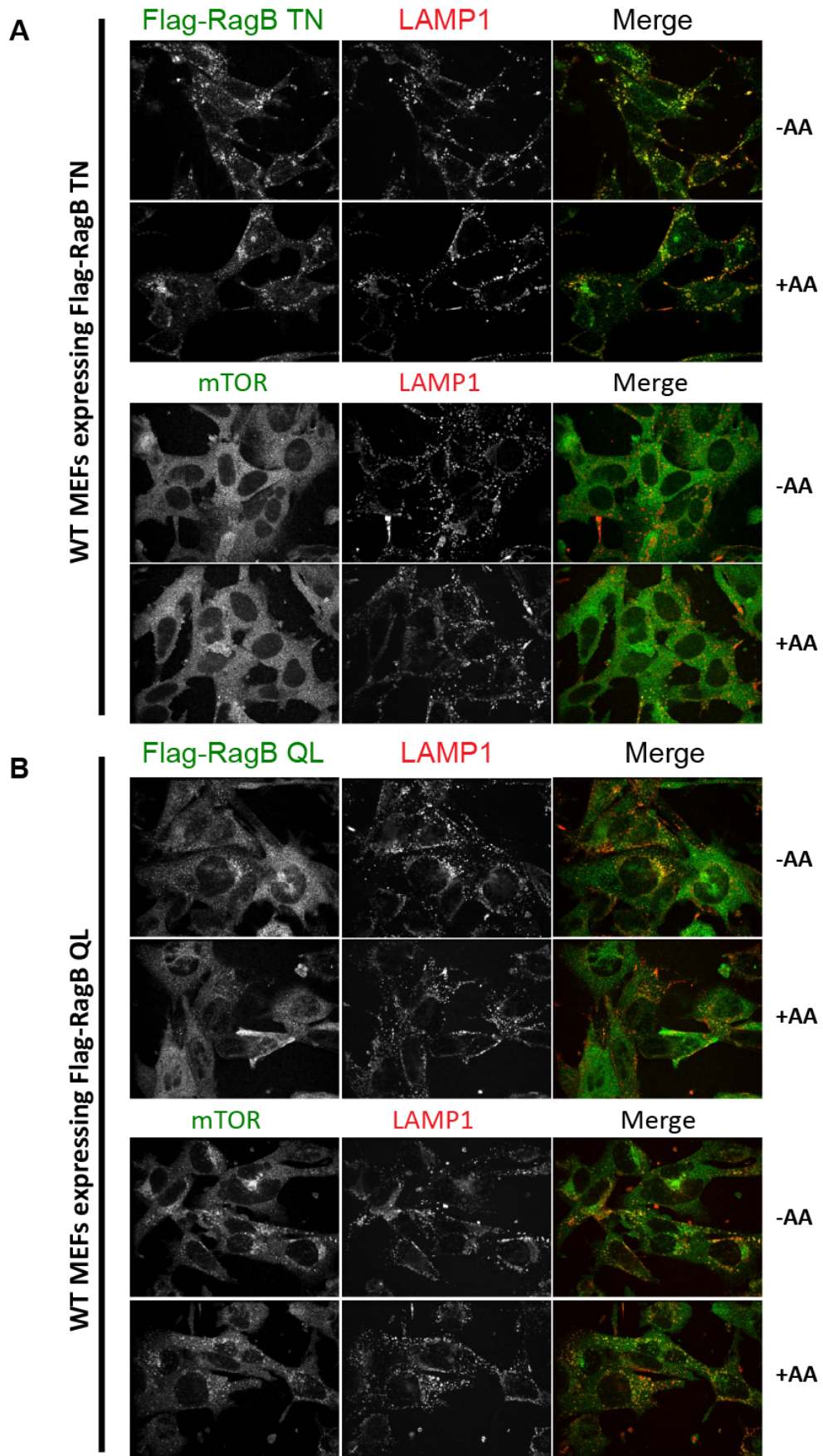
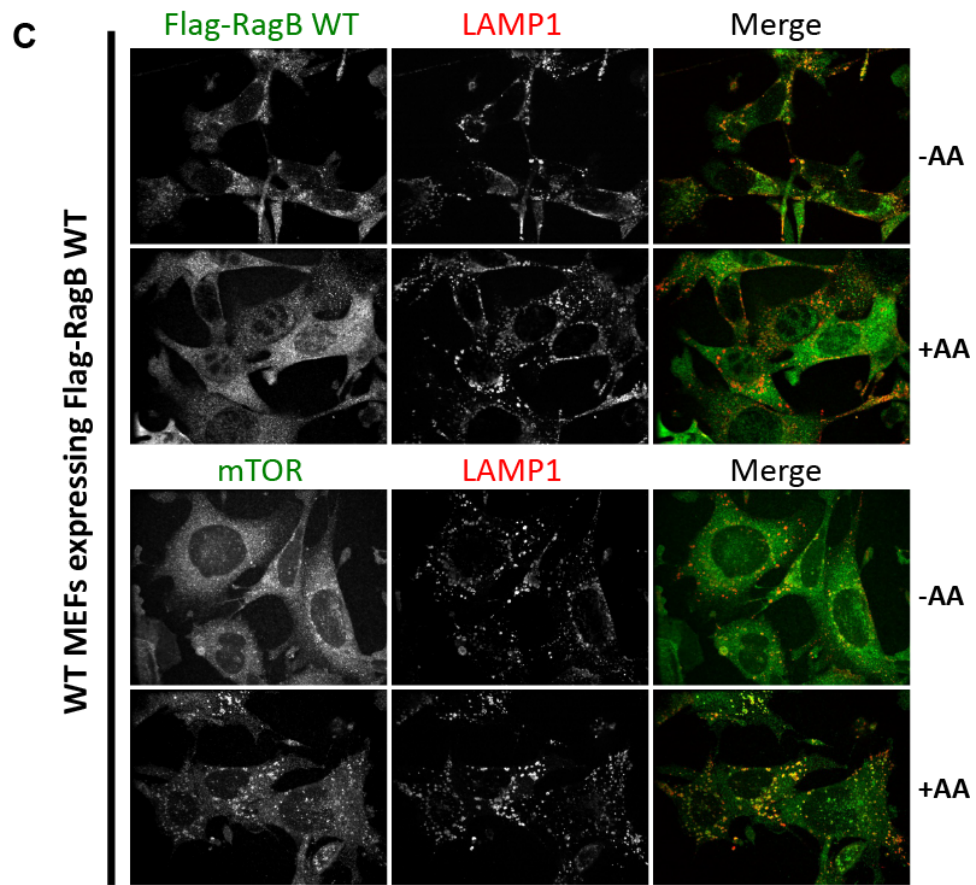


Figure 3.3 (Continued)



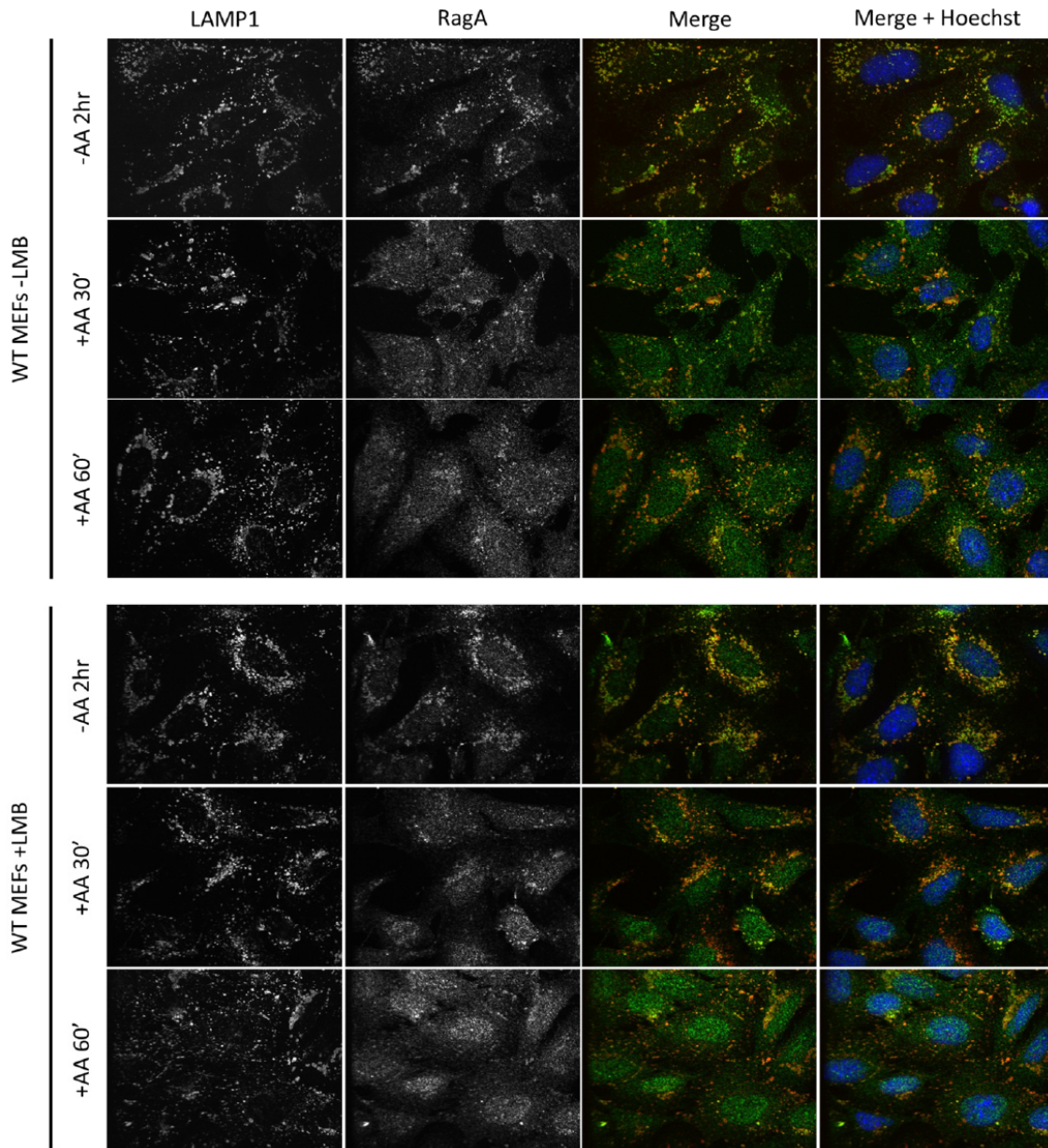


Figure 3.4 RagA enters the nucleus following amino acid stimulation. Cells were either starved of amino acids for 2 hours (-AA 2hr), starved for 90 minutes and stimulated with amino acids for 30 minutes (+AA 30'), or starved for 60 minutes and stimulated with amino acids for 60 minutes (+AA 60'), such that all experimental conditions lasted 2 hours. Experimental conditions were performed in the presence or absence of leptomycin B, an inhibitor of nuclear export. Cells were fixed, stained with the indicated antibodies, and imaged via confocal microscopy.

The Rag proteins enter the nucleus following amino acid stimulation

The staining pattern observed for RagA/B following amino acid stimulation/constitutive GTP binding did not seem to indicate a specific destination for RagA/B away from the lysosome. To investigate the possibility of Rag movement into the nucleus, I used an inhibitor of nuclear export, Leptomycin B (LMB). Following stimulation with amino acids, LMB treatment caused an increase in RagA staining in the nucleus, indicating that RagA enters the nucleus in amino acid stimulation conditions (Figure 3.4).

To investigate a role for nuclear shuttling of the Rag proteins in mTORC1 activation, I looked at mTORC1 activation following amino acid stimulation in the presence of LMB. mTORC1 activation was only slightly decreased in cells treated with LMB, suggesting that nuclear shuttling of the Rags is not necessary for mTORC1 activation (Figure 3.5 A). To investigate this further, I designed an experimental scheme in which I starved and stimulated cells twice, in the presence of LMB (Figure 3.5 B). The goal was to test the hypothesis that nuclear entry of the Rag proteins is required for “recycling” of the Rag proteins back to the lysosome; that without nuclear entry following the Rag diffusion, they might not be able to localize back to the lysosome or recruit mTORC1. So I performed a double starve/stimulation experiment, outlined in Figure 3.5 B, to test whether trapping Rags in the nucleus with LMB prevents subsequent mTORC1 activation. I found this was not the case, since cells that were doubly starved and stimulated in the presence of LMB were just as capable of activating mTORC1 as cells that were only singly starved and stimulated (Figure 3.5 B). These results do not provide a strong argument that nuclear entry or shuttling of the Rag proteins is necessary for mTORC1 activation.

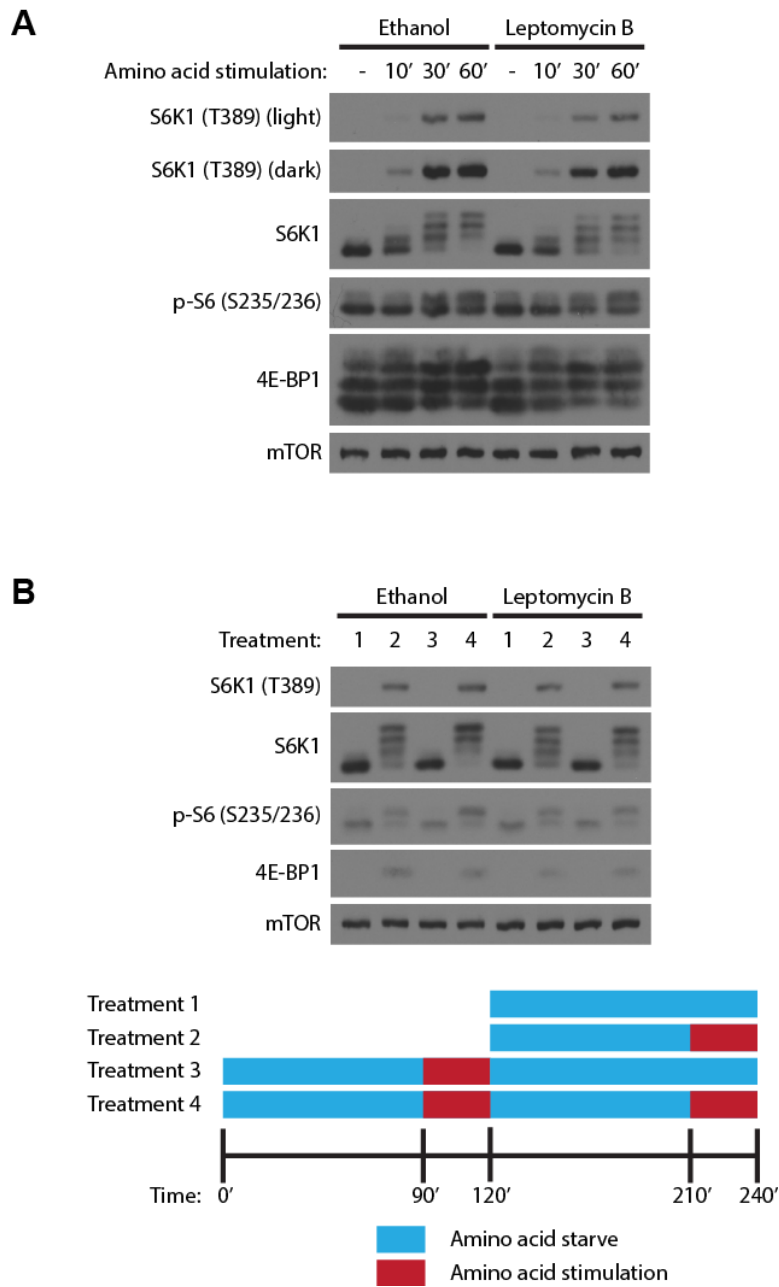


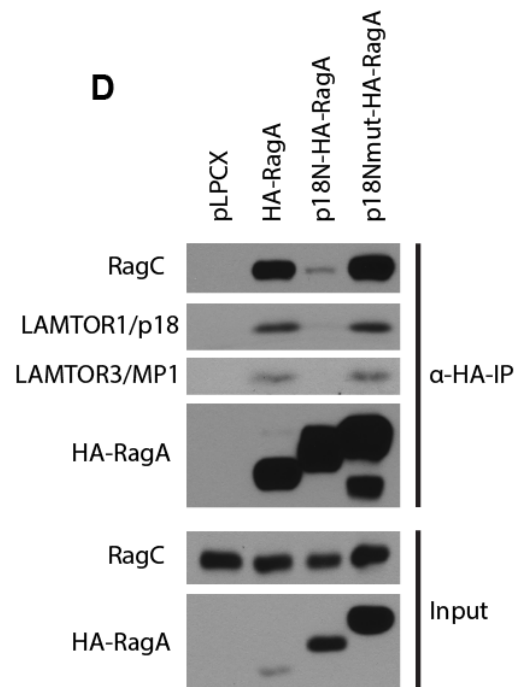
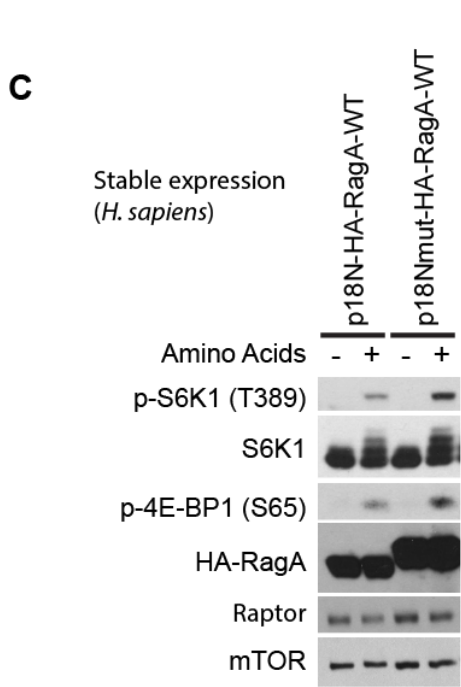
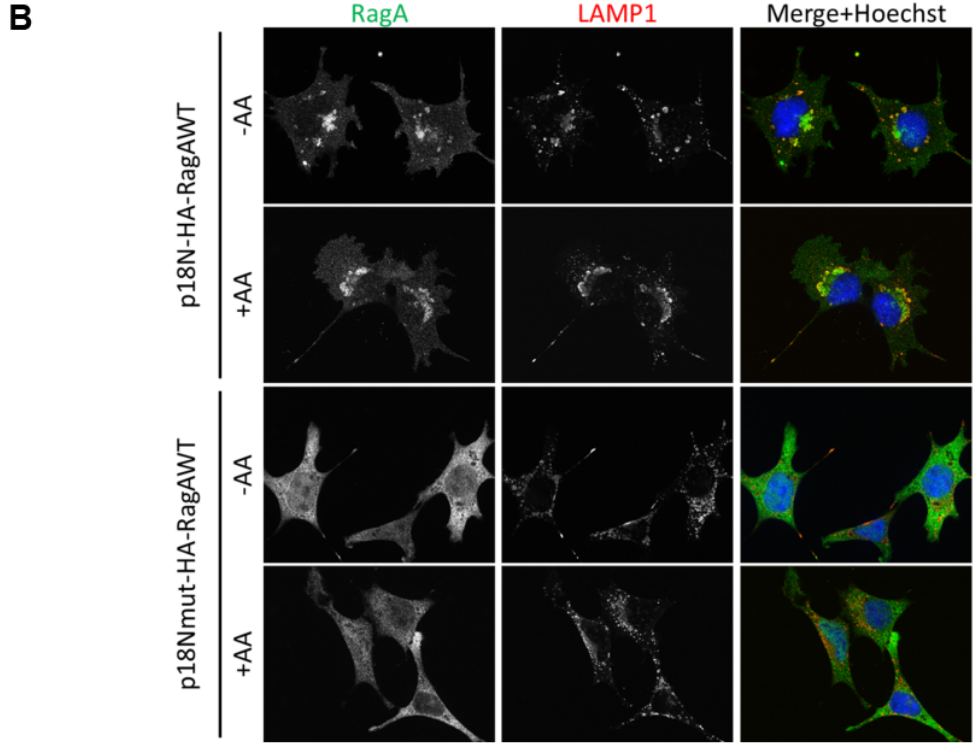
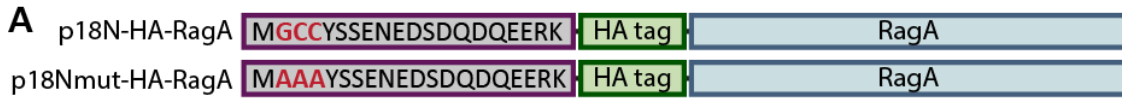
Figure 3.5 RagA entry into the nucleus does not appear to be necessary for mTORC1 activation. (A) MEFs were starved and stimulated as described in *Figure 3.4*, in the presence or absence of Leptomycin B. Cell lysates were probed with the indicated antibodies. **(B)** MEFs were treated according to the treatment schemes are illustrated in the bottom panel. Cell lysates were probed with the indicated antibodies (top panel).

N-terminal modification/lipidation results in dysfunctional RagA protein

To attempt to interrogate the role of Rag movement in mTORC1 activation, I designed a scheme to force the localization of the Rag proteins to the lysosome. To force-localize the Rags specifically to the lysosome, I considered various lysosomal proteins and their localization sequences that I could fuse to the Rags in order to artificially achieve lysosomal localization. I decided to use the N-terminal 20 amino acids of the Ragulator component p18/LAMTOR1 (late endosomal/lysosomal adaptor, MAPK and MTOR activator 1), which contain myristoylation and palmitoylation sites that allow for localization of the protein to the lysosomal membrane (Nada et al., 2009). Localization of p18/LAMTOR1 allows for lysosomal localization of the entire Ragulator complex as well as the Rag heterodimer (Bar-Peled et al., 2012; Sancak et al., 2010), so by fusing the N-terminal 20 amino acids of p18/LAMTOR1 to RagA (Figure 3.6 A), the goal is to bypass the need for the Ragulator in the localization of the Rags, and to eliminate any potential regulation of Rag localization that may be occurring. IF staining for RagA in MEFs stably expressing an HA-tagged p18-RagA fusion protein (referred to here as p18N-HA-RagA), indicated that the p18N-terminal fusion caused a majority of RagA to constitutively localize to lysosomes (Figure 3.6 B). In comparison, a control construct which contained the same HA tag and N-terminal p18/LAMTOR1 fusion as p18N-HA-RagA, but with the myristoylation and palmitoylation sites mutated to alanine (p18Nmut-HA-RagA) (Figure 3.6 A), was observed in a largely cytoplasmic staining pattern (Figure 3.6 B). In these MEFs expressing p18N-HA-RagA or p18Nmut-HA-RagA, I additionally knocked down the endogenous RagA with siRNA to be able to more specifically look at the constitutively localized RagA, and looked at phosphorylation of mTORC1 targets to assay mTORC1 activation. I found that expression of the constitutively localized RagA caused a decrease in the level of activation of mTORC1, compared to expression of p18Nmut-HA-RagA

Figure 3.6 N-terminal modification/lipidation of RagA results in dysfunctional protein. (A) Diagram illustrating the p18/LAMTOR fusion proteins used. p18N-HA-RagAWT is a fusion between the N-terminal 20 amino acids of p18/LAMTOR1 and RagA, with an HA tag in between the two. p18Nmut-HA-RagAWT has mutations in the residues of p18 that are normally lipidated. **(B)** Stable cell lines were generated expressing the fusion proteins described in **A**. Cells were starved and stimulated as described above and stained with antibodies to RagA and LAMP1. **(C)** The same stable cell lines used in **B** had endogenous RagA knocked down, and following amino acid starvation and stimulation, cells were probed with the indicated antibodies. **(D)** 293T cells transiently expressing an HA-tagged version of RagA, or the RagA constructs described above, were immunoprecipitated, and precipitates were probed with the indicated antibodies.

Figure 3.6 (Continued)



(Figure 3.6 C). Although this result would seem to suggest that RagA movement away from lysosomes is necessary for full activation of mTORC1, I was concerned that the modification to RagA was affecting the ability of RagA to function normally, beyond the effect of forced localization.

Since the activation of RagA is dependent on its binding to the Ragulator components (Bar-Peled et al., 2012), and its recruitment of mTORC1 is dependent on its binding to RagC/D (Sancak et al., 2008), I decided to test whether p18N-HA-RagA was able to bind these proteins when expressed in a cell. I immunoprecipitated HA-RagA, p18N-HA-RagA, and p18Nmut-HA-RagA from 293T cells, and probed the precipitates for RagC and the Ragulator components p18/LAMTOR1 and MP1/LAMTOR3. While HA-RagA and p18Nmut-HA-RagA both co-precipitated RagC and the regulator components, these proteins were barely detectable in the p18N-HA-RagA precipitates (Figure 3.6 D), indicating that this fusion protein is not able to function as normal RagA would at the lysosome. For this reason, I cannot make any meaningful interpretations of the result from Figure 3.6 C or any other experiments using this fusion protein, since it is not only constitutively localized to the lysosome, but it is also at least partially non-functional, explaining the observed decrease in mTORC1 activation in Figure 3.6 C.

As an alternative approach to the N-terminal p18 fusion, I also made versions of RagA with a C-terminal fusion to a portion of Rheb containing its CAAX motif (C=cysteine, A=aliphatic, X=terminal amino acid), which has been shown to be lipidated at the cysteine residue of the CAAX motif, and is sufficient for its localization (Hanker et al., 2010; Sancak et al., 2010). The drawback to this approach is that Rheb displays a much broader distribution than p18 (Hanker et al., 2010), so I knew these constructs would likely result in results that

are harder to interpret. I stably expressed the HA-RagA-WT-CAAX construct, as well as a mutant with the cysteine of the CAAX mutated to serine (referred to here as SAAX). Interestingly, expression of HA-RagA-WT-CAAX displayed lower mTORC1 activation than expression of HA-RagA-WT-SAAX under amino acid stimulation conditions. However, expression of HA-RagA-WT-CAAX appeared to slightly rescue the effects of amino acid starvation compared to expression of HA-RagA-WT-SAAX (Figure 3.7) for unknown reasons.

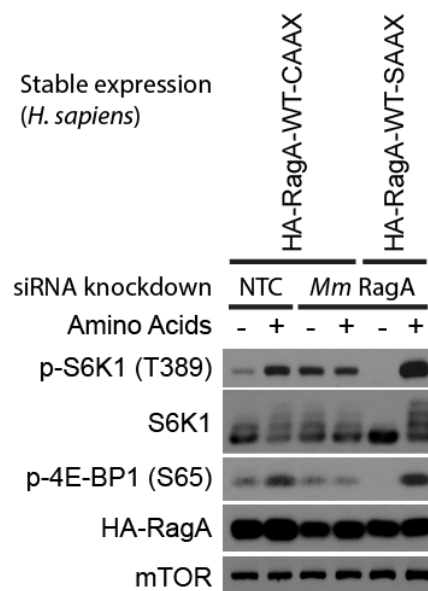


Figure 3.7 RagA forced localization with the Rheb CAAX motif inhibits activation of mTORC1 to its full extent, but partially rescues mTORC1 activity in amino acid starve conditions. *H. Sapiens* versions of HA-RagA-WT-CAAX and HA-RagA-WT-SAAX were stably expressed in MEFs, and the endogenous, mouse forms of RagA were knocked down with siRNA.

Discussion

I describe here the novel phenomenon of Rag movement from the lysosome following amino acid stimulation (Figure 3.1). I find that this movement is independent of mTORC1 activity (Figure 3.2), and that this localization is governed by the GTP/GDP binding status of RagA/B (Figure 3.3). RagA can be observed in the nucleus, but a role for Rag nuclear

translocation in mTORC1 activation is unclear (Figure 3.4, Figure 3.5). There may be a different sub-cellular location to which the Rag proteins are relocating, or there may be a non-mTORC1 related function for the Rags in the nucleus. Unfortunately, the p18 fusion approach I took to force the localization of the Rag proteins to the lysosome caused problems in the functionality of RagA, so I was unable to use this approach to assess the role of Rag movement in mTORC1 activation. I also show data here with a Rheb fusion approach to attempt to localize RagA via the CAAX motif of Rheb. However, we had difficulty determining the exact localization of the Rheb construct, and detailed analysis will be necessary to establish the functionality of these constructs. It will be possible to attempt to force the localization of the Rag proteins through other analogous methods, however, it is likely that similar N-terminal fusion methods may yield the same issue as with the p18 fusion. I have also made the construct p18N-HA-RagC, which is analogous to p18N-HA-RagA, but detailed analysis will be necessary to determine if this construct is able to function or if the p18 fusion causes it to be dysfunctional as well.

There are a number of possibilities why the Rag proteins may alter their location in response to amino acid stimulation. It has been proposed that potentially due to the large size of mTORC1, the Rags may leave the lysosome in order to bind mTORC1 and bring it to the lysosome (Bar-Peled et al., 2012), however there is currently no published data to support this model. I question this model because it implies that RagA would need to leave and then return to the lysosome in the time it takes mTOR to arrive at the lysosome. Based on our quantitation of the mTOR and RagA localization data, by an hour following amino acid stimulation, a majority of RagA is localized away from the lysosome while a majority of mTOR is localized at the lysosome. It may be that the stoichiometric ratio of Rag proteins in a cell compared to mTORC1 complexes is very high, and only a small proportion of the total Rag proteins may be necessary to bind mTORC1 at the lysosome. If this is the case, while I am

observing a net decrease in Rag localization at the lysosome, perhaps only a small proportion of the Rags are needed to shuttle out and back to the lysosome. To address this, it might be useful to assess the movement of the Rag proteins in a live cell to determine if the Rag proteins are indeed shuttling back and forth from the lysosome following amino acid stimulation.

Another possibility is that the Rag proteins diffuse away from the lysosome following binding to mTORC1, and that the Rag proteins have a role in mediating mTORC1 phosphorylation of substrates. Most mTORC1 substrates are found at locations other than the lysosome, so a question exists as to whether substrates need to come in proximity of the lysosome, or whether mTORC1 shuttles to the locations of substrates, and returns to the lysosome to keep being activated by Rheb. If the latter case is true, then perhaps the Rag proteins play a role in this shuttling to substrates, or perhaps prolonging the activation of mTORC1 while it is away from the lysosome and not bound to Rheb.

A third possibility is that Rag movement is involved in the inactivation of mTORC1. The data with HA-RagA-WT-CAAX suggests that forced localization of Rag to the cellular compartment of Rheb inhibits the ability of mTORC1 to turn off in situations of amino acid deficiency. It is likely that the Rags and Rheb occupy distinct locations, and only a portion of them overlap. The Rags may have a function that requires localizing more specifically with Rheb. For example, they may have to shuttle mTORC1 to Rheb, or facilitate mTORC1 binding to Rheb. If this is the case, then the HA-RagA-WT-CAAX construct would allow for decreased inactivation of mTORC1 in amino acid starve cases because it would be unable to leave the Rheb subcellular location, and would continue to activate mTORC1 even under amino acid starve conditions. However, another possibility is that the altered location of RagA fused to

the Rheb CAAX causes it to have decreased access to its GEF and GAP, leaving it in a neutral state, irrespective of amino acid status.

A fourth possibility as to why the Rag proteins move is to function in another amino acid-regulated process unrelated to mTORC1 activity, and yet a fifth possibility is that the Rag movement serves no functional role at all. The Ragulator not only serves as the docking point for the Rags, but it also serves as a guanine nucleotide exchange factor (GEF) for RagA/B (Bar-Peled et al., 2012; Sancak et al., 2010). Detailed binding affinities for Ragulator toward RagA/B have not been determined, but initial analysis of the Rags and detailed analysis of other Ras-related GTPases have revealed that GEFs bind the nucleotide free form of their GTPases more strongly than a nucleotide-bound form (Bar-Peled et al., 2012; Klebe et al., 1995; Sprang and Coleman, 1998). This alone could explain the observed localization of RagB in Figure 3.3, with decreased binding affinity leading to decreased association. However, analysis of the interaction between Ran and its GEF RCC1, showed that the binding affinities of RCC1 to Ran-GDP and Ran-GTP are similar (Klebe et al., 1995); if this is also the case for the association of Ragulator with RagA/B-GDP and RagA/B-GTP, then binding affinity of Ragulator toward RagA/B might not be sufficient to explain the change in localization I observe. More detailed studies of the binding affinities of Ragulator toward the specific nucleotide-bound Rag proteins will be needed to determine this.

Chapter 4

Discussion and future directions

General Discussion

I have described in this thesis two distinct mechanisms by which amino acids can regulate aspects of the mTORC1 pathway in novel ways. In Chapter 2, I describe how a relatively mild insult to mTORC1, that of deprivation of arginine or leucine, allows for reactivation of mTORC1 for a period of several hours. In Chapter 3, I describe how amino acid stimulation leads to movement of the Rag proteins away from their known location, the lysosome. The mechanisms of mTORC1 regulation by amino acids are complex and are still far from well understood. Although there is still much to study and decipher in regard to the mechanisms of regulation I describe above, it is my hope that these topics will be integrated into the overall understanding of mTOR regulation and function to help clarify what is still unknown.

The mTORC1 recovery response

The biggest question I would like to address in regard to the mTORC1 recovery response described in Chapter 2 is the cellular function of the response. My thought is that the response is a temporary adaptation response, to allow the cells to maintain essential cellular functions so they can survive the period of nutrient loss. However, in my attempts to block the recovery response through pharmacological inhibition of mTORC1, I have failed to observe any significant reproducible phenotypes in regard to cell death or apoptotic markers (data not shown). Preliminary data shows that rapamycin treatment causes an increase in nuclei size that is specific to the arginine deprivation condition; rapamycin treatment in growing cells induces a decrease in nuclei size (Figure A 1). While this may be indicative of mTORC1 reactivation inducing changes in cell cycle, additional studies are needed to confirm

this. Further, why this cell cycle phenotype may be beneficial for the cell under arginine deprivation conditions is not clear.

Another possibility as to why cells reactivate mTORC1 following arginine or leucine withdrawal is related to autophagy and its regulation by mTORC1. It might be possible that arginine or leucine deprivation causes induction of autophagy, but that long-term induction of autophagy is detrimental to the cells. This may lead to the cells turning on mTORC1 with the goal of inhibiting autophagy through ULK1, DAP1, or other mechanisms (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Koren et al., 2010) to limit the extent of cell “self-eating”.

A related question that might help provide clues as to the function of the mTORC1 recovery response is, what happens when you give back arginine to a cell that has been lacking it? Does the mTORC1 recovery response allow for these cells to return to a “normal” state more quickly than a cell that has not had mTORC1 active during the deprivation period? To fully address this question, a multi-level approach may be necessary in order to assess the metabolic and signaling changes that occur when arginine is added to cells following prolonged deprivation, either in the presence of Rapamycin or in cells where the recovery response is allowed to occur. It is likely that the cells that undergo the mTORC1 recovery response will have an advantage, either in viability, functionality, or in the general quickness that homeostasis is returned to normal.

In addition to questions about the function of the mTORC1 recovery response, questions still remain in regard to the full mechanism of the recovery response. I present data to suggest a role for Akt activation in the mTORC1 recovery response (Figure 2.4, Figure 2.5), however, it does not seem that Akt is solely responsible for the increase in mTORC1 activity

during the recovery response, as inhibition of Akt does not completely eliminate the response (Figure 2.4B). Addition of an SGK1 inhibitor allows for more complete inhibition of the recovery response, however, this also causes a potent decrease in the levels of mTORC1 activity in the absence of arginine starvation (Figure 2.8), suggesting that this effect is more general to mTORC1 activation and not specific toward the reactivation phase. Secondly, data from the Rictor KO MEFs also brings question to the model of Akt activation leading to mTORC1 recovery; the Rictor KO MEFs display robust mTORC1 recovery despite having no phosphorylation of Akt at S473 (Figure 2.6). It could be argued that Akt activation through phosphorylation of T308 by PDK is enough to activate Akt and then mTORC1 downstream, however, the WT littermate control cells do not display increased T308 phosphorylation during the recovery response (Figure 2.6), suggesting they are able to induce mTORC1 recovery through Akt-independent mechanisms.

In considering other mechanisms that may be contributing to the mTORC1 recovery response, several possibilities are immediately discernable. One possibility is that other signaling pathways known to regulate mTORC1 are also being activated in response to arginine or leucine deprivation, and these pathways are able to cause mTORC1 reactivation. I have tested a number of known mTORC1-regulatory pathways (data not shown), none of which have affected the mTORC1 recovery. However, there are a number of stress pathways whose involvement in mTORC1 regulation are not well characterized, which may be worth looking into for their role in the mTORC1 recovery response. However, western blotting for phosphor-p38 and JNK, two well-known stress kinases (Han et al., 1994; Hibi et al., 1993), did not show any correlations that might explain the mTORC1 recovery response (Figure A 4). Another possibility is that metabolic changes that occur following arginine or leucine withdrawal could be contributing to the recovery. A plot of the fold changes in amino acid

levels following arginine withdrawal and leucine withdrawal show changes in the levels of most amino acids (Figure A 2, and Figure A 3). It is possible that an increase in the levels of certain amino acids can somehow “stand in” for the lacking arginine or leucine. Initial experiments with double dropout media (lacking two amino acids, ie. arginine and glutamine, or leucine and glutamine), were unable to block the recovery response (Figure A 4). However, before we can rule out this possibility as a contributor to the mTORC1 recovery response, a more systematic approach needs to be taken, looking at each amino acid, and potentially blocking intracellular synthesis if the amino acids are being generated endogenously.

It is worth noting that another group recently published data suggesting that the expression Sestrin2, a sensor of leucine (Chantranupong et al., 2014; Saxton et al., 2016a), is induced by the transcription factor ATF4, downstream of GCN2 activation (Ye et al., 2015). GCN2 is an eIF2 α kinase which is activated by low levels of amino acids, which it detects by binding uncharged tRNAs (Wek et al., 1995). Through its induction of ATF4 and Sestrin2, GCN2 is able to decrease mTORC1 activity. However, the authors found that this inhibition was specific to later timepoints, and was necessary for continued suppression of mTORC1, but not the initial decrease in mTORC1 activity. Although the authors do not describe the recovery response per se, their data is consistent with what I see in my experiments. The data published by Ye et al. suggests that the Sestrin2 induction limits the mTORC1 recovery response, but it seems that the recovery response itself is independent of Sestrin2. This study is significant also because it highlights what seems to be a growing theory that mTORC1 regulation by amino acids seems to be biphasic, with acute starvation being signaled to mTORC1 through mechanisms that are quite distinct from longer-term starvation. There is some data to suggest that these two phases are regulated by distinct amino acids (Jewell et

al., 2015), however, more work will need to be done to understand the complexities of this regulation.

Rag movement from lysosomes

In Chapter 3 of this thesis, I present data showing that RagA proteins move away from their canonical subcellular location, the lysosomal membrane (Figure 3.1) in an mTORC1-independent manner (Figure 3.2). I observe RagA in the nucleus (Figure 3.4), but I am unsure of the potential role for nuclear entry of RagA (Figure 3.5) or Rag movement from the lysosome to any other subcellular location. I summarize my attempts to force the localization of the Rag proteins, which were largely unsuccessful (Figure 3.6). The crystal structure of the Gtr1p-Gtr2p heterodimer, the yeast homolog of the RagA/B-RagC/D heterodimer has been solved (Gong et al., 2011; Jeong et al., 2012), and may explain some of the difficulties I have faced with my attempts to create an N-terminal fusion protein. The structure reveals that the Gtr proteins each consist of two main domains: an N-terminal GTPase domain, and a C-terminal dimerization domain. The N-terminal GTPase domains undergo structural rearrangements following GTP hydrolysis which regulates binding to Raptor, and the C-terminal domain seems to be important for both heterodimerization and localization to the lysosome through interactions with the Ragulator complex (Gong et al., 2011; Jeong et al., 2012). The architecture of these proteins suggests that their orientation is extremely important for their ability to function, and also that both the domains are involved in important interactions. Based on what we can tell from the structure, it may be that the N-terminal fusion of RagA with the segment of p18/LAMTOR1 caused RagA to be turned sideways at the membrane, in such a way that it is unable to interact with the Ragulator

components, and less efficiently bind RagC/D or Raptor. In terms of allowing for proper orientation of the heterodimer, it does seem that a perhaps less invasive approach would be C-terminal modification. However, C-terminal modification of either RagA/B or RagC/D may block the ability of the Ragulator complex to bind the heterodimer, which would cause RagA/B to be inhibited due to lack of GEF activity from Ragulator.

The question of where the Rag proteins are going and the function of the movement is still unanswered. There has been data published that substantiates my findings of Rag movement in accordance with GDP/GTP binding (Bar-Peled et al., 2012; Sekiguchi et al., 2001), however, a lack of additional reports in this regard suggests that other groups have also encountered difficulty in studying the issue further. Curiously, multiple groups have published data arguing that the Rag GTPases serve functions independent of mTORC1 regulation, and it seems that these functions are related to lysosomal function (Kim et al., 2014; Shen et al., 2016). Part of this phenotype is likely due to the downregulation of mTORC1 and the subsequent upregulation of lysosomal biogenesis genes through TFEB (Peña-Llopis et al., 2011; Settembre et al., 2011, 2012; Yu et al., 2010), however, this does not explain the entire phenotype for Rag loss. Rather, it seems that Rag mutation allows for normal autophagy initiation, but defects in lysosomal acidification and degradation (Kim et al., 2014; Shen et al., 2016). In my attempts to understand Rag localization changes, I did some IF experiments looking at the localization of RagA and RagC compared to Rheb. These experiments were technically challenging, partially due to the fixation required prior to immunostaining, which would often lead to a fuzzy, unclear staining pattern of Rheb and Rags. However, in some experiments, as shown in Figure A 5, we were able to obtain a clearer view of the localization pattern. Although it is not clear whether RagA or RagC colocalization with Rheb increases following amino acid stimulation, it does appear that the Rags move to

another endomembrane structure in the amino acid stimulation condition, and not simply diffuse away from the lysosome. It is possible that these structures are tubules involved in autophagic lysosome reformation or another process that is involved in maintenance of lysosomal function. Future studies with additional cellular markers may help identify the structures to where Rags are relocating, and may help determine whether the function of the Rag movement may be in lysosomal function or in mTORC1 signaling, or another possibility altogether.

Chapter 5

Materials and methods

Materials and Methods

Cell culture and transfections

All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) at 37°C with 5% CO₂. TSC2^{-/-} mouse embryonic fibroblasts (MEFs) lacking p53 (p53^{-/-}) and littermate control MEFs were a generous gift from Dr. David Kwiatkowski (Brigham and Women's Hospital, Boston, MA). In most experiments, except those experiments done side by side with knockout (KO) MEFs described below, the littermate controls to the TSC2^{-/-} MEFs were used as "wild type" (WT) MEFs. ATG5^{+/+} and ATG5^{-/-} MEFs were obtained from Dr. N. Mizushima. The Akt1/Akt2 double-knockout (DKO) MEFs and littermate controls were kindly provided by Dr. M. Birnbaum. 293A RagA and RagB CRISPR/Cas9 knock out cells and 293A control cells were a kind gift from Dr. K-L Guan (University of California, San Diego). Rictor KO MEFs and littermate controls were a kind gift of Dr. David Sabatini (MIT). siRNAs targeted against Akt3 and negative control were purchased from Sigma-Aldrich: SASI_Mm01_00200790 (Akt3 #1) and SASI_Mm01_00200791 (Akt3 #2) and SIC001 (Mission siRNA Universal Negative Control). siRNA used for RagA knockdown and control were purchased from Qiagen: #SI01407826 (Mm_Rraga_2), #SI01407833 (Mm_Rraga_3), and #1027281 (AllStars Neg. Control siRNA). siRNA transfections were performed with lipofectamine RNAiMax (LifeTechnologies).

Amino acid deprivations and stimulations

Cells were plated two days prior to harvesting, with cell confluency for western blot experiments being around 90% at time of harvest. To avoid cell detachment during media changes, 20 µg/mL fibronectin (Corning #356009) in phosphate buffered saline (PBS) was added to the dish for 1 hr prior to cell plating. Medias used for amino acid deprivations

in Chapter 2 were purchased from the Memorial Sloan Kettering Cancer Center Media Core (New York, NY), formulated as normal high glucose (4.5 g/L) DMEM (exact formulation can be found on the ThermoFisher website), lacking only the indicated amino acid. Medias used for amino acid deprivations in Chapter 3 were made by hand according to the same DMEM formulation mentioned above. Amino acid-deficient medias were supplemented with 10% dialyzed FBS (dFBS) (Sigma-Aldrich) instead of normal FBS to avoid inadvertently adding amino acids to the media. During amino acid deprivation experiments, except where indicated differently, experimental conditions specified as “0’” starves were placed in complete amino acid media, also purchased from the Sloan Kettering Media Core and supplemented with dFBS, for the same duration as the longest deprivation condition in the given experiment. Experimental conditions with intermediate-length amino acid-deprivations (for example, 45’ or 3hr) were treated as the 0’ starve conditions until the beginning of the indicated starve, at which point they were placed in the indicated starve media. Unless indicated otherwise, for experiments in Chapter 2, all conditions received fresh media with fresh dFBS at each time point. For amino acid stimulations performed in Chapter 3, stimulations were either performed by changing the media to full-amino acid media, or by adding the appropriate amount of a 50x stock solution of amino acids made by hand according to the formulation of DMEM. Unless indicated otherwise, stimulations were performed such that the total treatment (starvation + stimulation) lasted 2 hours, with the starvation time being appropriately shortened based on the length of the stimulation.

Antibodies and reagents

The following antibodies were purchased from Cell Signaling Technologies: p-4E-BP1 S65 (#9451), p-S6K1 T389 (#9234), S6K1 (#2708), mTOR (#2983), p-TSC2 T1462

(#3611), p-Akt T308 (#13038), p-Akt S473 (#4060), p-ULK S757 (#6888), 4E-BP1 (#9644), LC3B (#3868), ubiquitin (#3933), ATG5 (#12994), Akt1 (#2983), Akt2 (#3063), Akt3 (#14982), pan-Akt (#4691), p-GSK3 β S9 (#9336), p-NDRG1 T346 (#5842), RagA/B (#4357), ATF4 (#11815), p-PRAS40 T246 (#2997), p-Tyr (#8954), RagC (CST 3360), LAMTOR1/p18 (CST 8975), and LAMTOR3/MP1 (CST 8168). Antibodies to vinculin (V9264), GAPDH (G8795) and FLAG (M2) were purchased from Sigma. The anti-puromycin antibody clone 12D10 (MABE343) and p-Grb10 S501/S503 (07-1520) were purchased from EMD-Milipore. The HA-tag antibody used for western blotting was from Santa Cruz (# 7392), The LAMP1 antibody (#553792) was purchased from BD biosciences. Rapamycin and MG132 were purchased from Calbiochem, MK2206 was purchased from Medchem express, GSK690693 and GDC0941 were purchased from Selleck Chemicals, Chloroquine was purchased from Invitrogen, puromycin and cycloheximide were purchased from Sigma-Aldrich. 14g was a kind gift of Dr. Jose Baselga (Memorial Sloan Kettering).

Cell Lysis and western blotting

Cell lysis was performed on ice following a wash with cold PBS. The lysis buffer contained 10 mM KPO₄, 1mM EDTA, 5 mM EGTA, 10 mM MgCl₂, 0.5% NP-40, 0.1% Brij-35, 0.1% deoxycholate, 1 mM sodium vanadate, 50 mM beta-glycerophosphate, 400 uM PMSF, 0.02 ug/uL Leupeptin, 0.1 ug/uL pepstatin A, and 0.02 ug/uL aprotinin. Lysates were cleared by centrifugation at 13,000g for 25 minutes at 4°C. Protein concentration of lysates was determined by Bio-Rad Protein Assay (Bio-Rad). Cellular lysates were run on large SDS-PAGE gels overnight and transferred to 0.2 um nitrocellulose membranes at 4°C for at least 4 hours. Membrane blocking was performed with 5% milk in TBST. Primary antibody incubations were

performed overnight in TBST with 7% BSA. Secondary antibody (HRP from GE healthcare) incubations were performed in 5% milk in TBST at room temperature for 2 hours.

Immunoprecipitations

293T cells were transfected with the indicated HA-RagA constructs and incubated for 48 hrs. Cells were washed with cold PBS, then harvested in Buffer A (10mM KPO₄, 1.27mM EDTA, 5mM EGTA, 10mM MgCl₂, 0.5% Igepal CA-360, 0.1% Brij-35, 1mM Na₃VO₄, 50 mM β-glycerophosphate, 0.4mM PMSF, 0.02μg/μL Leupeptin, 0.01μg/μL Pepstatin A, 0.02μg/μL Aprotinin). Following centrifugation, 90% of lysates were immunoprecipitated with immobilized α-HA antibody (Roche Anti-HA Affinity Matrix product no. 11815016001). Immunoprecipitates were washed 3 times with Buffer A, then subjected to 11% SDS-PAGE, transferred to nitrocellulose membrane, and subjected to western blotting as described above.

Microscopy

Glass coverslips (Fisherbrand Microscope Cover Glass 12-545-100 18CIR.-1) were incubated in 20 μg/mL fibronectin (Corning #356009) in PBS for 1 hr prior to cell plating. Experimental conditions and cell fixation were performed two days after plating. Cells were fixed in 4% formaldehyde in PBS for 10 minutes on ice followed by 25 minutes at room temperature. Cells were washed 3 times with 0.05% saponin in PBS to permeabilize. Cells were blocked in a 50/50 mix of odyssey blocking buffer (LI-COR Biosciences 927-50150) and a 0.05% saponin in PBS solution at 4° C for at least one hour. Primary antibody incubations were performed overnight in a fresh solution of 50/50 odyssey blocking buffer and 0.05% saponin in PBS. mTOR (CST #2983), RagA (CST #4357) and RagC (CST #3360) were diluted 1:100,

LAMP1 (BD biosciences # 553792) was diluted 1:500, Rheb (Abnova H00006009-M01) was diluted 1:1000. Cells were washed three times with the 50/50 solution described above. Alexa Fluor secondary antibodies (purchased from Life Technologies) were diluted 1:1,000 in the 50/50 mix and incubated with the cells for 2 hours with gentle agitation. Cells were washed four times with PBS, the third wash containing Hoechst stain at 1:10,000 (Life Technologies, H3569). Glass coverslips containing the stained cells were mounted on glass slides (VWR 48312-003) using Dako fluorescence mounting medium.

Images were collected with a Yokagawa CSU-X1 spinning disk confocal on a Nikon Ti motorized inverted microscope with a Nikon Plan Apo 60x 1.4 NA at the Nikon imaging center at Harvard Medical School. RagA or mTOR labeled with Alexa488 and LAMP1 or Rheb labeled with Alexa568 were excited with a Spectral Applied Research LMM-5 laser merge module with AOTF controlled solid state lasers, 488nm (100mW) and 561nm (100mW), and collected with a 405/491/561/642 Quad mirror, and a 525/50 emission filter (RagA and mTOR), or 620/60 emission filter (LAMP1). Hoechst-stained nuclei were imaged using a Lumencor SOLA fluorescence light source using a 395/25 excitation filter and collected with a 400lp mirror and a 460/25 emission filter. Images were acquired with a Hamamatsu ORCA-AG cooled CCD camera (confocal images of fluorophores labeling RagA, mTOR, and LAMP1) and Hamamatsu ORCA-R2 cooled CCD camera (widefield images of Hoechst stained nuclei) using MetaMorph image acquisition software.

Colocalization quantitation was performed using Imaris 7.5.2 with guidance at the Image and Data Analysis Core (IDAC) at Harvard Medical School. Thresholds were calculated via algorithms described by Costes et al., 2004.

Generation of Rag fusion proteins

RagA was PCR amplified off of a pRK7 backbone using the following primers to simultaneously add the N-terminal 20 amino acids of p18 and and HA tag onto RagA (see below for color-coding).

p18N-HA-RagA forward primer:

**5'GATCCTCGAGATGGGGTGCTGCTACAGCAGCGAGAACGAGGACTCGGACCAGGACCGAGAGG
AGCGGAAGTACCCATACGATGTTCCAGATTACGCTATGCCAAATACAGCCATGAAG 3'**

To create a mutant that is not able to be myristoylated or palmitoylated, the glycine and cysteines following the methionine at the beginning of the p18 segment were mutated to alanine:

P18Nmut-HA-RagA forward primer:

**5'GATCCTCGAGATGGCGGCCGCTACAGCAGCGAGAACGAGGACTCGGACCAGGACCGAGAGG
AGCGGAAGTACCCATACGATGTTCCAGATTACGCTATGCCAAATACAGCCATGAAG 3'**

For generation of both p18N-HA-RagA and p18Nmut-HA-RagA, the following reverse primer was used:

5' GCGGCCGCTCAACGCATAAGGAGACTGTG 3'

Extra bases for efficient restriction digestion

XhoI restriction site

N-terminal 20 amino acids of p18 (purple indicates mutation to alanine)

HA tag

RagA

NotI restriction site

The PCR products were digested with *XhoI* and *NotI* and ligated into a pLPCX vector digested with the same enzymes. Constructs were sequence verified.

The FLAG-tagged RagB constructs were previously described (Kim et al., 2013b).

To create a version of RagA fused to the C-terminal 15 amino acids of Rheb (containing the CAAX motif), I used the following reverse primer:

RagA reverse primer:

**5'GATCATCGATTCACATCACCGAGCATGAAGACTTGCCTTGTGAAGCTGCCCCGTCCATACGCATA
AGGAGACTGTGCTT -3'**

To create a mutant that lacks the cysteine required for modification (mutated to serine) I used the following primer:

RagA reverse primer:

**5'GATCATCGATTACATCACCGAGCTTGAAGACTTGCCTTGTGAAGCTGCCCCGTCCATACGCATA
AGGAGACTGTGCTT -3'**

Extra bases for efficient restriction digestion

C-terminal 15 amino acids of Rheb1, containing the CAAX motif (purple indicates mutation to serine)

***Clal* site**

RagA (minus the stop codon)

The RagA-CAAX and RagA-SAAX constructs were cloned into a version of pLPCX that had an HA-tag pre-cloned into the N-terminus. A RagA forward primer was designed based on the specific sequence of the modified pLPCX vector.

Generation of stable cell lines

Retroviruses were produced using the HEK293-GPG packaging cell line. HEK293-GPG cells were washed with OptiMEM media (Life Technologies 31985-088) and transfected with expression constructs using Lipofectamine (Life Technologies). Seven hours following transfection, DMEM with 10% FBS was added to the cells. After 22 hours, media was changed to fresh DMEM with 10% FBS. Starting 3 days after transfection, continuing until 6 days after transfection, supernatant containing the retrovirus was collected and stored at -80°C. Prior to use, virus supernatant was thawed, filtered through a .45 µm filter, and aliquoted into 1 mL Eppendorf tubes, and refrozen at -80°C.

Stable MEF cell lines were generated through three rounds of infection with the virus supernatant in the presence of polybrene (Sigma-Aldrich H9268-5G) (media was replaced with fresh media containing fresh FBS, polybrene, and virus supernatant every 24 hours). Three days after the initial infection, cells were trypsinized, moved to a larger dish, and 3 µg/mL puromycin was added immediately to select cells.

The TSC2 constructs were obtained from Dr. Brendan Manning and were described previously (Menon et al., 2014).

Metabolite extraction

Cells were washed once with cold PBS on ice, then flash frozen in liquid nitrogen and placed on dry ice. Cells were stored at -80°C. 1.5 mL of 80% methanol was added to each 6-cm plate and cells were incubated at -80°C for 1 hour. Plates were scraped as for cell harvesting and centrifuged at 4°C. The supernatant was dried by vacuum under centrifugation, and the metabolites were resuspended in water and measured via GC-MS by the Mass Spectrometry Core at Beth Israel Deaconess Medical Center, Boston, MA 02115.

SUnSET Translation Assay

Cells were deprived of amino acids as indicated above. 10 minutes prior to harvesting cells, 10 ug/mL Puromycin was added to each condition. Cells were harvested and cell lysates were processed for western blotting as described above.

Nuclei isolation for flow cytometry

Cells from a 10-cm plate were washed in PBS and re-suspended in Solution I (10 mM NaCl, 3.4 mM sodium citrate, and 0.03% NP-40) and incubated in the dark for 1 hour. To the cell suspension, 1 mL of Solution II (78 mM citric acid, 250 mM sucrose) was added. Suspensions were spun down for 5 min at 1000 rpm, and a majority of the supernatant was aspirated. Cells were re-suspended in the remaining solution and analyzed on a BD Accuri flow cytometer.

Appendix A
Supplemental figures

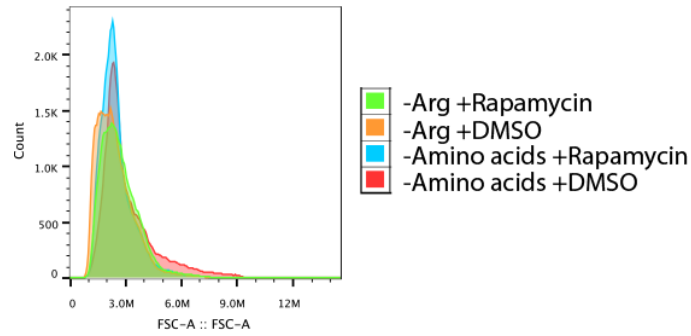


Figure A 1 Rapamycin induces a large nuclei phenotype under arginine deprivation conditions. MEFs were deprived of arginine for 72 hours. At 45' of starvation, either DMSO or 20 nM Rapamycin was added to the media to block mTORC1 reactivation. Nuclei were isolated and nuclei size was analyzed via flow cytometry with the assistance of my colleague, Tanya Schild. The plot shown is a representative set of samples performed in triplicate.

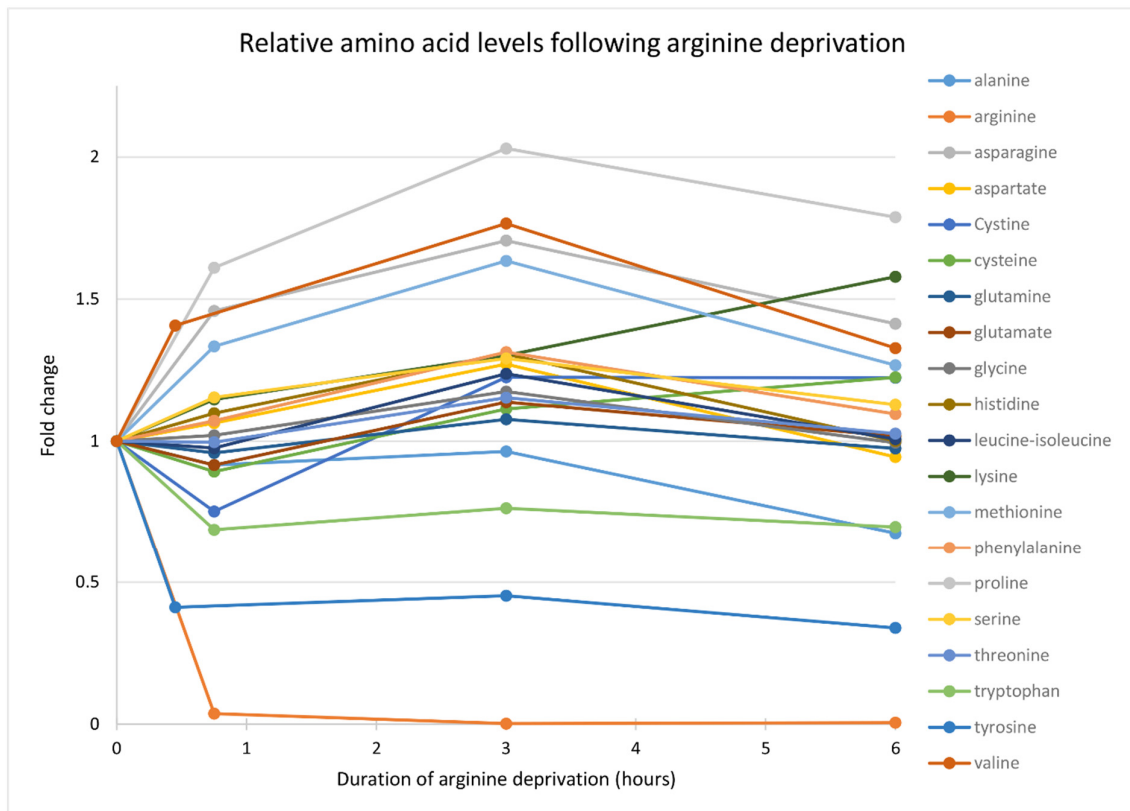


Figure A 2 Amino acid levels following arginine removal. WT MEFs were deprived of arginine for 0 minutes, 45 minutes, 3 hr, or 6 hr, and intracellular metabolite levels were analyzed via mass spectrometry. The graph shows the results of a representative experiment performed a total of 4 times.

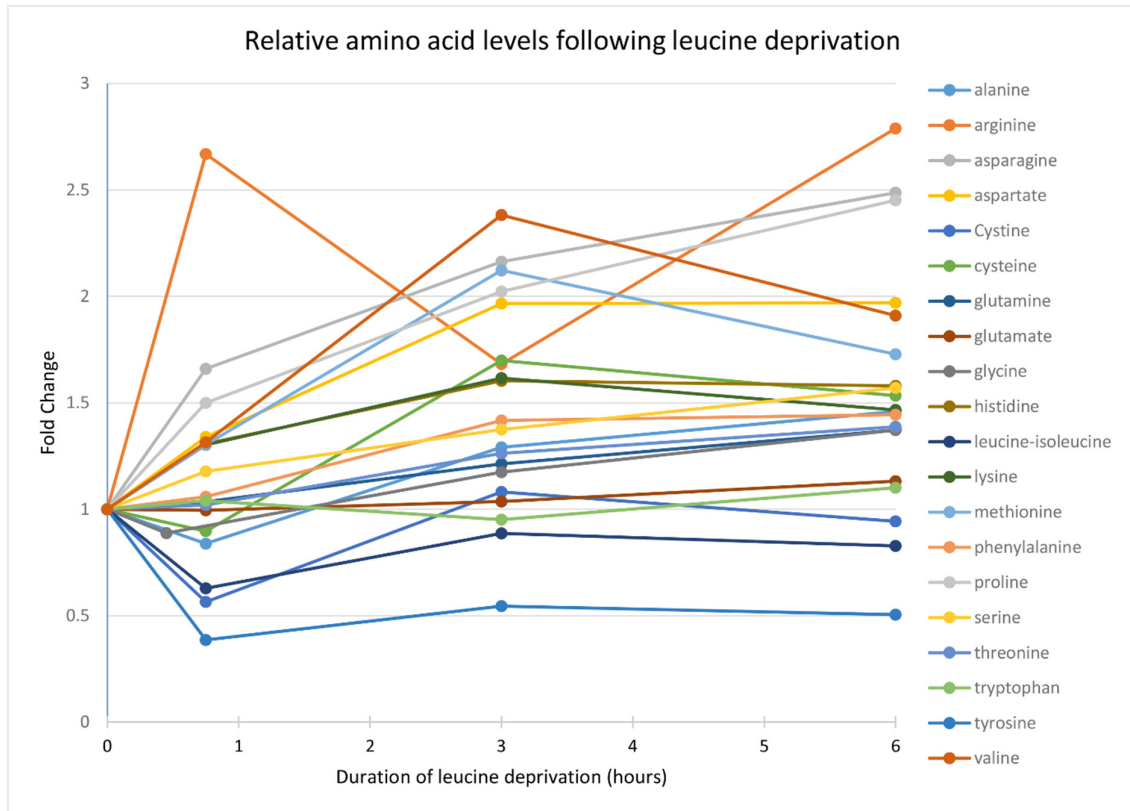


Figure A 3 Amino acid levels following leucine removal. WT MEFs were deprived of arginine for 0 minutes, 45 minutes, 3 hr, or 6 hr, and intracellular metabolite levels were analyzed via mass spectrometry. The graph shows the results of a representative experiment performed a total of 4 times.

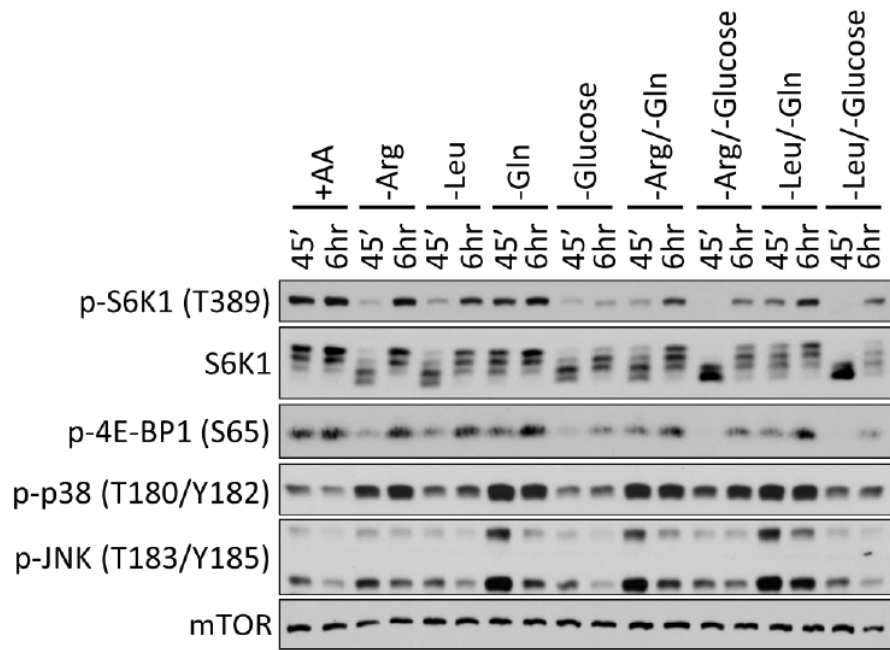


Figure A 4 mTORC1 recovery cannot be blocked through additional deprivation of glutamine. WT MEFs were deprived of arginine, leucine, glutamine, or glucose either singly or in the combinations shown.

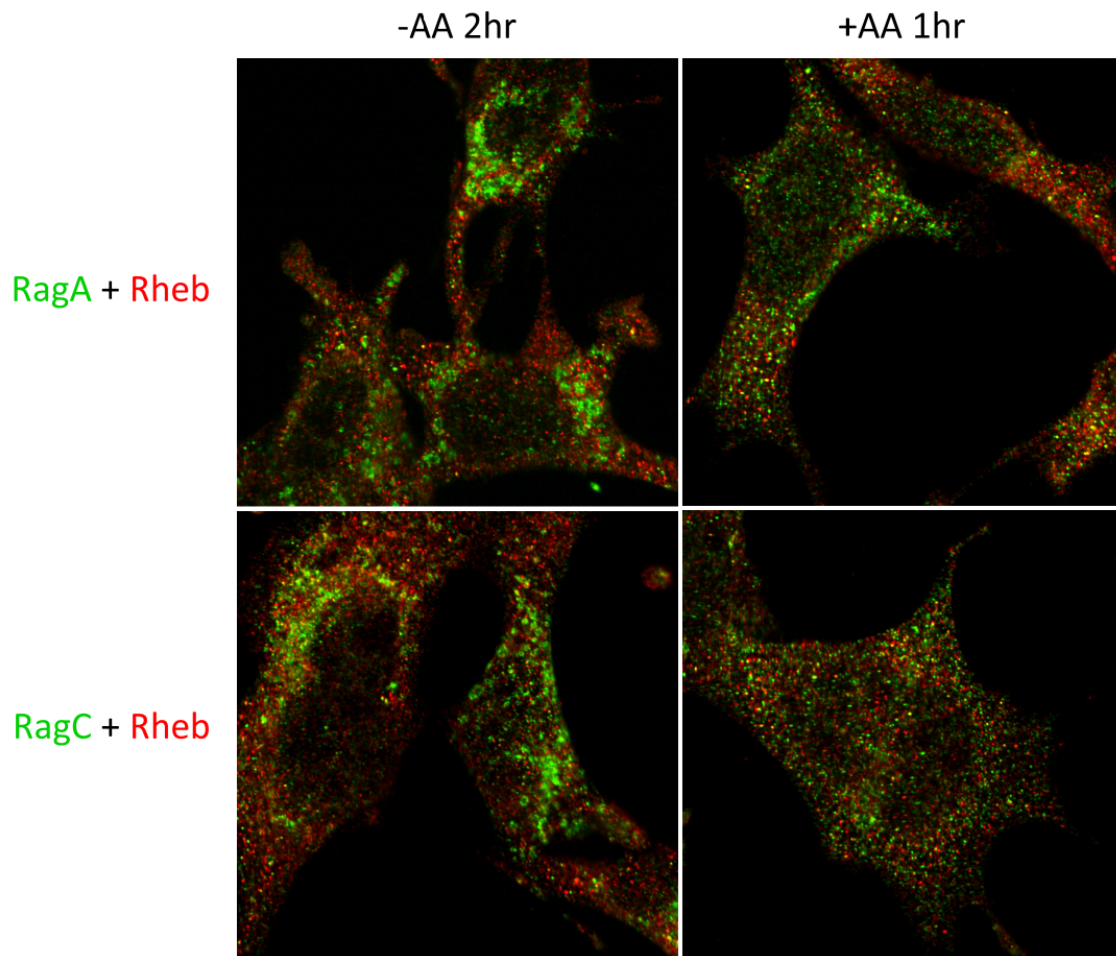


Figure A 5 Rags appear to be shifting from lysosomes to another endo-membrane structure. MEFs starved of amino acids or starved and then stimulated with amino acids were fixed and stained with the indicated antibodies, and were imaged by confocal microscopy.

References

- Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* *15*, 6541–6551.
- Arsham, A.M., Howell, J.J., and Simon, M.C. (2003). A Novel Hypoxia-inducible Factor-independent Hypoxic Response Regulating Mammalian Target of Rapamycin and Its Targets*. *J. Biol. Chem.* *278*, 29655–29660.
- Aylett, C.H.S., Sauer, E., Imseng, S., Boehringer, D., Hall, M.N., Ban, N., and Maier, T. (2015). Architecture of human mTOR complex 1. *Science* *351*, 48–52.
- Bar-Peled, L., Schweitzer, L.D., Zoncu, R., and Sabatini, D.M. (2012). Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. *Cell* *150*, 1196–1208.
- Bar-Peled, L., Chantranupong, L., Cherniack, A.D., Chen, W.W., Ottina, K.A., Grabiner, B.C., Spear, E.D., Carter, S.L., Meyerson, M., and Sabatini, D.M. (2013). A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. *Science* *340*, 1100–1106.
- Ben-Sahra, I., Howell, J.J., Asara, J.M., and Manning, B.D. (2013). Stimulation of de Novo Pyrimidine Synthesis by Growth Signaling Through mTOR and S6K1. *Science* *339*, 1323–1328.
- Ben-Sahra, I., Hoxhaj, G., Ricoult, S.J.H., Asara, J.M., and Manning, B.D. (2016). mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science* *351*, 728–733.
- Blommaart, E.F.C., Luiken, J.J.F.P., Blommaart, P.J.E., Van Woerkom, G.M., and Meijer, A.J. (1995). Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. *J. Biol. Chem.* *270*, 2320–2326.
- Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* *369*, 756–758.
- Brown, E.J., Beal, P.A., Keith, C.T., Chen, J., Shin, T.B., and Schreiber, S.L. (1995). Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature* *377*, 441–446.
- Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A.,

Ellisen, L.W., and Kaelin, W.G. (2004). Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev.* *18*, 2893–2904.

Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C., Jr., and Abraham, R.T. (1997). Phosphorylation of the Translational Repressor PHAS-I by the Mammalian Target of Rapamycin. *Proc. Natl. Acad. Sci. U. S. A.* *94*, 99–101.

Bun-Ya, M., Harashima, S., and Oshima, Y. (1992). Putative GTP-binding protein, Gtr1, associated with the function of the Pho84 inorganic phosphate transporter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* *12*, 2958–2966.

Burgering, B.M., and Coffey, P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH Kinase signal transduction. *Leit. Nat.* *376*, 599–602.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., and Sabatini, D.M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. U. S. A.* *95*, 1432–1437.

Cargnello, M., Tcherkezian, J., and Roux, P.P. (2015). The expanding role of mTOR in cancer cell growth and proliferation. *Mutagenesis* *30*, 169–176.

Castel, P., Ellis, H., Bago, R., Toska, E., Razavi, P., Carmona, F.J., Kannan, S., Verma, C.S., Dickler, M., Chandarlapaty, S., Brogi, E., Alessi, D.R., Baselga, J., and Scaltriti, M. (2016). PDK1-SGK1 Signaling Sustains AKT-Independent mTORC1 Activation and Confers Resistance to PI3K α Inhibition. *Cancer Cell* *30*, 229–242.

Chantranupong, L., Wolfson, R.L., Orozco, J.M., Saxton, R.A., Scaria, S.M., Bar-Peled, L., Spooner, E., Isasa, M., Gygi, S.P., and Sabatini, D.M. (2014). The Sestrins Interact with GATOR2 to Negatively Regulate the Amino-Acid-Sensing Pathway Upstream of mTORC1. *Cell Rep.* *9*, 1–8.

Chantranupong, L., Scaria, S.M., Saxton, R.A., Gygi, M.P., Shen, K., Wyant, G.A., Wang, T., Harper, J.W., Gygi, S.P., and Sabatini, D.M. (2016). The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway. *Cell* *165*, 153–164.

Chen, R., Zou, Y., Mao, D., Sun, D., Gao, G., Shi, J., Liu, X., Zhu, C., Yang, M., Ye, W., Hao, Q., Li, R., and Yu, L. (2014). The general amino acid control pathway regulates mTOR and autophagy during serum/glutamine starvation. *J. Cell Biol.* *206*, 173–182.

Chen, Y., Chen, H., Rhoad, A.E., Warner, L., Caggiano, T.J., Failli, A., Zhang, H., Hsiao, C.-L.,

Nakanishi, K., and Molnar-Kimber, K.L. (1994). A Putative Sirolimus (Rapamycin) Effector Protein. *Biochem. Biophys. Res. Commun.* *203*, 1–7.

Chiu, M.I., Katz, H., and Berlin, V. (1994). RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc. Natl. Acad. Sci. U. S. A.* *91*, 12574–12578.

Choo, A.Y., Yoon, S.-O., Kim, S.G., Roux, P.P., and Blenis, J. (2008). Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc. Natl. Acad. Sci. U. S. A.* *105*, 17414–17419.

Choo, A.Y., Kim, S.G., Vander Heiden, M.G., Mahoney, S.J., Vu, H., Yoon, S.O., Cantley, L.C., and Blenis, J. (2010). Glucose Addiction of TSC Null Cells Is Caused by Failed mTORC1-Dependent Balancing of Metabolic Demand with Supply. *Mol. Cell* *38*, 487–499.

Costes, S. V, Daelemans, D., Cho, E.H., Dobbin, Z., Pavlakis, G., and Lockett, S. (2004). Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys. J.* *86*, 3993–4003.

Dadehbeigi, N., and Dickson, A.J. (2013). Application of a nonradioactive method of measuring protein synthesis in industrially relevant chinese hamster ovary cells. *Biotechnol. Prog.* *29*, 1043–1049.

Demetriades, C., Doumpas, N., and Teleman, A.A. (2014). Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. *Cell* *156*, 786–799.

Demetriades, C., Plescher, M., and Teleman, A.A. (2016). Lysosomal recruitment of TSC2 is a universal response to cellular stress. *Nat. Commun.* *7*, 10662.

Deyoung, M.P., Horak, P., Sofer, A., Sgroi, D., and Ellisen, L.W. (2008). Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev.* *22*, 239–251.

Dibble, C.C., and Cantley, L.C. (2015). Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol.* *25*, 545–555.

Dibble, C.C., Asara, J.M., and Manning, B.D. (2009). Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1. *Mol. Cell. Biol.* *29*, 5657–5670.

- Dibble, C.C., Elis, W., Menon, S., Qin, W., Klekota, J., Asara, J.M., Finan, P.M., Kwiatkowski, D.J., Murphy, L.O., and Manning, B.D. (2012). TBC1D7 Is a Third Subunit of the TSC1-TSC2 Complex Upstream of mTORC1. *Mol. Cell* 47, 535–546.
- Ding, M., Bruick, R.K., and Yu, Y. (2016). Secreted IGFBP5 mediates mTORC1-dependent feedback inhibition of IGF-1 signalling. *Nat. Cell Biol.* 18, 319–327.
- Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E., and Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science* 314, 467–471.
- Duan, S., Skaar, J.R., Kuchay, S., Toschi, A., Kanarek, N., Ben-Neriah, Y., and Pagano, M. (2011). mTOR Generates an Auto-Amplification Loop by Triggering the β TrCP- and CK1 α -Dependent Degradation of DEPTOR. *Mol. Cell* 44, 317–324.
- Düvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S., Vander Heiden, M.G., MacKeigan, J.P., Finan, P.M., Clish, C.B., Murphy, L.O., and Manning, B.D. (2010). Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol. Cell* 39, 171–183.
- Efeyan, A., Zoncu, R., Chang, S., Gumper, I., Snitkin, H., Wolfson, R.L., Kirak, O., Sabatini, D.D., and Sabatini, D.M. (2013). Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. *Nature* 493, 679–683.
- Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* 294, 1942–1945.
- Feun, L., You, M., Wu, C.J., Kuo, M.T., Wangpaichitr, M., Spector, S., and Savaraj, N. (2008). Arginine Deprivation as a Targeted Therapy for Cancer. *Curr. Pharm. Des.* 14, 1049–1057.
- Franke, T.F., Yang, S.-I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., and Tsichlis, P.N. (1995). The Protein Kinase Encoded by the Akt Proto-Oncogene Is a Target of the PDGF-Activated Phosphatidylinositol 3-Kinase. *Cell* 81, 727–736.
- Frias, M.A., Thoreen, C.C., Jaffe, J.D., Schroder, W., Sculley, T., Carr, S.A., and Sabatini, D.M. (2006). mSin1 Is Necessary for Akt/PKB Phosphorylation, and Its Isoforms Define Three Distinct mTORC2s. *Curr. Biol.* 16, 1865–1870.
- Ganley, I.G., Lam, D.H., Wang, J., Ding, X., Chen, S., and Jiang, X. (2009). ULK1·ATG13·FIP200 complex mediates mTOR signaling and is essential for autophagy. *J. Biol. Chem.* 284, 12297–12305.

Gao, D., Inuzuka, H., Tan, M.-K.M., Fukushima, H., Locasale, J.W., Liu, P., Wan, L., Zhai, B., Chin, Y.R., Shaik, S., Lyssiotis, C.A., Gygi, S.P., Toker, A., Cantley, L.C., Asara, J.M., Harper, J.W., and Wei, W. (2011). mTOR Drives Its Own Activation via SCF β TrCP-Dependent Degradation of the mTOR Inhibitor DEPTOR. *Mol. Cell* 44, 290–303.

Gaubitz, C., Oliveira, T.M., Prouteau, M., Leitner, A., Karuppasamy, M., Konstantinidou, G., Rispal, D., Eltschinger, S., Robinson, G.C., Thore, S., Aebersold, R., Schaffitzel, C., and Loewith, R. (2015). Molecular Basis of the Rapamycin Insensitivity of Target Of Rapamycin Complex 2. *Mol. Cell* 58, 977–988.

Gong, R., Li, L., Liu, Y., Wang, P., Yang, H., Wang, L., Cheng, J., Guan, K.L., and Xu, Y. (2011). Crystal structure of the Gtr1p-Gtr2p complex reveals new insights into the amino acid-induced TORC1 activation. *Genes Dev.* 25, 1668–1673.

Gwinn, D.M., Shackelford, D.B., 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. *Mol. Cell* 30, 214–226.

Vander Haar, E., Lee, S.-I., Bandhakavi, S., Griffin, T.J., and Kim, D.-H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat. Cell Biol.* 9, 316–323.

Hall, M.N. (2013). On mTOR nomenclature. *Biochem. Soc. Trans.* 41, 887–888.

Halland, N., Schmidt, F., Weiss, T., Saas, J., Li, Z., Czech, J., Dreyer, M., Hofmeister, A., Mertsch, K., Dietz, U., Strübing, C., and Nazare, M. (2015). Discovery of N-[4-(1H-Pyrazolo[3,4-b]pyrazin-6-yl)-phenyl]-sulfonamides as Highly Active and Selective SGK1 Inhibitors. *ACS Med. Chem. Lett.* 6, 73–78.

Han, J., Lee, J.D., Bibbs, L., and Ulevitch, R.J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265, 808–811.

Han, J., Li, E., Chen, L., Zhang, Y., Wei, F., Liu, J., Deng, H., and Wang, Y. (2015). The CREB coactivator CRTC2 controls hepatic lipid metabolism by regulating SREBP1. *Nature* 524, 243–246.

Hanker, A.B., Mitin, N., Wilder, R.S., Henske, E.P., Tamanoi, F., Cox, A.D., and Der, C.J. (2010). Differential requirement of CAAX-mediated posttranslational processing for Rheb localization and signaling. *Oncogene* 29, 380–391.

Hara, K., Yonezawa, K., Weng, Q., Kozlowski, M.T., Belham, C., and Avruch, J. (1998). Amino Acid Sufficiency and mTOR Regulate p70 S6 Kinase and eIF-4E BP1 through a Common

Effector Mechanism. *J. Biol. Chem.* **273**, 14484–14494.

Hara, K., Maruki, Y., Long, X., Yoshino, K. ichi, Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110**, 177–189.

Harrington, L.S., Findlay, G.M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N.R., Cheng, S., Shepherd, P.R., Gout, I., Downes, C.P., and Lamb, R.F. (2004). The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J. Cell Biol.* **166**, 213–223.

Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P.M., Olefsky, J.M., and Kobayashi, M. (2000). A Rapamycin-Sensitive Pathway Down-Regulates Insulin Signaling via Phosphorylation and Proteasomal Degradation of Insulin Receptor Substrate-1. *Mol. Endocrinol.* **14**, 783–794.

He, C., and Kilonsky, D.J. (2009). Regulation Mechanisms and Signaling Pathways of Autophagy. *Annu. Rev. Genet.* **43**, 67–93.

Heitman, J., Movva, N.R., and Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**, 905–909.

Henske, E.P., Jóźwiak, S., Kingswood, J.C., Sampson, J.R., and Thiele, E.A. (2016). Tuberous sclerosis complex. *Nat. Rev. Dis. Prim.* **2**, 16035.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein-responsive and UV-responsive protein-kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* **7**, 2135–2148.

Hirose, E., Nakashima, N., Sekiguchi, T., and Nishimoto, T. (1998). RagA is a functional homologue of *S. cerevisiae* Gtr1p involved in the Ran/Gsp1-GTPase pathway. *J. Cell Sci.* **111**, 11–21.

Holz, M.K., Ballif, B.A., Gygi, S.P., and Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* **123**, 569–580.

Hosokawa, N., Hara, T., Kaisuka, T., Kishi, C.C., Takamura, A., Miura, Y., Iemura, S., Natsume, T., Takehana, K., Yamada, N., Guan, J.-L., Oshiro, N., and Mizushima, N. (2009). Nutrient-dependent mTORC1 Association with the ULK1-Atg13-FIP200 Complex Required for Autophagy. *Mol. Biol. Cell* **20**, 1981–1991.

Hsu, P.P., Kang, S.A., Rameseder, J., Zhang, Y., Ottina, K.A., Lim, D., Peterson, T.R., Choi, Y., Gray, N.S., Yaffe, M.B., Marto, J.A., and Sabatini, D.M. (2011). The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science* 332, 1317–1322.

Huang, C.-C., Tsai, S.-T., Kuo, C.-C., Chang, J.S., Jin, Y.-T., Chang, J.-Y., and Hsiao, J.-R. (2012). Arginine deprivation as a new treatment strategy for head and neck cancer. *Oral Oncol.* 48, 1227–1235.

Hudson, C.C., Liu, M., Chiang, G.G., Otterness, D.M., Loomis, D.C., Kaper, F., Giaccia, A.J., and Abraham, R.T. (2002). Regulation of hypoxia-inducible factor 1 α expression and function by the mammalian target of rapamycin. *Mol. Cell. Biol.* 22, 7004–7014.

Huffman, T.A., Mothe-Satney, I., and Lawrence, J.C. (2002). Insulin-stimulated phosphorylation of lipin mediated by the mammalian target of rapamycin. *Proc. Natl. Acad. Sci. U. S. A.* 99, 1047–1052.

Iiboshi, Y., Papst, P.J., Kawasome, H., Hosoi, H., Abraham, R.T., Houghton, P.J., and Terada, N. (1999). Amino Acid-dependent Control of p70 s6k. *J. Biol. Chem.* 274, 1092–1099.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.-L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* 4, 648–657.

Inoki, K., Li, Y., Xu, T., and Guan, K.-L. (2003a). Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* 17, 1829–1834.

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

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rügge, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell Biol.* 6, 1122–1128.

Jefferies, H.B.J., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B., and Thomas, G. (1997). Rapamycin suppresses 5' TOP mRNA translation through inhibition of p70s6k. *EMBO J.* 16, 3693–3704.

Jeong, J.H., Lee, K.H., Kim, Y.M., Kim, D.H., Oh, B.H., and Kim, Y.G. (2012). Crystal structure of the Gtr1pGTP-Gtr2pGDP protein complex reveals large structural rearrangements triggered by GTP-to-GDP conversion. *J. Biol. Chem.* 287, 29648–29653.

- Jewell, J.L., Kim, Y.C., Russell, R.C., Yu, F., Park, H.W., Plouffe, S.W., Tagliabracci, V.S., and Guan, K. (2015). Differential regulation of mTORC1 by leucine and glutamine. *Science* *347*, 194–198.
- Jung, C.H., Jun, C.B., Ro, S.-H., Kim, Y.-M., Otto, N.M., Cao, J., Kundu, M., and Kim, D.-H. (2009). ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery. *Mol. Biol. Cell* *20*, 1992–2003.
- Kantidakis, T., Ramsbottom, B.A., Birch, J.L., Dowding, S.N., and White, R.J. (2010). mTOR associates with TFIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proc. Natl. Acad. Sci.* *107*, 11823–11828.
- Kawase, T., Orikasa, M., Ogata, S., and Burns, D.M. (1995). Protein tyrosine phosphorylation induced by Epidermal Growth Factor and Insulin-Like Growth Factor-I in a rat clonal dental pulp-cell line. *Arch. Oral Biol.* *40*, 921–929.
- Kelly, M.P., Jungbluth, A.A., Wu, B.W., Bomalaski, J., Old, L.J., and Ritter, G. (2012). Arginine deiminase PEG20 inhibits growth of small cell lung cancers lacking expression of arginosuccinate synthetase. *Br. J. Cancer* *106*, 324–332.
- Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* *110*, 163–175.
- Kim, D.H., Sarbassov, D.D., Ali, S.M., Latek, R.R., Guntur, K.V.P., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2003). GβL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol. Cell* *11*, 895–904.
- Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T.P., and Guan, K.-L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. *Nat. Cell Biol.* *10*, 935–945.
- Kim, S.G., Buel, G.R., and Blenis, J. (2013a). Nutrient regulation of the mTOR Complex 1 signaling pathway. *Mol. Cells* *35*, 1–11.
- Kim, S.G., Hoffman, G.R., Poulogiannis, G., Buel, G.R., Jang, Y.J., Lee, K.W., Kim, B.Y., Erikson, R.L., Cantley, L.C., Choo, A.Y., and Blenis, J. (2013b). Metabolic Stress Controls mTORC1 Lysosomal Localization and Dimerization by Regulating the TTT-RUVBL1/2 Complex. *Mol. Cell* *49*, 172–185.
- Kim, Y.C., Park, H.W., Sciarretta, S., Mo, J.-S., Jewell, J.L., Russell, R.C., Wu, X., Sadoshima, J.,

and Guan, K.-L. (2014). Rag GTPases are cardioprotective by regulating lysosomal function. *Nat. Commun.* *5*, 4241.

Klebe, C., Prinz, H., Wittinghofer, A., and Goody, R.S. (1995). The kinetic mechanism of Ran-nucleotide exchange catalyzed by RCC1. *Biochemistry* *34*, 12543–12552.

Koltin, Y., Faucette, L., Bergsma, D.J., Levy, M.A., Cafferkey, R., Koser, P.L., Johnson, R.K., and Livi, G.P. (1991). Rapamycin sensitivity in *Saccharomyces cerevisiae* is mediated by a peptidyl-prolyl cis-trans isomerase related to human FK506-binding protein. *Mol. Cell. Biol.* *11*, 1718–1723.

Koren, I., Reem, E., and Kimchi, A. (2010). DAP1, a Novel Substrate of mTOR, Negatively Regulates Autophagy. *Curr. Biol.* *20*, 1093–1098.

Laplante, M., and Sabatini, D.M. (2012). mTOR signaling in growth control and disease. *Cell* *148*, 274–293.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P.C., and Semenza, G.L. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol. Cell. Biol.* *21*, 3995–4004.

Li, S., Brown, M.S., and Goldstein, J.L. (2010). Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. *Proc. Natl. Acad. Sci. U. S. A.* *107*, 3441–3446.

Li, S., Ogawa, W., Emi, A., Hayashi, K., Senga, Y., Nomura, K., Hara, K., Yu, D., and Kasuga, M. (2011). Role of S6K1 in regulation of SREBP1c expression in the liver. *Biochem. Biophys. Res. Commun.* *412*, 197–202.

Liu, L., Cash, T.P., Jones, R.G., Keith, B., Thompson, C.B., and Simon, M.C. (2006). Hypoxia-Induced Energy Stress Regulates mRNA Translation and Cell Growth. *Mol. Cell* *21*, 521–531.

Liu, P., Gan, W., Chin, Y.R., Ogura, K., Guo, J., Zhang, J., Wang, B., Blenis, J., Cantley, L.C., Toker, A., Su, B., and Wei, W. (2015). PtdIns(3,4,5)P3-Dependent Activation of the mTORC2 Kinase Complex. *Cancer Discov.* *5*, 1194–1209.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M.N. (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell* *10*, 457–468.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005). Rheb binds and regulates the mTOR kinase. *Curr. Biol.* *15*, 702–713.

Luiken, J.J.F.P., Blommaart, E.F.C., Boon, L., van Woerkorn, G.M., and Meijer, A.J. (1994). Cell swelling and the control of autophagic proteolysis in hepatocytes: involvement of phosphorylation of ribosomal protein S6? *Biochem. Soc. Trans.* *22*, 508–511.

Ma, X.M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. *Nat.Rev.Mol.Cell Biol.* *10*, 307–318.

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P.P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk: Implications for tuberous sclerosis and cancer pathogenesis. *Cell* *121*, 179–193.

Ma, X.M., Yoon, S.O., Richardson, C.J., Jülich, K., and Blenis, J. (2008). SKAR Links Pre-mRNA Splicing to mTOR/S6K1-Mediated Enhanced Translation Efficiency of Spliced mRNAs. *Cell* *133*, 303–313.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the Tuberous Sclerosis Complex-2 Tumor Suppressor Gene Product Tuberin as a Target of the Phosphoinositide 3-Kinase/Akt Pathway. *Mol. Cell* *10*, 151–162.

Martel, R.R., Klicius, J., and Galet, S. (1977). Inhibition of the immune response by rapamycin, a new antifungal antibiotic. *Can. J. Physiol. Pharmacol.* *55*, 48–51.

Mayer, C., Zhao, J., Yuan, X., and Grummt, I. (2004). mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev.* *18*, 423–434.

Menon, S., Dibble, C.C., Talbott, G., Hoxhaj, G., Valvezan, A.J., Takahashi, H., Cantley, L.C., and Manning, B.D. (2014). Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. *Cell* *156*, 771–785.

Nada, S., Hondo, A., Kasai, A., Koike, M., Saito, K., Uchiyama, Y., and Okada, M. (2009). The novel lipid raft adaptor p18 controls endosome dynamics by anchoring the MEK-ERK pathway to late endosomes. *EMBO J.* *28*, 477–489.

Nakashima, N., Noguchi, E., and Nishimoto, T. (1999). *Saccharomyces cerevisiae* putative G protein, Gtr1p, which forms complexes with itself and a novel protein designated as Gtr2p, negatively regulates the Ran/Gsp1p G protein cycle through Gtr2p. *Genetics* *152*, 853–867.

Oshiro, N., Takahashi, R., Yoshino, K.I., Tanimura, K., Nakashima, A., Eguchi, S., Miyamoto, T., Hara, K., Takehana, K., Avruch, J., Kikkawa, U., and Yonezawa, K. (2007). The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1. *J. Biol. Chem.* *282*, 20329–20339.

Pearce, L.R., Huang, X., Boudeau, J., Pawłowski, R., Wullschleger, S., Deak, M., Ibrahim, A.F.M., Gourlay, R., Magnuson, M.A., and Alessi, D.R. (2007). Identification of Protor as a novel Rictor-binding component of mTOR complex-2. *Biochem. J.* *405*, 513–522.

Pearce, L.R., Komander, D., and Alessi, D.R. (2010). The nuts and bolts of AGC protein kinases. *Nat. Rev. Mol. Cell Biol.* *11*, 9–22.

Peña-Llopis, S., Vega-Rubin-de-Celis, S., Schwartz, J.C., Wolff, N.C., Tran, T.A.T., Zou, L., Xie, X.-J., Corey, D.R., and Brugarolas, J. (2011). Regulation of TFEB and V-ATPases by mTORC1. *EMBO J.* *30*, 3242–3258.

Peng, M., Yin, N., and Li, M.O. (2014). Sestrins Function as Guanine Nucleotide Dissociation Inhibitors for Rag GTPases to Control mTORC1 Signaling. *Cell* *159*, 122–133.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S., and Sabatini, D.M. (2009). DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival. *Cell* *137*, 873–886.

Peterson, T.R., Sengupta, S.S., Harris, T.E., Carmack, A.E., Kang, S.A., Balderas, E., Guertin, D.A., Madden, K.L., Carpenter, A.E., Finck, B.N., and Sabatini, D.M. (2011). mTOR Complex 1 Regulates Lipin 1 Localization to Control the SREBP Pathway. *Cell* *146*, 408–420.

Petit, C.S., Roczniak-Ferguson, A., and Ferguson, S.M. (2013). Recruitment of folliculin to lysosomes supports the amino acid-dependent activation of rag gtpases. *J. Cell Biol.* *202*, 1107–1122.

Porstmann, T., Santos, C.R., Griffiths, B., Cully, M., Wu, M., Leever, S., Griffiths, J.R., Chung, Y.-L.L., and Schulze, A. (2008). SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth. *Cell Metab.* *8*, 224–236.

Potter, C.J., Pedraza, L.G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. *Nat. Cell Biol.* *4*, 658–665.

Raught, B., Peiretti, F., Gingras, A.-C., Livingstone, M., Shahbazian, D., Mayeur, G.L., Polakiewicz, R.D., Sonenberg, N., and Hershey, J.W. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. *EMBO J.* *23*, 1761–1769.

Robitaille, A.M., Christen, S., Shimobayashi, M., Cornu, M., Fava, L.L., Moes, S., Prescianotto-Baschong, C., Sauer, U., Jenoe, P., and Hall, M.N. (2013). Quantitative Phosphoproteomics Reveal mTORC1 Activates de Novo Pyrimidine Synthesis. *Science* 339, 471–484.

Rousseau, A., and Bertolotti, A. (2016). An evolutionarily conserved pathway controls proteasome homeostasis. *Nature* 536, 184–189.

Roux, P.P., Ballif, B.A., Anjum, R., Gygi, S.P., and Blenis, J. (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc. Natl. Acad. Sci. U. S. A.* 101, 13489–13494.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* 78, 35–43.

Sabers, C.J., Martin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G., and Abraham, R.T. (1995). Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* 270, 815–822.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. *Mol. Cell* 25, 903–915.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320, 1496–1501.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* 141, 290–303.

Sarbassov, D.D., Ali, S.M., Kim, D.-H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a Novel Binding Partner of mTOR, Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton. *Curr. Biol.* 14, 1296–1302.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307, 1098–1101.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and

Akt/PKB. *Mol. Cell* 22, 159–168.

Saxton, R.A., Knockenhauer, K.E., Wolfson, R.L., Chantranupong, L., Pacold, M.E., Wang, T., Schwartz, T.U., and Sabatini, D.M. (2016a). Structural basis for leucine sensing by the Sestrin2-mTORC1 pathway. *Science* 351, 53–58.

Saxton, R.A., Chantranupong, L., Knockenhauer, K.E., Schwartz, T.U., and Sabatini, D.M. (2016b). Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. *Nature*.

Schalm, S.S., and Blenis, J. (2002). Identification of a Conserved Motif Required for mTOR Signaling. *Curr. Biol.* 12, 632–639.

Schmidt, E.K., Clavarino, G., Ceppi, M., and Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. *Nat. Methods* 6, 275–277.

Schürmann, A., Brauers, A., Maßmann, S., Becker, W., and Joost, H.G. (1995). Cloning of a novel family of mammalian GTP-binding proteins (RagA, RagBs, RagB1) with remote similarity to the Ras-related GTPases. *J. Biol. Chem.* 270, 28982–28988.

Sekiguchi, T., Hirose, E., Nakashima, N., Li, M., and Nishimoto, T. (2001). Novel G Proteins, Rag C and Rag D, Interact with GTP-binding Proteins, Rag A and Rag B. *J. Biol. Chem.* 276, 7246–7257.

Settembre, C., Di Malta, C., Polito, V.A., Garcia Arencibia, M., Vetrini, F., Erdin, S., Erdin, S.U., Huynh, T., Medina, D., Colella, P., Sardiello, M., Rubinsztein, D.C., and Ballabio, A. (2011). TFEB links autophagy to lysosomal biogenesis. *Science* 332, 1429–1433.

Settembre, C., Zoncu, R., Medina, D.L., Vetrini, F., Erdin, S., Erdin, S., Huynh, T., Ferron, M., Karsenty, G., Vellard, M.C., Facchinetti, V., Sabatini, D.M., and Ballabio, A. (2012). A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J.* 31, 1095–1108.

Sheen, J.H., Zoncu, R., Kim, D., and Sabatini, D.M. (2011). Defective Regulation of Autophagy upon Leucine Deprivation Reveals a Targetable Liability of Human Melanoma Cells In Vitro and In Vivo. *Cancer Cell* 19, 613–628.

Shen, K., Sidik, H., and Talbot, W.S. (2016). The Rag-Ragulator Complex Regulates Lysosome Function and Phagocytic Flux in Microglia. *Cell Rep.* 14, 547–559.

Shor, B., Wu, J., Shakey, Q., Toral-Barza, L., Shi, C., Follettie, M., and Yu, K. (2010).

Requirement of the mTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. *J. Biol. Chem.* **285**, 15380–15392.

Shoshani, T., Faerman, A., Mett, I., Zelin, E., Tenne, T., Gorodin, S., Moshel, Y., Elbaz, S., Budanov, A., Chajut, A., Kalinski, H., Kamer, I., Rozen, A., Mor, O., Keshet, E., Leshkowitz, D., Einat, P., Skaliter, R., and Feinstein, E. (2002). Identification of a Novel Hypoxia-Inducible Factor 1-Responsive Gene, RTP801, Involved in Apoptosis. *Mol. Cell. Biol.* **22**, 2283–2293.

Singh, K., Sun, S., and Vezin, C. (1979). Rapamycin (AY-22,989), a new antifungal antibiotic. Mechanism of action. *J. Antibiot. (Tokyo)*. **53**, 160.

Sofer, A., Lei, K., Johannessen, C.M., and Ellisen, L.W. (2005). Regulation of mTOR and Cell Growth in Response to Energy Stress by REDD1. *Mol. Cell. Biol.* **25**, 5834–5845.

Sprang, S.R., and Coleman, D.E. (1998). Invasion of the nucleotide snatchers: Structural insights into the mechanism of G protein GEFs. *Cell* **95**, 155–158.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J., and Meyuhas, O. (2001). Amino Acid-Induced Translation of TOP mRNAs Is Fully Dependent on Phosphatidylinositol 3-Kinase-Mediated Signaling, Is Partially Inhibited by Rapamycin, and Is Independent of S6K1 and rpS6 Phosphorylation. *Mol. Cell. Biol.* **21**, 8671–8683.

Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C., and Blenis, J. (2003). Tuberous Sclerosis Complex Gene Products, Tuberin and Hamartin, Control mTOR Signaling by Acting as a GTPase-Activating Protein Complex toward Rheb. *Curr. Biol.* **13**, 1259–1268.

Teleman, A.A., Chen, Y.-W., and Cohen, S.M. (2005). 4EBP functions as a metabolic brake used under stress conditions but not during normal growth. *Genes Dev.* **19**, 1844–1848.

Thedieck, K., Polak, P., Kim, M.L., Molle, K.D., Cohen, A., Jen, P., Arrieumerlou, C., and Hall, M.N. (2007). PRAS40 and PRR5-like protein are new mTOR interactors that regulate apoptosis. *PLoS One* **2**, e1217.

Toschi, A., Lee, E., Xu, L., Garcia, A., Gadir, N., and Foster, D.A. (2009). Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin. *Mol. Cell. Biol.* **29**, 1411–1420.

Tsun, Z.Y., Bar-Peled, L., Chantranupong, L., Zoncu, R., Wang, T., Kim, C., Spooner, E., and Sabatini, D. (2013). The folliculin tumor suppressor is a GAP for the RagC/D GTPases that signal amino acid levels to mTORC1. *Mol. Cell* **52**, 495–505.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J., and Thomas, G. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* *431*, 200–205.

Vezina, C., Kudelski, A., and Sehgal, S.N. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot. (Tokyo)*. *28*, 721–726.

Wang, B.T., Ducker, G.S., Barczak, A.J., Barbeau, R., Erle, D.J., and Shokat, K.M. (2011). The mammalian target of rapamycin regulates cholesterol biosynthetic gene expression and exhibits a rapamycin-resistant transcriptional profile. *Proc. Natl. Acad. Sci. U. S. A.* *108*, 15201–15206.

Wang, L., Harris, T.E., Roth, R.A., and Lawrence, J.C. (2007). PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *J. Biol. Chem.* *282*, 20036–20044.

Wang, S., Tsun, Z.-Y., Wolfson, R.L., Shen, K., Wyant, G.A., Plovanich, M., Yuan, E., Jones, T., Chantranupong, L., Comb, W., Wang, T., Bar-Peled, L., Zoncu, R., Straub, C., Kim, C., Park, J., Sabatini, B., and Sabatini, D.M. (2015). Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science* *347*, 188–194.

Wek, S.A., Zhu, S., and Wek, R.C. (1995). The Histidyl-tRNA Synthetase-Related Sequence in the eIF-2 α Protein Kinase GCN2 Interacts with tRNA and Is Required for Activation in Response to Starvation for Different Amino Acids. *Mol. Cell. Biol.* *15*, 4497–4506.

Wolfson, R.L., Chantranupong, L., Saxton, R.A., Shen, K., Scaria, S.M., Cantor, J.R., and Sabatini, D.M. (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science* *351*, 43–48.

Wullschleger, S., Loewith, R., Oppliger, W., and Hall, M.N. (2005). Molecular Organization of Target of Rapamycin Complex 2. *J. Biol. Chem.* *280*, 30697–30704.

Yang, H., Rudge, D.G., Koos, J.D., Vaidialingam, B., Yang, H.J., and Pavletich, N.P. (2013). mTOR kinase structure, mechanism and regulation. *Nature* *497*, 217–223.

Yang, Q., Inoki, K., Ikenoue, T., and Guan, K.L. (2006a). Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev.* *20*, 2820–2832.

- Yang, Q., Inoki, K., Kim, E., and Guan, K.-L. (2006b). TSC1/TSC2 and Rheb have different effects on TORC1 and TORC2 activity. *Proc. Natl. Acad. Sci. U. S. A.* *103*, 6811–6816.
- Ye, J., Palm, W., Peng, M., King, B., Lindsten, T., Li, M.O., Koumenis, C., and Thompson, C.B. (2015). GCN2 sustains mTORC1 suppression upon amino acid deprivation by inducing Sestrin2. *Genes Dev.* *29*, 2331–2336.
- Yecies, J.L., Zhang, H.H., Menon, S., Liu, S., Yecies, D., Lipovsky, A.I., Gorgun, C., Kwiatkowski, D.J., Hotamisligil, G.S., Lee, C.H., and Manning, B.D. (2011). Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. *Cell Metab.* *14*, 21–32.
- Yip, C.K., Murata, K., Walz, T., Sabatini, D.M., and Kang, S.A. (2010). Structure of the Human mTOR Complex I and Its Implications for Rapamycin Inhibition. *Mol. Cell* *38*, 768–774.
- Yoon, M.S., Rosenberger, C.L., Wu, C., Truong, N., Sweedler, J. V., and Chen, J. (2015). Rapid mitogenic regulation of the mTORC1 Inhibitor, DEPTOR, by phosphatidic acid. *Mol. Cell* *58*, 549–556.
- Yu, L., McPhee, C.K., Zheng, L., Mardones, G. a, Rong, Y., Peng, J., Mi, N., Zhao, Y., Liu, Z., Wan, F., Hailey, D.W., Oorschot, V., Klumperman, J., Baehrecke, E.H., and Lenardo, M.J. (2010). Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature* *465*, 942–946.
- Yu, Y., Yoon, S.-O., Poulgiannis, G., Yang, Q., Ma, X.M., Villén, J., Kubica, N., Hoffman, G.R., Cantley, L.C., Gygi, S.P., and Blenis, J. (2011). Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science* *332*, 1322–1326.
- Zhang, Y., Nicholatos, J., Dreier, J.R., Ricoult, S.J.H., Widenmaier, S.B., Hotamisligil, G.S., Kwiatkowski, D.J., and Manning, B.D. (2014). Coordinated regulation of protein synthesis and degradation by mTORC1. *Nature* *513*, 440–443.
- Zhao, J., Zhai, B., Gygi, S.P., and Goldberg, A.L. (2015). mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. *Proc. Natl. Acad. Sci.* *112*, 15790–15797.
- Zhao, Y., Xiong, X., and Sun, Y. (2011). DEPTOR, an mTOR Inhibitor, is a physiological substrate of SCF β TrCP E3 ubiquitin ligase and regulates survival and autophagy. *Mol. Cell* *44*, 304–316.
- Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., and Sabatini, D.M. (2011). mTORC1

Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H⁺-ATPase. *Science* 334, 678–683.