

UPSTREAM COMPONENTS OF MTORC1

by

Li Li

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Doctoral Committee:

Professor Kunliang Guan, Chair
Professor Randal J. Kaufman
Associate Professor Anne B. Vojtek
Associate Professor Martin G. Myers
Assistant Professor Diane C. Fingar

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This work is dedicated to my parents. Without their support and guidance, I would not be where I am today. I also dedicate this work to my husband, for pushing me to the best I can. Last, and most importantly, I would like to dedicate this work to my fifteen months son for being well behaved during the last few months. You are the treasure of my life.

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ABSTRACT

The target of rapamycin (TOR) is an evolutionally conserved protein kinase that belongs to the phosphoinositide-3-kinase-related protein kinase family. It resides in two distinct protein complexes which in mammals are referred to as mTORC1 and mTORC2. mTOR pathway plays a central role in cell growth and metabolism by integrating extracellular and intracellular signals, including nutrients, growth factors, energy and stresses to regulate a wide array of cellular processes through its target proteins such as S6K, 4EBP1, ULK1, AKT, SGK and PKC. Dysregulation of mTOR is involved in many human diseases, including cancer, cardiovascular disease, autoimmunity, and metabolic disorders. Therefore, understanding of mTOR regulation and function will shed light on developing therapeutics targeting those diseases.

Significant emphasis has been placed on how mTOR activity is regulated by different signals. Recent biochemical and genetic studies made great progress demonstrating the Rag family GTPases, which belong to the ras superfamily, are key mediators between amino acids and mTORC1 activation. The Rag heterodimer can directly bind to raptor, a key subunit of mTORC1. It was proposed that amino acids promote the translocation of mTORC1 to the surface of the lysosome compartment in a Rag-dependent manner, where mTORC1 can colocalize with its activator, RHEB. In addition, a protein complex called Ragulator, which interacts with Rag GTPases, is reported to be responsible for mTORC1 translocation and necessary for its amino acid-dependent activation.

In this thesis, I summarize my research in the past few years in three aspects. First, I identified Rab and Arf family GTPases as indispensable mediators of mTORC1 activity. Second, I identified MARK4 as an important negative regulator of mTORC1

activity. Although these studies did not fully answer the question where are these proteins positioned on the mTOR pathway, my research raises many important questions and will inspire further scientific inquiries.

CHAPTER 1

INTRODUCTION

The story of rapamycin began in 1965, when it was isolated from a bacterial strain in soil and showed a potent anti-fungal property(1). Today, rapamycin is a FDA approved drug for immunosuppression and a drug for late stage renal cancer. Since its discovery, rapamycin keeps bringing surprises and excitement to the scientific world. In 1990s, a series of study in yeast and mammals identified a large 250 kDa protein as its target, named target of rapamycin (TOR)(2-6). TOR is a member of the phosphoinositide-3-kinase-related protein kinase family, which includes ATM, ATR, DNA-PK, and TOR. Upon entering cells, Rapamycin binds to FBKP12; the complex then interacts with and perturbs the function of TOR(4, 6). Using rapamycin as a tool, scientists showed TOR is a central regulator of cell growth.

The mammalian target of rapamycin (mTOR) signaling pathway integrates various signals including nutrients, growth factors, stress and energy to regulate cell growth and metabolism(7). A twist in the TOR story is the finding that mTOR forms two distinct structural and functional complexes, a nutrient and rapamycin sensitive complex mTORC1 and a nutrient and rapamycin insensitive complex mTORC2(8). mTORC1 consists of mTOR, raptor, mLST8, Deptor and PRAS40(9-14) while mTORC2 consists of mTOR, rictor, sin1, Deptor, mLST8(15-18). Characterized substrates of mTORC1 include the ribosomal S6 kinase (S6K), eukaryote initiation factor 4E binding protein (4EBP1), and the Unc-51 like kinase 1 ULK1(19-22). Therefore, mTORC1 plays a critical role in translation, cell growth, and autophagy. In contrast, mTORC2 is required for phosphorylation of AKT, SGK, and conventional PKC, suggesting that the two TOR

complexes have different physiological functions(23-26). This chapter will mainly discuss the regulation of mTORC1 by various signals.

The known components of mTORC1 are mTOR, which is the catalytic subunit of the complex; regulatory-associated protein of mTOR (raptor); G protein beta subunit like (GbetaL, also known as mLST8); proline rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor). Among these components, PRAS40 and Deptor are negative regulators of mTORC1 activity, but the exact functions of these components are far from well understood. Studies continue to bring new insights about how mTORC1 senses different signals and leads to the biological output.

mTORC1 Activation by Multiple Signals:

Amino acids are one of the most potent signals for mTORC1 activation(27). In recent years, scientists in the field have made significant achievement in understanding how amino acids regulate mTORC1. I will discuss this part in detail in later chapter.

Growth factors mediated mTORC1 activation is so far the most studied and thus well-characterized mTORC1 regulator. Growth factors and hormones, such as insulin, regulate mTORC1 through the PI3K pathway. The ligand-receptor interaction triggered intermolecular phosphorylation of the receptor and the subsequent recruitment of IRS1 and IRS2 to the cell membrane. PI3K then bound to IRS and converts PIP2 in the cell membrane to PIP3. PIP3 serves as a docking site for PDK1 and Akt, resulting in the phosphorylation and activation of Akt by PDK1(28). AKT has been shown to phosphorylate TSC2 at multiple sites, which is the major upstream negative regulator of mTORC1(29, 30). Studies showed that the phosphorylation creates a binding site for 14-3-3(31). The TSC1/TSC2 complex has GTPase activating protein (GAP) activity towards Rheb, which is a Ras family small GTPase(32-34). Binding of TSC2 to 14-3-3 disrupts the TSC1-Rheb interaction and activates Rheb. Rheb directly and potently activates mTORC1 when it is in a GTP bound status(12, 35). Additionally, AKT phosphorylates PRAS40, an mTORC1 component(11, 12). The phosphorylated PRAS40 dissociated from mTORC1 and lost its inhibition on mTORC1.

TSC1 and TSC2 are tumor suppressors mutated in the tuberous sclerosis, a genetic disorder characterized by benign tumor formation in a wide range of tissues(36). Given the fact that TSC1/TSC2 are phosphorylated by multiple signaling kinases, they may serve as an integration point to receive information from multiple pathways to modulate cell growth by regulating mTORC1 activity. Upon growth factor stimulation, the activated RAS signaling pathway may also lead to mTORC1 through ERK1/2 phosphorylation of TSC2(37). In TSC1 or TSC2 mutant cells, mTORC1 is constitutively active and is no longer regulated by growth factors, indicating a critical role of TSC1/TSC2 in mediating the growth factor signal to mTORC1 activation(30, 38).

AMP-activated protein kinase (AMPK) is the master player relaying energy status to mTORC1. In response to energy depletion, the regulatory subunits sense the drop in ATP: AMP level and activate the catalytic subunit. AMPK phosphorylates at least two residues on TSC2(39). The phosphorylation enhances TSC2 GAP activity towards Rheb and thus inhibits mTORC1 activation. The fact that in cells lacking TSC2, mTORC1 is still partially suppressed following AMPK activation suggests that additional AMPK substrates may directly or indirectly modulate mTORC1 activity. Recently, studies from Shaw lab showed AMPK directly phosphorylates raptor on two well-conserved serine residues, and this phosphorylation induces 14-3-3 binding to raptor and affects mTORC1 kinase activity(40). However, studies have to address the importance of each phosphorylation event in a physiological context.

Oxygen level is essential for cellular metabolism. It is not surprising that mTORC1 activity is also sensitive to oxygen deprivation. Upon exposure to hypoxia, inhibition of mTOR can occur through HIF1 (hypoxia inducible factor alpha) - independent pathways as well as HIF1-dependent pathways. In response to chronic hypoxia, the reduction of cell ATP level activates AMPK, which will then inhibit mTORC1 activity through the previous described mechanisms(41, 42). HIF1alpha is a transcription factor that is stabilized during hypoxia. Hypoxia can also activate TSC1/TSC2 through HIF1 driven expression of REDD1(43, 44). Recent studies suggest that REDD1 competes with TSC2 for 14-3-3 binding which will re-activate TSC1-TSC2

by releasing TSC2 from its growth factor-induced or energy-induced association with 14-3-3(45). Another point in mTOR signal where hypoxia may exert its effect is interaction between mTOR and RHEB. Several reports show that hypoxia inhibits mTOR activity through interfering with mTOR-RHEB interaction. During hypoxia, the promyelocytic leukaemia tumour suppressor (PML) has been reported to bind mTOR and inactivate it through sequestration in nuclear bodies(46). Similarly, the hypoxia-inducible proapoptotic protein BNIP3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3) is reported to regulate mTOR by direct binding to RHEB and disruption of its interaction with mTOR(47).

The plasma membrane is a semi-permeable membrane. A change in the solute concentration around a cell may cause the movement of water across cell membrane. Thus, it is not surprising that all organisms, especially unicellular organisms, which will be directly exposed to extracellular environment, have evolved a mechanism to respond to osmotic stress. It has been known for decades that osmotic stress induced by sorbitol treatment decreases S6K phosphorylation(48, 49). Yet, the detailed mechanism is still largely unknown. Some studies suggest the involvement of a phosphatase, since the phosphatase inhibitor calyculin A (but not other phosphatase inhibitors) was shown to prevent osmotic stress-induced inhibition of S6K(50). Studies also show that sorbitol's effect on mTOR is independent of AKT(50), TSC2(51), Rheb(33) and p38 MAPK(50, 52), a kinase that is activated by a variety of stress stimuli including osmotic stress and UV radiation. Another suggested mechanism is that osmotic stress might affect a subpopulation of mTORC1 that localize to mitochondria. Under hyperosmotic conditions, the mitochondrial proton gradient is perturbed. This affects the mTORC1 activity that associates with the outer mitochondrial membrane(53).

There are some other cellular conditions and signal inputs that were also shown to regulate mTORC1 activity, including DNA damage, ROS, mechanical stress, heat shock, inflammation and viral infection. For example, genotoxic stress activated p53 can activate AMPK through unknown mechanisms, which will in turn inhibit mTORC1 through mechanisms discussed above(54). In addition, p53 regulates the transcription of

two key negative regulators of mTORC1, PTEN and TSC2, thus negatively regulates mTORC1 activity(54, 55).

Recent advance on amino acid activation of mTORC1:

One of the most potent signals for mTORC1 activation is amino acid (27). How cell growth is coordinated with amino acids is a fundamental issue in cell biology. In the absence of amino acids, mTORC1 cannot be efficiently activated by other stimuli, such as insulin (27). There is conflicting evidence whether amino acids act through Rheb to stimulate mTORC1 (32, 56-58). However, neither TSC1 nor TSC2 is essential for amino acid-induced mTORC1 activation because amino acid deprivation still inactivates mTORC1 in TSC1^{-/-} or TSC2^{-/-} cells (57). However, the amino acid-stimulated mTORC1 activation is sensitive to wortmannin, indicating a possible role of PI3K in amino acid response. Vacuolar protein sorting 34, Vps34, is a class III PI3K, which is inhibited by wortmannin, and has been implicated in mTORC1 activation by amino acids. Knockdown of Vps34 suppressed mTOR activation by amino acids (58). Similarly, expression of the FYVE domain, which may function dominant-negatively by binding to phosphatidylinositol 3-phosphate (the product catalyzed by Vps34), blocked mTORC1 activation by amino acids. However, the function of Vps34 in amino acid signaling is disputed by genetic studies in *Drosophila*, in which mutation of Vps34 had no effect on mTORC1 activation by amino acids (59). Therefore, further experiments are needed to clarify whether Vps34 is indeed involved in amino acid signaling to mTORC1.

RNA interference screen using *Drosophila* cells has identified the MAP4K3 protein kinase as a potential regulator in dS6K phosphorylation in response to amino acids (60). A similar role of MAP4K3 has also been verified in mammalian cells. Interestingly, amino acids rapidly stimulated MAP4K3 activity, further supporting a role in amino acid response. The effect of MAP4K3 on mTORC1 is independent of TSC1 and TSC2, results consistent with other reports that amino acids regulate mTORC1 in a manner independent of TSC1/TSC2. However, MAP4K3 overexpression only partially delayed dephosphorylation of S6K upon amino acid withdrawal and the effect of

MAP4K3 knockdown on mTORC1 substrate phosphorylation was modest. Therefore, MAP4K3 may have a limited role in mTORC1 activation by amino acids.

1. Rag GTPases and amino acid-induced mTORC1 activation

The Ras family GTPases function as molecular switches in many signaling pathways. As discussed earlier in this chapter, for example, Rheb activates mTORC1 directly when in GTP form. These GTPases cycle between active GTP-bound state and inactive GDP-bound state. The Ras family GTPases are regulated by GEF (Guanine nucleotide exchange factors) and GAP (GTPase activating protein), which promote the activation and inhibition of target GTPases, respectively. Because the intrinsic GTPase activity of Ras family is rather low, GTP hydrolysis and inactivation of Ras family proteins are greatly enhanced by GAP. Similarly, GEF accelerates the nucleotide exchange, therefore activation of GTPases. In the mTOR pathway, TSC2 inhibits mTORC1 by acting as a GAP towards Rheb (32-34).

Among the signals that activate mTORC1, amino acids are one of the most potent, but the mechanism of mTORC1 activation by amino acid is largely unknown. Rag GTPases have been brought to attention recently because of their critical role in mediating amino acid signals to mTORC1 activation (61, 62). Rag GTPases (four in human, named RagA, B, C, and D) are distantly related to Ras. RagA and RagB were previously identified in efforts of cloning novel members of Ras GTPases, but their physiological functions were unclear. Rag A and Rag B share around 97% amino acid sequence identity (63). RagC and RagD were identified by virtue of their ability to form heterodimer with RagA or RagB in a yeast two hybrid screen. RagC and RagD are highly homologous to each other (around 81% sequence identity) while the homology between RagA/B and RagC/D is rather low (less than 30% identity in the N-terminal GTPase domain. The overall sequence identity is even lower) (64). Different from most of the Ras family members, the Rag GTPases are much bigger. In addition to their GTPase domain, Rags also have a C-terminal domain, which is important for dimerization. RagA/B forms heterodimer with RagC/D and the dimerization is independent of GTP/GDP loading (63). Most Ras family GTPases are modified by

isoprenylation in their C-terminal cysteine residues for membrane association. However, Rag GTPases lack the C-terminal cysteine for lipid modifications although they may associate with intracellular membrane.

Both genetic and biochemical studies support a conserved role of Rag in amino acid signaling. The function of Rag in mTOR signaling was discovered by an RNA interference-based screen for GTPases that were important for S6K phosphorylation (61). In parallel, RagC was identified as a protein co-purified with mTOR (62). Knockdown of dRagA or dRagC strongly decreased TORC1 activation in *Drosophila* S2 cells, as determined by a decreased phosphorylation of TORC1 substrate dS6K. Similarly, knockdown of RagA and RagB (both have redundant function) or RagC and RagD dramatically reduced the phosphorylation of S6K1 and 4EBP1 in mammalian cells. The Rag knockdown effect on mTORC1 was particularly dramatic in the presence of amino acids. Consistently, overexpression of the active GTP bound form of RagA/B activated mTORC1 even in the absence of amino acids. These data suggest that active Rag GTPases can substitute the amino acid signals and are sufficient to activate mTORC1. In contrast, expression of a dominant-negative RagA or RagB (GDP form mutant) potently inhibited mTORC1 activation even in the presence of amino acids, indicating that Rag is necessary for mTORC1 activation. Together, it is proposed that Rag GTPases mediate the signal from amino acids to mTORC1 activation (61, 62).

The function of Rag in TORC1 regulation is further supported by cell size analysis in both *Drosophila* and mammalian cells (61, 62). Expression of active dRagA in *Drosophila* fat body significantly increased cell size, indicating activation of TORC1. The effect of active dRagA on cell size was less obvious in the presence of sufficient nutrients but was enhanced by nutrient starvation. This is likely because TORC1 is already activated in the presence of sufficient nutrients. However, under nutrient starvation condition, TORC1 activity is low; therefore ectopic expression of active dRagA activates TORC1 and increases cell size. Genetic inactivation of dRagC also decreased fat body cell size in *Drosophila*, but the phenotype was evident only under nutrient sufficiency. Similar effect of RagB/D on mammalian cell size was observed.

Autophagy is a physiological response to nutrient starvation. Further supporting a role of Rag in nutrient signaling is the observation that active dRagA suppressed autophagy even under nutrient starvation. In mammalian cells, LC3 cleavage and lipid modification is a well-characterized marker for autophagy. Ectopic expression of active RagA inhibited LC3 cleavage while expression of dominant negative RagA promoted LC3 cleavage. These data not only indicate that the Rag GTPases play a key role in TORC1 activation but also demonstrate that the effect of Rag is specific to nutrient signaling (61).

The heterodimer formation of Rag GTPases has at least two functional consequences. Within the heterodimer, the two GTPases stabilize each other. In addition, the heterodimer is more active than each subunit to activate mTORC1. Interestingly, within a heterodimer, the two GTPases bind different guanine nucleotides, one binds GTP and the other binds GDP (65). The active form of Rag heterodimer consists of the GTP form of RagA/B and the GDP form of RagC/D. However, the contribution of RagA/B and RagC/D to mTORC1 activation is not equal (61). RagA/B have a dominant role over RagC/D to activate mTORC1. In other words, the Rag heterodimer activates mTORC1 as long as RagA/B is in GTP form regardless of the nucleotide status of the associated RagC/D. Complementarily, the Rag heterodimer suppresses mTORC1 activity as long as RagA/B is in GDP form regardless of the nucleotide status of the associated RagC/D (61, 62).

Unlike Rheb, which can directly activate mTORC1, Rag does not activate mTORC1 *in vitro* (61). However, Rag likely has a direct role in mTORC1 regulation because Rag interacts with raptor. Actually, Rag interaction with raptor is significantly stronger than Rheb. In Rag heterodimer, RagA/B is likely to be directly responsible for raptor binding. The interaction depends on GTP binding and the effector domain of RagA/B while the GTP binding status of RagC/D is not important for raptor interaction (unpublished observation). These results are consistent with the functional data that RagA/B act dominantly over RagC/D in mTORC1 activation.

Rag GTPases must be regulated by amino acids if they are critical in mediating amino acid signals. Indeed, amino acids stimulate RagB GTP binding and RagD GDP

binding, therefore activate the RagB/D heterodimer (62). As predicted, the interaction between Rag and raptor was also stimulated by amino acids because RagB GTP binding is required for its interaction with Raptor. The exact mechanism of Rag GTPases in mTORC1 activation is largely unclear. However, Rag GTPases are proposed to affect the subcellular localization of mTORC1. In response to amino acids, the active Rag heterodimer may tether mTORC1 and be transported to the vicinity of Rheb, where mTORC1 is activated by Rheb binding (62). This model nicely explains the interdependent relationship of mTORC1 activation by insulin and amino acids. Insulin activates Rheb via the PI3K-AKT-TSC1/2 pathway while amino acids activate Rag by a mechanism yet to be characterized. Under physiological conditions, mTORC1 is activated only when both Rheb and Rag are active.

The function of Rag GTPases in amino acid-induced TORC1 activation is conserved in *Saccharomyces cerevisiae* (66), which has two Rag homologs, Gtr1 and Gtr2. Gtr1 exhibits approximately 52% sequence identity with RagA/B and Gtr2 exhibits 46% identity with RagC/D (64). Similar to Rag, Gtr1 and Gtr2 also form a stable complex (67). By genetic study, GTR1 was initially identified as a gene that was important for phosphate transport (68). Later, GTR1 and GTR2 were implicated to negatively regulate the Ran GTPase cycle (69). More recent studies have shown that Gtr1/2 are key components of the EGO complex, which is a protein complex associated with vacuolar membrane and is required for microautophagy during exit from rapamycin-induced growth arrest in budding yeast (70). In one study, Gtr1/2 is shown to play important roles in proper sorting of Gap1 (general amino acid permease) from late endosome to plasma membrane (65). Another study shows that Gtr1 and Gtr2 are important for recovery from rapamycin treatment induced-growth arrest (70). Collectively, these studies imply a possible connection between GTR1/2 and TOR function.

The function of Gtr1/2 in amino acid-mediated mTOR activation has recently been demonstrated in yeast (66). Gtr1 and Gtr2 act in a manner similar to mammalian Rag GTPases. The Gtr1-GTP and Gtr2-GDP heterodimer was most active to stimulate

phosphorylation of Sch9, which is a substrate of yeast TORC1 and homologous to the mammalian S6K. Deletion of GTR1 or GTR2 decreased Sch9 phosphorylation. In addition, the other two EGO complex components, EGO1 and EGO3, were also required for TORC1 activation, demonstrating the function of EGO complex in TOR activation. Gtr1-GTP but not Gtr1-GDP bound to TORC1 component Tco89 and Kog1 (the yeast homolog of mammalian Raptor). In addition, the interaction between wild type Gtr1 and TORC1 was sensitive to amino acids while the interaction between the constitutively active Gtr1 mutant and TORC1 was insensitive to amino acids. Therefore, a model has been proposed that in yeast amino acids activate Gtr1/Gtr1 and promote their interaction with TORC1, therefore resulting in TORC1 activation (66).

Although yeast Gtr1 and Gtr2 are structurally and functionally similar to human Rag GTPases, the subcellular localization of TORC1 components, such as Tor1 and Tco89, were not affected by amino acids. In addition, Gtr1/Gtr2 localization was not altered in response to amino acid treatments either (66). These data are different from the observations made with mammalian Rag and mTORC1 (62), which are reported to be translocated to Rab7 positive vesicle in response to amino acid stimulation. Further studies are needed to clarify whether the mechanisms of TORC1 activation by Rag GTPases are different between yeast and mammalian cells or the amino acid-induced subcellular translocation is not critical for TORC1 activation by Rag GTPases.

2. Vam6 as a Rag GEF in amino acid-induced TORC1 activation

There is no evidence that Rag GTPases directly sense amino acids. Then, how is Rag activated by amino acids? A key advancement on this issue is from the yeast genetic study that identified Vam6 (also known as Vps39) as a Gtr1 GEF (66). Vam6 is a component of HOPS complex (homotypic fusion and vacuole protein sorting), which associates with the vacuolar membrane and is involved in vacuole fusion and vacuole protein sorting. The yeast Vam6 is known to have GEF activity towards Ypt7 (the yeast Rab7 homolog) (71). A systematic, genome-wide synthetic dosage lethal screen showed that loss of VAM6 yielded a strong synthetic growth defect when combined with overexpression of GTR1-GDP. Loss of VAM6 resulted in a growth defect phenotype

similar to that caused by mutation of EGO complex components. Overexpression of Vam6 suppressed the defect caused by the dominant negative mutant Gtr1 overexpression. Further genetic study placed VAM6 upstream of GTR1 in TORC1 signaling, possibly as a GEF of Gtr1. Biochemistry analysis showed that Vam6 bound to Gtr1 and facilitated GDP release from Gtr1, providing direct and convincing evidence that Vam6 has Gtr1 GEF activity. Furthermore, loss of VAM6 decreased Gtr1-GTP level in the cell, supporting the conclusion that Vam6 acts as a Gtr1 GEF in vivo (66).

Vam6/Vps39 is highly conserved in other species. In fact, knockdown of Vam6 in *Drosophila* S2 cells or human HEK293 cells also decreased the phosphorylation of S6K and S6, indicating that the function of Vam6 is conserved in higher eukaryotes (unpublished observation). One may speculate that Vam6 could play a key role in mediating amino acid signals, either directly or indirectly, to Rag activation, which then in turn promotes TORC1 activation.

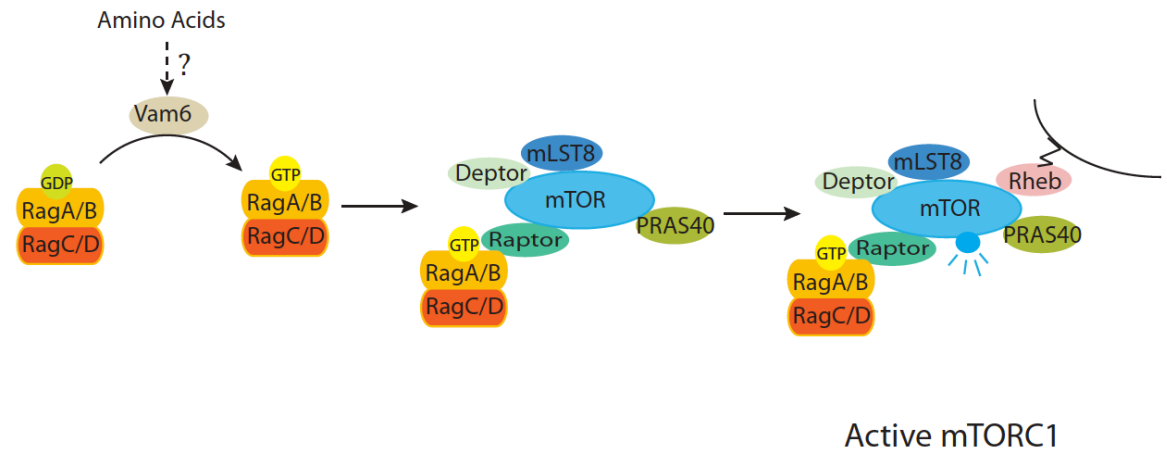


Figure 1.1. Proposed function of Rag GTPases in TORC1 activation by amino acids.

The Rag heterodimer is activated by the Vam6 GEF, which may mediate the amino acid signals by an unknown mechanism. The active Rag heterodimer binds raptor and recruits the mTORC1 to Rheb-associated vesicles, where mTORC1 is activated. This model suggests that mTORC1 activation requires both Rheb and Rag.

3. Raptor interacts with both upstream regulators and downstream substrates

Raptor forms a stable complex with mTOR and is required for mTOR signaling (9, 13). It contains a highly conserved amino-terminal region named RNC (Raptor N-terminal conserved) domain, followed by three HEAT repeats and seven WD40 repeats at the C-terminal part of the protein. Raptor is ubiquitously expressed in many tissues with high levels of expression in brain, kidney and muscle. It interacts with amino terminus HEAT repeats of mTOR, but the mTOR binding region in Raptor has not been clearly defined, possibly because Raptor uses multiple sites to contact mTOR. Both genetic knockout and knockdown have unequivocally shown that Raptor is indispensable for mTORC1 function (72).

The Raptor-mTOR interaction is dynamic and very sensitive to detergent (9). Even mild detergent, such as Triton X-100 and NP40, disrupts the mTOR complex and kinase activity towards substrate whereas mTOR autophosphorylation is enhanced by detergents. The mTORC1 complex is preserved in no detergent or CHAPS buffer and retains kinase activity towards 4EBP1 and S6K. Therefore, special care should be taken when isolating mTORC1 complex. One characterized function of Raptor is to serve as a scaffold protein to recruit substrates for phosphorylation by mTOR in the mTORC1. Raptor directly binds to a short amino acid sequence called the TOS (mTOR signaling) motif found in the C-terminal region of 4EBP1 and the N-terminal region of S6 kinases (73-75). The TOS motif in S6K and 4EBP1 is required for mTORC1 dependent phosphorylation of both proteins.

Raptor may also serve as a signal receiver for mTORC1 by directly interacting with upstream activators. For example, Rheb binds to the C-terminal WD domain of Raptor and this interaction may be important for Rheb to activate mTORC1 (35). Furthermore, RagA strongly binds to Raptor (62). This interaction depends on the RagA effector domain and GTP binding, demonstrating that Raptor is a physiological downstream effector of RagA. Therefore, raptor serves a dual role, both as signal receiver and transmitter, in mTORC1. Raptor controls mTORC1 function by interacting

with upstream regulators, such as Rheb and RagA, and downstream substrates, such as S6K and 4EBP1.

Recent studies have indicated that Raptor function is also regulated by phosphorylation in response to upstream signals. Under energy stress condition, AMPK is activated (76). Active AMPK can directly phosphorylate Raptor on two conserved sites (Ser722, Ser792) (40). This phosphorylation creates 14-3-3 binding sites and possibly changes mTORC1 kinase activity, as cells with phosphorylation defective Raptor mutant show a defective mTORC1 inhibition by energy starvation. Raptor can also be phosphorylated by RSK on RXXRXXpS/T motifs (77). This phosphorylation likely stimulates mTORC1 activity. Thus, mitogenic growth factors stimulate mTORC1 not only through PI3K-Akt pathway to phosphorylate TSC2, but also through ERK-RSK to phosphorylate Raptor. Moreover, Raptor phosphorylation by mTOR has also been reported. The mTOR dependent Raptor phosphorylation is stimulated by insulin and is necessary for insulin mediated mTOR activation (78).

4. RalA in nutrient-induced mTORC1 activation

Ral GTPases, including RalA and RalB, belong to the Ras superfamily. They have been implicated in multiple cellular processes, including cell transformation, migration, membrane dynamics, and transcription regulation (79). For example, Ral and RalGDS, which is a Ral GEF, are known to be important for Ras-induced cellular transformation (80). In fact, RalGDS is a direct downstream effector of Ras (81). Maehama et al reported that RalA has an essential role in amino acid-induced mTORC1 activation (82). Knockdown of RalA or RalB decreased mTORC1 activity in HeLa cells. Notably, RalA knockdown strongly blocked phosphorylation of S6K and 4EBP1 in response to amino acid or glucose but had a much weaker effect on the phosphorylation of mTORC1 substrates in response to insulin stimulation. These data indicate a specific role of RalA in nutrient signaling. Further supporting the role of Ral in nutrient response, knockdown of RalGDS also decreased mTORC1 activity. Interestingly, amino acids increased the accumulation of active GTP form of RalA, indicating a direct involvement of RalA in amino acid response.

The relationship between RalA and Rheb is different from that between Rag and Rheb. Rag and Rheb act parallel and upstream of mTORC1 (61). Dominant negative Rag did not block mTORC1 activation by Rheb overexpression. Consistently, mutation in dRagC did not block the growth stimulating effect caused by dRheb overexpression. In contrast, RalA knockdown suppressed Rheb S16H (a hyperactive Rheb mutant)-induced mTORC1 activation, suggesting that RalA might work downstream of Rheb (82). The function of RalA in nutrient signal could be indirect, as no interaction between RalA and mTORC1 was detected. Furthermore, not all data are consistent with a direct role of Ral in amino acid signaling as overexpression of the active RalA did not substitute the stimulating effect of amino acids on mTORC1. Amino acid still potently activated mTORC1 in cells expressing both dominant negative Rheb and constitutively active Ral. mTORC1 activity is likely to be affected indirectly by multiple Ras family GTPases. For example, we have observed that other Ras family GTPases, such as the Rab family, are also important for mTORC1 activation by amino acids (unpublished observations). We speculate that intracellular vesicular trafficking, which could be affected by many GTPases, such as Rag, Rab, and Ral, may play a critical role in mTORC1 activation.

The coordination between cell growth and nutrient availability is a fundamental question in cell biology. As a key cell growth regulator, mTORC1 is activated by nutrients, such as amino acids, to stimulate protein synthesis and cell growth. Recent studies have implicated possible roles of VPS34, MAP4K3, RalA, and Rag GTPases in mTORC1 activation in response to amino acids. The exact role of these proteins and the relationship among them to mediate amino acid signals to mTORC1 are largely unknown. However, the Rag GTPases emerge as the key mediator between amino acid sufficiency and mTORC1 activation. This conclusion is supported by convincing genetic and biochemical data from yeast, *Drosophila*, and mammalian cells (61, 62, 66). However, many key questions remain to be answered. What is the amino acid sensor? How does Rag activate mTORC1 and what is the relationship between Rag and Rheb? Future research in amino acid signaling and mTORC1 activation will significantly

advance our understanding not only of normal cell growth and development, but also pathological abnormality in diseases, such as diabetes and cancer.

In the past decade, scientists have made significant progress toward identifying new components of the mTOR pathway and understanding the connections between those components and the connection to the environmental input. However, a lot of gaps still remain. We are now beginning to appreciate the complexity of the signaling network and trying to elucidate the function of the pathway in the context of whole body physiological conditions.

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CHAPTER 2

REGULATION OF mTORC1 BY THE RAB AND ARF GTPASES

Abstract

The mammalian target of rapamycin (mTOR) is a key cell growth regulator, which forms two distinct functional complexes (mTORC1 and mTORC2). mTORC1, which is directly inhibited by rapamycin, promotes cell growth by stimulating protein synthesis and inhibiting autophagy. mTORC1 is regulated by a wide range of extra and intra cellular signals, including growth factors, nutrients, and energy levels. Precise regulation of mTORC1 is important for normal cellular physiology and development, and dysregulation of mTORC1 contributes to hypertrophy and tumorigenesis. In this study, we screened *Drosophila* small GTPases for their function in TORC1 regulation and found that TORC1 activity is regulated by members of the Rab and Arf family GTPases, which are key regulators of intracellular vesicle trafficking. In mammalian cells, uncontrolled activation of Rab5 and Arf1 strongly inhibit mTORC1 activity. Interestingly, the effect of Rab5 and Arf1 on mTORC1 is specific to amino acid stimulation while glucose-induced mTORC1 activation is not blocked by Rab5 or Arf1. Similarly, active Rab5 selectively inhibits mTORC1 activation by Rag GTPases, which are involved in amino acid signaling, but does not inhibit the effect of Rheb, which directly binds and activates mTORC1. Our data demonstrate a key role of Rab and Arf family small GTPases and intracellular trafficking in mTORC1 activation, particularly in response to amino acids.

Introduction

The target of rapamycin (TOR) is a large (approximately 280kDa) protein kinase that regulates cell growth and cell size(1). The function of TOR in promoting cell growth is conserved from yeast to mammals. TOR forms two structurally and functionally distinct complexes, TORC1 and TORC2(2). Only TORC1, but not TORC2, is directly inhibited by rapamycin. The mammalian TORC1, mTORC1, directly phosphorylates and activates the ribosomal S6 kinase, S6K(3). Because direct kinase assay of mTORC1 is rather challenging, phosphorylation of S6K is the most frequently used readout for mTORC1 activation.

As a key cell growth regulator, TORC1 activity is tightly regulated by mitogenic growth factors, the availability of amino acids, and cellular ATP levels(1). Extensive studies have elucidated the signaling mechanisms of growth factors and cellular energy levels in mTORC1 activation. The TSC1 and TSC2 tumor suppressors are key upstream negative regulators of mTORC1(4). TSC1 and TSC2 form a complex and function as a GTPase activating protein (GAP) to inhibit the Rheb GTPase, which can directly bind to and activate mTORC1(5-9). AKT, which is a protein kinase activated by numerous growth stimulating signals, such as growth factors, can phosphorylate and inhibit TSC2, therefore relieve the inhibitory effect of TSC2 on mTORC1(10,11). Furthermore, AKT can directly phosphorylate PRAS40, which is a subunit of mTORC1, and thus contribute to mTORC1 activation(9). The mechanism of mTORC1 regulation by cellular energy status has been elucidated. The AMP activated protein kinase, AMPK, is a key cellular energy sensor and regulates a large number of cellular responses upon energy starvation. AMPK inhibits the mTORC1 pathway by phosphorylating TSC2 and raptor, a key subunit of mTORC1, thereby coordinates cell growth with the availability of cellular energy(12,13).

Amino acids are the most potent stimulator of mTORC1. In the absence of amino acids, mTORC1 activation by insulin is severely compromised(14). We have recently shown that the Rag family GTPases play an essential role in mTORC1 activation in response to amino acid stimulation(15,16). In mammalian cells, the RagA or RagB form heterodimers with either RagC or RagD and the resulting heterodimers strongly bind to

raptor in a manner depending on GTP binding of RagA or RagB(16). It has been suggested that amino acids modulate the subcellular translocation of mTORC1 through Rag GTPases(16). These results indicate that intracellular trafficking may be important in mTORC1 regulation. In this report, we demonstrate that members of the Rab and Arf family GTPases play important roles in mTORC1 activation.

Materials and Methods

Antibodies, plasmids and chemicals

Anti-Drosophila S6 kinase antibody was provided by Mary Stewart (North Dakota State University, Fargo, ND). Anti-phospho Drosophila S6K, anti-S6K, anti-phospho S6K, anti-Akt, anti-phospho Akt and anti-phospho 4EBP1 antibodies were from Cell Signaling. Anti-Myc, Anti-HA and anti-Flag antibodies were from Santa Cruz Biotechnology, Covance and Sigma, respectively. RagA/C constructs were made as described previously. Rab5A, Rab7A, Rab10, Rab11A, Rab22, Rab31, Ran constructs were obtained from Drs. X. Chen and A. Saltiel (University of Michigan). Rab5A was subcloned into pBABE-puro retroviral vector. All other DNA constructs, including HA-S6K, Myc-4EBP1, GST-Akt, and Myc-Rheb, were from laboratory stock. Insulin and brefeldin A were obtained from Sigma.

Cell culture

Drosophila S2 cells (Invitrogen) were cultured in Drosophila serum-free medium (Invitrogen) supplemented with 18mM L-glutamine and maintained at 28 °C. HEK293 cells and HeLa cells were cultured in DMEM supplemented with 10% FBS. PC3 cells were cultured in F-12K medium supplemented with 10% FBS. Amino-acid-containing (SDMK) or -free (SDMK-AA) media used for Drosophila S2 cells were made using Schneider's Drosophila medium (Invitrogen) formulation as described previously(15). Amino-acid-containing (DMEMK) or amino acid-free (DMEMK-AA) media used for HEK293 and HeLa cells were made using DMEM medium (Invitrogen, Cat. No.12430) formulation.

RNA interference

Drosophila RNA interference (RNAi) experiments were performed as described previously(17).

Transfection and cell lysis- Transfection was performed in serum-free conditions using Lipofectamine reagent (Invitrogen) as described by the manufacturer. Cells were lysed in SDS lysis buffer (1% SDS, .1M Tris pH7.5).

Drosophila genetics and histology

Clonal knockdown of Arf1 in larval fat body cells was performed using the dsRNA UAS line GD12522 from the Vienna Drosophila RNAi Center. This line was coexpressed with UAS-dicer to increase RNAi efficiency. Spontaneous flippase-mediated induction of GFP-marked cells, and staining with Texas Red-phalloidin and LysoTracker Red (Invitrogen) was performed as previously described (15). Cell area measurements were determined from confocal images of fixed fat body tissues using Adobe Photoshop, as previously described(15). mCherry-Atg8a was expressed and analysed as previously described(18).

Membrane fractionation

HEK293 cells grown in 10-cm plates were collected in fractionation buffer (50mM HEPES, pH7.4, 1mM DTT, and 1X protease inhibitor) and passed 10 times through a 25-gauge needle on ice. The lysates were centrifuged for 5 min at 1,000g, and the post-nuclear supernatants were centrifuged for 20 min at 53,200 rpm at 4 °C. The cytosolic supernatants were collected and the remaining pellets were lysed in mild lysis buffer (1% NP-40, 10mM Tris pH7.5, 2mM EDTA, 100mM NaCl, 50mM NaF) and centrifuged for 20 min at 53,200 rpm at 4°C. Membrane supernatants were collected. Each fraction with equal protein amounts were analysed by western blotting.

Immunofluorescence assays

Cos7 cells were plated on 12 well plates, 16 hours after plating; cells were transfected with indicated plasmids. 24 hours later, cells were split and plated on glass coverslips in 24-well tissue culture plates. Another 24 hours later, cells were rinsed with PBS once and fixed for 5 minutes with 4% paraformaldehyde in PBS. The coverslips were rinsed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 minutes. After rinsing twice with PBS, the coverslips were blocked for one hour in blocking buffer (0.2% BSA in PBS) and incubated with primary antibody in blocking buffer overnight at 4°C, rinsed twice with blocking buffer and incubated with secondary antibodies (diluted in blocking buffer 1:1000) for one hour at room temperature in dark. The coverslips were mounted on glass slides using Prolong gold mounting medium (Invitrogen) and imaged with a 63X objective using confocal microscopy.

Immunoprecipitation

HEK293 cells transfected with indicated plasmids were lysed in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 2mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, and 0.3% CHAPS and one tablet of EDTA-free protease inhibitors (Roche) per 10.5 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,200 rpm for 10 minutes by centrifugation in a microfuge. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation for 1.5 hours at 4°C. 10 µl of 50% slurry of protein G-sepharose was then added and the incubation continued for an additional 1 hour. Immunoprecipitates were washed four times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 µl of sample buffer and boiling for 5 minutes, resolved by SDS-PAGE, and analyzed by immunoblotting as described.

Results and Discussion

Knockdown of Rab and Arf decrease TORC1 activity in Drosophila S2 cells.

To search for GTPases that may regulate TORC1, we used RNAi to knockdown all putative small GTPases predicted in the Drosophila genome. Examples of GTPase knockdown on dS6K phosphorylation are shown in figure 1. Besides Ras, Rheb, and Rag, which are known to regulate TORC1, knockdown of several other small GTPases also significantly decreased dS6K phosphorylation (Fig. 2.1). Ran GTPase is essential for nuclear import and export(19). In cells treated with double stranded RNA against Ran, both dS6K protein and phosphorylation were decreased dramatically, possibly due to a reduction of total cell numbers. However, the remaining dS6K displayed a fast migration, indicating that dS6K was dephosphorylated in Ran knockdown S2 cells.

We found that knockdown of Rab5 and Rab11 similarly decreased both dS6K protein and phosphorylation (Fig. 2.1). Due to a dramatic reduction in dS6K protein, it is unclear whether the relative dS6K phosphorylation was decreased by knockdown of these two GTPases. However, knockdown of Rab1 caused a significant decrease of dS6K phosphorylation as indicated by both the phosphorylation specific antibody and an increased electrophoretic mobility. Similar results were observed with Arf1 knockdown, and the effects of Rab1 and Arf1 knockdown on dS6K phosphorylation were similar to that caused by RagA knockdown. These results strongly indicate that Rab1 and Arf1 are important for TORC1 activity in Drosophila S2 cells.

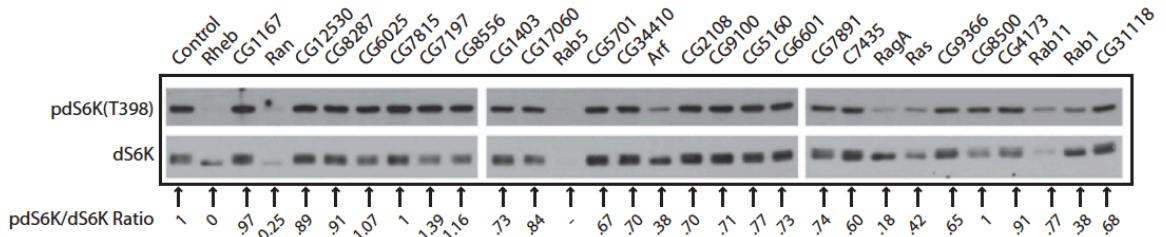


Figure 2.1. Rab and Arf proteins are indispensable in regulating TORC1 activity in *Drosophila* S2 cells.

Drosophila S2 cells untreated (lane 1) or treated with the double stranded RNA against individual genes (as indicated by the *Drosophila* genome CG numbers) were starved of amino acids for 1 h followed by amino acid stimulation for 30 min. Phosphorylation and protein levels of dS6K were determined by immunoblotting with the indicated antibodies. Signals detected by anti-pdS6K and anti-dS6K were quantified and the ratio was calculated. The control ratio is set to be 1 and all other ratio is the comparison with the control. – means protein signal not detectable.

Constitutive activation of Rab inhibits mTORC1. In mammalian cells, there are large numbers of Rab and Arf family GTPases that often have overlapping functions in intracellular trafficking(20,21). The data from *Drosophila* S2 cells indicate that intracellular vesicle trafficking may play a key role in TORC1 activation. To test this possibility, we overexpressed several constitutively active Rab GTPases in 293 cells to determine their effect on mTORC1 in mammalian cells. As positive controls, expression of either constitutive RagAQL or RhebL64 increased S6K phosphorylation (Fig. 2.2A). We observed that expression of active mutants of Rab5, Rab7, Rab10, and Rab31 potently inhibited S6K phosphorylation. In contrast, expression of active Rab11 had little effect while Rab22 had a minor inhibitory effect on S6K phosphorylation. The inhibitory effect of the Rab GTPases on S6K phosphorylation was surprising because knockdown of these GTPases in *Drosophila* S2 cells also decreased dS6K phosphorylation. However, it is well documented that disruption of GTP cycling of Rab proteins can disrupt normal cellular trafficking(20,22). Our data suggest that normal intracellular trafficking is critical for proper mTORC1 activation.

To further elucidate the effect of Rab proteins on mTORC1, we picked Rab5 as a representative for further characterization. We tested the effects of wild type, constitutively active, and dominant negative Rab5 on the phosphorylation of S6K and 4EBP1, two direct substrates of mTORC1(23). We found that both the wild type and constitutively active Rab5 strongly inhibited the phosphorylation of co-transfected S6K and 4EBP1 although the Rab5QL was more potent (Fig. 2.2B). The dominant negative Rab5TN also inhibited S6K phosphorylation but significantly less effectively than the active Rab5QL. In addition, the endogenous S6K phosphorylation was also inhibited by either Rab5QL or Rab5TN overexpression (Fig. 2.3A). These data support a notion that too much or too little Rab5 activity inhibits mTORC1, suggesting that continuous intracellular trafficking is important for mTORC1 activation. We also determined the effect of Rab5 on AKT phosphorylation, which is phosphorylated by mTORC2 but not mTORC1(24), and found that neither the constitutively active nor the dominant negative Rab5 affected AKT phosphorylation (Fig. 2.2B, 2.3A), indicating that the effect of Rab5

is specific on mTORC1. In PC3 cells, which have elevated PI3K pathway activity, Rab5 overexpression still inhibited S6K phosphorylation. (Fig. 2.2.C).

We tested if Rab5 interacts with mTORC1 by co-immunoprecipitation. Our data showed that Rab5 could not co-immunoprecipitate with either Raptor or mTOR while the positive control RagA/RagC interacted with both (Fig. 2.3.C). Immunofluorescence of transfected Rab5 showed that a significant fraction of endogenous mTOR co-localized with Rab5 (Fig. 2.3D), indicating Rab5 and mTOR can exist in the same subcellular compartment.

We then examined the importance of Rab5 membrane localization, which is required for its function in vesicle trafficking(25), in mTORC1 inhibition. Expression of the Rab5QL-2CS, which has the two C-terminal membrane targeting essential cysteines substituted by serines and not associated with membrane (Fig. 2.3E), failed to inhibit S6K phosphorylation (Fig. 2.2D), supporting that membrane localization is essential for Rab5 to influence mTORC1 activity. Next, we tested the importance of Rab5 signaling in mTORC1 inhibition. Mutations of F57, W74, or Y89 of Rab5 are known to compromise Rab5 signaling in vivo(26). Our data showed that mutations of these residues abolished the ability of Rab5 to inhibit mTORC1 (Fig. 2.2D). Together, these observations further support the notion that a proper Rab5 signaling is important for physiological mTORC1 regulation.

To further investigate a role of Rab5 in mTORC1 regulation, GAPex, a GEF for Rab5 and Rab31(27), was coexpressed with S6K1. We reasoned that hyperactivation of endogenous Rab5 might also inhibit mTORC1. In deed we observed that GAPex overexpression decreased S6K1 phosphorylation (Fig. 2.2E). The above data are consistent with a critical role of endogenous Rab5 and Rab31 in mTORC1 regulation.

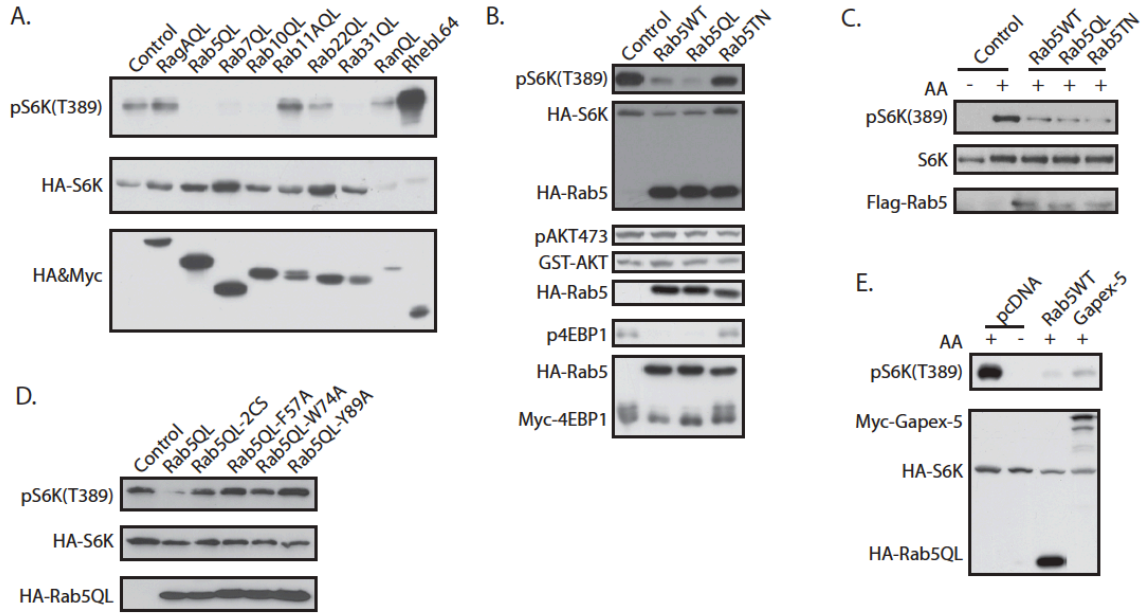


Figure 2.2. Several mammalian Rab proteins inhibit mTORC1 activity.

(A) Constitutively active Rab proteins inhibit S6K phosphorylation. Each mammalian Rab construct was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(B) Rab5 regulates TORC1 but not TORC2 activity. Each indicated Rab protein construct was co-transfected with either HA-S6K, Myc-4EBP1 or of GST-Akt. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(C) Rab5 regulates TORC1 activity in PC3 cells. PC3 cells stably express pBABE-puro vector, and corresponding Rab5 constructs were generated and the protein and phosphorylation levels were determined by immunoblotting with the indicated antibodies.

(D) Rab5's effect on mTORC1 is dependent on its membrane localization and effector binding. Each Rab5 mutant construct was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(E) GAPex overexpression inhibits S6K phosphorylation. Either Rab5 or GAPex construct was transfected into HEK293 cells together with HA-S6K. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

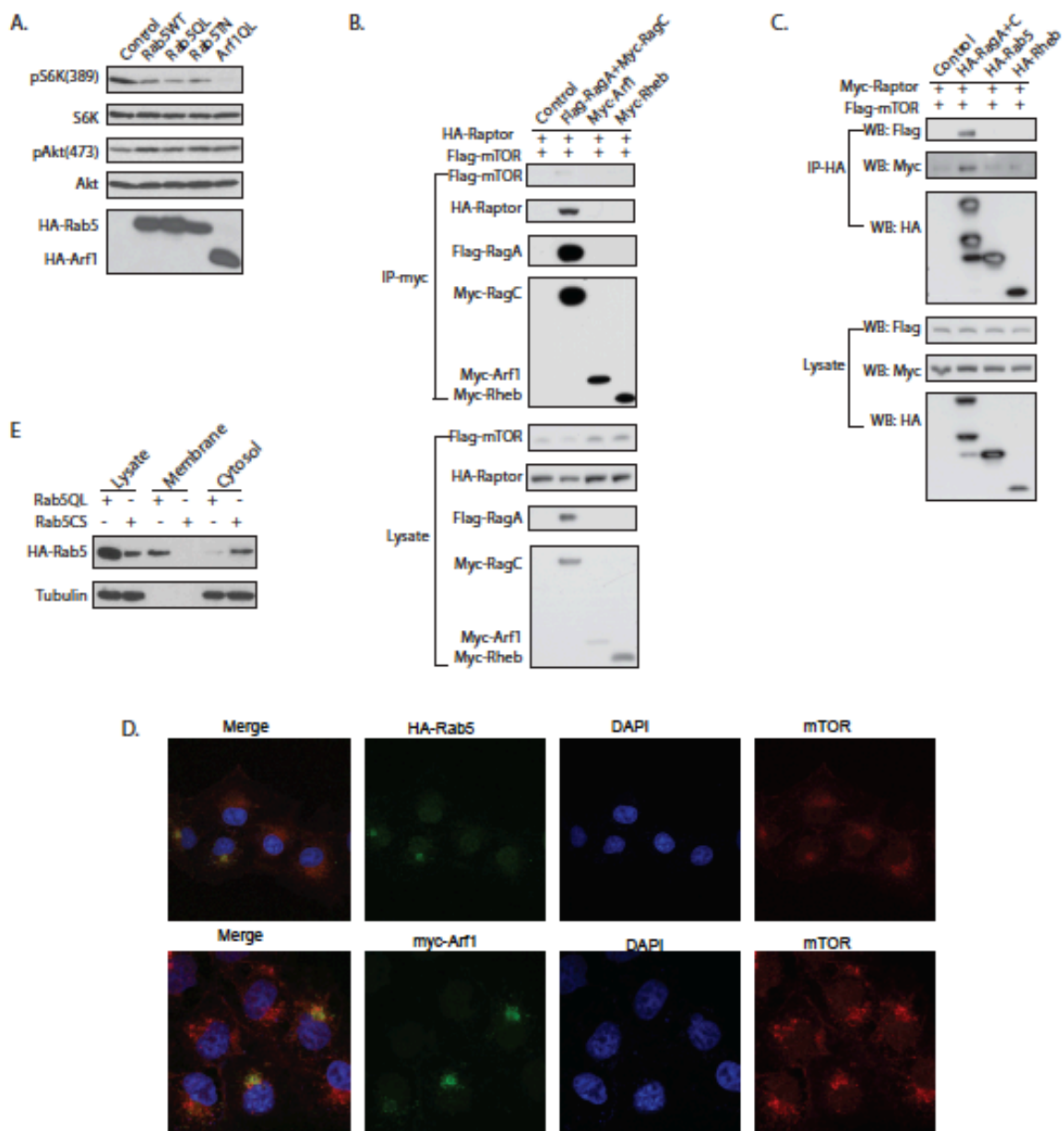
Figure. 2.3. Supplemental figure for Figure. 2.2.

(A) Rab5 and Arf1 regulate TORC1 but not TORC2 activity. Each indicated Rab5 and Arf1 protein construct was transfected into HEK293 cells and the protein and phosphorylation levels of S6K and Akt were determined by immunoblotting with the indicated antibodies.

(B,C) Rab5 and Arf1 do not form stable complex with TORC1 complex. HEK293 cells were transfected with indicated protein constructs, cell lysates were prepared and lysates and HA- or myc- immunoprecipitates were analyzed by immunoblotting for the indicated tagged proteins.

(D) Overexpressed Rab5 and Arf1 partially colocalized with mTOR. Cos7 cells transfected with HA-Rab5 or myc-Arf1 are processed in an immunofluorescence assay to detect HA/myc (green), mTOR (red), costained with DAPI for DNA content (blue), and imaged.

(E) Rab5CS mutant lost the membrane targeting signal. HEK293 cells transfected with Rab5QL or Rab5CS mutants were fractionated and each fraction was immunoblotted with the indicated antibodies.



Rab5 specifically affects amino acid or Rag-induced but not glucose or Rheb-induced mTORC1 activation. It has been proposed that amino acids activate TORC1 possibly by affecting intracellular mTORC1 localization through Rag GTPases(16). We tested the effect of Rab5 on mTORC1 activation by amino acids. Co-expression of Rab5QL inhibited S6K phosphorylation in response to amino acid stimulation (Fig. 2.4.A). In contrast to the amino acid stimulation, Rab5QL had no inhibitory effect on S6K phosphorylation when cells were stimulated by glucose (Fig. 2.4.B). These results show that the effect of Rab5 is pathway specific and consistent with the model that amino acids stimulate mTORC1 activation by regulating its intracellular localization.

Previous studies have shown that transient overexpression of Rheb-induced mTORC1 activation is insensitive to amino acid deprivation while Rag mediates amino acid signals to regulate mTORC1 localization(16). We tested the relationship between Rab5 and RagA or Rheb. Co-expression of Rab5QL strongly inhibited mTORC1 activation by active RagAQL (Fig. 2.4.C). On the other hand, Rab5QL only had a minor effect on S6K phosphorylation in the presence of active RhebS16H mutant. These data demonstrate that Rab5 is particularly important for mTORC1 activation by amino acids and Rag GTPase, but not required for mTORC1 activation by glucose and Rheb. Similar observations were made with active Rab7, Rab10, and Rab31 (data not shown). Given the fact that both amino acids and Rag affect mTORC1 localization(16), our data support that intracellular trafficking, at least the steps that regulated by Rab5, Rab7, Rab10 and Rab31, is important for mTORC1 activation in response to amino acid stimulation.

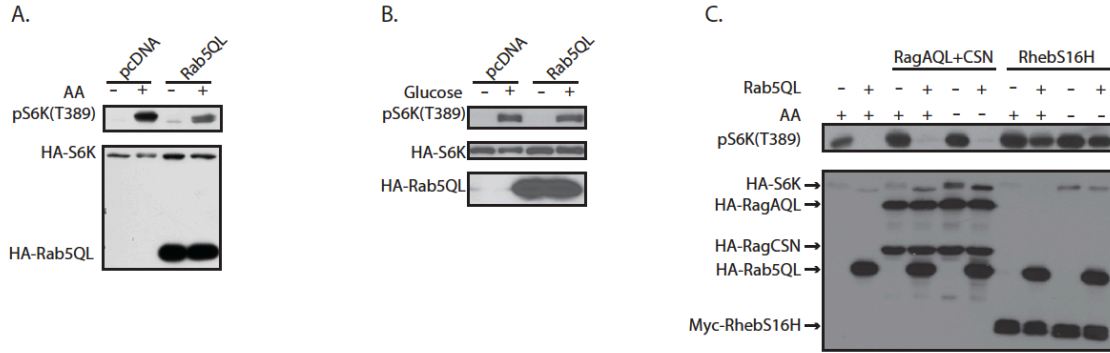


Figure 2.4. The active Rab5QL blocks amino acid-stimulated but not glucose or Rheb-induced mTORC1 activation.

(A) Rab5 inhibits mTORC1 activity induced by amino acids. Rab5 construct was co-transfected with HA-S6K into HEK293 cells. Cells were starved for amino acids for 1 h followed by amino acid stimulation for 30 min before harvesting. Amino acid stimulation is denoted as AA+. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibody.

(B) Rab5 does not affect glucose induced mTORC1 activity. Rab5 construct was co-transfected with HA-S6K into HEK293 cells. Cells were deprived glucose for 1 h followed by glucose stimulation for 30 min before collection. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(C) Rab5 blocks Rag but not Rheb induced S6K phosphorylation. Rab5 was transfected into HEK293 cells with or without RagA/C or Rheb construct as indicated. S6K was included in the co-transfection. Phosphorylation and protein levels of the transfected proteins were determined by immunoblotting with the indicated antibodies.

Arf1 regulates mTORC1 in both *Drosophila* and mammalian cells. The RNA interference screen revealed that knockdown of Arf1 homolog in S2 cells significantly decreased S6K phosphorylation (Fig. 2.1). Arf family GTPases are also involved in intracellular vesicle trafficking(23). We tested the effect of Arf1 in mammalian cells. Co-expression of the constitutively active Arf1QL inhibited S6K phosphorylation as strongly as Rab5QL (Fig. 2.5.A). Similar to the results observed with Rab5 mutants, the constitutively active Arf1QL was more potent than the wild type or dominant negative Arf1TN in inhibiting S6K1 phosphorylation (Fig. 2.5.A). We also found that a significant fraction of mTOR was co-localized with the transfected Arf1 (Fig. 2.3.D). On the other hand, Arf1 did not show a direct interaction with either Raptor or mTOR (Fig. 2.3.B) by our co-immunoprecipitation experiments.

Brefeldin A is a drug that inhibits Arf1 GEF, thereby inhibiting Arf1 activity(28,29). We tested the effect of brefeldin A on S6K1 phosphorylation and found that it potently inhibited endogenous S6K phosphorylation in a dose dependent manner (Fig. 2.5.B). The inhibitory effect was rather rapid, as an almost complete inhibition of S6K1 phosphorylation was observed with 60-minutes brefeldin A treatment. These data support a role of endogenous Arf in mTORC1 regulation.

Two well characterized cellular functions of TORC1 are to promote cell growth and to inhibit autophagy(1,30). We investigated the role of Arf1 in cell size regulation in *Drosophila*. Knockdown of Arf1 in clones of GFP marked cells in the larval fat body resulted in a 40% reduction in cell area (Fig. 2.5.C), consistent with a decreased TORC1 activity in these cells. In parallel, we examined the effect of Arf1 on autophagy, which is inhibited by high TORC1 activity. Arf1 knockdown caused a strong induction of autophagy, as indicated by punctuate localization of mCherry-Atg8a and LysoTracker Red, which mark early (autophagosome) and late (autolysosome) stages of autophagy, respectively (Fig. 2.5.D, E). These data further support that Arf1 plays an important role in TORC1 regulation in vivo.

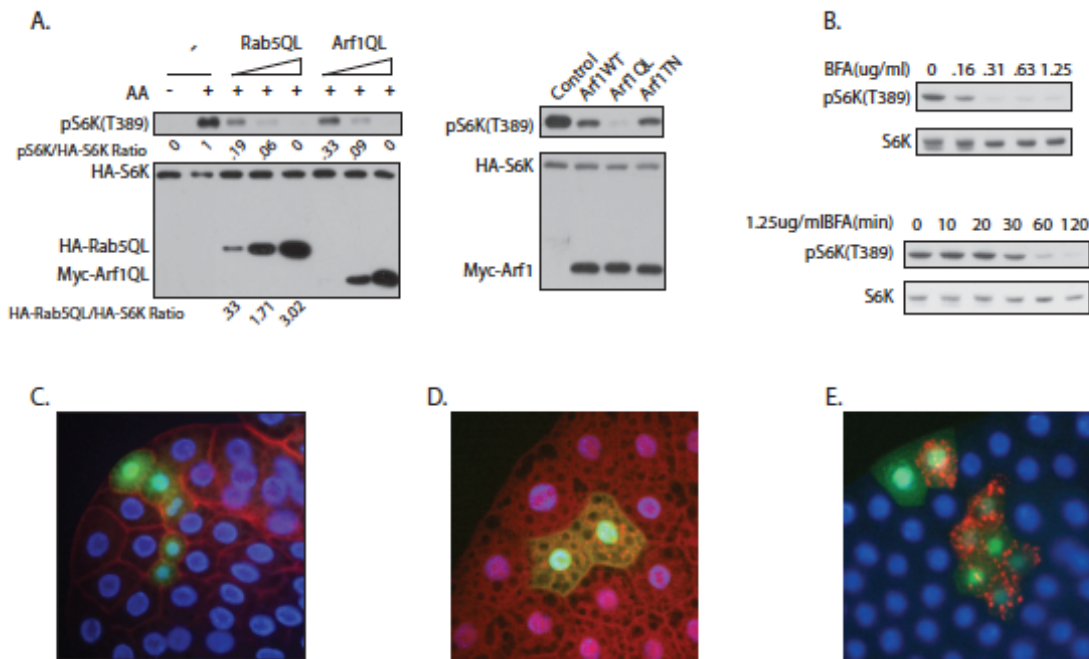


Figure 2.5. Regulation of TORC1 by Arf1.

(A) Arf1 inhibits S6K phosphorylation. HA-S6K was co-transfected with increasing amount of Rab5QL (50, 100, 200ng) or Arf1QL (50, 100, 200ng) into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies. Signals detected by anti-pS6K, anti-HA-S6K and anti-HA-Rab5QL were quantified and the ratio was calculated.

(B) Brefeldin A inhibits S6K phosphorylation. MEF cells were treated with indicated concentrations or times of Brefeldin A. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(C) Downregulation of Arf1 expression reduces cell size. Fat body cells expressing UAS-Arf1-RNAi (GFP positive clone) have a decreased area comparing to surrounding wild type control cells (GFP negative). DAPI (blue) labels nuclei; phalloidin staining of F-actin (red) helps to visualize cell boundary .

(D, E) Arf1 downregulation induces autophagy. Clonal expression of UAS-Arf1-RNAi results in punctate staining of mCherry-Atg8a (D) and Lysotracker Red (E) under fed conditions.

The TORC1 complex has an essential role in cell growth in response to various growth promoting and inhibitory signals(1). Two GTPase families, Rheb and Rag, have previously been shown to stimulate mTORC1 activation(8,9,15,16). However, the mechanisms of these GTPases in mTORC1 regulation are different. Rheb, acting downstream of TSC1/TSC2, binds to and activates mTORC1(5-9). Therefore, the effect of Rheb on mTORC1 is direct. On the other hand, the Rag GTPases act downstream of amino acids to promote mTORC1 activation(15,16). Although RagA and RagB bind directly to raptor with high affinity they cannot directly activate mTORC1 without the help of Rheb(16). A model has been proposed that Rag GTPases may regulate the intracellular localization of mTORC1 in response to amino acids. When amino acids are sufficient, the active RagA and RagB recruit mTORC1 to subcellular compartments where Rheb is localized(16). Therefore, active Rag presents mTORC1 to location where it can be activated by Rheb. This model suggests that intracellular trafficking would be important for mTORC1 activation.

In this study, we have discovered Rab and Arf GTPases as having an important function in mTORC1 activation. However, the roles of these GTPases in mTORC1 regulation are different from Rag or Rheb. Neither Rab5 nor Arf1 is sufficient to activate mTORC1 and they do not interact with mTORC1 (Fig. 2.3.B,C). Their effects on mTORC1 are likely to indirectly influence the subcellular localization of mTORC1 or its regulators. Consistently, both Rab and Arf are key regulators of intracellular trafficking. Unlike Rheb or Rag, which promote mTORC1 activation when in GTP-bound form and inhibit mTORC1 in GDP-bound form, overexpression of both the GTP-bound and GDP-bound forms of Rab5 or Arf1 inhibit mTORC1. These results are consistent with an important role of GTPase cycling of Rab or Arf for proper intracellular vesicle trafficking. Overexpression of either GTP-bound or GDP-bound forms of these proteins might result in trapping of target proteins in a certain step and disruption of normal cycling. It is worth noting that Rab5 selectively blocks the stimulating effect of RagA but not Rheb on mTORC1 activation. Furthermore, Rab5 inhibits the effect of amino acids but not glucose on mTORC1 activation. These data support a model that intracellular trafficking is critical for amino acids signaling to mTORC1 activation that is

mediated by the Rag GTPases. Future studies are needed to clarify the subcellular compartment where mTORC1 localizes.

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Footnotes

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CHAPTER 3

MARK4 IS A NEGATIVE REGULATOR OF MTORC1

Abstract

The mammalian target of rapamycin (mTOR) is a central cell growth regulator, which resides in two distinct protein complexes which in mammals are referred to as mTORC1 and mTORC2. mTORC1, which is directly inhibited by rapamycin, promotes cell growth by stimulating protein synthesis and inhibiting autophagy. mTORC1 is regulated by a wide range of extra and intra cellular signals, including growth factors, nutrients, and energy levels and various stress conditions. Precise regulation of mTORC1 is important for normal cellular physiology and development, and dysregulation of mTORC1 contributes to many human diseases, including cancer, cardiovascular disease, autoimmunity, and metabolic disorder. In this study, we identified MARK4, an AMPK related kinase, as a new negative regulator of mTORC1. In drosophila S2 cells and mammalian cells, knockdown of MARK family member increase mTORC1 activity while overexpression of MARK4 in mammalian cells significantly inhibit mTORC1 activity. Interestingly, MARK4 selectively inhibits mTORC1 activation by Rag GTPases, which are involved in amino acid signaling, but does not inhibit the effect of Rheb, which directly binds and activates mTORC1. In addition, we find out that MARK4 phosphorylates Raptor, a key component of mTORC1. The phosphorylation inhibits mTORC1 kinase activity and affect Raptor-Rag interaction. Our data demonstrate MARK as a negative regulator of mTORC1.

Introduction

The target of Rapamycin is an evolutionary conserved protein kinase that regulates a wide span of cellular processes(1). It forms two structurally and functionally distinct complexes, a nutrient and rapamycin sensitive complex TORC1 and a nutrient and rapamycin insensitive complex TORC2(2). Well-characterized mTORC1 substrates include S6K and 4EBP1(3).

mTORC1 integrates various signals including nutrient, growth factors, stress and energy to regulate cell growth and metabolism(1). Huge amount of efforts have been put on exploring mTORC1 regulation by growth factors and cellular energy levels, both go through the key upstream regulator TSC1 and TSC2 tumor suppressors. Growth factors activate PI3K pathway(1). The activated Akt phosphorylate TSC2 and inhibit TSC1/2's GAP activity towards Rheb(4, 5). Additionally, AKT phosphorylates PRAS40, an mTORC1 component. The phosphorylated PRAS40 dissociated from mTORC1 and lost its inhibition on mTORC1(6, 7). Energy level regulates mTORC1 through AMPK(1). AMPK sensed low energy status and phosphorylate TSC2 and activate TSC complex GAP activity(8). AMPK also phosphorylate Raptor, posing another regulation node on mTORC1(9). Recently, several reports identified Rag GTPases as important mediator that relay amino acids to mTORC1 activation(10, 11). It was shown that through interaction with Raptor, Rag GTPases affect the localization of mTORC1(11).

While significant emphasis has been placed on how mTORC1 activity is set, there are still a lot of unanswered questions. There are still gaps left on how other signals regulate mTORC1. For example, in addition to Rag GTPases, studies also show that VPS34, MAP4K3 and RalA have essential role in mTORC1 activation in response to amino acids, but the exact mechanism remains unknown(12-15). Rab and Arf GTPases also shown to regulate mTORC1 possibly through regulation of trafficking of the pathway components(16, 17). Here, we report that MARK4 is a possible negative regulator of mTORC1. Our data indicate that Raptor Ser722/792 phosphorylation is an important node in mTORC1 activation.

Materials and Methods

Antibodies, plasmids and chemicals

Anti-Drosophila S6 kinase antibody was provided by Mary Stewart (North Dakota State University, Fargo, ND). Anti-phospho Drosophila S6K, anti-S6K, anti-phospho S6K, anti-Akt, anti-phospho Akt and anti-phospho 4EBP1 antibodies were from Cell Signaling. Anti-Myc, Anti-HA and anti-Flag antibodies were from Santa Cruz Biotechnology, Covance and Sigma, respectively. HA-raptor S722/792A were made using strategene mutagenesis kit. RagA/C constructs were made as described previously. All other DNA constructs, including HA-GST-MARK4, HA-S6K, Myc-4EBP1, GST-Akt, and Myc-Rheb, were from laboratory stock. Insulin was obtained from Sigma.

Cell culture

Drosophila S2 cells (Invitrogen) were cultured in Drosophila serum-free medium (Invitrogen) supplemented with 18mM L-glutamine and maintained at 28 °C. HEK293 cells and HeLa cells were cultured in DMEM supplemented with 10% FBS. Amino-acid-containing (SDMK) or -free (SDMK-AA) media used for Drosophila S2 cells were made using Schneider's Drosophila medium (Invitrogen) formulation as described previously(10). Amino-acid-containing (DMEMK) or amino acid-free (DMEMK-AA) media used for HEK293 and HeLa cells were made using DMEM medium (Invitrogen, Cat. No.12430) formulation.

RNA interference

Drosophila RNA interference (RNAi) experiments were performed as described previously(11).

Transfection and cell lysis

Transfection was performed in serum-free conditions using Lipofectamine reagent (Invitrogen) as described by the manufacturer. Cells were lysed in SDS lysis buffer (1% SDS, .1M Tris pH7.5).

Immunoprecipitation

HEK293 cells transfected with indicated plasmids were lysed in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 2mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, and 0.3% CHAPS and one tablet of EDTA-free protease inhibitors (Roche) per 10.5 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,200 rpm for 10 minutes by centrifugation in a microfuge. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation for 1.5 hours at 4°C. 10 µl of 50% slurry of protein G-sepharose was then added and the incubation continued for an additional 1 hour. Immunoprecipitates were washed four times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 µl of sample buffer and boiling for 5 minutes, resolved by SDS-PAGE, and analyzed by immunoblotting as described.

Kinase assay:

For the MARK4 kinase assays, HEK293 cells were transfected with HA-MARK4 or HA-AMPKa, myc-AMPKb, myc-AMPK. 48 hours post-transfection, cells were lysed with lysis buffer [50mM HEPES (pH 7.5), 150mM NaCl, 1mM EDTA, .3% CHAPS, 10mM pyrophosphate, 10mM glycerophosphate, 50mM NaF, 1.5mM Na₃VO₄, protease inhibitor cocktail (Roche), 1mM DTT, 1mM PMSF], and immunoprecipitated with anti-HA antibodies. The immunoprecipitates were washed 3 times with lysis buffer,

followed by once with wash buffer [40mM HEPES, 200mM NaCl] and once with kinase assay buffer [30mM HEPES, 50mM potassium acetate, 5mM MgCl₂]. The immunoprecipitated proteins was subjected to a kinase assay in the presence of 500μM cold ATP, 10μCi [γ -³²P]ATP, and 1μg GST-raptor⁷⁹² expressed and purified from E.coli as substrate. The reaction mixtures were incubated at 30°C for 30 min, terminated with SDS sample buffer, and subjected to SDS-PAGE and autoradiography. The same procedure was used for mTOR kinase assay except using different kinases and substrates.

Results and discussion:

Knockdown of Par-1 increases TORC1 activity in Drosophila S2 cells:

Kinases have profound effects in almost every cellular process. They are particularly important in signal transduction because of highly regulated feature and their ability to amplify signal cascade. In TOR signaling pathway, several kinases are involved in regulating pathway activity such as AMPK and AKT. We hypothesized that there might be more protein kinases involved in TORC1 regulation. Based on that, we performed an RNAi screen of 431 annotated Drosophila Kinases using Drosophila S2 cells, looking for Kinases whose silencing affect the phosphorylation of dS6K in S2 cells. As shown in Fig. 3.1, in amino acid deprivation condition, which has low basal S6K phosphorylation, knockdown of Par-1 significantly increase dS6K phosphorylation to the same extent as PTEN, which is a known upstream negative regulator of TORC1 in amino acid deprived contition. Par-1 was first identified in *c. elegans* as one of the six Par genes essential for the asymmetric division of zygotes(18). In drosophila, Par-1 is reported to have a similar function as in *c. elegans*. The Par-1 mutant showed defective anterior-posterior (A-P) axes of the oocyte and embryo and problems with oocyte fate determination and maintenance. In addition, PAR-1 regulates the microtubule cytoskeleton in Drosophila, and is also shown to be implicated in both the canonical and noncanonical Wnt signaling pathways(19).

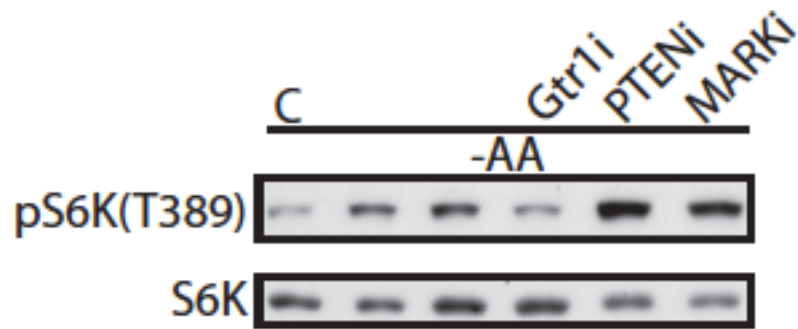


Figure 3.1. *par-1* negatively regulates TORC1 activity in *Drosophila* S2 cells.

Drosophila S2 cells untreated (lane 1) or treated with the double stranded RNA against individual genes (as indicated above) were starved of amino acids for 1 h before lysis. Phosphorylation and protein levels of dS6K were determined by immunoblotting with the indicated antibodies.

Mammalian Par-1 homolog MARK4 regulates mTORC1:

We next examined the function of Par-1 homologs in mammalian cells. Human has four Par-1 homolog, MARK1, MARK2, MARK3, and MARK4. Each showed around 30-40% sequence identity with drosophila Par-1. Knockdown of MARK1, 2, 3 does not have a dramatic effect on S6K phosphorylation while knockdown of MARK4 significantly increased S6K phosphorylation in normal culture condition as shown by both phospho-antibody and gel mobility shift (Fig. 3.2.A). So we chose MARK4 to further explore its function on mTOR pathway.

Expression of wide-type MARK4 significantly decreased S6K phosphorylation (Fig. 3.2.B), while the kinase dead mutant of MARK4 showed no inhibitory effect on S6K phosphorylation (Fig. 3.2.C). To further examine the role of MARK on mTORC1 regulation, we determined the phosphorylation status of another well characterized mTORC1 substrate, 4EBP1. As shown in fig. 3.2.D, wide type MARK4 significantly decreased 4EBP1 phosphorylation, while the kinase dead mutant showed no effect. On the other hand, MARK4 had little effect on Akt Ser473 phosphorylation, a TORC2 substrate (Fig. 3.2.E). These results support the notion that MARK4 specifically inhibits mTORC1 but not mTORC2.

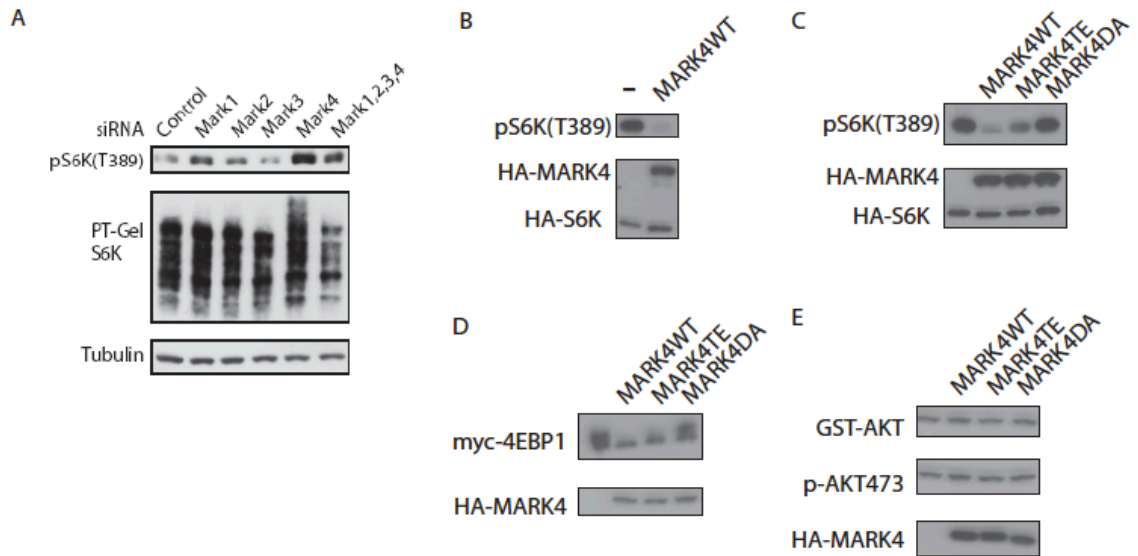


Figure 3. 2 MARK4 regulates mTORC1 activity in mammalian cells.

(A) Knockdown of MARK4 proteins increase S6K phosphorylation. Each MARK siRNA pool was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(B) MARK4 inhibits S6K phosphorylation. MARK4 was co-transfected with HA-S6K into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(C-E) MARK4WT, not the MARK4 kinase dead mutant, regulates TORC1 but not TORC2 activity. Each indicated MARK protein construct was co-transfected with either HA-S6K, Myc-4EBP1 or of GST-Akt. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

MARK4 specifically affects Rag-induced but not Rheb-induced mTORC1 activation:

To investigate the role of MARK4 in mTORC1 regulation, we tested if MARK4 overexpression specifically inhibits a sub-group of activation signal. In contrast, we saw that MARK4 blocks amino acids, glucose, and insulin induced mTORC1 activation (Fig. 3.3.A, B, C). In addition, co-expression of MARK4 strongly inhibited active RagA/C induced mTORC1 activation; suggesting MARK4's position in the pathway is either paralleled of these different signals or directly on mTORC1 complex activity. On the other hand, MARK4 had no effect on S6K phosphorylation in the presence of active RhebS16H mutant, consistent with Rheb's function as direct activator of mTOR (Fig. 3.3.D).

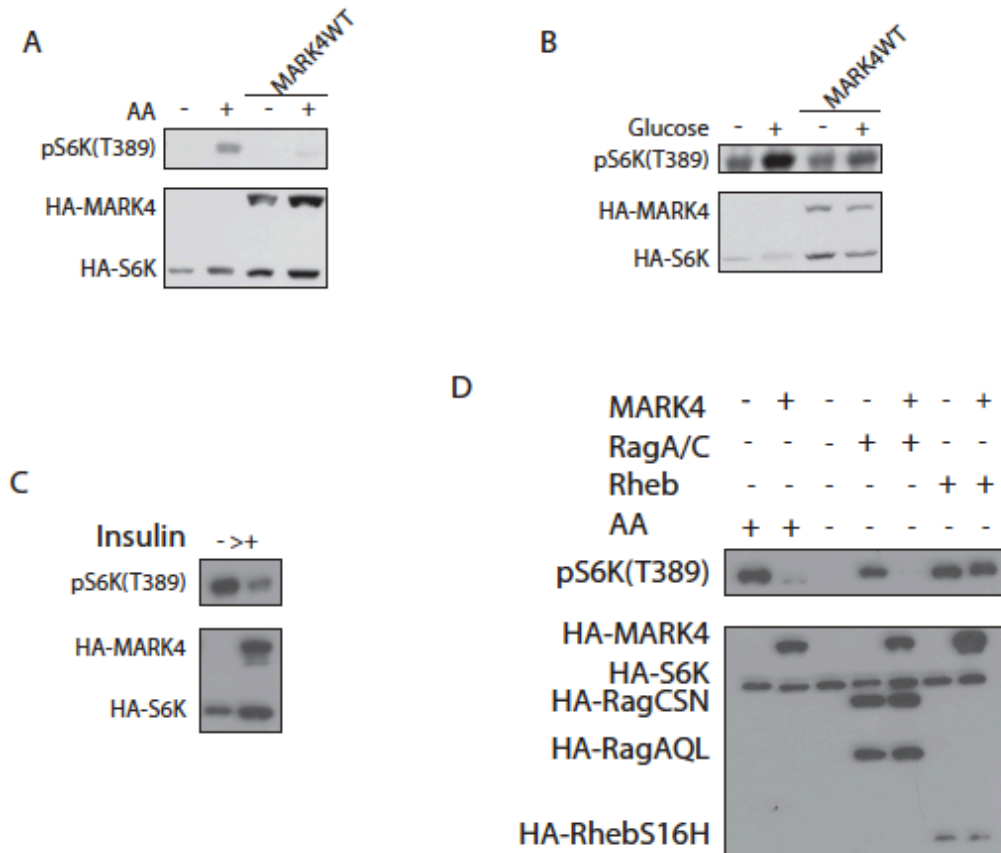


Figure 3.3. MARK4 blocks Rag-stimulated but not Rheb-induced mTORC1 activation.

(A) MARK4 inhibits mTORC1 activity induced by amino acids. MARK4 construct was co-transfected with HA-S6K into HEK293 cells. Cells were starved for amino acids for 1 h followed by amino acid stimulation for 30 min before harvesting. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibody.

(B) MARK4 inhibits mTORC1 activity induced by glucose. MARK4 construct was co-transfected with HA-S6K into HEK293 cells. Cells were deprived glucose for 2 h followed by glucose stimulation for 30 min before collection. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(C) MARK4 inhibits mTORC1 activity induced by insulin. MARK4 construct was co-transfected with HA-S6K into HEK293 cells. Cells were deprived serum for 5 h followed by insulin stimulation for 30 min before collection. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(D) MARK4 blocks Rag but not Rheb induced S6K phosphorylation. MARK4 was transfected into HEK293 cells with or without RagA/C or Rheb construct as indicated. S6K was included in the co-transfection. Phosphorylation and protein levels of the transfected proteins were determined by immunoblotting with the indicated antibodies.

MARK4 phosphorylate Raptor on 722/729:

Recently, AMPK was shown to directly phosphorylate raptor on two well-conserved serine residues in response to energy stress(9). The phosphorylation of Raptor induces 14-3-3 binding to raptor and inhibit mTORC1 kinase activity(9). In AMPK α 1/ α 2 double knockout MEFs, AICAR or 2-DG induced raptor phosphorylation is compromised but sorbitol induced raptor phosphorylation persists, suggesting there's other kinase that can phosphorylate Raptor in other stress conditions (Fig 3.6.A).

MARK4 is a well-known AMPK related kinase. We then try to figure out if MARK4 can also phosphorylates raptor. Overexpression of MARK4 strongly induced raptor phosphorylation as indicated by both mobility gel shift assay and phospho-antibodies, and the raptor S722/792A mutant is resistant to the MARK4 induced mobility shift, indicating 722/792 are the only MARK4 phosphorylation sites (Fig. 3.4.A,B). In vitro kinase assay using a fragment raptor ser792 residue as substrate showed that MARK4 could phosphorylate the raptor fragment as potently as AMPK (Fig. 3.4.C,D). In addition, MARK4 knockdown slightly decreased the basal raptor phosphorylation level (Fig. 3.4.E). Although these data indicate that MARK4 can phosphorylate Raptor on Ser722/792 in vivo and in vitro, we were not able to see the direct interaction between MARK4 and any of the mTORC1 components including raptor (data unshown).

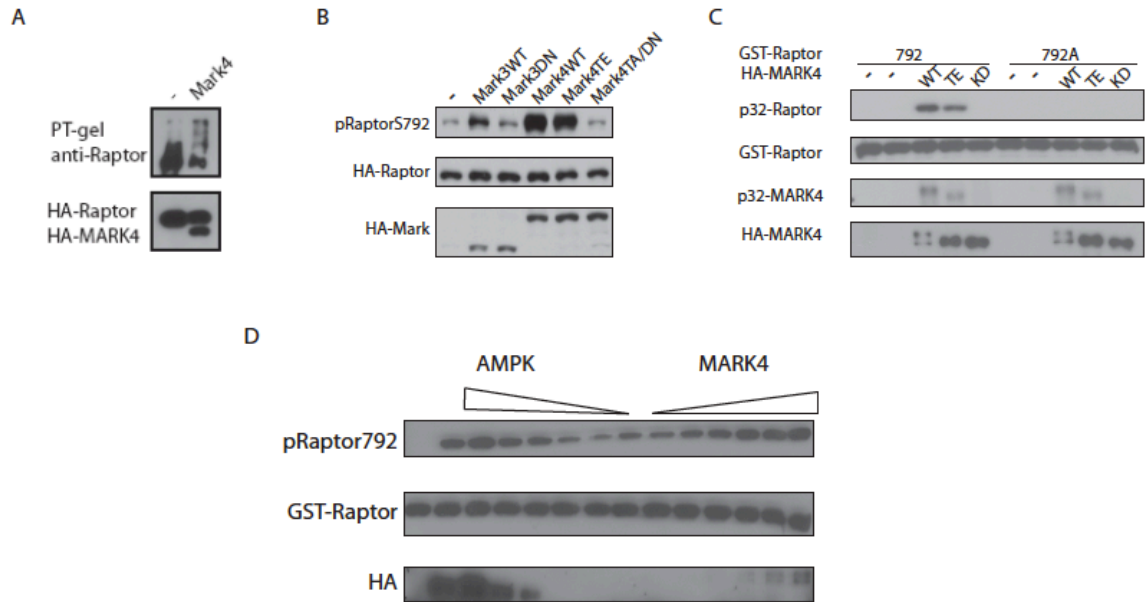


Figure 3. 4. MARK4 phosphorylates Raptor in vivo and in vitro.

(A) MARK4 phosphorylates Raptor. MARK4 construct was co-transfected with HA-Raptor into HEK293 cells. Phosphorylation was determined through mobility shift and protein levels were determined by immunoblotting with the indicated antibody.

(B) MARK4 phosphorylates Raptor on ser792. Different MARK construct was co-transfected with HA-Raptor into HEK293 cells. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(C) MARK4 phosphorylates Raptor on ser792 in vitro. HA-MARK4 was immunoprecipitated from transfected HEK293 cells. *In vitro* kinase assay was performed using purified GST-Raptor fragment containing Ser792 as a substrate in the presence of ^{32}P -ATP. GST-Raptor792A mutant was used as a negative control.

(D) MARK4 phosphorylates Raptor as potent as AMPK. HA-MARK4 or HA-AMPK was immunoprecipitated from transfected HEK293 cells. *In vitro* kinase assay was performed using purified GST-Raptor fragment containing Ser792 as a substrate in the presence of cold ATP. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

Raptor 722/792 phosphorylation affect mTORC1 activity:

Because AMPK phosphorylation on Raptor 722/792 residues were shown to inhibit mTORC1 kinase activity, we want to know if MARK4's effect on mTORC1 inhibition goes through raptor phosphorylation. As shown in Fig. 3.5.A, Mark4 overexpression only slightly decreases mTORC1 activity in in vitro kinase assay, which is not consistent with its potent effect on S6K phosphorylation in vivo. In vitro kinase assay using Raptor722/792A mutant showed no decrease of mTORC1 kinase activity under MARK4 overexpression condition (Fig. 3.5.B). These data suggested that MARK4 might have some other effect on mTORC1 other than affecting its kinase activity.

It is recently reported that RagA/C interact with raptor(11). And it is proposed that in response to amino acid, raptor/mTOR moved to lysosome compartment where rheb is localized, thus is activated by rheb(11, 20). We thought to test whether raptor 722/792 phosphorylation affect binding with RagA/C. It was shown that the interaction between RagA/C and wild type raptor is decreased upon MARK4 overexpression, while the interaction between RagA/C and raptor SA mutant is resistant to MARK4 overexpression (Fig. 3.5.C).

Figure 3. 5. Regulation of TORC1 by MARK4.

(A) MARK4 overexpression slightly decreases mTORC1 kinase activity. Raptor was immunoprecipitated in CHAPS buffer and assayed for mTORC1 kinase activity using purified S6K1 as a substrate. IP-kinase assays were immunoblotted for phosphorylation of purified S6K1 substrate using phospho-Thr389 S6K1 antibody as well as for level of immunoprecipitated raptor, mTOR.

(B) MARK4 overexpression does not affect the mTORC1 kinase activity containing Raptor 722/792 mutant. Raptor722/792A was immunoprecipitated in CHAPS buffer and assayed for mTORC1 kinase activity using purified S6K1 as a substrate. IP-kinase assays were immunoblotted for phosphorylation of purified S6K1 substrate using phospho-Thr389 S6K1 antibody as well as for level of immunoprecipitated raptor, mTOR.

(C) MARK4 decreases Raptor-Rag interaction. RagA/C and Raptor were co-transfected into HEK293 cells with or without MARK4. The interaction was determined by co-immunoprecipitation with SE (short exposure) and LE (long exposure)

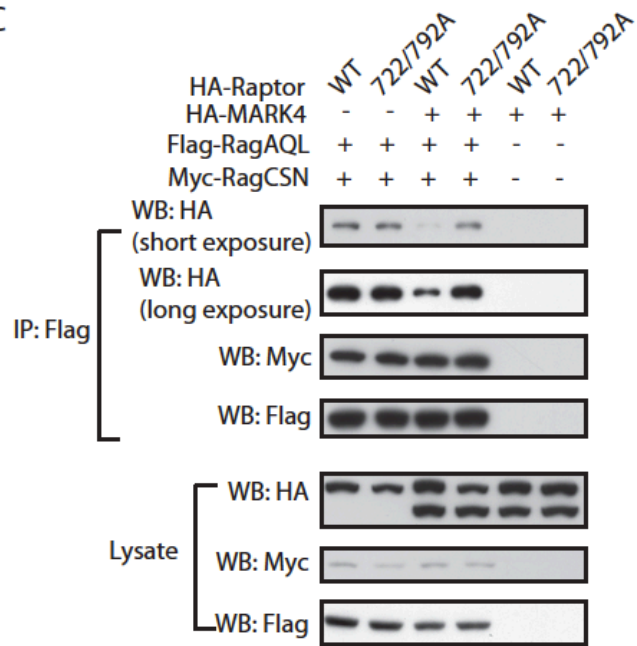
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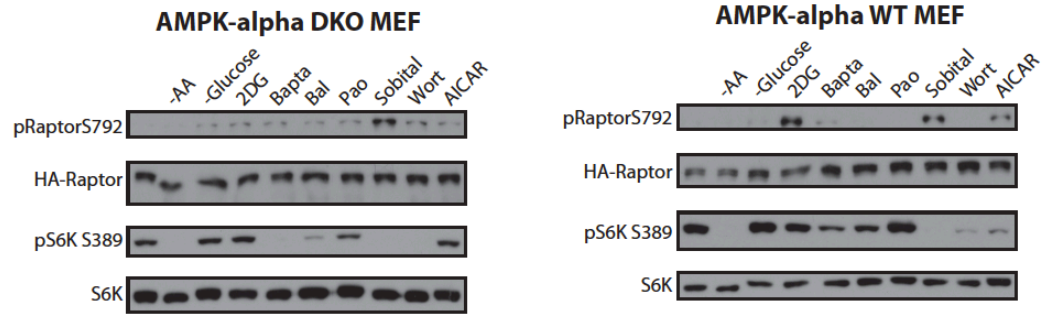


C



Together with AMPK phosphorylating the same site, we propose that Raptor 722/792 phosphorylation might serve as another integration point to receive different upstream signals to modulate mTORC1 activity in addition to TSC1/TSC2. We tried to figure out what signal goes through MARK4. Using AMPK double knockout MEFs, we screened several stress conditions for signals that can specifically induce raptor792 phosphorylation (Fig. 3.6.A). We noticed that Sorbitol can induce raptor792 phosphorylation in knockout MEFs. When knockdown MARK4, sobitol induced Raptor792 phosphorylation is greatly compromised yet, sorbitol's effect on mTORC1 activity as indicated by S6K phosphorylation still remains, suggesting that sorbitol's effect on mTORC1 at least not soly goes through MARK4 (Fig. 3.6.B).

A



B

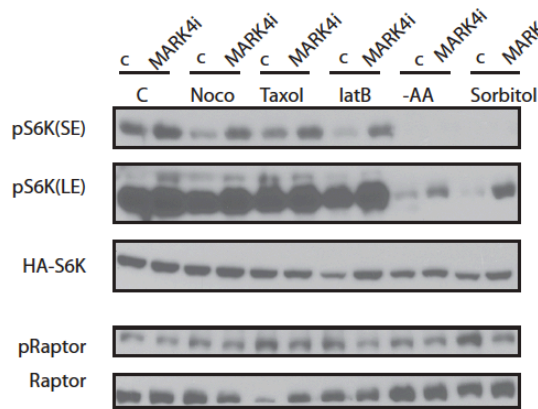


Figure 3. 6. MARK4 might regulate Sorbitol effects.

(A) Sorbitol induces Raptor792 phosphorylation in AMPK double knockout MEFs. AMPK WT and DKO MEFs were treated with indicated drugs. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

(B) Sorbitol's effect on Raptor792 phosphorylation goes through MARK4. Control and MARK4 siRNA were co-transfected with HA-S6K into HEK293 cells. Before collection, cells were treated with the indicated drugs. Phosphorylation and protein levels were determined by immunoblotting with the indicated antibodies.

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CHAPTER 4

CONCLUSION

How cells coordinate environmental input to regulate growth and metabolism is a fundamental question in cell biology. As a central growth regulator, the TOR pathway has attracted a wide attention and many progresses have been made to illustrate the complexity of this pathway. As has been shown that every signal uses several different pathways to regulate mTOR activity, which further strengthens the significance of the pathway. In addition to the core signaling components, there are several new members emerged as indispensable factors in the pathway. Collectively, insulin activates mTORC1 through PI3K pathway; glucose mainly regulates mTORC1 through AMPK; and amino acids go through Rag GTPases to activate mTORC1. Based on our data, we proposed an mTORC1 regulation model (Fig. 4.1), in which we added Rab and Arf GTPases and MARK4. We speculate that Rab and Arf GTPases regulate the trafficking of mTORC1 or its regulators and possibly act downstream of amino acids and Rag GTPases. MARK4 phosphorylates Raptor on Ser722/792, and therefore inhibits mTORC1 kinase activity by inhibiting the Rag-Raptor interaction. MARK4 may partially mediate sorbitol-induced mTORC1 inhibition, and there might be some other signals that go through MARK4.

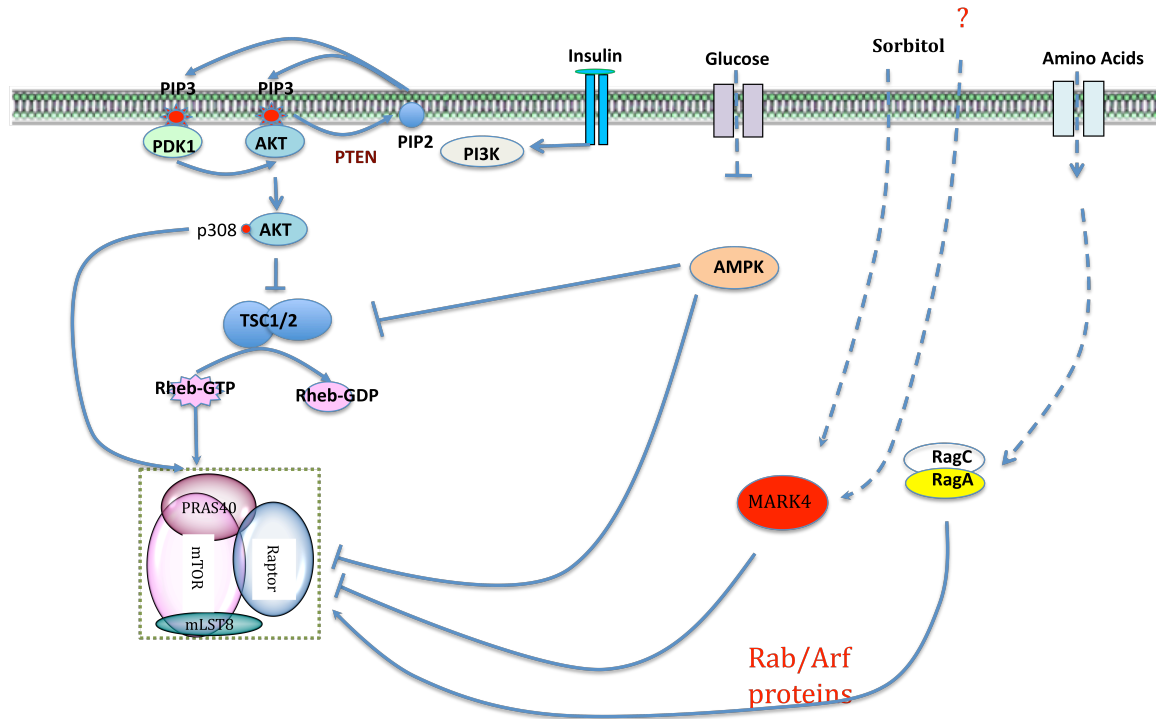


Figure 4.1. Proposed model of mTORC1 regulation.

Rag GTPases

In 2008, two groups independently reported Rag GTPases as critical mediators between amino acid sufficiency and mTORC1 activation(3, 6). RagA or RagB forms stable heterodimers with RagC or RagD via their respective C-terminal regions, and it is the heterodimer that fully activates mTORC1. Within the heterodimer, the two GTPases bind guanine nucleotides in opposite manners; one binds GTP and the other binds GDP. Only when RagA/B is in GTP form and RagC/D is in GDP form is the heterodimer fully active and able to stimulate TORC1. In addition, RagA/B has a dominant role over RagC/D in TORC1 activation. The active Rag heterodimer can directly bind to raptor, which is a key subunit in TORC1. This interaction between Rag and raptor depends on the GTP binding status of RagA/B. However, unlike Rheb, which can activate TORC1 kinase activity by binding, Rag is unable to activate TORC1 by binding alone. It has been proposed that Rag may activate TORC1 by transporting TORC1 to the vicinity of Rheb.

Last year, work from Sabatini lab identified three proteins - MAPK scaffold protein 1 (MP1), p14 and p18 - that form a complex and tether Rag GTPases to lysosome, through protein purification approaches(7). The complex, termed Ragulator, co-immunoprecipitates with Rag GTPases, and p18 interacts directly with Rag GTPases in vitro. In p14- null or p18-null MEFs, the subcellular localization of Rag GTPases and mTORC1 are perturbed, resulting a decreased mTORC1 activity. When reconstituted with wide type p14 or p18, the cells showed normal Rag GTPase and mTORC1 localization and mTORC1 activity. The authors also show that expression of a modified p18 protein that is targeted to mitochondria in the p18-null MEFs leads to the mitochondrial localization of Rag GTPases. Together, these findings suggest that the Ragulator complex is necessary and sufficient to target Rag GTPases to their subcellular location but the recruitment of mTORC1 is regulated by Rag GTPases.

In addition, using raptor fusion protein that is constitutively targeted to lysosomal membranes, the pathway becomes insensitive to amino acid stimulation or deprivation and independent of Rag and Ragulator, but still requires Rheb function, further dictating that unlike other stimuli's that mainly regulate Rheb activity, amino acids induce mTORC1 movement toward Rheb and thus activated by Rheb.

It would be interesting to see if p18-MP1-p14-RagA/C form a big complex or p18 forms dimer or oligmer, with each p18 molecule interacting with MP1/p14 or RagA/C. Since p14/MP1 complex has been previously shown to be critical for the spatial and temporal specificities of the MAP kinase signaling pathway (8), it would be interesting to see if MAP kinase signaling pathway and mTOR pathway have some crosstalk in the lysosome compartment and if these two pathway crosstalk has physiological significance in the *in vivo* conditions. Rag GTPases are the only small GTPase that has a long C-terminal domain and forms a heterodimer. Additionally, within the herterodimer, the two GTPases bind guanine nucleotides in opposite manners. It would also be interesting to know the significance of the Rag heterodimer on mTORC1 activation and why it requires an active RagA and an inactive RagC in the dimmer to activate mTORC1. Raptor has been shown to be the RagA effector. It would be interesting to identify the RagC effector and see if it mediates a different signaling pathway or negatively regulates mTORC1. It has been shown in many cases for small GTPases (especially Rab) cascades that Rab binds a GEF as effector that activates the next Rab, and it also binds a GAP that inactivates the previous Rab. Whether Rag GTPases follow the same manner of regulation is still under investigation. A key remaining issue is the activation/nucleotide exchange of Rag GTPases in response to amino acid signal. Notably, VAM6, also known as VPS39, has been suggested as a guanine nucleotide exchange factor for Gtr1p in yeast(9). Whether Vam6 shows GEF activity towards Rag GTPases in mammalian cells is still an unanswered question. Future study of amino acids in regulating nucleotide exchange of Rag GTPases will shed new light on this important signaling pathway in cell growth regulation.

Rab and Arf family members

Studies in Yeast and *Drosophila* have indicated a connection between endocytic trafficking and TOR signaling. Tor has been shown to be localized on endocytic vesicles in Yeast, fly and mammalian cells(1-3). In addition, amino acids are shown to drive localization of mTOR to late endosomes in mammalian cell culture condition(3).

Recently, two reports, including work from our lab, further characterized the role of endocytic trafficking in mTORC1 activation (4, 5). It was shown that perturbation of early to late endosome trafficking by either overexpression of Rab5CA (constitutive active mutant) or knockdown of a key member of the HOPS complex, hVps39 inhibits mTORC1 activity possibly by affecting mTORC1 localization. In addition, our results show that several other Rab and Arf family GTPases also affect mTORC1 activity in *Drosophila* S2 cells and in mammalian cells. Although the exact mechanism remains unknown, we speculate that they might indirectly influence the subcellular localization of mTORC1 or its regulators. By overexpression of constitutively active Rab5 mutant, we show that Rab5 inhibits amino acids but not glucose induced mTORC1 activity. In addition, Rab5 inhibits active RagA/C induced mTORC1 activity. These results are consistent with the notion that amino acids mainly affect the mTORC1 localization, and Rab and Arf proteins are important molecules in distinct trafficking steps and may affect the mTORC1 localization upon activity change. The fact that several but not all the Rab and Arf family GTPases affecting mTORC1 raises the question that which trafficking steps are involved in mTORC1 activation. It has been shown that mTORC1 is localized to lysosome compartment in nutrient rich condition. Upon amino acids starvation, mTORC1 is diffused in the cytosol. The mTORC1 lysosome \leftrightarrow cytosol localization change might be regulated by the indicated Rab and Arf proteins. To test this idea, we should determine the localization of mTORC1 in different Rab or Arf expression cells using immunofluorescence. According to our model, different Rab or Arf protein overexpression traps the mTORC1 in various vesicular compartments, indicated by organelle markers, and not capable of going to lysosome compartment. In addition, those Rab protein that show no effect on mTORC1 should not affect the mTORC1 subcellular

localization although they might affect certain steps of the trafficking pathway. Another possibility is that Rab and Arf proteins regulate the localization of upstream regulators of mTORC1. For example, they might affect the amino acid transporters, Rheb lysosome localization, and Akt or PDK1 membrane association. The fact that Rab5 overexpression can inhibit active RagA/C induced mTORC1 activation tells us that at least Rab5's effect on mTORC1 is not through affecting amino acid transporter trafficking in the cell. We also show that Rab5 does not affect the mTORC2 activity, indicating that Rab5 may not affect Akt or PDK1 membrane association. It should be taking into consideration that although Rab5 and Arf1 having similar inhibitory effect on mTORC1 upon overexpression and we speculate that their effect on mTORC1 is through trafficking pathway, they might have different mechanisms on regulating mTORC1. For example, using immunofluorescence assay, we noticed that Rab5 overexpression cells show different mTOR localization pattern than those neighbouring cells that do not have Rab5 overexpression, with the Rab5 overexpression cells show perfect mTOR colocalization with overexpressed Rab5 but the neighbouring cells show more broad area of mTOR staining. In contrast, overexpressed Arf1 shows partial colocalization with mTOR and Arf1 overexpression does not seem to affect mTOR localization. These data suggest that mTOR localization change in the cell involves the trafficking step that dependent on Rab5, yet Arf1 might not regulate mTOR localization but rather the trafficking of some other upstream regulators of mTORC1. Although Rab and Arf family GTPases affect the mTORC1 activity likely through their effect on general trafficking pathway, the exact role of different Rab and Arf proteins on mTORC1 regulation need to be clarified using immunofluorescence studies.

MARK4

MARK protein kinases were originally identified by their ability to phosphorylate a serine motif critical for microtubule binding in the microtubule-associated protein (MAP) tau(10). It is an AMPK related kinase that shares a similar domain structure. The physiological roles of the MARK/Par-1 kinases come from the study of orthologous

genes in yeast, nematodes and flies(10). Par-1 is first identified in *c. elegans* as one of the six Par genes essential for the asymmetric division of zygotes, later, its function expanded to a wider range including cell polarity, microtubule stability, and possibly cell cycle control and Wnt signaling(10).

Our recent work identified MARK4 as an important negative regulator of mTORC1. We show that in *Drosophila* S2 cells and mammalian cells, knockdown of MARK family member increases mTORC1 activity while overexpression of MARK4 in mammalian cells significantly inhibits mTORC1 activity. MARK4 phosphorylates Raptor, a key component of mTORC1. The phosphorylation inhibits mTORC1 kinase activity and disrupts Raptor-Rag interaction. However, till now we can't rule out the possibility that MARK4 might affect some other aspects of mTORC1 signaling. Since MARK4 has been shown to phosphorylate the microtubule-associated protein (MAP) tau, and affect the microtubule stability, we tested whether MARK4 affects mTORC1 through its regulation of microtubule function. As shown in Fig. 3.6.B, Nocodazole, a drug that has the similar function as MARK4 to destabilize microtubule, shows the similarly inhibitory effect on S6K phosphorylation, which is consistent with MARK4 effect on mTORC1. But when we treated the cells with Taxol, a drug that stabilizes microtubule, which should show opposite effect as MARK4, we still observed inhibitory effect on S6K phosphorylation (Fig. 3.6.B). These drug effects are consistent with the note that affecting trafficking pathway would inhibit mTORC1, but not consistent with the suspicion that MARK4 may affect mTORC1 through its effect on microtubule. So it is likely that the mTORC1 inhibitory effect of MARK4 is mediated through some other unidentified substrate other than tau, eg. Raptor.

AMPK has been shown to phosphorylate TSC2 and Raptor in mTOR pathway to inhibit mTORC1. Given that MARK4 is the same family member with similar consensus sites, one might speculate that MARK4 could also phosphorylate TSC2 and Raptor. We've shown in Chapter 3 that MARK4 can phosphorylate Raptor. We also tested if MARK4 can phosphorylate TSC2. Using gel shift assay, we did not see TSC2 mobility shift change upon MARK4 overexpression, indicating that MARK4 does not

phosphorylate TSC2. The more interesting aspect of MARK4 story is to identify the upstream of MARK4. Using AMPK double knockout MEFs, we screened several stress conditions for signals that can specifically induce raptor792 phosphorylation (Fig. 3.6.A). We noticed that sorbitol could induce raptor792 phosphorylation in AMPK knockout MEFs. When MARK4 was knockdown, sobitol induced Raptor792 phosphorylation was greatly compromised yet, sorbitol's effect on mTORC1 activity as indicated by S6K phosphorylation still remained, suggesting that sorbitol effect on mTORC1 at least not soly goes through MARK4 (Fig. 3.6.B). Till now most of the MARK4 studies focused on its function in neuron cells. MARK4S is predominantly expressed in the brain (11). It would be interesting to see if there're any neuron specific stresses that might go through MARK4 to regulate mTORC1 activity in neuronal cells. In addition, tau, the only identified MARK4 substrate in the cell has long been implicated in Alzheimer disease (12). Recent studies highlighted an interrelation between mTOR signaling and tau in the pathology of Alzheimer disease (13). It would be interesting to see the MARK4 activity in various Alzheimer disease samples. Using animal model of AD, one might answer whether MARK4 contributes to AD disease progression. Recently, several studies have linked MARK4 to cancer progression (14,15). By studying MARK4 and mTOR activity in these cancer cell lines and determining MARK4's contribution to mTOR activation and tumor progression, we may learn whether MARK4 is a upstream component of mTOR pathway in the pathophysiological context.

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