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mTOR signaling: RAG GTPases transmit the amino acid signal

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Abstract

mTOR (mammalian target of rapamycin) is a highly conserved nutrient-responsive regulator of cell growth that is found in all eukaryotes. The mechanism by which amino acids signal to mTOR has remained one of the largest outstanding questions in the field. Two recent complimentary studies provide compelling evidence that the Rag family of small GTPases is both necessary and sufficient to transmit a positive signal from amino acids to mTOR.

mTORC1 is regulated by nutrients and growth factors

The coupling of nutrient availability to cellular growth is essential for all organisms and, in eukaryotes, the target of rapamycin (TOR; or mTOR in mammals) Ser/Thr kinase is a key regulator of cell growth that is acutely sensitive to both growth factor and nutrient levels, including glucose and amino acids [1]. Consistent with its central role in controlling cellular growth, the mTOR pathway is hyperactivated in a broad spectrum of human cancers and in metabolic disease. mTOR, like its budding yeast orthologs, exists in two biochemically and functionally discrete signaling complexes. Signaling from mTOR complex 1 (mTORC1) is nutrient-sensitive, acutely inhibited by the bacterial macrolide rapamycin and functions as a master regulator of cell growth, angiogenesis and metabolism. mTORC1 comprises four known subunits: (i) mTOR; (ii) mammalian lethal with Sec13 protein 8 (mLST8), which is also called Gbl; (iii) proline-rich Akt substrate of 40 kD (PRAS40); and (iv) the WD40 repeat-containing subunit Raptor. Raptor, a scaffold, recruits downstream substrates including eIF4E-binding protein (4E-BP1) and ribosomal S6 kinase (S6K1) to the mTORC1 complex. mTORC2 is neither sensitive to nutrients, nor acutely inhibited by rapamycin. It lacks Raptor and PRAS40 and instead contains the scaffolding subunit Rictor in addition to mLST8, mammalian stress-activated protein kinase interacting protein 1 (mSin1) and Protor, which is also called PRR5; (proline rich 5 [renal]) [2].

Approximately five years ago, genetic studies in *Drosophila melanogaster* and mammalian cells identified the tuberous sclerosis complex (TSC) tumor suppressors as crucial upstream inhibitors of mTORC1 [3]. TSC2 contains a GTPase activating protein (GAP) domain at its carboxyl terminus that inactivates the Rheb GTPase, which associates with and activates mTORC1 *in vitro*. Phosphorylation of TSC1 and TSC2 forms an integration point for a wide variety of environmental signals that regulate mTORC1. Mitogen-activated kinases including Akt, extracellular signal-regulated kinase (Erk) and ribosomal S6 kinase (Rsk) directly phosphorylate TSC2, leading to its inactivation and Akt also directly phosphorylates the inhibitory protein PRAS40, resulting in mTORC1 activation [4,5]. In contrast to these pro-

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growth stimuli, glucose deprivation activates the growth-suppressive kinase AMP activated kinase (AMPK) to directly phosphorylate both TSC2 [6,7] and Raptor [8], thereby inhibiting mTORC1. Despite the many recent advances in our understanding of the molecular details of the mTOR pathway, one major area without a clear molecular understanding is how amino acids regulate mTORC1 activity.

The Rag GTPases: mediators of amino acid signaling to mTOR

Now using different approaches, two research teams have identified the Rag GTPases as essential mediators of amino acid signals to mTORC1. Sabatini and colleagues searched for proteins that immunoprecipitated with epitope-tagged Raptor in human embryonic kidney (HEK)293 cells and found RagC, a small GTPase in the Ras superfamily [9]. Given that the Rheb GTPase is a key regulator of mTORC1 [3], Guan and colleagues performed a focused RNA interference (RNAi) screen in *Drosophila* cells to search for GTPases required for amino acids to induce TOR-mediated S6K1 phosphorylation [10]. By means of this strategy, they independently discovered the *Drosophila* orthologs of the RagA and RagC GTPases.

Mammalian cells contain four members of the Rag subfamily of Ras small GTPases [11]. These proteins are orthologs of the budding yeast Gtr1p and Gtr2p GTPases [12], which interestingly were shown to regulate microautophagy in concert with TOR signaling, although they were thought to function in parallel or downstream of TOR in the initial genetic studies [13]. They exist as an obligate heterodimer in all eukaryotes examined, each containing a Gtr1p-like (RagA and Rag B) partner and a Gtr2p-like (RagC and RagD) partner.

Using RNAi, both new studies convincingly demonstrate that Rag GTPases are required for amino acids to acutely stimulate TORC1 in both *Drosophila* and mammalian cells. Because the signal from amino acids is required for insulin or growth factors to maximally stimulate mTOR, RNAi against Rag GTPases also reduces insulin-induced mTORC1 activation. Moreover, mutants of RagB locked in the GTP-bound (active) state confer resistance to amino acid withdrawal in mammalian cells, indicating that the Rag GTPases are sufficient to transmit the amino acid signal to mTORC1 [9,10].

In an elegant *in vivo* proof of this concept, the Neufeld laboratory, in collaboration with the Guan laboratory, demonstrated that *Drosophila* expressing a constitutively active RagA in cells of the fat body or the wing showed dramatically increased cell size under nutrient-limited conditions [10]. In addition, cells expressing the constitutively active RagA were completely resistant to starvation-induced autophagy, reinforcing the concept that active Rags alone are sufficient to transmit a nutrient replete signal to mTOR. Strikingly, expression of the activated RagA in the fat body alone also ameliorated the ability of flies to survive under starvation conditions, perhaps owing to its suppression of autophagy, despite the shortage of nutrient availability [10].

But what is the molecular mechanism by which Rag GTPases activate mTORC1? The Sabatini laboratory demonstrated that, in contrast to Rheb, Rag GTPases directly bind Raptor and showed that this association is stimulated by amino acids, the requirement of which is overcome with constitutively active RagB mutants. Despite this direct association, Rag GTPases, unlike Rheb, cannot stimulate mTORC1 kinase activity *in vitro* [9]. Epistasis experiments in the fly indicated that Rheb is either downstream or parallel to the Rag GTPases, because a constitutively active *Rheb* allele is sufficient to promote cell growth in the absence of Rag GTPases, whereas constitutively active Rag-promoted growth requires Rheb [10]. A final key insight provided by the Sabatini laboratory is that endogenous mTOR relocates to Rab7-positive perinuclear vesicular structures upon re-addition of amino acids to starved cells. RNAi revealed that this mTOR re-localization relied on Raptor and the Rag GTPases, but was not inhibited by rapamycin, indicating that it did not require mTORC1 kinase activity [9].

Interestingly, Rheb also seemed to localize to Rab7-positive vacuolar structures under both starvation and amino acid re-addition conditions. Taken together, these findings led the authors to propose a model whereby amino acids signal to induce RagA or RagB–GTP binding, thereby stimulating their binding to Raptor. The Rags then mediate mTORC1 relocation to vesicles that might contain Rheb; this association results in increased mTORC1 kinase activity (Figure 1). The potential ability of amino acids to direct mTORC1 to the proper location to receive the growth-factor-mediated signal from Rheb also provides a molecular explanation for why insulin signaling to mTORC1 requires the positive input from amino acids.

Concluding remarks and future perspectives

Despite this incredible leap forward in our knowledge, many questions remain. How do amino acids signal to increase GTP loading of the Rag GTPases? As remains the case for Rheb, the search for guanine-nucleotide-exchange factors that mediate the temporal or spatial activation of the Rag GTPases will now become a major focus of the field. How GTP-bound Rag stimulates Raptor and mTOR relocation to Rab7-positive structures also requires further investigation. Importantly, these findings demonstrate that mTORC1 localization might be dynamically regulated by upstream stimuli. Given that the regulation of mTOR localization remains poorly understood, further analyses are required to examine the temporal and spatial dynamics of endogenous mTORC1 and mTORC2 subcellular localization and that of their upstream signaling components. Such investigation might reveal further insight into how different upstream stimuli (Figure 1) are coordinated to regulate these complexes and whether discrete post-translational modifications and complex assemblies are compartmentalized within the cell. Such a mechanism could keep inhibitors such as TSC and PRAS40 in distinct localizations in response to different stimuli. The recent findings that the TSC complex can directly associate with mTORC2 [14], along with recent cell fractionation studies [15], indicate the existence of further spatial and temporal control of the crosstalk between mTOR complexes. In addition, recent studies revealed that both Akt-mediated PRAS40 phosphorylation [4] and AMPK-mediated Raptor phosphorylation [8] induce 14–3–3 binding, which often triggers the relocation of its binding partners. Because several other mTORC1 pathway components, including TSC2 and regulated in development and DNA-damage response 1 (REDD1), are also 14–3–3 binding partners [16], altered localization or shuttling might help to regulate mTORC1 through several upstream cues.

Similarly to Rheb, whether other Rag GTPase effectors exist beyond mTORC1 will be an interesting area to watch. Previous studies in yeast implicated the Rag GTPases in the control of vesicle trafficking [12], which is consistent with the effects seen in mammalian cells by the Sabatini laboratory. Interestingly, yeast studies also reported that Yrb2p is a direct interactor and effector of the Rag GTPases [17]. The closest mammalian homolog of Yrb2p, Ran-binding protein 3 (RanBP3), is a substrate for both Akt and Rsk kinases [18]. This connection offers another potential crosstalk mechanism between growth-factor-mediated and amino acid mediated control of mTOR (Box 1).

As with all major breakthroughs, the opening of this door creates a series of additional doors, the opening of which will surely reveal exciting insights into how eukaryotic cells couple their growth to nutrient availability in their environment. Combining the central position of mTORC1 in the regulation of cell growth and metabolism with the fact that rapamycin analogs are in >50 ongoing clinical trials, the elucidation of the Rag GTPase signaling pathway has immediate therapeutic consequences [2]. Knowing whether the Rag GTPases themselves represent excellent therapeutic targets for cancer or metabolic disease will represent another key step forward.

Box 1

Other connections from amino acids to mTOR

Before the findings reported in the papers of Sancak *et al.* [9] and Kim *et al.* [10], one of few proteins ever implicated in mediating the signal from amino acids to mTORC1 was the vacuolar protein-sorting-associated protein 34 (Vps34) type III PtdIns-3 kinase [19–21]; however, its involvement in regulating TOR was recently questioned in *Drosophila* [22]. Intriguingly, Vps34 has also been reported to regulate Rab7-mediated vesicular trafficking [23,24]. Given the Rag-dependent relocalization of mTORC1 to Rag7-positive structures, this connection could provide a potential hypothesis for how Vps34 depletion could interfere with the amino acid control of mTORC1 activity. A previous RNAi screen in *Drosophila* for kinases that mediate signaling from amino acids to TOR identified the sterile20 family member MAP4K3 [25]. How MAP4K3 signaling connects to the Rag GTPases will also be an important future question.

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Figure 1.

mTORC1 activation by nutrients and growth factors. mTOR is found in two distinct protein complexes. mTORC1 (mTOR, Raptor, PRAS40, mLST8) is acutely rapamycin sensitive and is regulated by both growth factors and nutrient cues. By contrast, mTORC2 (mTOR, Rictor, Sin1, Protor, mLST8) is not acutely regulated by rapamycin nor is it nutrient-sensitive. Growth factors stimulate mTORC1 via activating PI3-kinase downstream of growth-factor-binding receptor tyrosine kinases (RTKs), such as the insulin receptor. PI3-kinase stimulates phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P₃] production, which recruits Akt to the plasma membrane and results in its activation through phosphoinositide-dependent kinase 1 (PDK1)-mediated activation loop phosphorylation and hydrophobic motif phosphorylation by the mTORC2 complex. Akt and other growth-factor-dependent kinases not depicted converge to phosphorylate several residues in the TSC2 tumor suppressor, resulting in its inactivation. TSC2 and TSC1 form an obligate heterodimer that functions as a GAP for the Rheb GTPase. Hence, activation of PI3-kinase or Ras results in TSC complex inactivation and an increase in GTP-bound Rheb, which then binds mTORC1, stimulating its kinase activity. Glucose stimulates mTORC1, at least in part, through its inactivation of AMPK. Under conditions of low intracellular ATP, such as during glucose- or oxygen-deprived conditions, AMPK is activated in a manner dependent on both its direct binding to AMP and phosphorylation by its upstream kinase LKB1. AMPK in turn inhibits mTORC1 by directly phosphorylating both TSC2 and Raptor, thereby decreasing mTORC1 kinase activity. Two new studies identified the Rag GTPases as crucial mediators of amino acid signaling to the mTORC1 complex. The addition of amino acids to starved cells increases the levels of GTP-

bound RagB, which increases its affinity for Raptor. The Rag-bound mTORC1 complex then relocalizes to Rab7-positive perinuclear vesicular structures in the cell (also the site of Rheb localization). Localized recruitment of Rag-bound mTORC1 enables Rheb to associate with mTORC1 and stimulate its kinase activity. Active mTORC1 then phosphorylates its downstream substrates 4EBP1 and S6K1, thus, stimulating protein synthesis and cell growth. The Vps34 class III PtdIns-3 kinase and its binding partners Vps15 and Beclin were previously reported to modulate Rab7 vesicle trafficking; this finding could provide an explanation for previous studies that identified Vps34 as a component of the pathway from amino acids to mTORC1 activation. Further studies are needed to validate several different aspects of the model. The crucial role this pathway has in human cancer is underscored by the fact that several human tumor suppressors (blue with red text) that are inactivated in a variety of human cancers are found in this pathway. In addition, four different human oncoproteins (yellow) are found bearing activating mutations in a wide variety of human cancers. In addition, mTORC1 hyperactivation occurs in patients with metabolic syndrome. Color code: AMPK, pink; mLST8, brown; mSin1, mid blue; mTOR, green; Rag proteins, red; Raptor, purple; Rheb, orange; PRAS40, beige; Protor, dark blue; Rictor, bright pink; S6K1, gold; Vps proteins, gray; 4E-BP1, light blue.