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Regulation of the mTOR pathway by amino acid sufficiency

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Trang Huong Phan

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ABSTRAST

The target of rapamycin (TOR), highly conserved protein complexes across the eukaryotic kingdom, is a central pathway that coordinates numerous signaling cascades in the cells. The TOR signaling pathway enables to simultaneously sense different factors such as nutrient, energy, stress and, in metazoan, growth factors, thus controlling the cell growth and proliferation. Mammalian TOR (mTOR) exists in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which are structurally and functionally different. Owing to its ability to sense amino acid availability, mTORC1 governs a network of signaling cascade and plays as a key regulator of protein synthesis and autophagy. mTORC1 controls protein synthesis by phosphorylating the downstream substrates 4E-binding proteins (4E-BPs) and ribosomal protein S6 kinase-1 (S6K1). Defects in mTORC1 have been reported as causes of many severe diseases, such as cancer and diabetes. In this review, the biochemical mechanisms that stimulate mTORC1 activity in response to amino acid signal will be emphasized. During the last decade, there has been a plethora of studies revealing a number of signaling regulators implicated in transferring the amino acid signal to mTORC1. This review will especially focus on the processes that modulate mTORC1 activity by modulating intracellular amino acid level such as the regulation of amino acid transports and protein degradation. The complexity of intracellular mediators that sense amino acid signals and trigger mTORC1 activities including the Rag GTPases, Ragulator complex, Rheb, MAP4K3, the vacuolar H^+-ATPase and hVps34 will be discussed. Although it is not fully understood yet, the significant role of branched-amino acids, especially leucine, in activating mTORC1 will be covered.

1. General introduction to TOR and its amino acid regulation

Cell growth is the biological term that indicates cellular development in terms of mass (and number). This process is tightly regulated through the control of anabolic and catabolic pathways in response to a wide range of nutritious conditions and growth factors. When cells are in plentiful nutrients and in the presence of growth factors, anabolic processes are promoted, synthesizing new cellular components, such as proteins, lipids and nucleic acids. In contrast, when cells are starving or suffer from stress factors, the reverse mechanisms, such as protein synthesis suppression and protein degradation, are operative to provide an internal supply of metabolites and thereby maintain fundamental metabolism for survival. During the long history of evolution, cells have developed distinct signaling mechanisms to couple external changes and intracellular responses.

The target of rapamycin (TOR) Ser/Thr kinase is a key signaling pathway component, which is highly conserved across the eukaryotic kingdom. The discovery of TOR originated from the finding of a new bacteria strain *Streptomyces hygroscopicus,* and its metabolic product, rapamycin (Vézina et al., 1975). This substance was found to possess anti-fungal property. Later on, it was observed that rapamycin is able to inhibit cell proliferation of higher eukaryotes. These striking findings have paved the way for further studies regarding the molecular mode of rapamycin in cells. In the last decades, the studies in the field have strongly confirmed the crucial role of TOR signaling in controlling a cellular balance between anabolic and catabolic processes, adapting to various changes of nutrients and growth factors in the diverse environments. As an example to illustrate for its irreplaceable role in cell growth, mammalian TOR deregulation is frequently observed in human diseases, such as cancer, metabolic disorders as obesity, type 2 diabetes, and aging. Therefore, understanding of how mTOR connects extracellular factors and intracellular regulation is of importance in this field, which can further lead to pharmacological applications.

Amino acids have been well-known for their central roles as building block of proteins and as intermediates in metabolism. The first observation that amino acids have impact on TOR signaling came from research in yeast *Saccharamyces cerevisiae*. Yeast mRNA translation was shown to significantly decrease either when the cells were treated with rapamycin or when TOR genes were mutated (Barbet et al., 1996a), which was accompanied with the promotion of catabolic pathways such as autophagy. Similarly, later on, investigations in multicellular organism strengthened this view. In *Drosophila melanogaster*, the decrease in cell size and endoreplication that result from the complete loss of TOR function are similar to those observed in amino acid-starved cells (Oldham et al., 2000a). In *Caenorhabditis elegans*, TOR deficiency leads to a suppression of global mRNA translation, further causing a developmental arrest which is comparable to the response to nutritious limitation (Long et al., 2002a). For tissue culture cells, amino acid withdrawal does not only lead to remarkable decrease in processes that are sensed through the TOR pathway but also renders them in certain aspects insensitive to growth factors (Hara et al., 1998a). Therefore, amino acids are speculated to play a positive role in

regulation of TOR signaling (Kimball and Jefferson, 2002). Thanks to extensive research, an inclusive picture of how TOR is regulated by growth factors has been generated. In contrast, given its comparable importance, the questions of how amino acids are sensed by cells and which knots connect signals in amino acids-facilitated TOR pathway have been unanswered.

In this thesis, I would like to summarize our current understandings of the mode of action of TOR signaling pathway with focus on its regulative relation by amino acids. We firstly start out by introducing the TOR structure and its roles in intracellular signaling. The amino acid-mediated TOR pathway will follow with discussions of amino acid transporters system on cell membranes, which are the first composed gate of the TOR signaling. How the upstream and downstream signals of TOR work will be explained. In addition, the particular influence of leucine on this pathway will be discussed in more detail. To sum up our current understanding of amino acid-mediated TOR signaling, we would like to draw a picture of TOR signaling regulation by amino acid and to discuss the remaining questions that need to be tackled in the future.

2. mTOR structure and organization

The mammalian TOR (mTOR) is a large protein of 289 kDa, which is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKK) family. Studies on yeast and higher eukaryotic models, using genetic and biochemical approaches, discovered that there exists two conserved mTOR multiple-protein complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Loewith et al., 2002). Given that a number of TOR components are shared by both mTORC1 and mTORC2, each of them also contains its distinct constitutions.

2.1. Structure and general roles of TORC1

mTORC1 comprises of five elements: the main core mTOR which is a Ser/Thr kinase; mammalian orthologue of yeast Lethal with Sec13 protein 8 (mLST8, also referred as GβL); DEP-domain-containing mTOR-interacting protein (DEPTOR); regulatory-associated protein of mTOR (RAPTOR); proline-rich AKT substrate 40kDa (PRAS40) (**Figure 1**). The three former are commonly shared by mTORC2 and the two latter define mTORC1. However, detailed functions of each component have been not defined yet. In mTORC1, mLST8 function is not clearly known yet. It has been shown that mLST8 is not required for mTORC1 activity as mLST8 deficiency does not affect mTORC1 pathway (Guertin and Sabatini, 2007; Guertin et al., 2006). DEPTOR is found to be an inhibitor of mTOR complexes and its expression is tightly coupled to mTOR function. mTOR phosphorylates DEPTOR, which in turn promotes a release of DEPTOR from the complexes. DEPTOR deficiency causes an increase in TOR activities, which further reduces DEPTOR expression (Timothy Peterson, Cell 2009). RAPTOR serves as a scaffold protein for mTORC1, which is essential for mTOR assembly and gaining its catalytic function (Hara et al., 2002; Kim et al., 2002; Nojima et al., 2003). PRAS40 binding to kinase domain of mTOR and preferentially to RAPTOR plays a role of mTORC1 substrate inhibitor. While PRAS40 overexpression will subsequently reduce the

cell size, its silence will result in an increase in mTORC1 substrate activities. Phosphorylation of PRAS40 leads to release of PRAS40 from complex and its loss of effect on mTORC1(Oshiro et al., 2007; Sancak et al., 2007a; Vander Haar et al., 2007).

mTORC1, not mTORC2, is sensitive to rapamycin, which is defined by their unique elements, RAPTOR and RICTOR, respectively(Jacinto et al., 2004; Loewith et al., 2002; Sarbassov et al., 2004). When rapamycin enters cells, it binds to the small protein 12kDa FK506-binding protein (FKBP12), founding rapamycin-FKBP12. This complex consequently inhibits RAPTOR-mTOR interaction by disassembling RAPTOR from mTORC1 and inhibiting mTORC1 (Kim et al., 2002).

Figure 1: Components of mTORC proteins. Mammalian target of rapamycin (mTOR) complex 1 and mTORC2 hare 3 elements in common: mTOR, mammalian orthologue of yeast Lethal with Sec13 protein 8 (mLST8) and DEP-domain-containing mTOR-interacting protein (DEPTOR). Regulatory-associated protein of mTOR (RAPTOR) and proline-rich AKT substrate 40kDa (PRAS40) are unique for mTORC1; rapamycin-insensitive companion of mTOR (RICTOR), protein-observed with RICTOR (PROTOR) and mammalian stress-activated protein kinase interacting protein (mSIN1) are specific to

Owing to mTORC1 intrinsic rapamycin-sensitivity, many studies of its biological function have been conducted. A number of evidences strongly emphasize mTORC1 central role in interplaying between external factors and internal processes during cell growth and proliferation (**Figure 2**). Many roads of external factors lead to mTORC1, including amino acids, growth factors, energy and stress status and oxygen. In response to such signals, mTORC1 regulates its downstream effectors to promote anabolic processes such as protein and lipid synthesis and ribosome biogenesis or to limit catabolic mechanisms such as autophagy. Downstream regulator of mTORC1 will be discussed below. The remainder of this thesis aims to dig in how amino acids regulate mTORC1 pathway.

2.2. Structure and general role of TORC2

mTORC2 has six component proteins: mTOR, DEPTOR, mLST8, which are commonly shared with mTORC1; rapamycin-insensitive companion of mTOR (RICTOR), protein-observed with RICTOR (PROTOR), mammalian stress-activated protein kinase interacting protein (mSIN1) (**Figure 1B**). Playing as similar role as in mTORC1 complex, DEPTOR negatively regulates mTORC2 activity (Peterson et al., 2009). Although being a shared element in both mTOR complexes, mLST8 functional importance is different between in two complexes. In fact, mLST8 is required to remain interaction of RICTOR to mTORC2 complex. The interaction of RICTOR to mTORC2 is completely abrogated upon the loss of mLST8, consequently leading to impair mTORC2 functions (Guertin et al., 2006). PROTOR exists in two isoforms, PROTOR1 and PROTOR2. Either single or double mutant of PROTOR1 and PROTOR2 does not

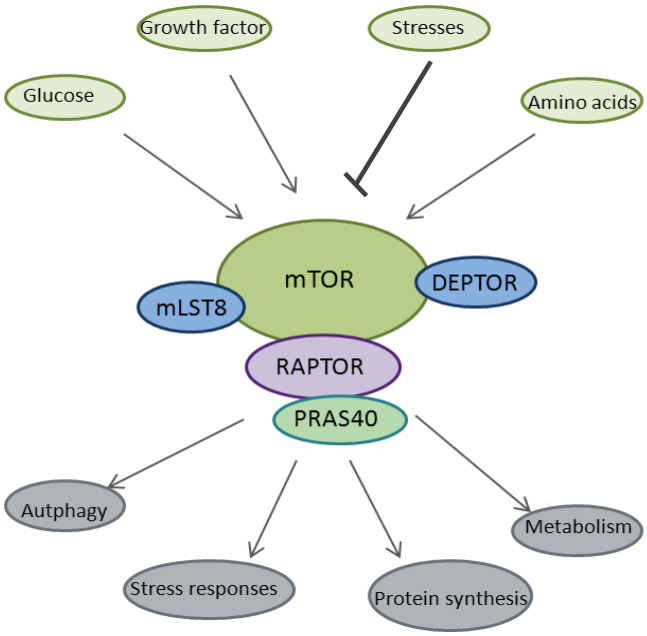

Figure 2: mTORC1 is a master regulator in cellular signaling pathway. mTORC1 integrates extracellular stimuli and changes and converts into cellular process for cell adaptation. External factors include amino acids, stresses, glucose and growth factor. Cellular processes induced by mTORC1 are protein synthesis, metabolic processes, autophagy, stress responses

have impact on mTORC2 assembly and other components (Pearce et al., 2011). In the same study, it was indicated that PROTOR1 may help mTORC2 to effectively phosphorylate its substrate SGK1 as its loss results in the reduction of SGK1 phosphorylation. RICTOR does not only help assembly of mTORC2 but also acts to define rapamycin-insensitivity of mTORC2, which is contrast to RAPTOR in mTORC1 complex (Jacinto et al., 2004; Sarbassov et al., 2004). There is some evidence that mTORC2 assembly and its catalytic phosphorylation is facilitated by mSIN1 (Frias et al., 2006; Lu et al., 2011).

In contrast to largely understanding of mTORC1 downstream and upstream signals, relatively little is known about mTORC2. The limited understanding of mTORC2 is due to two reasons. Firstly, mTORC2 deficiency is lethal and secondly, there is no inhibitors of mTORC2 have known currently. Nevertheless, numerous genetic approaches utilized recently have revealed different roles of mTORC2 in cell survival, proliferation and cytoskeleton organization. However, environmental factors and signal pathways that lead to mTORC2 have been not well determined. In fact, there is no involvement of mTORC2 in sensing amino acid signals. Further studies need conducting to better understanding mTORC2 upstream and downstream effectors.

2.3. mTORC1 as a central regulator of cell growth and metabolism

Serving as a master nutritional sensor within cell, mTORC1 senses the availability of amino acids as well as the presence of growth factors and in turn responses to these signals by controlling different steps of protein synthesis. Two direct targets of mTORC1, which have been studied intensively over last decades, include eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal S6 kinase (S6K1).

4E-BP1, which is phosphorylated by mTORC1, is well known for its inhibiting function to translation. Hypophosphorylated 4EBP1 inhibits translational initiation by competing with eIF4G to strongly bind to translational initiation factor eIF4E and therefore preventing the formation of translational pre-initiation complex (PIC). Once being phosphorylated by mTORC1, hyper-phosphorylated 4EBP1 disassociates from eIF4E, releasing the binding site on eIF4E for eIF4G and subsequently promoting the formation of PIC by recruitment of other components such as eIF3 and eIF4A (Gingras et al., 2001, 1998).

S6K1 kinase, an AGC family kinase, is another phosphorylation-mediated substrate of mTORC1. To gain fully activated mode, S6K1 kinase need phosphorylating at multiple sites, of which T389 and T229 are essential (Martin and Blenis, 2002). mTORC1 directly phosphorylates S6K1 kinase at Thr389, causing S6K1 kinase conformational change and increasing accessibility of another kinase phosphoinositide-dependent kinase 1 (PDK1) towards S6K1. As a result, S6K1 Thr229 is phosphorylated and consequently obtains its full activation (Martin and Blenis, 2002). In turn, S6K1 kinase leads to numerous changes by phosphorylating its downstream substrates. Phosphorylated eIF4B at Ser422 is among substrates of S6K1 kinase, which facilitates its recruitment to eIF3:40S ribosomal subunit of translation pre-initiation complex (Gingras et al., 1998; Raught et al., 2004). In addition, phosphorylated eIF4B boosts helicase catalysis of eIF4A which is normally at low processivity (Rogers et al., 2002). Another substrate of S6K1

kinase is programmed cell death 4 (PDC4D), which is shown as tumor suppressor. PDC4D functions to suppress helicase capable of eIF4A. Upon being phosphorylated at Ser67, PDC4D becomes a target of ubiquitin ligase, a family of proteasome. Hence, S6K1 kinase prevents eIF4A from inhibition of PDC4D (Dorrello et al., 2006; Yang et al., 2003).

Besides directly regulating translation factors, S6K1 kinase also magnifies protein synthesis by controlling ribosomal machinery. Indeed, S6K1 kinase activates rDNA transcriptional factor UBF by phosphorylation of UBF carboxy-terminal domain. Phosphorylated UBF in turn interacts with another basal transcriptional factor SL-1 and form a stable initiation complex at rDNA promoter (Hannan et al., 2003). Moreover, it was shown that S6K1 kinase targets to TIF-IA, a regulatory factor of RNA polymerase I (RNA Pol I), stimulating RNA Pol I activity(Mayer et al., 2004).

Targeting 4E-BP1 and S6K1 kinase as two distinct pathways to control protein synthesis suggests critical role of mTORC1 in tightly and efficiently regulating protein synthesis, which is one of the most important checkpoint for cell growth and division. mTORC1 stimulates protein synthesis multiple levels such as mRNA translation regulation and ribosomal machinery enhancement, which will overall increases protein synthesis efficiency upon stimuli and nutritional changes.

3. Regulation of intracellular amino acids levels

3.1. Cellular amino acid homeostasis

Intracellular amino acid homeostasis is affected by different factors including the availability of environmental amino acids, cellular uptake, cellular utilization and cellular recycling activity. This section of the thesis will discuss the interplay between mTORC1 signaling and processes that determine amino acid level of cell.

One of the interesting questions concerning the regulation of mTORC1 by amino acids is that whether intracellular or extracellular amino acids play dominant role in regulating mTORC1 signalling pathway. It is well observed that the changes of amino acids in the culture medium have significant effect on cell growth and development (Barbet et al., 1996b; Hara et al., 1998b; Long et al., 2002b; Oldham et al., 2000b). On the other hand, there is extensive evidence suggesting that the intracellullar amino acid concnetration plays an important role in controlling the mTORC1 pathway. In the study performed on *Xenopus* by Christie, the influence of intracellular amino acid on mTORC1 activity was investigated. The increased level of intracellular amino acid without outer supplement, which is conducted by adding cycloheximide, an inhibitor of protein synthesis, resulted in a remarkable increase in phosphorylation of 4E-BP1 and S6K1 kinase (Christie et al., 2002). A similar effect was observed when amino acid-deprived mammalian cells were treated with four different types of protein synthesis inhibitors (Proud, 2004). The increase in intracellular amino acids resulting from inhibition of protein translation is in part dependent on autophagy: addition of 3-MA, an autophagy inhibitor, to amino acid-deprived cells led to a significant decrease in cycloheximide-induced phosphorylation of 4E-BP1 and S6K1 (Beugnet et al., 2003). Taken together, it appears that intracellular amino acids pool, which is determined by multiple factors, such as an import of environmental amino acids, provision by protein recycling and the consumption rate due to protein synthesis, plays a superior role in regulating mTOR (**Figure 3**).

Importance of Leucine

It is well established that different amino acids have a different impact on stimulating TORC1. Leucine, in particular, has a profound positive effect on mTORC1 signaling pathway. A comparable suppressive effect on mTORC1 activity was observed in leucine-deprived and total amino acid-deprived cells, whereas removal of other single amino acid showed various extents of effect (Hara et al., 1998b). There are consistent results revealing the preeminent effect of leucine withdrawal in various cell types (Avruch et al., 2008a). Moreover, leucine was found to stimulate phosphorylation of S6K1 kinase and 4E-BP1, subsequently promoting protein synthesis (Kimball and Jefferson, 2006). In human skeletal muscle, it is well documented that leucine and other branched-chain amino acids account for one-third of muscle protein and function as positive stimuli towards protein synthesis (Dodd and Tee, 2012; Drummond et al., 2010). The significant effect of leucine in regulating TORC1 is also demonstrated by studies conducted in *Xenopus laevis*. Christie et al showed that neither an increase in extracellular leucine nor

total amino acid level is capable of activating TORC1 here (Christie et al., 2002). Intriguingly, a clear effect on TORC1 activity as indicated by an up-regulation of phosphorylated S6K1 and 4E-BP1, was seen following amino acid or leucine addition to the extracellular medium of oocytes over-expressing system L transporters. Also directly injecting leucine into oocytes activated mTOR, indicating that intracellular levels are determining the response of mTOR.. Despite supportive evidence for its role, understanding of how leucine is activating mTOR only became clearer recently.

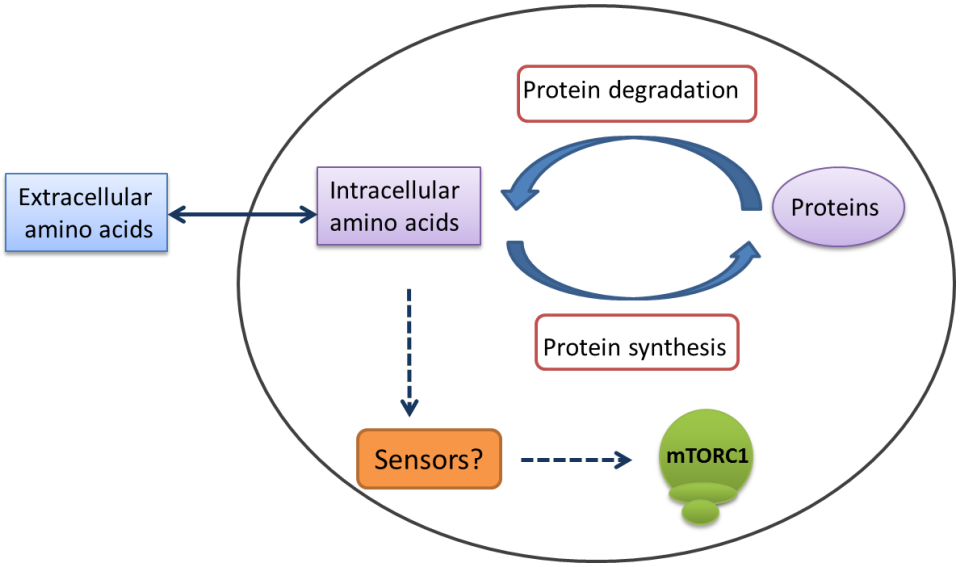

Figure 3: Multiple factors affect intracellular amino acid level. Intracellular amino acid level is reduced by protein synthesis consuming and is increased by autophagy. Among extra- and intra-cellular amino acid, a number of studies support the critical impact of intracellular amino acid on mTORC1 activity.

3.2. Regulation of amino acids transportation

The crucial role of amino acids in modulating the mTORC1 pathway, which is conserved across eukaryotes, has become well recognized. The first and foremost node of the mTORC1 signaling cascade is amino acid transporter where amino acids enter the cell and initiate the pathway. Owing to the numerous studies focusing on this topic, we have gained better understanding of how amino acids are transported into the cells. Current knowledge of amino acid transports in different research models will be addressed in this part.

Bidirectional amino acid transport system

Recently, in studies on HeLa cells, Nicklen et al. have discovered that the cell surface transporter of L-glutamine and L-leucine SLC7A5/SLC3A2 acts in a bidirectional manner to regulate mTORC1 activity (Nicklin et al., 2009). It was shown that pre-loading cells with glutamine is essential to prime mTORC1 activation in response to leucine. In contrast, the presence of only glutamine is ineffective in triggering mTORC1. Using both pharmacological inhibitors and short-interfering RNAs (siRNAs), it was revealed that SLC1A5, a Na^+-dependent system A, is in charge of taking up glutamine and other neutral amino

acids into cells. SLC1A5 works in combination with SLC7A5-SLC3A2, a heterodimeric system L transporter, which is required for leucine and other branched amino acids import in exchange for glutamine export. The proposed mechanism is that SLC1A5 and SLC7A5-SLC3A2 together control amino acids transport in two steps. SLC1A5 generates a high intracellular L-glutamine level, which is utilized as an efflux substrate by SLC7A5-SLC3A2 to import L-leucine (**Figure 4**). Once SLC1A5 is inhibited, subsequent addition of L-leucine to cell culture has no effect on mTORC1 activity while autophagy, a proteolysis process, is triggered. Inhibition of SLC7A5-SLC3A2 restrains activation of mTORC1 induced by leucine and glutamine with no effect on glutamine uptake. Either SLC1A5 or SLC7A5-SLC3A2 knockdown is sufficient to suppress mTORC1 pathway and consequently cause a significant reduction in cell size. It is noteworthy that SLC7A5 is expressed at high level in many tumor cell lines, suggesting its critical involvement in promoting anabolic processes in tumors.

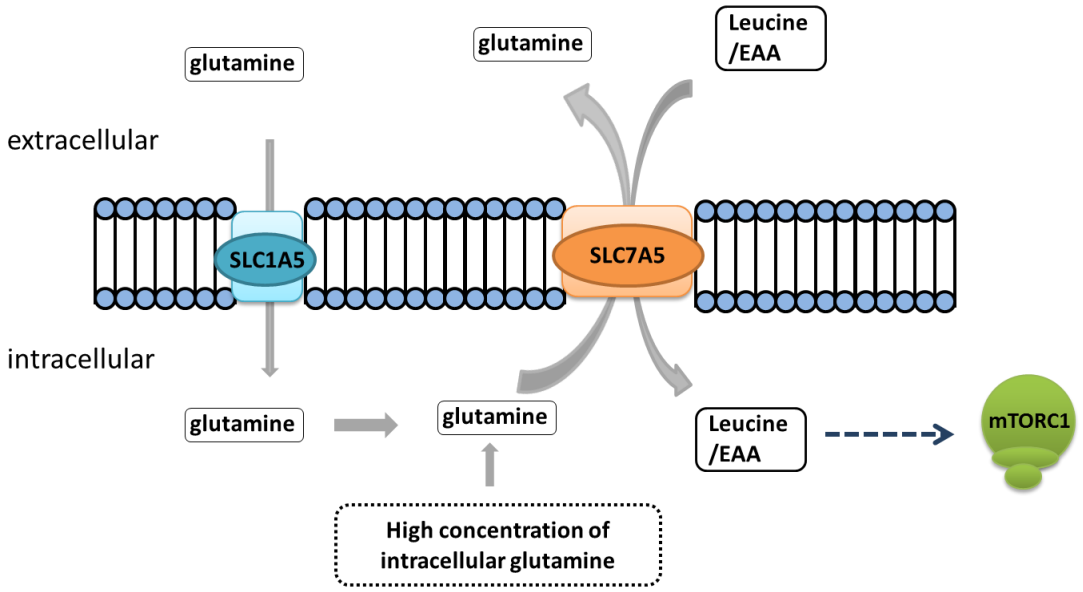

Figure 4: Bidirectional transport of amino acids regulates mTORC1. SLC1A5 and SLC7A5 work in pair to take up essential amino acids. Intracellular glutamine is maintained at high concentration by SLC1A5, which is used by SLC7A5 as efflux to shuffle extracellular leucine. This in the end activates mTORC1 activity.

The study by Nicklen is in line with several researches conducted on skeletal muscle cell lines. It was reported that there are two types of transporter systems related to mTORC1 regulation on cell surfaces (Drummond et al., 2010; Liu et al., 2004). LAT1, a system L type transporter, which is similar to SLC7A5-SLC3A2 found in HeLa cells, functions to import branched amino acid in exchange with other neutral amino acids. Working in couple with LAT1 is SNAT2, a Na^+-linked system A transporter, which carries glutamine from outside to the cytoplasm. Like SLC1A5, SNAT2 transporters induce a high concentration of intracellular glutamine, facilitating amino acids exchange. In parallel, LAT1 uses the high concentration of intracellular glutamine as exchanger to promote leucine import. mTORC1 activity corresponds to expression levels of both transporters types (Drummond et al., 2010). Using S6K1 and

4E-BP1 phosphorylation as indicators of mTORC1 activity, a strong suppression of mTORC1 signaling was observed when SNAT1 was deleted (Evans et al., 2008). When amino acids are plentiful, LAT1 and SNAT1 expression are elevated. This is explained as consequence of up-regulation of mTORC1 activity, leading to the higher rate of protein synthesis. The feedback here illustrates for an adaptive mechanism of cells that they quickly increase their sensitivity of amino acid influx.

In *Drosophila melanogaster*, two transporter classes, which appear to be involved in amino acid-regulated activity of TOR include *minidiscs* and *slimfast* (Colombani et al., 2003; Martin et al., 2000). Minidiscs belongs to the heterodimeric family of transporters and slimfast encodes cationic amino acid transporters. Being present at high concentration in the fat body, these transporters have been ascribed to sense amino acid availability and control body size via dTOR (Colombani et al., 2003). Strikingly, both transporters share high homology with members of SLC7 family; for example, 48% of minidisc sequence is similar to that of SLC7A5 (Martin et al., 2000). This raises a high possibility that eukaryotic evolution has conserved the bidirectional flux of amino acids to regulate TOR and cell growth. Taken together, it is evident that two transporter systems of glutamine and leucine actively contribute to mTORC1 regulation processes. It is clear that glutamine acts upstream of leucine to regulate mTORC1 activity, confirming the important roles of amino acids, especially leucine and glutamine, in this key signaling pathway within cells.

Proton-assisted amino acid transporter system

Proton-assisted transporters (PATs), another class of amino acid transporters, have been revealed in both *D. melanogaster* and human cell lines (Goberdhan et al., 2005; Heublein et al., 2010). The presence of PATs is not only found at cell surface but also in endosomal compartments such as lysosome and early endosome (Boll, 2002; Rubio-Aliaga et al., 2004). PATs were demonstrated to act as growth activators by promoting TOR signaling cascade. In *D. melanogaster*, one of PAT members, PATH, can facilitate cellular growth in vivo and activate S6K1. Interestingly, unlike above-mentioned amino acid transporter systems such as SLC1A5 and SLC7A5, PATH is characterized by its low capacity of transporting amino acids (Goberdhan et al., 2005). Likewise, studies on human MCF-7 breast cancer and HEK-293 embryonic kidney cell lines using siRNA screening revealed that PAT1 and PAT4 are required for mTORC1 activation (Heublein et al., 2010). Knockdown of either PAT1 or PAT4 reduces phosphorylation of S6K1 and 4E-BP1, indicating the reduction of mTORC1 activity. Given that PAT1 is highly concentrated in intracellular compartments, including endosomes, their roles in regulating mTORC1 are clear These findings utter questions of PATs intracellular function: do they promote TORC1-mediated cellular growth through modulating the local concentration of amino acids or activate an as yet undefined amino acid-independent pathway? One proposal is that PATs are implicated to intracellular amino acid-sensing mechanism. In other words, they are called *transceptors*, referring to a group of receptors that are structurally similar to transporters and function to activate mTORC1 by loading amino acids to a subcellular complex (Goberdhan et al., 2005). Indeed, this model well explains for the low capacity of

PATs transporter, their condensation on subcellular compartments and their ability of positively regulating TORC1. Given the observed important role of leucine, subsequently interesting questions might include that how PATs model couples with cytosolic leucine to regulate TORC1. Goberdhan *et al.* proposed that intracellular amino acids, such as leucine, act via transceptor mechanism and therefore can facilitate activity of PATs (Goberdhan et al., 2009). Future studies need to configure different mechanisms of how amino acids are sensed by TORC1 and conditions where they are utilized.

3.3. Cellular recycling: Autophagy

Autophagy is an evolutionarily conserved mechanism that degrades unnecessary or dysfunctional cellular components upon nutrient limitation by driving them to lysosome. The breakdown of cellular components can provide energy and building units such as amino acids for fundamental cellular processes, thus ensuring cellular survival. In yeast, the regulatory protein complex of autophagy process includes Atg1, Atg13, Atg17, Atg29 and Atg31 (Kabeya et al., 2005). Its mammalian homolog composes ULK1, ATG13, FIP200 and ATG101 (Ganley et al., 2009). The balance of cellular energy and amino acid availability is well maintained owing to a tightly controlled crosstalk between biosynthesis orchestrated by mTORC1 and proteolysis promoted by AMP-dependent protein kinase (AMPK) and ULK1 (or ATG1 in yeast) (Jung et al., 2010). The suppressive crosstalk between mTORC1 and ULK1 has been a subject of intense research over past years.

In mammalian cells, ULK1 is a homologue of Atg1 and functions to accelerate autophagosome complex formation. Similarly, it has been shown that there exists a strictly controlled interaction between mTORC1, ULK1 and AMPK in response to nutrient and energy condition. In nutritious environment, mTORC1 is active and autophagy is strongly suppressed. This is a consequence of ULK1 inhibition by mTORC1-mediated phosphorylation at Ser758 (Ganley et al., 2009; Hosokawa et al., 2009). Once cells have a shortage of nitrogen, ULK1 undergoes activation and in turn triggers autophagy. ULK1 is also initiated by AMPK-induced phosphorylation at its Ser317 and Ser777 under carbon unavailability (Kim et al., 2011). In addition, a study conducted by Elaine et al. in HEK-293 cells revealed that ULK1 inhibits mTORC1 signaling by phosphorylating Raptor, a scaffold component of mTORC1 complex, at multiple sites including Ser859, Ser792, Ser855 (Dunlop et al., 2011). Phosphorylated Raptor shows no difference in its interaction with mTOR compared to un-phosphorylated Raptor. However, the phosphorylated form of Raptor exhibits a reduced binding affinity to its substrates, for instance, rpS6 and 4E-BP1. A significant up-regulation of mTORC1 signaling towards rpS6 and 4E-BP1 is observed upon loss of ULK1. In sum, the central role of ULK1 in nutrient and growth factor signaling has been highlighted. Owing to studies of dynamic interplay between mTORC1 complex and ULK1, the complicated looping mechanism between mTORC1 signaling and autophagy has brought to light (**Figure 5A**). However, further studies need to configure if there is any cross talk between ULK1 and other upstream mediators in mTORC1 signaling pathway such as Vps34, MAP4K3, Rag proteins, RalA.

In yeast, Atg1 is a conserved serine/threonine kinase which plays a key role in forming pre-autophagosome structures and triggering autophagy. Atg1 activity is enhanced by nutrient limitation or rapamycin treatment (Kamada et al., 2009; Scott et al., 2007). When mTORC1 is switched on, Atg13 is phosphorylated by downstream TORC1 signaling kinase?. This brings about a reduction in Atg13 binding affinity to Atg1, repressing autophagosome formation and subsequently autophagy (Kabeya et al., 2005; Kamada et al., 2009; Kawamata et al., 2008). Conversely, when cells are in starving condition, TORC1 is inhibited by Snf1, a yeast orthologue of AMP-dependent protein kinase (AMPK) (Orlova et al., 2006) or by amino acid starvation signals. In these conditions, hypo-phosphorylation form of Atg13 promotes autophagosome formation and trigger autophagy (Kamada et al., 2009). In brief, Atg1 complex places in the center of the negative regular mechanism between mTORC1 signaling and autophagy (**Figure 5B**).

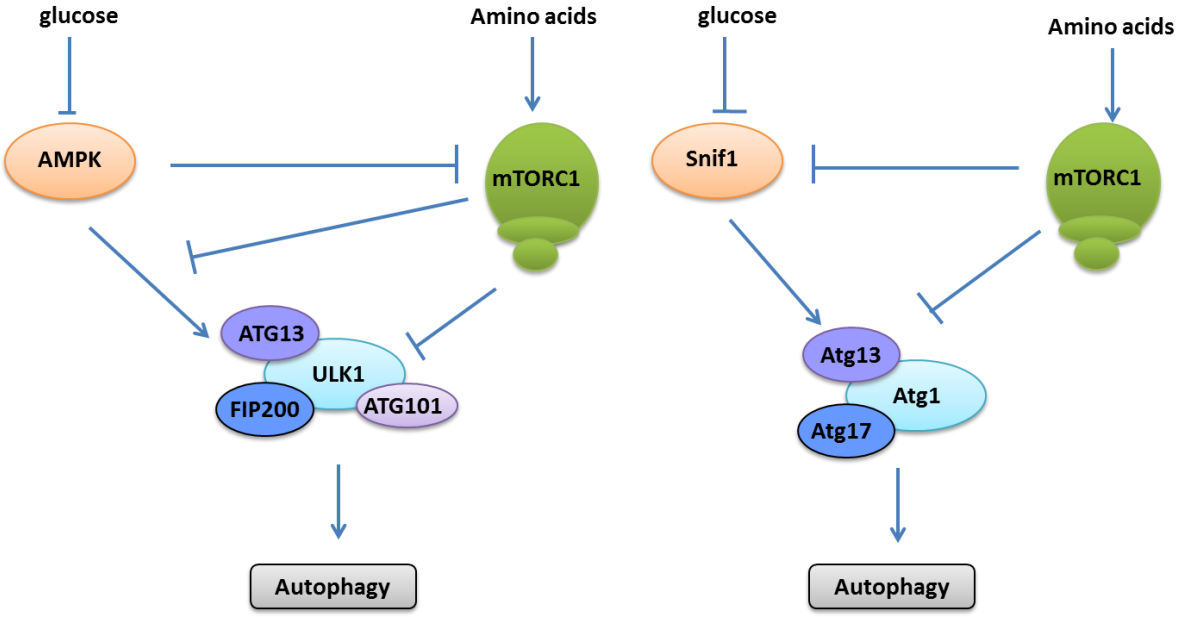

Figure 5: mTORC1 is tightly controlled by carbon and nitrogen availability. 5A: In yeast, mTORC1 is tightly controlled by carbon and nitrogen availability, through the Snif1 and Atg complex, respectively. The yeast regulatory complex of autophagy consists of Atg1, Atg13, Atg17, Atg29 and Atg31. This complex is negatively controlled by AMPK and mTORC1; **5B**: In mammalian cells, mTORC1 is tightly controlled by carbon and nitrogen availability, through the AMPK and ULK1, respectively. The mammalian regulatory complex of autophagy composes ULK1, ATG13, FIP200 and ATG101. This complex is negatively controlled by AMPK and mTORC1.

The tangled interplay among three protein kinases of mTORC1, AMPK and ULK1 has gradually become clearer. The next focus in this topic needs to address whether any other facets of autophagy machinery is regulated by mTORC1 like ULK1. It is equally important to understand if amino acids generated by autophagy have any role in mTORC1 acceleration.

3.4. The G Protein-Couple Taste Receptor T1R1/T1R3 is a direct regulator of mTORC1 pathway

The involvement of taste receptor in controlling mTORC1 activation has been recorded for the first time by Wuason *et al.* (Wauson et al., 2012). It has been well-recognized that the taste-specific T1R1 and T1R3 G-protein couples together to form a heterodimer receptor, which senses amino acid taste in

gustatory neurons. This receptor responds to twenty L-amino acid stimuli and is broadly expressed in various types of culture cells, in mouse tissues and human islets as well. The potential role of T1R1/T1R3 in transmitting amino acid signals to mTORC1 was first noticed in the studies of MIN6 cells lacking of either T1R1 or T1R3. The ability of amino acid to induce the phosphorylation of S6K1 and to initiate translation is dramatically reduced in these cells. Similarly, RNA interference targeting either T1R1 or T1R3 in mouse heart cells significantly impaired amino-acid stimulated phosphorylation of S6 protein on S235 and S236. Moreover, the capacity of growth factor EGF and FBS to induce S6 kinase in the presence of amino acid reduced in correlation with the reduced expression of T1R1 in H9C2 cells. It is well established that the presence of amino acid contributes to the localization of mTORC1 to lysosomal membranes where the interaction between amino acids, Rag small GTPase and the Ragulator complex is triggered (Sancak and Sabatini, 2009; Sancak et al., 2010). Interestingly, reduced T1R3 expression prevents amino acids from triggering mTOR mobilization onto lysosomal membranes while mTORC1 activity is reduced by 25% in T1R3 knockout mice compared to that in wild-type. These clear findings support the novel involvement of T1R1/T1R3 in integrating amino acid signaling to mTORC1 in various types of cell lines.

The group also gave an explanation of how T1R1/T1R3 transfers amino acid signals to mTORC1 by providing evidence for the involvement of ERK1/2 kinase and a calcium channel PLCβ in this pathway (Wauson et al., 2012). Firstly, by blocking MEK1/2, the upstream kinase of ERK1/2, in several different cell lines, the authors found that amino acid-stimulated ERK1/2 and S6 phosphorylation were precluded. Secondly, it was observed that inhibiting PLCβ prevented an intracellular release of calcium by amino acids and consequently suppressed amino acid-activated mTORC1 activity. In fact, silencing T1R3 lessened the calcium influx induced by amino acids, indicating that calcium influx was essential for the maximum mTORC1 activation. These findings together reveals that the novel pathway that T1R1/T1R3 senses amino acids signals, activates PLCβ and ERK1/2 and transduces signals to activate mTORC1 (**Figure 6**).

It has been observed that the taste knockdown cells compensate for their incompleteness by several mechanisms. Although that there was no change in the internal amino acid level of T1R1 knockdown cells compared to that in control cells, there was a clear increase in mRNA level of amino acid transporters SLC7A11 and SLC3A2. However, up-regulated mRNA level of amino-acid transporters was accompanied by a reduced effect of amino acid-activated mTORC1, which has been further explained by neither a decreased expression nor an impaired functionality of these transporters?. Furthermore, knockdown of T1R1/T1R3 caused an increase in autophagy, which was observed by an accumulation of the ubiquitin-like protein LC3 in the autophagosomes. Thus, T1R1/T1R3 knockdown cells apparently attempt to counterbalance decreased mTORC1 activity by up-regulating mRNAs encoding transporter and increasing autophagy rate. However, it is well-recorded that internal amino acid level plays massive role in regulating mTORC1 activity (Christie et al., 2002; Proud, 2004). Therefore, what is still lacking in this study is to address several concerns. Firstly, which factor among internal amino acid and external

amino acid has more impact on regulating mTORC1? Secondly, is there any crosstalk between intracellular and extracellular amino acid regulating mode of action of taste receptor? While these findings add another branch to the complexity of mTORC1 regulation, it reflects that gaining optimal mTORC1 activation demands a merging of multiple factors.

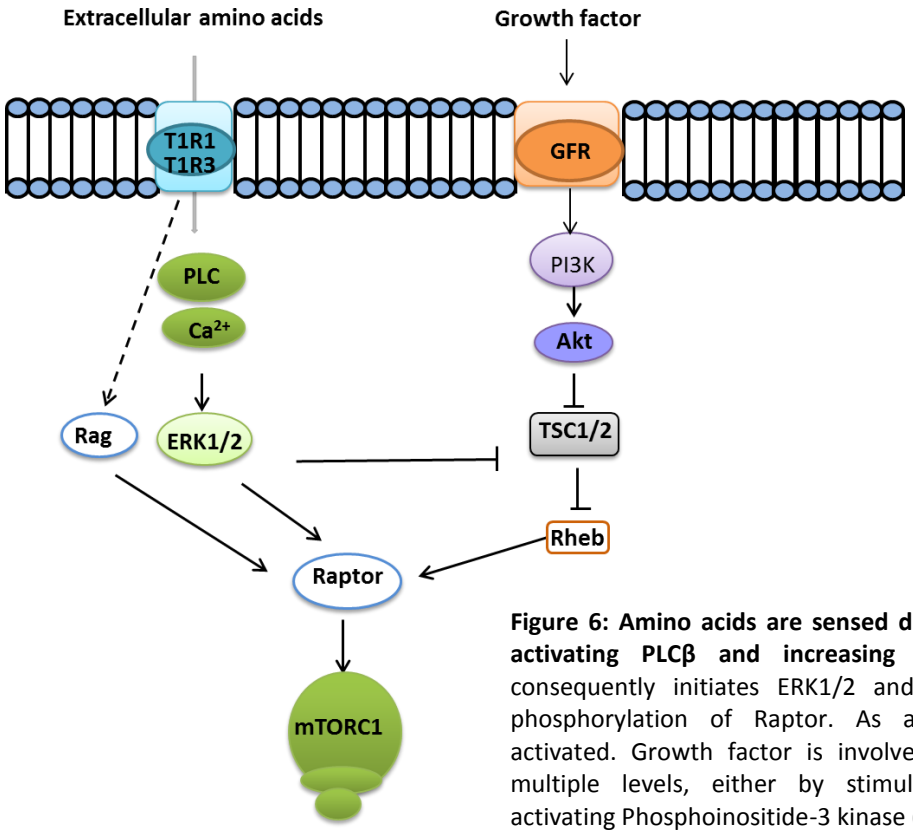

Figure 6: Amino acids are sensed directly via T1R1/T1R3, activating PLCβ and increasing calcium influx. This consequently initiates ERK1/2 and RSK, which induces phosphorylation of Raptor. As a result, mTORC1 is activated. Growth factor is involved in this pathway at multiple levels, either by stimulating ERK1/2 or by activating Phosphoinositide-3 kinase (PI3K) cascade.

4. Intracellular amino acid signaling to mTORC1

The next questions one may ask include how amino acid signals bring about the activation of mTORC1 and what are mediators for this process. Over the last ten years, these questions have been investigated intensively. A number of breakthrough findings provide us with a better understanding of these puzzling points. This part will present and discuss our current knowledge of several important mediators, including Rag GTPAses, Rheb, Ragulator complex. in amino acid-mediated regulation of mTORC1.

4.1. Rag GTPases are key mediators of amino acid signaling to mTORC1

The Rag GTPases belong to a superfamily of Ras-related small guanosine triphosphatases. The function of Rag GTPases dicsoverd by investigations of the Kun-Liang Guan lab, who used an RNAi screening approach for 132 annotated *Drosophila* GTPases, and by Sabatini lab by employing a biochemical approach in mammalian HEK-293T cells (Kim et al., 2008; Sancak et al., 2008). The finding that Rag GTPases function as a mediator in amino acid-induced mTORC1 signaling is an important achievement in this field, setting the stepping stone for later researches.

There are four isoforms of Rag GTPases. In mammalians, RagA and RagB, which are orthologues of Gtr1p in budding yeast, are identical in properties, whereas RagC and RagD, which are orthologues of Gtr2p in yeast, share similarities. Rag GTPases function as a heterodimer and only activate mTORC1 when GTP-bound RagA or RagB is coupled with GDP-bound RagC or RagD. The studies by Sancak *et al.* showed that heterodimer of $RagB^{GTP}$-$RagD^{GDP}$ interact with mTORC1 through binding with Raptor, consequently enhancing mTORC1-mediated phosphorylation of S6K1 kinase (Sancak et al., 2008). In contrast, there is no binding affinity between $RagB^{GDP}$-$RagD^{GTP}$ heterodimer that has negative effect on S6K1 phosphorylation. The combination of $RagB^{GTP}$ and $RagD^{GDP}$ shows highest binding affinity towards mTORC1 while the stable expression of $RagB^{GTP}$ could overcome the sensitivity of mTORC1 pathway to leucine or total amino acid withdrawal. The findings imply that $RagB^{GTP}$ serves as a primary determinant for mTORC1 activation. Furthermore, they outlined several evidences supporting that Rag GTPaese lay downstream in this amino acid signaling cascade. Firstly, when intracellular amino acids level was increased by protein synthesis inhibitor cycloheximide, mTORC1 activity was only deactivated by the presence of $RagB^{GDP}$-$RagD^{GTP}$ but not by leucine starvation. Secondly, employing reversible chemical cross-linker approach, they showed that the presence of amino acids lead to a remarkable increase in GTP-loaded RagB. Hence, the presence of Rag proteins is essential and sufficient for mTORC1 stimulation by amino acids. However, it remained mysterious till recently how GTP is bound to RagB under amino acid stimulation. The mechanism underlying the molecular regulation of Rag nucleotide state will be discussed in detailed in the next part.

Rag GTPases indirectly activate mTORC1 by determining its intracellular translocation. The localization of endogenous mTORC1 depends on amino acid status. mTORC1 is in a spatially limited location throughout the cytoplasm in starved cells, while mTORC1 localizes to peri-nuclear region and to large

vesicular substructures marked by lysosome-associated membrane protein 2 (LAMP2) and Rab7. Overexpression of RagBGTP induces the localization of mTORC1 on the Rab7-positive structure in amino acid-depleted cells. This implies that amino acid activates and localizes mTORC1 in a Rag-dependent manner.

4.2. Ragulator interacts with Rag GTPases to activate mTORC1

The discovery of Rag GTPases was crucial to our understanding of amino acid-facilitated mTORC1 activation, however little was clear about activation mechanism of Rag proteins. In fact, Rag GTPases contain no lipid-targeting domain which is necessary for its anchoring on the lysosome surface. This raised a suspicion that there exists an assisting Rag-binding protein which does bindto membranes. Indeed, the work by Sabatini lab later demonstrated Ragulator as an upstream component of Rag GTPases capable of targeting Rag to the lysosomal surface. Immunoaffinity purification followed by mass spectrometry was used to identify Rag-interacting proteins, which revealed three proteins including p18, p14 and MP1 encoded by LAMTOR1, LAMTOR2 and LAMTOR3 respectively. These three proteins interact together and form a trimeric complex, which is named Ragulator. To confirm the specific interaction of Ragulator and Rag GTPases, they co-expressed three elements with RagB and RagD and the result gratifyingly showed an immunoprecipitation of both RagB and RagD. Collectively, the results implied the unambiguous interaction between Rag GTPases and Ragulator and the biological contribution of this interaction was discovered.

The underlying mechanism of how Ragulator mediates Rag GTPases recruitment to lysosome membrane was further addressed. Employing biochemical and immunoprecipitation approaches, the group demonstrated that Rag GTPases directly interact with p18 but not p14 or MP1. In addition, it has been well-documented that because of its N-terminal myristoylation and palmitoylation, p18 appears to facilitate lysosomal localization of the trimeric complex (Nada et al., 2009). Thus, p18 functions as a key player in forming the binding between Ragulator and Rag GTPases and further with mTORC1 complex. The p18-mediated interaction of Ragulator towards Rag proteins subsequently leads to localization of Rag proteins onto lysosomal surface. To illustrate, RNA interference of p18 precluded Rag proteins from mobilizing to lysosome as RagC distributed throughout cytoplasm. In cells with artificially modified p18 that only allowed p18 to restrict on lysosome, mTORC1 activity was notably reduced as detected by S6K1 phosphorylation. Significantly, depletion of any Ragulator component disrupted mTORC1 recruitment to lysosome upon amino acid stimulation, consequently reducing mTORC1 activity while at the same time autophagy was triggered. These data together confirm that Ragulator is the essential upstream mediator for amino acid-induced recruitment of Rag GTPases and thus of mTORC1.

In attempt to explain the weak interaction between Rag heterodimers and purified Ragulator, the same group discovered two novel component proteins, namely HBXIP and C7orf59. In complementary experiments, these proteins were unambiguously co-immunopreciated with endogenous Rag proteins and were identified as additional components of Ragulator. P18 serves as a scaffold protein for

interaction between heterodimer of HBXIP-C7orf59 and herodimer MP1-p14, which forms a pentameric Ragulator (Bar-Peled et al., 2012). The presence of HBXIP and C7orf59 in pentameric Ragulator strengthened its interaction with Rag proteins to a much greater level and facilitated the mTORC1 sensitivity upon amino acid addition. In line with previous established data, all component of pentameric Ragulator are required to mediate the recruitment and localization of Rag GTPases and mTORC1 to the lysosome. An illustration for the essential role of the pentameric Ragulator is that in HBX1P and C7orf59 depleted cells, amino acids were incapable of activating mTORC1 activity.

In addition to enhancing affinity of Ragulator to Rag proteins, the HBXIP and C7orf59 presence in extended complex determines a novel function of Ragulator as a guanine nucleotide exchange factor (GEF) for RagA or RagB in mTORC1 signaling cascade (**Figure 7**). Possessing a characteristic like other GEFs, Ragulators represents a much stronger affinity for binding nucleotide-free than that for nucleotide-loaded GTPases (Bos et al., 2007). Indeed, Ragulator has a strong preference towards RagA and RagB, but not RagC or RagD. The preferential differences were defined by the distinctness between switch I and switch II regions of RagA/B and RagC/D. During amino acid stimulation, the GTP loading of RagA/B stimulated by Ragulator results in activation of Rag GTPases; and as a consequence, this activates mTORC1 activity. The entire complex of Ragulator is required for its GEF function towards RagA/B since depletion of any component prevented the effect on GTP association to RagB.

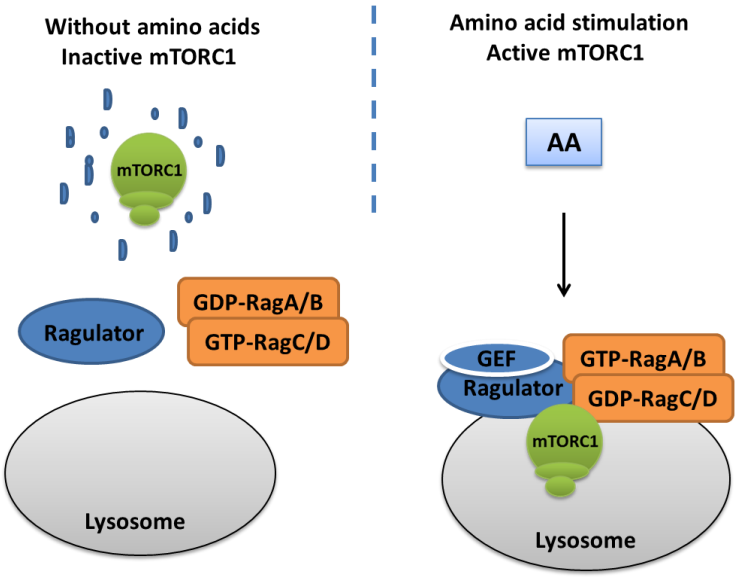

Figure 7: Model for role of Rag GTPases and Ragulator in signalling amino acid availability to mTORC1 in mammalian cells. Without amino acids, mTORC1 is inactive and dispersed throughout the cytoplasm. The presence of amino acid promotes the formation of the active configuration of the Rag GTPases complex at lysosomal surface. The GEF activity of Ragulator for Rag GTPases is switched on, promoting the conversion of GDP-RagA/B to GTP-RagA/B. mTORC1 binds to Rag complex and is localized to lysosome membrane, where it becomes activated. Figure is adjusted from (Dodd and Tee, 2012)

The finding of pentameric Ragulator has enlarged our knowledge regarding the key event of activating Rag GTPases by conversing GDP-bound RagA/B to GTP-bound form. However, several issues still need to be clarified by further intensive study. First, studies on Rag proteins and Ragulators implicate the importance of lysosome as a sensing site for activation of Rag-Ragulator complex. The question of what is an unknown function of lysosome would be interesting to address Additionally, how amino acids and especially leucine or arginine, acting upstream of Ragulator are precisely sensed is still not elucidated at the molecular level.

Up to date, it has been shown that there is no identified Ragulator orthologues in yeast. In fact, in this organism, TORC exists in a complex with Ego1, Ego3, Gtr1 and Gtr2 locating at the vacuolar membrane, which is called Ego complex (EGOC) (Ashrafi et al., 1998; Kogan et al., 2010). Similarly to p18, Ego1 is a palmitoylated and myristoylated protein and therefore functions to tether Grt1-Grt2 and TORC1 at the vacuolar membrane. Notably, the binding of TORC1 to Ego1 is dependent on amino acid signaling. However, there are certain divergences in TORC1 activation manner between mTORC1 and yeast TORC1. During amino acid deprivation, mTORC1 is dispersed throughout the cytoplasm in mammalian cells, whereas TORC1 is always located at the vacuole. Moreover, Vam6, a GEF of Gtr1 in yeast, is homologous to VPS39 in mammalian cells, which does not serve as GEF for RagA/B (Valbuena et al., 2012). Given differences of tethering machinery, it appears that the localization of mTORC1 at mammalian lysosome or TORC1 at yeast vacuole is the important event in activating its function.

4.3. Rheb - a direct activator of mTORC1 pathway

Rheb (Ras homolog enriched in brain) belongs to Ras superfamily of GTP-binding protein, which is a lipid-anchored and cellular membrane protein. Its name refers to the remarkable upregulation of expression seen in rat brain, following induction of seizures (Yamagata et al., 1994). Later, it was recognized as a novel regulator of growth in both *Drosophila* and mammalian cells. The overexpression of Rheb often leads to a remarkable increase in cell size and cell progression (Saucedo et al., 2003), thus making it an interesting protein to many researches in the field.

A wealth of studies has demonstrated that Rheb acts as a positive regulator of the mTORC1 signaling pathway. However, the underlying biochemical mechanism has not been constitutive yet. In the complicated signaling network, Rheb functions downstream of the tuberous sclerosis heterodimeric complex (TSC1-TSC2), which is an inhibitory control point of growth signaling pathways such as PI3K/AKt or Ras/MAPK pathways but not amino acids stimulation. A number of studies has shown that TSC1-TSC2 is an inhibitor of Rheb and that the phosphorylation of TSC induced by growth factors leads to an inhibition of GTPase-activating function towards Rheb (Zhang et al., 2003). Moreover, inactivation of TSC leads to dramatic activation of S6K1 phosphorylation, which is indicator of increased mTORC1 activity.

The positive contribution of Rheb to mTORC1 pathway was attributed to a direct interaction of Rheb with TORC1. Although there was a hypothesis that Rheb would function to stimulate amino acid uptake, hence facilitating TORC1, it was shown to not be biochemical mechanism employed by Rheb in the study

performed by Nobukuni (Nobukuni et al., 2005). Evidence supporting this conclusion was that overexpression of Rheb and TSC1-TSC2 brought about no effect on the steady state levels of intracellular amino acids. Moreover, when intracellular amino acid level was declined by lowering extracellular amino acids, neither overexpression of Rheb nor of TSC1-TSC2 assists to protect TOR1 activity against the decline. Thus, Rheb does not activate TORC1 by stimulating amino acids uptake.

The now widely held view is that Rheb activates TORC1 by directly interacting with TOR. Indeed, studies by Long and colleagues proved that Rheb binds directly to the TOR kinase domain (mTOR 2148-2300) in TORC1 (Long, 2005; Sancak et al., 2007b). Although it is unclear which part of Rheb is responsible for the binding to mTORC1, mutations in the switch I and switch II region of Rheb restrain the capacity of Rheb to prevent upregulating mTORC1in the presence of amino acids. However, the binding affinity between Rheb and TOR is very weak. While an interaction between endogenous TOR and endogenous Rheb has been uncertain, *in vitro* experiments showed that nucleotide-free recombinant Rheb binds to a much higher extent with mTORC1 than wild-type Rheb does. Furthermore, GTP-binding to Rheb does not selectively enhance binding to mTOR, which contrast the situation for other Ras-like GTPases and their immediate downstream effectors. Interestingly, the stronger binding affinity of nucleotide-deficient Rheb mutant towards Rheb does not lead to a higher kinase activity of mTORC1. In fact, mTORC1 only achieves its catalytic competence when it interacts with GTP-charged Rheb, implying that GTP binding is essential for a catalytic interaction.

Based on these findings, it has been proposed that the direct interaction of wild-type, GTP-loadeded Rheb to mTORC1 initiates conformational changes of the complex, which is necessary for the complex obtain its catalytic phosphorylation (Long, 2005). Although the GTP-loaded state of Rheb loosens the association between Rheb and TORC1, its configuration promotes the TOR complex to gain the correct form that is physiologically active. However, this explanation should be proven by further structural modeling. Moreover, it is also important to address several concerning question: Which intracellular compartment, lysosomes or endosomes, is Rheb located to? Is there any other protein that mediates the binding of Rheb to TORC1 or the interaction is indeed direct? How do amino acids activate Rheb and how is the Rheb-TORC1 binding important in maintaining TORC1 activity once it is activated.

Rheb can indirectly activate TORC1 signaling pathway via different mediators. The indirect pathway occurs via the synthesis of phosphatidic acid (PA) by Phospholipase D-catalyzed hydrolysis of phosphatidylcholine (PC) (Groenewoud and Zwartkruis, 2013; Sun et al., 2008). The study by Sun *et al.* revealed that in vitro, Rheb interacts and modulates the activity of PL-D1 in a GTP-dependent manner. As a result, the PL-D1-mediated generation of PA leads to an increase in mTORC1 activity through a direct interaction of PA with the mTOR FRB domain. Indeed, reduced activity of mTORC1 was observed when PA accumulation was inhibited as a consequence of PL-D1 depletion.

4.4. The vacuolar H^+-ATPase

Our knowledge regarding mTORC1 activation upon amino acids stimulation so far has emphasized an undeniable importance of lysosome as an organelle for localization and activation of Ragulator, Rag and mTORC1. This view is further strengthened by the interesting observation that in cell-free a system containing purified mTORC1 and isolated RAG GTPase-bound lysosomes, addition of amino acids propagated Rags-mTORC1 interaction (Zoncu et al., 2011). So, what makes lysosome become a special compartment where the activation of mTORC1 happens? Is there any lysosome-associated protein or process that initiates mTORC1 upon amino acid addition? These wonders were well explained by a research of Zoncu *et al.* that revealed the involvement of vacuolar H^+-ATPase (v-ATPase) in the mTORC1 activation in amino acid-dependent manner.

Employing double strand RNAs interference screening for lysosomal biogenetic and functional genes, they found that vhaAC39, vha16, vha100-1 and vha100-2, components of the v-ATPase, were implicated in the mTORC1 activation pathway. Decreased expression of these genes reduced dS6K phosphorylation to a level equal to that caused by down regulation of RagC. Furthermore, chemical inhibitors against v-ATPase such as ConA or SalA significantly decreased S6K1 phosphorylation. These findings are clear proof that v-ATPase is an activator of the mTORC1 activity.

However, our understanding of v-ATPase structure and function is not complete yet. Structurally, v-ATPase composes of two domains which build up a multisubunit complex. The V1 domain, which carries out ATP hydrolysis, is a peripheral complex. The V0 domain, which is responsible for proton flux from the cytoplasm into the lysosome, is an integral complex (Nishi and Forgac, 2002). Functionally, ATP is hydrolyzed by V1 domain, which drives proton transport from cytoplasm to the lumen by V0 domain (Kawasaki-Nishi et al., 2003). Consequently, this process maintains cytosolic pH and generates an acidified environment inside lysosome.

When it comes to address the precise role of v-ATPase in mTORC1 activation, it is vital to understand where in this cascade the v-ATPase is located: whether v-ATPase functions downstream of amino acids or owing to its function, v-ATPase controls intracellular amino acids transportation to the compartments where amino acids are sensed? Because alcohol ester derivatives of amino acids are able to diffuse across membranes and are hydrolyzed by esterases on lysosome and cytoplasm, they were employed to assess the role of v-ATPase. In fact, amino acid esters activated mTORC1 to a comparable level as native amino acids do. In addition, ConA and SalA showed similar inhibitory effects on mTORC1 activation upon ester amino acids stimulation (Zoncu et al., 2011). Thus, v-ATPase functions downstream of amino acid signal in the pathway and it has no effect on amino acid transportation.

Being downstream of amino acids in activating mTORC1, the v-ATPase is involved in the translocation of mTORC1 mediated by Rag GTPase and Ragulator. Using immunoaffinitiy purification followed by mass spectrometry, it has been pinpointed that Ragulator serves as a physical and functional connection between Rag GTPases and v-ATPase (Zoncu et al., 2011). While Ragulator interacts with V0 and V1

domain of v-ATPase, Rags only binds to V1 subunits with lower affinity. The interaction of V1 domain with Rags and Ragulator is strengthened or weakened by amino acid starvation or stimulation, respectively, whereas the interaction between the V0 domain and Ragulator is insensitive to fluctuation of amino acids levels. In the cells treated with v-ATPase chemical inhibitors, these physical interactions become insensitive to amino acids changes. Moreover, v-ATPase is not essential for guiding the Rag GTP-ases to the lysosome surface. Inhibition of v-ATPase activity by introducing chemicals or RNAi had no effect on lysosomal mobilization of RagC. Expression of constitutively active RagB GTPase rescued the defect in mTORC1 lysosomal localization and S6K1 phosphorylation induced by ConA and SalA. Therefore, v-ATPase lays downstream of amino acids and upstream of Rag GTPase activation and the physical interactions between v-ATPase, Ragulator and Rag GTPases are controlled by amino acid signal.

The ability of v-ATPase to hydrolyze ATP plays an important role in mTORC1 activation pathway. Evidently, ionophore FCCP, which disrupts lysosome pH without affecting v-ATPase function, does not affect the binding of Raptor and Rags (Zoncu et al., 2011). Inhibitors that block ATPase activity of V1 and rotation of the talk and the V0 subunit consequently suppress the binding of RagB and Raptor induced by amino acids in a concentration-dependent manner. Collectively, these data suggested that the ATP hydrolysis and the rotation of the stalk and are essential in amino acids-mediated mTORC1 activation. What still needs to be elucidated is whether the role of v-ATPase in amino acids-mediated mTORC1 activation is to acidify the lysosome lumen or maintain optimal cytosolic pH for cell growth.

Based on these findings, a novel model, an inside-out mechanism of lysosomal communication to the v-ATPase, has been proposed to explain the v-ATPase role in activating mTORC1 (Zoncu et al., 2011) (**Figure 8**). In this model, intracellular amino acids need to accumulate in the lysosome to trigger the signaling which will be transferred to other mediators located in the cytoplasm. Supportively, C14-labeled amino acids fed to cells rapidly appear within lysososme. Permeabilization of lysosomal membrane by treatment of organelle with Streptolysin O, Trixton X-100 disclosed amino acids out of lysosome lumen and totally inhibited amino acids capacity to initiate the interaction of Raptor and RagB. However, the issue regarding how amino acids are transported into lysosome, whether lysosomal amino acids come from within the cell or are imported directly from extracellular environment, and how they are sensed inside lysosome to activate v-ATPase remain unsettled.

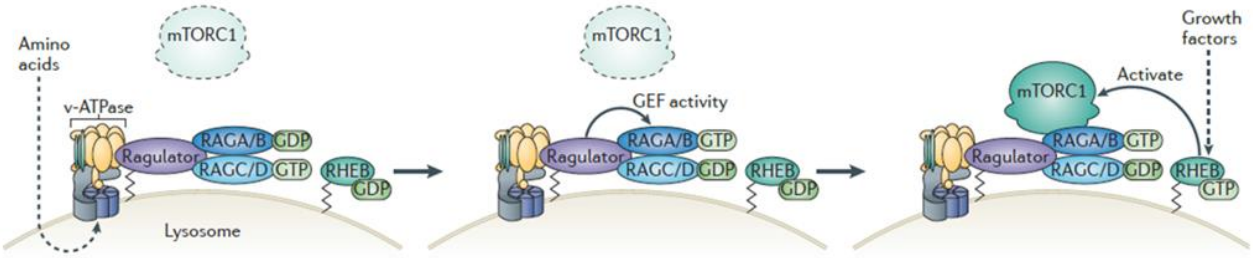

Figure 8: The involvement of v-ATPase in mTORC1 activation at the lysosome. Amino acids are supposed to accumulate within the lysosomal lumen and to signal to vacuolar H^+-ATPase (v-ATPase) through an "inside-out" mechanism. v-ATPase controls the Rag GTPase-Ragulator binding and the GTP- bound RagA/RagB. The active Rag complex binds to mTORC1 and recruits it to the lysosome and in a close distance with Rheb. Consequently, mTORC1 is activated. Figure is adopted from Jewell et al., 2013.

4.5. MAP4K3 is an effector of mTORC1 activation

MAP4K3 kinase is a Ste20 family member that was defined as an activator of mTORC1 in response to amino acids (Findlay et al., 2007). Initially, an RNAi screen for 200 protein kinases was carried out in *Drosophila* aimed to answer the question of which kinases are essential for optimal activity of TORC1. It revealed that suppression of *CG7097* led to decrease in dS6k1 phosphorylation. In human cells, MAP4K3 is an equivalent kinase of Drosophila *CG7097*. Suppressing MAP4K3 by RNAi significantly reduces phosphorylation of S6K1 at Thr389. In agreement with this view, overexpression of wild-type MAP4K3 increased phosphorylation of S6K1 to a significant level as amino acid stimulation does. Moreover, overexpression of MAP4K3 also increased phosphorylation of 4E-BP1. It was also shown that overexpression of MAP4K3 led to a remarkable increase in cell size. MAP4K3 activity is independent of stimulation of growth factors as insulin stimulation had no effect on MAP4K3 activity. These observations suggested that MAP4K3 is a downstream effector of amino acid stimulation and an upstream regulator of mTORC1.

Further study identified that phosphorylation of MAP4K3 at Ser170 is essential for its activity and mTORC1 activation (Yan et al., 2010). Amino acid sufficiency controls the phosphorylation of MAP4K3 at Ser170 without any effect on MAP3K4 localization. The removal of amino acids from medium suppressed Ser170 phosphorylation while re-supplementing amino acids stimulated Ser170 phosphorylation significantly. In addition, it was also observed that inhibition of RagC/D by RNAi impaired MAP4K3 ability to mediate S6K1 phosphorylation. However, there was no co-immunoprecipitation of Rag proteins and MAP4K3 was observed. Future works need to clarify how Rag GTPases and MAP4K3 activities are connected.

Given that amino acid withdrawal led to a remarkable decline of Ser170 phosphorylation within 5 mins, it was proposed that the dephosphorylation of Ser170 would be mediated by a phosphatase during amino acid insufficiency. Indeed, the inhibition of PP2A-type phosphatase protected MAP4K3 from de-

phosphorylation at Ser170, implying the involvement of PP2A as an inhibitor of MAP4K3 phosphorylation (Yan et al., 2010). Further immunoaffinity purification followed by mass spectrometry confirmed an interaction between a regulatory B-subunit of PP2A, PR61ε, and MAP4K3. Overexpression of PR61ε promotes MAP4K3 de-phosphorylation and therefore inhibits the MAP4K3 capacity of activating mTORC1. In line with this notice, inhibition of PR61ε prevents MAP4K3 from de-phosphorylation at Ser170. Collectively, these data imply that PP2A-PR61ε functions as a negative regulator of MAP4K3.

On the basis of these data, two different models describing how amino acids stimulate MAP4K3 and how PP2A is involved have been proposed. The first model considers a direct effect of amino acid sufficiency on MAP4K3 phosphorylation while PP2A acts in parallel to inhibit MAP4K3 phosphorylation. This model is supported by the dependency of MAP4K3-PP2A association on amino acids as the removal of amino acids enhances their association. The second model regards PP2A as a downstream effector of the amino acid signal. However, it remains unclear if amino acid withdrawal would propagate PP2A activity. Configuring an underlying mechanism should be a focus in the next steps of the field.

4.6. Leucyl-tRNA synthetase activates mTORC1 by sensing intracellular leucine

Recently, there were two independent studies reporting an involvement of leucyl-tRNA synthetase (LRS) as a direct sensor of intracellular leucine and as an activator of mTORC1 in yeast and mammalian cells (Bonfils et al., 2012; Han et al., 2012). However, the proposed models of LRS function in two studies are considerably different. LRS is a member of aminoacyl-tRNA synthetases (ARSs) which conjugate amino acids to their cognate transfer RNAs (tRNAs) for protein synthesis.

In mammalian cells, the identification that LRS works as an activator of mTORC1 signaling pathway stemmed from several observations (Han et al., 2012). First, upon amino acid stimulation, especially leucine, LRS was found to localize mTORC1 and Raptor to lysosomes, whereas amino acid withdrawal significantly decreased lysosomal localization of mTORC1 and LRS. LRS lysosomal localization occurred very quickly, only after 10 min of leucine supplement. Second, interference of LRS led to suppressed expression of S6K1 phosphorylation and to inhibited lysosomal localization of mTORC1 induced by amino acids. Lastly, LRS knockdown cells also had smaller sizes than control cells while autophagy was at uplifted level. Thus, LRS appears to be an effector in mTORC1 activation upon amino acid supplement.

Further experiments have resolved the manner how LRS activates mTORC1 pathway. LRS specifically interacts with heterodimer of RagD-GTPase with RagA or RagB in the presence of leucine or amino acids, which is determined by the C-terminus of RagD (spanning region of 371-400) and C-terminus of LRS (spanning region of 951-971) (Han et al., 2012). Mutations in these regions suppressed amino acid-induced mTORC1 activation by ablating the specific interaction between LRS and RagD-GTPase. The association of LRS to RagD-GTPase enables a GAP activity of LRS to promote the hydrolysis of RagD-GTPase into GDP-bound RagD. Evidently, amino acid or leucine addition increased RagD-GDP level whereas LRS knockdown lowered GTP hydrolysis of RagD induced by leucine. In addition, a mutation in

the putative LRS GAP motif prevented the hydrolysis of GTP-bound RagD. These results demonstrate that LRS activate mTORC1 pathway through hydrolyzing GTP-bound RagD which is carried out by its GAP motif. LRS is able to sense leucine presence and a variety of non-cognate amino acids owing to its conserved HIGH motif (Han et al., 2012) (**Figure 9**). Mutation of the conserved residues Phe50 and Tyr52 disables LRS to bind to RagD heterodimers and to activate the mTORC1 pathway. Ile could also be sensed by LRS but not IRS and its presence enables activation of mTORC1. The tRNA charging activity of LRS appears not to be required for activating mTORC1 while it has been unclear whether ATP recognition by LRS is implicated in mTORC1 activationThe studies by Sancak *et al.* emphasized the importance of amino acid signals in inducing GTP loading of RagB, which consequently leads to an interaction of RagB and mTORC1 (Sancak et al., 2008, 2010). Here, in this study the authors highlight that the transition RagD-GTP into RagD-GDP is a rate-limiting step for mTORC1 activation mediated by RagB (Han et al., 2012).

In yeast, LRS was shown to activate TORC1 but in a different manner (**Figure 9**). The presence of leucine and its binding towards LRS trigger a conformational change of LRS (Bonfils et al., 2012). Consequently, the interaction between LRS and GTP-bound Gtr1 (a homologue of RagA/B) inhibits GTP hydrolysis, maintaining Grt1 activated. It is likely that the binding of LRS towards Grt1-GTPase prevents Grt1-GTPase from activity of an unknown GAP. There is no similarity between the domains that are responsible for binding of Grt1-GTPase and RagD-GTPase (Jewell et al., 2013). While the amino acid sensing inside-out mechanism stressed on the importance of lysosomal amino acid accumulation, LRS can sense the availability of cytoplasmic amino acids. It will be worthy to address how two mechanisms are integrated in mammalian cells.

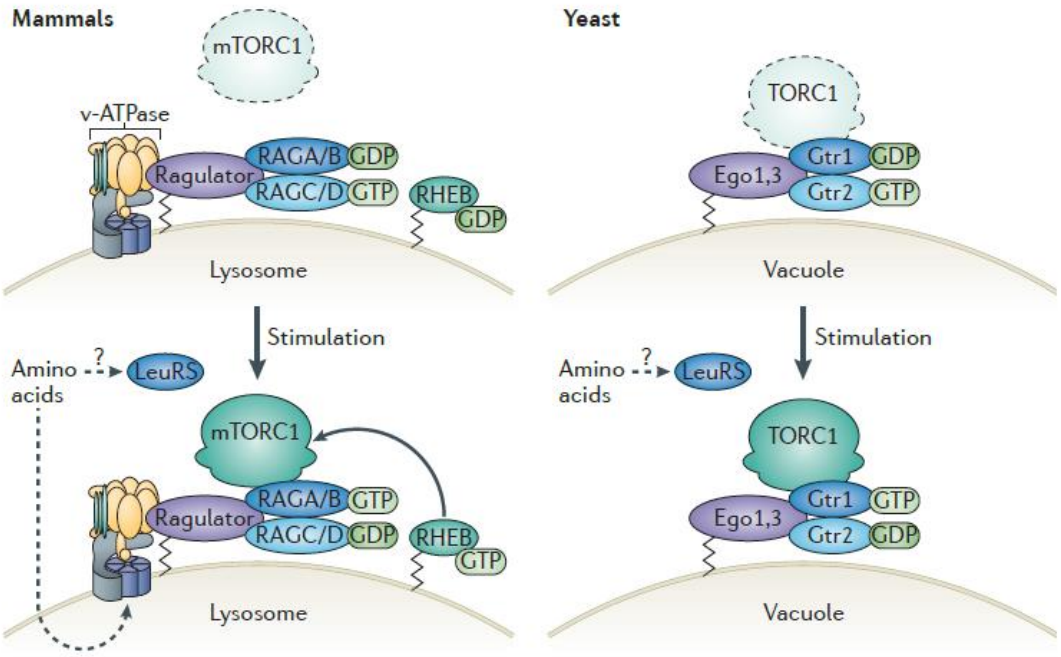

Figure 9: Leucyl-tRNA synthetase (LeuRS) serves as a sensor of leucine and amino acids in mTORC1 activation pathway. The formation of the active configuration of the Rag GTPase complex is promoted by amino acid presence, at the lysosome (left) in mammals. The orthologue of Rag GTPase complex in yeast is Gtr1-Gtr2 which is located at vacuole. At the basal condition, mTORC1 is inactive and is dispersed in mammalian in cytosol whereas TORC1 is always located at lysosome in yeast. Amino acids signal to v-ATPase, which is required for GEF activity of Ragulator. LeuRS acts as a direct sensor of leucine in cytoplasm and signals to activate mTORC1 in mammals. LeuRS is also linked to the activation of TORC1 in yeast.

4.7. Vps34

There has been a number of reports suggesting that type III PI 3-kinase, the homologue of the *S. cerevisiae* vps34, plays a positive role in amino acid-regulated TORC1 activity (Byfield et al., 2005; Nobukuni et al., 2005). Vps34 associates with its kinase partner, vps15 and this binding assists a recruitment of Vps34 to endosomal membranes. Vps34 is also well known for its role of activating autophagy. Vps34/Vps15 heterodimer is responsible for phosphorylation of PI at the 3'-OH group on endosomal membranes and this complex is a unique PI kinase in *S. cerevisiae*.

The human class III PI3K, hVps34, has been shown to be a wortmannin-sensitive target for amino acid signaling to mTORC1 (Nobukuni et al., 2005). Overexpression of hVps34 led to enhanced levels of mTORC1 signal transduction in the presence of amino acids, whereas knockdown of hVps34 by RNAi resulted in a reduction in S6K1 phosphorylation induced by amino acids. Because hVps15 kinase activity is essential for Vps34 activity, it is consistent that knockdown of hVps15 protein suppresses amino acid-induced S6K1 phosphorylation. Moreover, overexpression of FYVE domain, which is characterized by its capacity of competing for intracellular PI3P-docking sites, significantly inhibits S6K1 phosphorylation

both under basal or amino acid stimulation. These evidences place hVps34 as a downstream of amino acid signals which functions as a critical regulator of mTORC1.

Later studies revealed that hVps34 activates mTORC1 in a Ca^{2+}-dependent fashion owing to its Ca^{2+}-calmodulin binding domain (Gulati et al., 2008). The mechanism was explained by the fact that a rise of amino acid concentration leads to an increase in intracellular Ca^{2+}, which in turn raises the binding of Ca^{2+} to hVps34 through a conserved motif of hVps34. This interaction is considered to trigger hVps34 lipid kinase activity which is required for mTORC1 activation.

As documented, hVps34 is also involved in mediating macroautophagy by associating with a cellular beclin protein (Liang et al., 1999). Given the contradictory roles of hVps34 in activating two functionally opposite processes of autophagy and mTORC1, the real role of hVps34 is a matter of debate. One possible explanation is that the positive effect of Vps34 on activating mTORC1 results from an increase in intracellular amino acids mediated by autophagy (Dodd and Tee, 2012). Another alternative possibility is that there is existence of different intracellular pools of Vps34, each of them carries out specific role to promote autophagy or mTORC1 activation (Nobukuni et al., 2005). This model elegantly fits with the identification of at least two unlike Vps34 protein complexes (Kihara et al., 2001). Moreover, while all of cellular beclin exists in an association with hVps34, 50% of hVps34 is beclin-free. Later researches should elucidate what is a primary function of hVps34, as an activator of autophagy or a critical sensor of amino acid availability.

5. Remark questions

Cell balance the nutrient supplement and demand. The nutrient demand of cells is satisfied in part by mTORC1 pathway that accelerates protein synthesis and cell growth. Cells are supplied by different sources of nutrient via amino acid transporters or protein degradation during fasting period. In this thesis, the critical role of amino acids in regulating mTORC1 activity via a complexity of cellular mediators has been summarized.

There is a debate regarding whether extracellular amino acids or intracellular amino acids play critical role in regulating mTORC1 activity. The role of extracellular amino acids in the regulation of mTORC1 pathway is achieved via the G protein-couple taste receptor T1R1/T1R3 (Wauson et al., 2012). However, there is a wealth of documents stressing the dominant role of intracellular amino acids in regulating mTORC1 (Avruch et al., 2008b; Hara et al., 1998c; Long et al., 2002c). In the future, the study to understand if there is a direct sensor of intracellular amino acids should be one of main focuses in the field.

Lysosome is a cellular compartment where mTORC1 is primarily activated. This finding has led to significant findings of intracellular mediators that transfer signals from amino acids to activate mTORC1. There are two different models that describe the fashion how amino acids stimulate mTORC1 activity. In one model, the accumulation of amino acids in the cytoplasm is detected by LeuRS, which is critical in activating mTORC1 pathway (Han et al., 2012). Contradictorily, in the amino acid sensing inside-out model, amino acids must accumulate in the lysosome lumen and then initiate the signals (Zoncu et al., 2011). Given that radioactivity-labeled amino acids appear in the lysosome within 10 minutes while mTORC1 is activated quickly after 3 minutes of amino acid addition (Jewell et al., 2013), it appears that the presence of amino acids in the cytoplasm plays critical role in regulating mTORC1 pathway. It would be possible that there are existences of both pathways but how they are employed depends on specific cell types, cellular cycle state. Therefore, it is important to address how these two pathways are integrated.

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