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Minireview

The amino acid sensitive TOR pathway from yeast to mammals

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Abstract The target of rapamycin (TOR) is an ancient effector of cell growth that integrates signals from growth factors and nutrients. Two downstream effectors of mammalian TOR, the translational components S6K1 and 4EBP1, are commonly used as reporters of mTOR activity. The conical signaling cascade initiated by growth factors is mediated by PI3K, PKB, TSC1/2 and Rheb. However, the process through which nutrients, i.e., amino acids, activate mTOR remains largely unknown. Evidence exists for both an intracellular and/or a membrane bound sensor for amino acid mediated mTOR activation. Research in eukaryotic models, has implicated amino acid transporters as nutrient sensors. This review describes recent advances in nutrient signaling that impinge on mTOR and its targets including hVps34, class III PI3K, a transducer of nutrient availability to mTOR.
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1. Introduction: Insulin stimulation and mTOR signaling

The coordinated control of cell growth to produce a genetically predetermined cell size, organ shape and body plan is largely directed by the mammalian target of rapamycin (mTOR). A large protein of ~280 kDa, mTOR consists of a number of Huntington, EF3, A subunit of PP2A, and TOR1 repeats (HEAT repeats), common in protein–protein interaction; a large Frap, ATM, and TRAP PIKK-like domain (FAT domain); a FKBP12-rapamycin binding domain (FRB Domain) a C-terminal kinase domain; and two regulatory domains, termed the negative regulatory domain (NRD Domain) and FAT domain C-terminal (FAT/C Domain) (reviewed in [1]). The kinase domain is similar to the phosphatidylinositol 3OH-kinase (PI3K) domain and in mammals mTOR was originally considered a phosphotidylinositol-4 kinase [2]. Further research, however, showed that TOR was in fact a protein kinase, belonging to the PI3K-related family of protein kinases, which also includes ATM, ATR and DNA-dependent protein kinase [3].

Stimulation of PI3K by growth factors such as insulin results in the initiation of a number of signaling cascades that lead to growth and proliferation, a cellular phenomenon conserved throughout metazoans (Fig. 1). In mammals, receptor interaction with insulin results in recruitment of insulin receptor substrates (IRS) to the cell membrane [4]. Subsequently, recruitment and stimulation of the class I PI3K produces the phosphatidylinositol second messenger PIP_3 [5]. PIP_3 binds to the pleckstrin homology (PH) domain of several proteins, in particular to the Protein A, G and C (AGC) serine/threonine kinase PKB/AKT, a pro-growth, pro-survival kinase [6]. Binding of PIP_3 to its PH domain recruits PKB to the cell membrane where it is activated through phosphorylation by PDK1 and PDK2 [7,8]. Activated PKB phosphorylates the TSC1/2 complex resulting in their dissociation and degradation, thereby releasing the small GTPase Rheb from the inhibitory GAP activity of TSC2 [9–13]. Recent studies illustrate a direct interaction between Rheb and mTOR, which stimulates its kinase activity [14].

Two alleles, TOR1p and TOR2p, were originally described in a screen of yeast mutants resistant to toxic doses of the anti-fungal, bacterial macrolide, rapamycin [15]. In mammals, rapamycin blocks mTOR function by forming an inhibitory complex with the immunophilin FKBP12, which binds to and attenuates the ability of mTOR to phosphorylate downstream substrates [16,17], including S6K1 [3,18–20] as well as the 4E binding protein, 4E-BP1 [21–23], a repressor of translation initiation factor 4E [24]. In this rapamycin sensitive pathway mTOR is bound to two additional proteins, raptor and mLst8/GβL, to make Complex 1. Raptor and mLst8 are homologues of the yeast KOG1p and Lst8p, respectively [25–27]. mTOR interacts with downstream substrates through raptor, which recognizes mTOR substrates through their TOR signaling (TOS) motifs [28], whereas mLST8 is required to make a competent signaling complex that can respond to nutrient and energy inputs [27]. Recent studies show that mTOR also exists in a second signaling complex with mLST8 and a protein termed rictor, rather than raptor. Moreover, this second complex is proposed to directly control PKB phosphorylation and activation and, unlike Complex 1, Complex 2 is rapamycin resistant [8,29].

Activation of mTOR through both growth factor and nutrient sensitive pathways results in the upregulation of protein synthesis. Phosphorylation of 4E-BP1 by mTOR induces its dissociation from initiation factor eIF4E [23]. Relieved of 4E-BP1, eIF4E is free to interact with the eIF-4G subunit of the eIF-4F complex [30]. Once these associations are complete, the secondary structure of mRNA can be melted allowing the 40S ribosomal subunit to scan the mRNA until it encounters the first AUG initiation codon and promotes translation. In

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Abbreviations: HEAT domains, Huntington, EF3, A subunit of PP2A, and TOR1 Domains; FAT, Frap, ATM, and TRAP PIKK like domains; FAT/C, FAT domain C-terminal; FRB, FKBP12-Rapamycin Binding domain; NRD, Negative Regulatory Domain

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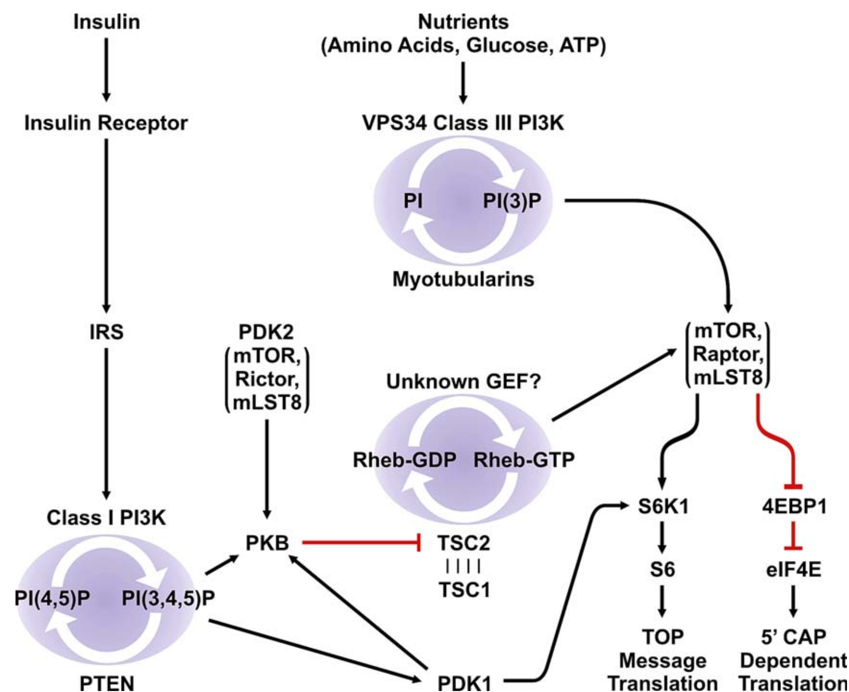

Fig. 1. Insulin and amino acid signals converge on mTOR through independent PI3K signals. Insulin binds to its receptor resulting in activation of the class I PI3K which recruits PKB to the cell membrane. Upon activation by PDK1 and PDK2 PKB inhibits TSC1/2 which releases Rheb from its intrinsic GTPase activity. Rheb-GTP bound to mTOR activates mTOR kinase activity resulting in phosphorylation of its downstream targets S6K1 and 4EBP1. Meanwhile amino acid availability positively regulates the activity of the class III PI3K hVPS34. hVPS34 produces the second messenger PI(3)P which activates mTOR through unknown mechanisms.

the case of S6K1, its first known substrate was identified as ribosomal protein S6, whose phosphorylation was earlier shown to be implicated in increased rates of protein synthesis [31]. The recent identification of four additional substrates is also consistent with a role in cell growth, including translation initiation factor 4B, which facilitates the unwinding of the 5′ ends of mRNA [32], eEF2 kinase, which mediates the phosphorylation of translation elongation factor 2, involved in controlling ribosomal transit rates [33], BAD, a pro-apoptotic protein [34] and most recently SKAR a nuclear protein proposed to couple transcription with pre-mRNA splicing and mRNA export [35]. The role of these targets in cell growth is consistent with the effect of the S6Ks on growth, as established in deletion studies in both *Drosophila* [98] and in the mouse [36,37].

The ability of nutrients to modulate mTOR signaling downstream to its substrates is relatively well documented, however little is known with regard to the identity of the upstream effectors of this branch of the signaling pathway. Evidence exists for both an intracellular and/or a membrane bound extracellular sensor for amino acid stimulation of the mTOR pathway. Research in yeast, *Drosophila*, and mammalian settings have implicated amino acid transporters as sensors of nutrient availability. Investigations in *Drosophila* and yeast have described amino acid transporters as genetically epistatic to TOR activity while, in a mammalian setting, mTOR activity is linked to amino acid transporter transcription, translation and turnover. Considering the well conserved nature of the TOR pathway in eukaryotes, it seems likely that membrane nutrient transporters are able to promote traditional signaling cascades beyond their well known function of regulating nutrient concentration within the cell. The importance of the nutrient branch of the mTOR signaling pathway is underscored by recent studies showing that nutrient overload, while driving cell growth, also acts to suppress insulin signaling, leading to hyperglycemia and insulin resistance [36,38]. Moreover, obesity and type 2 diabetes have recently been implicated as risk factors in a number of cancers [39,40], consistent with the clinical importance of rapamycin, and its derivatives in the treatment of solid tumors [1,41]. This review will describe recent advances in a variety of eukaryotic models of nutrient signaling that impinge on the mTOR kinase and its targets including the recent identification of hVps34, class III PI3K, as a transducer of nutrient availability to mTOR [42,43].

2. Yeast studies

Many advances in the field of cell growth control in the mammalian setting were initially described in yeast. The TOR1 and TOR2 alleles as well as the distinct TOR complexes are examples of the advantages that a simple eukaryotic system confers in describing novel pathways and potential interactions. In yeast, Torp and Lst8p have been implicated in the nutrient responsive, mitochondria-to-nucleus, retrograde signaling (Fig. 2). The most detailed reports on this pathway involve the de-repression of Rtg1p and Rtg3p target genes, many of which control the supply of intermediates necessary for the tricarboxylic acid cycle (reviewed in [44]). In brief, restriction of a suitable glutamate source results in Rtg1p:Rtg3p transcription complex being released from a repressed state leading to the subsequent upregulation of target gene families, including CIT1, CIT2, ACO1, IDH1 and IDH2 [45]. Glutamate or glutamine is sensed in yeast by the membrane bound SPS sensor which negatively regulates Rtg2p. Rtg2p is also a sensor of

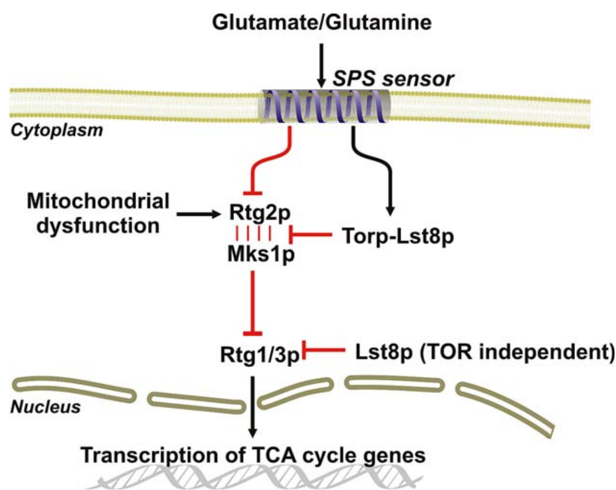

Fig. 2. The Rtg1p:Rtg3p mitochondrial retrograde response in yeast is directed by the SPS amino acid sensor, Torp, and Lst8p. The Rtg2p:Mks1p complex integrates signals from the SPS sensor, mitochondrial dysfunction and Torp to regulate the activity of the Rtg1p:Rtg3p transcription factors. Lst8p also negatively regulates Rtg1p:Rtg3p function through a Torp independent mechanism.

mitochondrial dysfunction and a positive regulator of the Rtg1p:Rtg3p transcription complex. When glutamine is available and mitochondrial function is normal the Rtg1p:Rtg3p transcription complex is sequestered in the cytoplasm and Rtg3p is phosphorylated at several sites. If the retrograde pathway is activated Rtg3p becomes partially dephosphorylated and Rtg1p and Rtg3p relocalize to the nucleus. Deletion of RTG2 results in both hyperphosphorylation of Rtg3p and cytoplasmic localization of the Rtg1p:Rtg3p transcription complex regardless of retrograde signals [46].

Genetic screens in yeast that bypass the requirement for Rtg2p in the retrograde response resulted in the identification of MKS1 by three independent laboratories [47–49]. Cells deficient in MKS1 have constitutively high CIT2 expression and are insensitive to extracellular glutamate [50,51]. Partially dephosphorylated Mks1p and Rtg2p are found in complex together during the retrograde response, but when retrograde signaling is suppressed Mks1p becomes hyperphosphorylated and is bound to the 14-3-3 yeast homologues Bmh1p and Bmh2p.

The Torp kinase in *Saccharomyces cerevisiae* is intimately linked with the retrograde response both directly and through Lst8p (Fig. 2). Treatment of yeast cells with rapamycin results in Rtg1p:Rtg3p complex dependent gene expression [52,53]. This rapamycin sensitive response depends on the activity of Rtg2p, placing the effect of Torp upstream, perhaps at the point of the SPS sensor. Crespo et al. [54] have also described a glutamine sensitive input to the Rtg1p:Rtg3p transcription complex, which is controlled by Torp. In more recent studies, Dilova et al. [47,55] have refined the independent glutamine/glutamate sensing event and Torp signaling events, arguing they converge on Mks1p, and not the SPS sensor, to regulate Rtg1p:Rtg3p complex activity. In these studies, glutamine/glutamate sensitivity were completely dominant to Torp signals in regards to Mks1p phosphorylation state [55].

Lst8p has also been linked to the retrograde response where it acts as a negative regulator of the pathway. Liu et al. [48] identified a series of LST8 mutants that increased CIT2 expression independent of glutamine availability. Some of these mutants were dependent on Rtg2p activity while others bypassed the Rtg2p requirement to activate the Rtg1p:Rtg3p transcription complex. This has led to the finding that Lst8p likely acts on the retrograde response at two sites, one upstream of Rtg2p, i.e., Rtg2p dependent, and one downstream of Rtg2p, i.e., Rtg2p independent. The Rtg2p-dependent LST8 mutation was originally described as a point mutation at the C-terminus that results in a general amino acid permease (Gap1p) sorting defect [56]. Since sublethal concentrations of rapamycin result in a Gap1p sorting defect similar to the LST8 mutant phenotype [57] it is tempting to speculate that this mutant is a modulator of the Torp activity that converges on Mks1p described by Dilova et al. [55]. Recent evidence supports a separation of glutamine sensing Torp-Lst8p regulation of the Rtg1p:Rtg3p transcription complex, and a Torp-independent, Lst8p-specific regulation of Rtg1p:Rtg3p complex activation through mitochondrial dysfunction [58].

The importance of the Torp pathway in nutrient uptake and regulation of amino acid permeases has also been described for *Schizosaccharomyces pombe* leucine auxotrophs [59]. Rapamycin or disruption of TOR1 results in defective leucine transport and downregulation of three putative amino acid permeases. Reduction of Tsc1p or Tsc2p, results in constitutive Rhebp and Torp activity, and causes a reduction in arginine uptake [60]. Similarly, Rhebp-deficient *S. cerevisiae* have an increased arginine uptake phenotype [61], whereas *S. pombe* deficient for Rhebp exhibit cell cycle arrest in G1 and a phenotype similar to that observed for nitrogen starvation [62]. Indeed, one could imagine a system where the availability of nitrogen and/or amino acids results in expression of their respective transporters for uptake mediated by the Torp pathway.

3. Mammalian cells

Observations of increased protein degradation due to amino acid deficiency and increased protein synthesis due to amino acid supplementation in cell culture were first reported in the early 1980s [63]. Amino acids as signaling molecules were first described in studies of cultured hepatocytes where amino acids could inhibit the progression of macroautophagy [64]. The same researchers determined that this inhibitory effect was dependent on phosphorylation of the small ribosomal subunit S6 [65]. Soon after, several laboratories demonstrated the specific regulation by phosphorylation of mTOR substrates S6K1 and 4EBP1 due to amino acid stimulation, in particular the branched chain amino acid leucine. Studies performed in adipocytes [66], Chinese Hamster Ovary (CHO) cells [67,68], hepatoma and myotubes [69] myoblasts [70], pancreatic β-cells [71] and hepatocytes [72] all show sensitivity of phosphorylation status of mTOR substrates to amino acid concentrations. That is, amino acid depletion results in a rapid dephosphorylation of S6K1 and 4EBP1 while addition of amino acids, especially leucine, can rescue this effect in a rapamycin-sensitive manner.

These studies spurred investigation intended to discover a mammalian amino acid sensor regulating mTOR activity. Researchers used several leucine analogues and catabolites to determine whether leucine itself or a leucine derivative was responsible for mTOR activation. Shigemitsu et al. [73] indicated leucine chirality (i.e., L-leucine), the four branched carbon structure and the primary amine as all necessary to stimulate S6K1 activity. Lynch et al. [74] described equivalent

results on 4EBP1 phosphorylation in adipocytes. Similar to rapamycin treatment, exposure of cells to the leucine derivative *N*-acetylleucine amide results in inhibition of S6K1 activation and G1 cell cycle arrest [75]. Since the cellular effects of rapamycin treatment and amino acid deprivation are paralleled by Ac-Leu-NH2 treatment, this compound will be a useful tool in determining the leucine-specific effects on mTOR signaling.

Fox et al. [76] showed a potent response of 4EBP1 phosphorylation in response to the addition of α-ketoisocaproate (KIC). KIC is a leucine intermediate metabolite resulting from the transamination of leucine by the branched chain amino acid transferase (BCAT). This is a reversible step in leucine metabolism and is coupled to the regulation of the rate-limiting irreversible activity of the branched chain α-keto acid dehyrdogenase (BCKDH) complex. KIC stimulation of 4EBP1 was reversed by addition of (Aminooxy)acetic acid, an inhibitor of BCAT activity, indicating that the observed stimulation was due to conversion of KIC to leucine. Still, due to the pleiotropic effect of AOAA on all amino acid transferases, the possibility remained that BCKDH activity induced by KIC was modulating mTOR. Lynch et al. [77] observed the requirement for much higher concentrations of leucine to stimulate BCKDH than was necessary to stimulate mTOR indicating that BCKDH was involved only in leucine oxidation and not mTOR signaling.

Earlier studies were able to illustrate a suppression of macroautophagy and proteolysis with leucine as well as a non-transportable multiple antigen peptide made of eight N-terminal leucine residues (MAP-Leu8) [78,79]. Although this cell-impermeant synthetic peptide clearly suppresses autophagy more recent investigations have shown that stimulation of S6K1 or 4EBP1 is not responsive to MAP-Leu8 [74,80]. These results indicate that the extracellular receptor that suppresses cellular autophagy is independent of the leucine receptor that signals to mTOR.

Considering the high affinity for leucine of the system L amino acid transporters, it is likely they are involved in initiating the mTOR signal. The system L transporter light chain subunit LAT1 is upregulated in several cancerous states when compared to normal tissue (reviewed in [81]). Increasing the expression of this obligatory transporter would give tumors a distinct growth advantage as they would be able to scavenge essential amino acids and provide increased signaling to the mTOR complex. In fact transformed cells in culture display increased uptake of amino acids compared to their parental lines [82]. The upregulation of LAT1 in tumors and subsequent upregulation of mTOR activity may constitute a positive feedback loop as transcription of LAT1 is induced by a rapamycin sensitive PDGF stimulation [83]. While sensitive to rapamycin the induction of LAT1 mRNA by PDGF is resistant to ERK, MEK, JNK and PI3K inhibitors implicating only mTOR in the transcriptional regulation of LAT1. The PDGF-induced increase in expression of LAT1 message is well correlated with heightened system L activity over the same period. Lynch et al. [74] and Kanazawa et al. [80] both employed the system L competitive inhibitor 2-amino-3-norbane carboxylic acid (BCH) to examine the system L contribution to leucine-induced mTOR signaling. Both research groups found no effect of BCH on leucine's ability to stimulate the mTOR dependent phosphorylation of S6K1 or 4EBP1. In fact, the ratio of hyperphosphorylated to unphosphorylated S6K1 appears to be greater after treatment with BCH and stimulation by leucine than with leucince stimulation alone [80]. Other groups have used translation inhibitors such as cycloheximide (CHX) to block protein synthesis and subsequently allow signaling to S6K1 by insulin even in the absence of amino acids [84]. These investigators conclude that inhibition of protein synthesis results in an increase in the intracellular amino acid concentrations allowing mTOR signaling to progress.

Considering the cellular consequences of MAP-Leu8, BCH and CHX on S6K1 and 4EBP1 in regard to mTOR signaling it is postulated that the signal from leucine to mTOR originates from an intracellular receptor. Iiboshi et al. [85] suggested that the inhibition of S6K1 phosphorylation during amino acid deprivation was the result of accumulation of uncharged tRNA. This group observed inhibition of S6K1 phosphorylation by amino acid alcohols which competitively inhibit tRNA synthetases. Other groups, however, have found no effect of amino acid alcohols on S6K1 or 4EBP1 phosphorylation state [86], and work from our lab indicates that amino acid deprivation does not induce accumulation of uncharged tRNA within the period necessary to abolish S6K1 activity [19]. Leucine-induced phosphorylation of 4EBP1 and S6K1 during BCH-blocked leucine transport argues for the presence of an amino acid receptor that acts at the membrane to sense intra and extracellular amino acids perhaps via interaction with amino acid transporters.

Gao et al. [87] examine the effect of TSC1/2 loss on the amino acid signal to mTOR. They report that the S6K1 is resistant to inhibition from amino acid deprivation in cells lacking TSC1/2. Recent data from our lab [42] and others [88] shows that TSC1/2 is not required to transduce the amino acid signal to mTOR. While Smith et al. [88] describes the response of TSC2 null cells to amino acid deprivation and stimulation, Nobukuni et al. [42] expands on this report with the use of TSC2 siRNA and TSC2 null cells. We show that S6K1 phosphorylation is completely resistant during serum starvation in either TSC2-reduced background compared to wild-type. On the other hand, S6K1 is still deactivated during amino acid deprivation in both TSC2 depletion settings. Similarly, overexpression of TSC1/2 or Rheb had no effect on amino acid concentrations within the cell during amino acid deprivation or stimulation, opposing the model proposed by Saucedo et al. [89]. Since amino acids signal to mTOR in a TSC1/2-deficient setting, the nutrient responsive pathway must exert its effects on mTOR downstream of TSC1/2.

An observation from earlier work in our lab and others, concerning amino acid stimulation of mTOR signaling was, the sensitivity of S6K1 and 4EBP1 phosphorylation to wortmannin. This phenomenon was attributed to a class I PI3K input to mTOR independent of PKB or off-target effects of wortmannin on mTOR. Likewise, Nobukuni et al. [42] show a lack of PKB phosphorylation in response to amino acid stimulation, even when a robust S6K1 phosphorylation is observed. While the insulin stimulation here is sensitive to rapamycin, it is also sensitive to concentrations of wortmannin well below those necessary to inhibit mTOR. Using siRNA to Class I PI3K we show that while insulin no longer results in stimulation of S6K1 or PKB, amino acid stimulation readily induces phosphorylation of S6K1. Therefore, amino acid stimulation of mTOR is not transduced through Class I PI3K or PKB.

Due to its sensitivity to wortmannin we chose the Class III PI3K hVPS34 as a candidate signaling intermediate between amino acids and mTOR. Unlike Class I PI3K, which produces the phosphatidylinositide triphosphate PI(3,4,5)P (PIP_3), the

Class III PI3K uses unphosphorylated phosphatidylinositol as a substrate to produce the monophosphate signaling molecule PI(3)P. Using a monoclonal antibody engineered to specifically recognize PI(3)P and confocal microscopy we showed a loss of PI(3)P signal during amino acid deprivation followed by a strong PI(3)P signal upon re-addition of amino acids to the cell culture media. This experiment was carried out in Hela, WT MEFs and TSC null MEFs indicating that the TSC1/2 complex was not controlling the fluctuation of PI(3)P in the cell. To ensure that the Class III PI3K was the enzyme responsible for the PI(3)P production, we used an in vitro PI3K assay to measure hVPS34 activity before, during and after amino acid deprivation and stimulation. Indeed, activity of hVPS34 was determined by amino acid availability in the culture media in each cell line tested.

Epitope-tagged S6K1 was expressed with increasing amounts of hVPS34, and, while phosphorylation of S6K1 (i.e., mTOR activity) was not resistant to amino acid deprivation, amino acid stimulation resulted in hVPS34-dose dependent phosphorylation of S6K1. Using expression of a hVPS34-dominant negative acting FYVE domain construct, which binds available cellular PI(3)P, we can block the amino acid-induced phosphorylation of co-expressed S6K1. The immunocytochemistry, in vitro kinase assay and gain-of-function overexpression assays detailed above make a very strong case for hVPS34 regulation of amino acid-induced mTOR activity. The loss-of-function siRNA experiments, however, are the vital experiments that prove our hypothesis. RNAi of hVPS34 blocked completely the phosphorylation of S6K1 in response to amino acid and insulin stimulation while retaining Class I PI3K-induced phosphorylation of PKB after insulin treatment. Not only can we block the activation of mTOR by amino acids by hVPS34 RNAi but RNAi of the hVPS34 adaptor hVPS15 (p150), a protein kinase with unknown targets, also results in a block of mTOR activity to S6K1 substrate. During the completion of this manuscript Byfield et al. [43] published similar results to our own describing a general nutrient regulation of hVPS34 activity.

Tassa et al. [90] previously reported an increase in hVPS34 activity associated with the amino acid starvation signal and implicated the activity of hVPS34 with the BECN-1 tumor suppressor in macroautophagy in C2C12 cells. While these results appear to be at odds with ours they can be explained by Kihara et al.'s [91] description of multiple hVPS34 complexes, where some control autophagy and others are associated with endocytic trafficking and sorting. Recently Zeng et al. [92] reported that those hVPS34 complexes that contain BECN-1 are specifically acting as modulators of autophagy, while hVPS34 complexes without BECN-1 were important for cellular trafficking events. Review papers on the subject of amino acid stimulation of mTOR five years before Nobukuni et al. [42] theorized that the wortmannin-sensitive stimulation of S6K1 could be due to hVPS34 [93]. Thanks to advances in our biochemical cell-based assays we were able to prove these theories correct.

The intimate relationship of amino acids, mTOR, hVPS34 and cellular trafficking is augmented by the research from Edinger and Thompson [94] regarding endocytosis of nutrient transporters during periods of growth factor withdrawal. Using an IL-3 dependent cell line these researchers were able to show that 4F2hc/CD98, the heavy chain of the LAT1 and LAT2 amino acid transporters, was localized to the lysosome during IL-3 withdrawal. This effect could be reversed upon expression of a constitutively active PKB construct and was reversed by rapamycin treatment implicating mTOR. In a follow-up study an exogenously expressed, activated allele of mTOR could also rescue 4F2hc/CD98 lysosomal breakdown during IL-3 withdrawal [95]. In fact cells expressing the activated mTOR were resistant to growth factor withdrawal-induced apoptosis but remained sensitive to nutrient withdrawal. Activity of the system A amino acid transporter also appears to be under control of leucine-mTOR signaling in a translation-dependent manner [96,97]. Thus mTOR signaling can influence amino acid transport at the transcriptional level (LAT1 light chain), the translational level (system A transport), and at the level of transporter turnover (LAT1 heavy chain – 4F2hc/CD98).

Recent research has also focused on the role of Rheb in mediating the amino acid signal to mTOR. Long et al. [14] described domain specific interactions between Rheb and mTOR, Rheb and raptor, and Rheb and mLST8. A follow-up article examines the Rheb–mTOR interaction and its dependency on extracellular amino acids and/or leucine [98]. While the results shown in both studies are clear, they employ immunoprecipitation (IP) with exogenously expressed constructs to illustrate interactions as opposed to IP of endogenous proteins. Whether Rheb and mTOR/raptor/mLST8 interact at in vivo levels in an amino acid-dependent manner remains to be seen. Roccio et al. [99] show that while Rheb activity (as measured by percentage of GTP-bound Rheb) is upregulated by insulin, this stimulation is blocked by amino acid depletion. Amino acids alone, however, were insufficient to modulate GTP loading on Rheb in a wild-type setting. Nobukuni et al. [42] also demonstrates the full activation of Rheb in a TSC2 null background regardless of amino acid depletion even though S6K1 dephosphorylation occurs. While exogenous Rheb can clearly regulate mTOR signaling its precise function in nutrient activation of mTOR remains unknown.

Regarding mTOR interactions, what is known however is that the raptor–mTOR interaction is nutrient regulated [26]. In the initial description, raptor–mTOR binding is only detected under specific mild lysing buffer conditions. During IP of mTOR at its N-terminus the altered nature of the interaction is displayed as a reduced amount of raptor seen in Western blot post-IP upon stimulation with leucine. However, if a reversible cross-linker is added before IP the amount of raptor that co-IPs with mTOR during leucine deprivation and stimulation remains the same. This indicates that a dynamic interaction between mTOR and raptor occurs upon nutrient stimulation where the actual amount of raptor bound remains stable but the nature of the interaction (i.e., number of binding sites) changes. More recently, Sarbassov and Sabatini [100] reported that the raptor–mTOR interaction is a redox-sensitive event where reducing reagents result in more raptor bound to mTOR even in amino acid replete conditions. Although this interaction is clearly dynamic it is currently considered an observed phenomenon due to nutrient availability and not a mechanism regulating mTOR activity.

4. Live animal models

Several studies since the 1970s have illustrated the relationship between supplementation of amino acids in diet with protein synthesis and restriction of dietary amino acids with protein degradation [101–103]. Since the discovery of the nutrient sensitive mTOR pathway several of the effects of dietary

amino acids have been linked to a rapamycin-sensitive response. Reiter et al. [104] observed a rapamycin-sensitive hyperphosphorylation of 4EBP1 upon leucine administration in rat liver after fasting. Similarly, phosphorylation of S6K1 and S6 was strong after leucine administration but was ablated by addition of rapamycin. Reiter et al. also observed that the increase in ribosomal proteins being actively translated on polysomes after leucine administration was rapamycin sensitive. Similar results have been recorded in mammalian skeletal muscle tissue [105] and adipose tissue [106]. Other researchers have shown that S6K1 phosphorylation is enhanced 3–4-fold after resistance exercise due to supplementation of leucine in human subjects [107]. Other reports in elderly human subjects reveal a dysregulation of S6K1 resulting in constitutively high phosphorylation before and after perfusion of insulin and amino acids. This dysregulation is correlated with an impaired protein synthesis response in elderly compared with young subjects [108]. VPS34 activity assays in mammalian live animal models have not yet been performed to determine the effect of dietary amino acid intake on VPS34 function. The results of aging on S6K1 signaling is especially intriguing considering the lifespan extension effects of nutrient restriction and/or expression of dominant negative constructs of TOR and S6K1 in *Drosophila* and *Caenorhabditis elegans* [109,110].

Research in *Drosophila* has revealed two distinct amino acid sensors/transporters that are linked to TOR function. Goberdhan et al. [111] identified a proton-assisted amino acid transporters (PATH) which genetically interacts with TOR in an amino acid-dependent way. PATH is especially important during fly development, controlling growth and specifically modulating TOR signaling. Slimfast, another amino acid transporter, was recently identified within the *Drosophila* fat body and appears to involve TSC/TOR signaling [112]. Downregulation of slimfast results in a global growth defect and demonstrates a humoral mechanism of growth control emanating from slimfast activity in the fat body. The involvement of amino acid sensing in mammalian development is also observed in blastocyst outgrowth, as embryonic implantation will not occur without transport of exogenous amino acids [113]. These researchers show that trophectoderm differentiation is dependent on the amino acid transport that stimulates mTOR and subsequent S6K1 phosphorylation. This data is recapitulated by data from mice in our own lab [114] and others [115] where mTOR deletion results in early embryonic lethality before implantation. As stated earlier several amino acid transporters, especially the system L transporters, are upregulated in different forms of cancer. In a recent report LAT1 expression is well correlated with poor survival in glioblastoma and expression of LAT1 was a good predictor of outcome independent of other variables [116]. These researchers also describe a dose-dependent in vitro and in vivo reduced growth response of rat glioma C6 cells treated with BCH. While one could extrapolate that increased expression of obligatory leucine transporters would result in increased mTOR signaling, correlations between increased system L transport activity and increased mTOR activity have yet to be made in a cancer setting or otherwise.

5. Conclusions

Activation of mTOR through stimulation of cellular signaling pathways by growth factors is well described relative to nutrients. In yeast the nutrient sensitive retrograde response has been linked to Torp function as has the regulation of amino acid permeases in response to nutrient availability. In mammalian systems amino acids, especially leucine, modulate mTOR phosphorylation of S6K1 and 4EBP1. No amino acid or leucine-specific receptor has been identified that results in a signaling cascade to activate mTOR. Evidence suggests, however, that this receptor is independent of the one responsible for suppressing autophagy. Our lab has identified the Class III PI3K hVPS34 as a regulator of mTOR activity in response to nutrients. In fact, the activity of hVPS34 is itself regulated by nutrient availability as illustrated by results from our lab and others. Results from live animal models generally recapitulate those found in cell culture and evidence from *Drosophila* and yeast models implicates amino acid transporters as important mediators of TOR signaling. The link between mTOR activity and nutrient transporter turnover through endocytosis as described by Edinger and Thompson [94,95], as well as the control of amino acid transporter expression at the level of transcription [83] and translation [96,97] through rapamycin sensitive mechanisms, is indicative of the intimate relationship between nutrient transporters and the mTOR pathway. Future studies that examine the link between amino acid transport, hVPS34 activity and mTOR activity will resolve the initial signaling events that result in activation of mTOR targets and provide insight into the opportunistic scavenging of nutrients by malignant cells.

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