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Growing roles for the mTOR pathway

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The mammalian TOR (mTOR) pathway is a key regulator of cell growth and proliferation and increasing evidence suggests that its deregulation is associated with human diseases, including cancer and diabetes. The mTOR pathway integrates signals from nutrients, energy status and growth factors to regulate many processes, including autophagy, ribosome biogenesis and metabolism. Recent work identifying two structurally and functionally distinct mTOR-containing multiprotein complexes and TSC1/2, rheb, and AMPK as upstream regulators of mTOR is beginning to reveal how mTOR can sense diverse signals and produce a myriad of responses.

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Introduction

Rapamycin has had a story book trajectory: emerging in the 1970s from the soil of Easter Island [1], playing the starring role in the discovery of a fundamental biological pathway and rising to its current status as an important drug. The study of its mechanism of action has been full of unexpected and exciting findings, beginning with the odd way in which it acts. Rapamycin binds to the FKBP12 protein to form a drug–receptor complex that then interacts with and perturbs a large protein kinase called TOR (target of rapamycin) [2–6]. Although the function of TOR is far from well understood, it is increasingly clear that TOR is the central component of a complex signaling network that regulates cell growth and proliferation as well as animal size. This article reviews new insights into the molecular mechanisms that regulate mammalian TOR (mTOR) and their role in growth and disease.

A tale of two mTOR complexes

Until the introduction of RNA interference technology, the majority of work on the mammalian TOR pathway relied on rapamycin to probe mTOR biology. We now

realize that rapamycin does not perturb all mTOR functions because mTOR exists in two distinct multi-protein complexes and only one binds to FKBP12–rapamycin (Figure 1). This complex is composed of mTOR as well as the GβL and raptor proteins, and rapamycin inhibits its kinase activity *in vitro* [7–10]. The rapamycin-insensitive complex also contains mTOR and GβL, but, instead of raptor, a different protein called rictor (also known as mAVO3) [11*,12*]. Raptor, rictor and GβL, like mTOR, contain repeated sequences, such as HEAT and WD40 domains, which suggest involvement in protein–protein interactions. The components of both complexes exist in all eukaryotes examined, but rictor is poorly conserved compared to the other proteins.

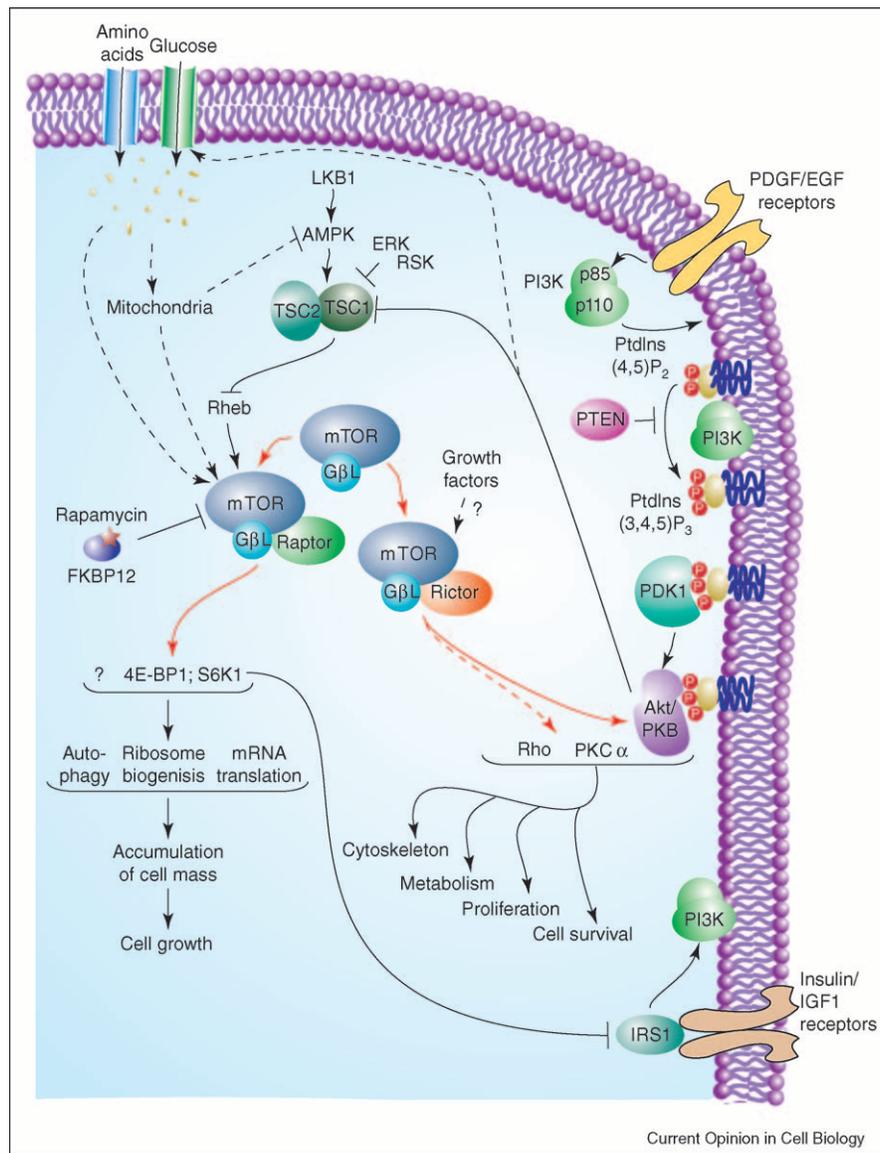
How FKBP12–rapamycin inhibits the kinase activity of the raptor–mTOR complex is not understood. The drug does not displace GβL or raptor from mTOR but does strongly destabilize the raptor–mTOR interaction [8]. This is a bit odd because FKBP12–rapamycin binds to a region adjacent to GβL and the mTOR kinase domain but >1000 amino acids away from where raptor binds to mTOR [7,8]. Perhaps FKBP12–rapamycin induces a conformational change in mTOR that weakens the binding of raptor and perturbs its capacity to recruit substrates (see below). It is also unclear why FKBP12–rapamycin does not bind the rictor-containing mTOR complex. Rictor or an unidentified component of the complex may block or occupy the FKBP12–rapamycin binding site or allosterically destroy the FKBP12–rapamycin binding pocket.

Growth control by raptor–mTOR

Extensive work with rapamycin indicates that the raptor–mTOR complex positively regulates cell growth and that its inhibition causes a large decrease in cell size. The raptor branch of the mTOR pathway modulates a stunning number of major processes, including mRNA translation (reviewed in [13]), ribosome biogenesis [14], nutrient metabolism [15] and autophagy (reviewed in [16]) (Figure 1). With few exceptions the components and mechanisms that link raptor–mTOR to these processes are not known. This is the case even in budding yeast, where several raptor–TOR-regulated processes, like autophagy and ribosomal protein synthesis [17], are relatively well understood. Two mammalian proteins, S6 Kinase 1 (S6K1) and 4E-BP1, are known to link raptor–mTOR to the control of mRNA translation.

S6K1 is a famous protein in the TOR field. It was the first component of the pathway to be identified — even before the cloning of the mammalian and yeast TOR genes —

Figure 1



A model of the mTOR and PI3K/Akt signaling pathways and their interconnections. Two mTOR-interacting proteins, raptor and rictor, define distinct branches of the mTOR pathway. The raptor–mTOR pathway regulates cell growth (accumulation of cell mass) through S6K1 and 4E-BP1 as well as unknown effectors. It responds to nutrients and growth factors in part through the upstream regulators TSC1/2 and rheb. The rapamycin-insensitive rictor–mTOR pathway regulates Akt/PKB, PKC α , Rho/Rac to control cell survival, proliferation, metabolism and the cytoskeleton. The binding of growth factors to cell surface receptors activates PI3K to generate PtdIns(3,4,5)P₃ and recruits the PDK1 kinase and Akt/PKB to the plasma membrane. Akt/PKB is activated by its phosphorylation on two different sites. The rictor–mTOR complex phosphorylates Akt/PKB on Ser473 in the hydrophobic motif which may facilitate the phosphorylation by PDK1 of the activation loop of Akt/PKB on Thr308. How the rictor–mTOR complex is regulated is unknown. Dashed lines indicate interactions that are likely not direct.

and its phosphorylation state is a convenient measure of the activity of the raptor branch of the pathway [18–20]. Raptor–mTOR activates S6K1, and likely the related S6K2, by phosphorylating it within the hydrophobic motif conserved in the AGC family of kinases [21]. Mice null for S6K1, but not those null for S6K2, have small cells, as do *Drosophila* lacking dS6K, the single S6 kinase gene found in this organism [22,23]. In most mammalian cells rapamycin reduces cell size to a greater extent than does

inhibition of S6K1 [8,24], and fly cells missing *Drosophila* TOR (dTOR) are smaller than those without dS6K [25,26]. This suggests that other growth regulators in addition to S6 kinase must exist downstream of the raptor branch of the TOR pathway. Interestingly, mammalian skeletal muscle may be an exception because skeletal muscle cells deficient for S6K1 (but not those deficient for S6K2) are very small and are not shrunken further by rapamycin [27*]. The raptor–mTOR pathway also has

other roles in skeletal muscle physiology, including regulating muscle hypertrophy [28,29] and myoblast fusion [30].

Despite its long history in the TOR field, exactly how S6K1 regulates cell size is unclear. It was thought to act by controlling the translation of an abundant class of mRNAs containing a 5' TOP sequence, but more recent work does not support this conclusion [22,31]. Several S6K1 substrates have been described, including the ribosomal S6 protein, and the translational regulators eEF2 kinase and eIF-4B [32,33]. In addition, S6K1 interacts with and may phosphorylate SKAR, a putative RNA-binding protein with potential roles in mRNA splicing and transport [34]. How these S6K1 substrates contribute to cell size control remains to be determined.

The function in translational control of 4E-BP1 is better understood and has been recently reviewed [13]. In its non-phosphorylated state 4E-BP1 represses cap-dependent mRNA translation by binding to the eIF-4E cap-binding protein and preventing it from interacting with the eIF-4G protein. Phosphorylation of 4E-BP1 by raptor-mTOR releases eIF-4E to restore cap-dependent translation, which is particularly important for the translation of mRNAs with highly structured 5' UTRs.

The paucity of direct substrates for raptor-mTOR remains a major obstacle to understanding how it connects at the molecular level to downstream growth processes, like ribosome biogenesis. The phosphorylation sites on S6K1 and 4E-BP1 are not conserved [21], suggesting that the mTOR kinase domain may not have inherent substrate specificity and that mTOR-associated proteins may determine substrate preference. Consistent with this notion, both S6K1 and 4E-BP1 contain a conserved short sequence called the TOS motif that raptor recognizes and that is required for efficient *in vitro* and *in vivo* phosphorylation by the raptor-mTOR complex [35–37]. The lack of conservation between the S6K1 and 4E-BP1 phosphorylation sites and the high frequency of TOS-motif-like sequences in many proteins makes it difficult to identify additional raptor-mTOR substrates using bioinformatic approaches. It is important to keep in mind that it is very difficult to prove that both S6K1 and 4E-BP1 are direct substrates of raptor-mTOR, although all evidence suggests that this is the case. We cannot rule out the possibility that a distinct kinase that is regulated by and associated with raptor-mTOR is the true kinase that phosphorylates S6K1 or 4E-BP1 in *in vitro* assays.

In addition to regulating cell size, the raptor-dTOR pathway also controls *Drosophila* organ and organism size by regulating cell proliferation in a non-cell-autonomous fashion [38]. A decrease in raptor-dTOR signaling in just the fat body (an organ that shares functions with both mammalian fat and liver tissue) causes a dramatic reduc-

tion in total body size. The mechanism likely involves the nutrient- and dTOR-dependent production by the fat body of soluble factors that enhance the activity of the brain-derived *Drosophila* insulin-like peptides (Dilps). The Dilps activate cell proliferation through the PI3K/Akt pathway — one of many examples where this pathway intersects with raptor-TOR.

Regulation of Akt/PKB by rictor-mTOR

As the rictor-mTOR complex cannot bind FKBP12-rapamycin, it is unlikely to mediate mTOR functions discovered through their sensitivity to acute treatment with the drug. The surprising finding that rictor-mTOR, instead of raptor-mTOR, is the hydrophobic motif kinase of a rapamycin-resistant mutant of S6K1 was critical to discovering a *bona fide* substrate for rictor-mTOR [39]. The S6K1 mutant has a C-terminal truncation that leaves its hydrophobic motif dangling at the end of the protein, in an analogous position to the hydrophobic motifs of SGK and Akt/PKB. This realization contributed to the discovery that rictor-mTOR is a long-sought hydrophobic motif kinase for Akt/PKB and plays an important role in Akt/PKB activation [40**] (Figure 1). Recent work in *Dictyostelium* confirms that rictor is necessary for Akt/PKB activation [41*]. Akt/PKB is a key component of the insulin/PI3K signaling pathway and modulates cell survival and proliferation through downstream substrates such as the FOXO class of transcription factors (reviewed in [42]) and the p53-regulator mdm2 [43,44]. It is also an important drug target, because Akt/PKB becomes hyperactive in cancer cells that lose the PTEN tumor suppressor or acquire an activating mutation in the PI3K α catalytic subunit. In addition to Akt/PKB, rictor-mTOR also regulates the actin cytoskeleton through unknown mechanisms that involve PKC α and Rho [11*,12*], a function that is conserved in budding yeast [45]. Thus, through the rictor- and raptor-containing complexes, mTOR affects cell size, shape and number, consistent with the essential roles of mTOR and dTOR in the early development of mice and *Drosophila*, respectively [25,26,46,47*,48*]. The separation of the developmental and organismal roles of mTOR into the raptor and rictor branches will have to await the creation of mouse mutants of these genes.

It is interesting to consider why rictor might be significantly less conserved than raptor. Perhaps this reflects the involvement of the rictor-mTOR complex in pathways that are unique to metazoans, such as the insulin-stimulated PI3K/Akt pathway, and the participation of the raptor-mTOR complex in nutrient-sensing pathways that are likely to be well conserved.

Upstream of mTOR

Using S6K1 and 4E-BP1 phosphorylations as readouts, many diverse signals have been identified that regulate the raptor-mTOR pathway, but until recently the mole-

cular mechanisms have been a black box. The signals known to regulate S6K1 and presumably raptor–mTOR are bewildering, with growth factors, amino acids, glucose, energy status, and many forms of stress (e.g. osmotic stress, DNA damage) all being well-documented regulators. Work initiated in *Drosophila* and confirmed in mammalian tissue culture cells revealed that the heterodimer consisting of the TSC1 (hamartin) and TSC2 (tuberin) tumor suppressors is an upstream integrator of many signals that regulate the raptor–mTOR pathway [49,50]. TSC1/TSC2 is the GTPase-activating protein (GAP) for the ras-family GTP-binding protein rheb [51–55], which has been reported to directly bind and activate the raptor–mTOR complex [56]. *Drosophila* contains one rheb gene while mammals have two: rheb1 and rheb2. Several kinases, including AMP-activated kinase (AMPK), Akt/PKB, RSK1 and ERK, signal to raptor–mTOR by phosphorylating TSC2 and regulating the stability or GAP activity of the TSC1/2 heterodimer (Figure 1).

Raptor–mTOR responds to two metabolism-related signals, at least in part through TSC1/2. Under conditions of energy deprivation that increase the AMP/ATP ratio, AMPK becomes active and phosphorylates TSC2 to stimulate its GAP activity [57], inhibiting rheb and presumably raptor–mTOR. Oxygen sensing by the raptor–mTOR pathway also requires the TSC1/2 complex, but functions through a distinct mechanism that involves the hypoxia inducible factor (HIF)-dependent expression of REDD1 and REDD2, two growth regulators first identified in *Drosophila* as Scylla and Charybdis [58,59,60,61]. Akt/PKB [62–64], RSK1 [65] and ERK [66,67] link raptor–mTOR to growth factor signaling by phosphorylating and inhibiting TSC2 function. Recent work suggests that Akt/PKB can also signal to raptor–mTOR by decreasing the AMP/ATP ratio and thus preventing AMPK from inhibiting TSC1/2 [68]. In addition, Akt/PKB regulates the influx of nutrients that activate the raptor–mTOR pathway [69]. Interestingly, placement of the raptor–mTOR complex upstream of Akt/PKB indicates that raptor–mTOR is an upstream regulator of its raptor–mTOR sibling. Although the study of the raptor–mTOR pathway has just begun, it is already clear that growth factors modulate raptor–mTOR activity towards Akt/PKB [40]. The mechanism is unknown and the potential roles of TSC1/2, rheb, AMPK and REDD1/2 have not been tested.

As TSC1 and TSC2 do not exist in budding yeast and yeast rheb does not appear to function in the TOR pathway, it is unlikely that all signals upstream of mTOR will flow through TSC1/2. It would be evolutionarily efficient if signals that regulate both mammalian and yeast TOR, like amino acids and glucose, are sensed, at least in part, by a conserved mechanism. Two recent findings suggest that nutrient-derived signals can directly affect the raptor–mTOR complex independently of

TSC1/2. First, the strength of the interaction between raptor and mTOR is regulated by the same nutrient signals that regulate the pathway [8] but is independent of TSC1/2 (D Sarbassov, S Ali and D Sabatini, unpublished). Second, nutrient levels change the capacity of the mTOR kinase domain to interact with rheb [70]. In both examples the mechanisms are unknown.

There is an increasing appreciation that the TSC1/2–raptor–mTOR module signals to the insulin/PI3K/Akt pathway. Early work indicated that the rapamycin-sensitive mTOR pathway represses insulin/PI3K/Akt signaling [71–73] and it is now known that S6K1 inhibits IRS1 by directly phosphorylating it [74,75]. This S6K1-mediated inhibitory loop exerts a significant negative effect on the activity of downstream components of the insulin/PI3K pathway, like Akt/PKB, and its deregulation may play a role in insulin-resistant diabetes. S6K1 has also recently been shown to directly phosphorylate mTOR but the functional consequences are not yet known [76,77].

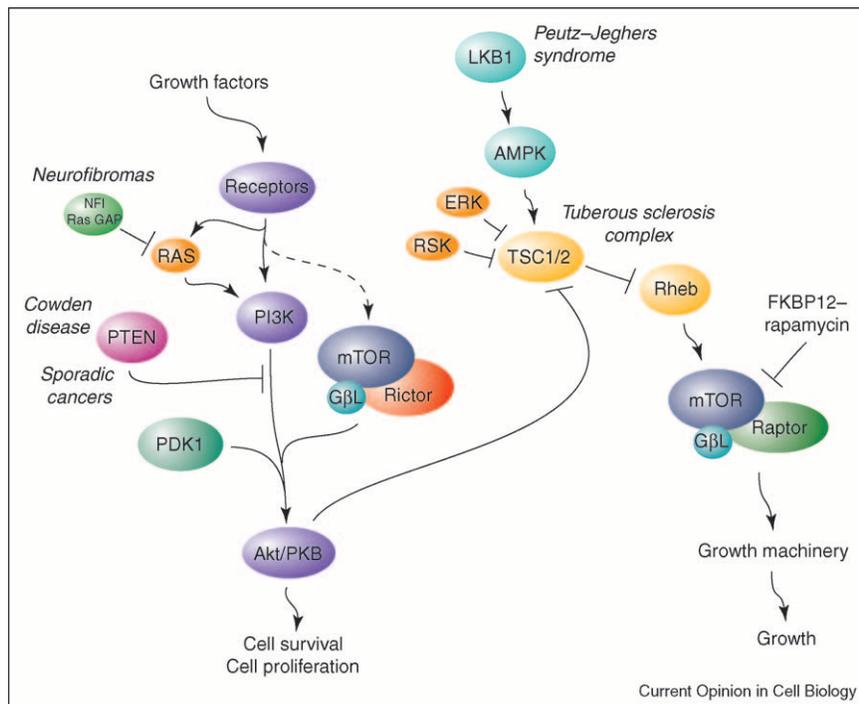
The mTOR pathway and disease

Deregulation of the mTOR pathway is emerging as a common theme in diverse human diseases and as a consequence drugs that target mTOR have therapeutic uses (Figure 2). Rapamycin is already used as an immunosuppressant to prevent the rejection of transplanted organs and also blocks restenosis after angioplasty. These uses have been reviewed [78,79] and will not be further covered here. In addition to rapamycin several analogues, including CCI-779, AP23573 and RAD001 (everolimus) are in clinical development.

The diseases most clearly associated with deregulation of the raptor–mTOR pathway are tuberous sclerosis complex (TSC) and lymphangioleiomyomatosis (LAM), both of which are likely caused by mutations in the TSC1 or TSC2 tumor suppressors. Patients with TSC develop slow-growing and usually benign tumors that when present in the brain, however, can cause seizures, mental retardation and death. LAM is a rarer disease in which patients develop seriously compromised lung function resulting from the abnormal proliferation of lung fibroblasts. In TSC1- or TSC2-null cells raptor–mTOR signaling is high, as reflected by an increase in S6K1 phosphorylation. Normalization of pathway activity with rapamycin should have beneficial effects and proof-of-concept work in a *Drosophila* model gives the hopeful possibility that this may be the case [80]. Inhibition of raptor–mTOR may also aid patients with the Peutz–Jeghers cancer-prone syndrome caused by mutations in the LKB1 tumor suppressor, a kinase that normally represses raptor–mTOR by phosphorylating and activating AMPK [81,82].

Raptor–mTOR modulation may also have a role in the treatment of sporadic human cancers. Inactivation of several tumor suppressors, in particular PTEN but also

Figure 2



Components of the mTOR and PI3K/Akt pathway implicated in cancer and related diseases. A simplified model of the mTOR and PI3K/Akt pathways is shown. Components implicated in disease have the disease name in italics next to the component name. Dashed lines indicate interactions that are likely not direct.

p53 and NF1, has been linked to raptor–mTOR activation. Interestingly, many cancer cells without PTEN function and, therefore, with hyperactive Akt/PKB signaling are highly sensitive to the anti-proliferative effects of rapamycin [83,84]. The reason is not clear but presumably the rapid division of these cells requires the activated raptor–mTOR that results from Akt/PKB inhibiting TSC1/2. Alternatively, it has been suggested that chronic treatment of cells with rapamycin may partially inhibit the rictor–mTOR complex to directly suppress hyperactive Akt/PKB signaling [40^{••}].

The wisdom of inhibiting the raptor–mTOR pathway in a solid tumor may vary depending on the activity state of the pathway. Raptor–mTOR will be highly active in well-vascularized tumor areas under the stimulation of nutrients and of tumor- and stroma-derived growth factors. In these cases, inhibitors like rapamycin may slow cell growth and proliferation and perhaps synergize with chemotherapeutics to induce cell death. On the other hand, in areas of poor blood flow raptor–mTOR activity is likely to be low because of the absence of necessary upstream signals like nutrients and oxygen. In such areas, the suppression of raptor–mTOR will decrease cell growth and induce autophagy to allow cells to conserve vital energy and nutrients until environmental conditions improve. One might imagine then that an activator of the

raptor–mTOR pathway could be therapeutically beneficial by driving cells to exhaust energy and nutrients so they can no longer maintain vital processes, such as the membrane potential.

Because of the existence of the negative signal from S6K1 to the insulin/PI3K/Akt pathway, it is important to keep in mind that inhibitors of raptor–mTOR, like rapamycin, can activate Akt/PKB. If this effect persists with chronic rapamycin treatment it could provide cancer cells with an increased survival signal that may be clinically undesirable. Interestingly, recent work indicates that tumors formed in mouse models of TSC may be relatively non-aggressive because activation of raptor–mTOR and S6K1 represses the PI3K/Akt pathway [85[•],86[•]]. Consistent with this, suppression of PTEN in TSC2 mutant cells reactivates the PI3K/Akt pathway to generate more aggressive tumors [85[•],86[•]]. Thus, from a clinical perspective, it is necessary to consider when and when not to use rapamycin as an anti-cancer therapy. In addition, it may be beneficial to develop therapies where rapamycin is used in combination with another drug to inhibit both branches of the mTOR pathway.

Conclusions

Despite its discovery over a decade ago, mTOR is only recently beginning to shed some of its mystery. We now

know that mTOR is part of at least two distinct multi-protein complexes that nucleate complex signaling pathways involved in regulating cell growth and proliferation by controlling many major cellular processes. Many outstanding questions remain to be answered in the TOR field. For example, what is the molecular nature of the nutrient-derived signal that controls raptor-mTOR? Do the raptor- and rictor-containing complexes mediate all mTOR functions? Does mTOR, like dTOR in *Drosophila*, play an important role in setting mammalian body size by regulating humoral factors? How is rictor-mTOR regulated and does it have additional substrates besides Akt/PKB? The increasing appreciation that mTOR deregulation occurs in human disease underscores the need to answer these questions and to understand how mTOR senses upstream signals to control diverse processes.

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