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Target of Rapamycin (TOR) Regulates Growth in Response to Nutritional Signals

RONIT WEISMAN
Department of Natural and Life Sciences, The Open University of Israel, Raanana, 435379, Israel

ABSTRACT All organisms can respond to the availability of nutrients by regulating their metabolism, growth, and cell division. Central to the regulation of growth in response to nutrient availability is the target of rapamycin (TOR) signaling that is composed of two structurally distinct complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2). The TOR genes were first identified in yeast as target of rapamycin, a natural product of a soil bacterium, which proved beneficial as an immunosuppressive and anticancer drug and is currently being tested for a handful of other pathological conditions including diabetes, neurodegeneration, and age-related diseases. Studies of the TOR pathway unraveled a complex growth-regulating network. TOR regulates nutrient uptake, transcription, protein synthesis and degradation, as well as metabolic pathways, in a coordinated manner that ensures that cells grow or cease growth in response to nutrient availability. The identification of specific signals and mechanisms that stimulate TOR signaling is an active and exciting field of research that has already identified nitrogen and amino acids as key regulators of TORC1 activity. The signals, as well as the cellular functions of TORC2, are far less well understood. Additional open questions in the field concern the relationships between TORC1 and TORC2, as well as the links with other nutrient-responsive pathways. Here I review the main features of TORC1 and TORC2, with a particular focus on yeasts as model organisms.

INTRODUCTION

A universal feature of all organisms is their ability to respond to nutrient availability by regulating growth and developmental programs. The identification of the target of rapamycin (TOR) pathway was a seminal discovery in the quest to understand the molecular mechanisms that govern such processes. TOR is an evolutionarily conserved serine/threonine kinase belonging to the family of phosphatidylinositol kinase-related kinases. Other members of this family include the mammalian DNA damage checkpoint kinases ATM and ATR,which are conserved from yeast to human, and DNA-PK and SMG1, which are not found in yeasts. TOR regulates growth (accumulation of mass), proliferation (accumulation in cell number), and survival in response to nutritional changes by diverse mechanisms that include regulation of anabolic and catabolic metabolism, nutrient uptake, protein translation and turnover, gene transcription, and the epigenome (reviewed in 1–3).

The first TOR genes were isolated in the budding yeast *Saccharomyces cerevisiae* as point mutations that conferred resistance to the growth-inhibiting effect of rapamycin (4–6). Rapamycin is a small hydrophobic molecule, a product of the soil bacterium *Streptomyces hygroscopicus* that was first isolated as an antifungal antibiotic but later proved to be a potent inhibitor of cellular growth in humans via inhibition of mammalian TOR (mTOR). The antiproliferative effect of rapamycin has proved beneficial for several clinical applications, including immunosuppression for the prevention of allograft rejection, treatment of cancer, and elution of stents to prevent restenosis after angioplasty (7, 8).

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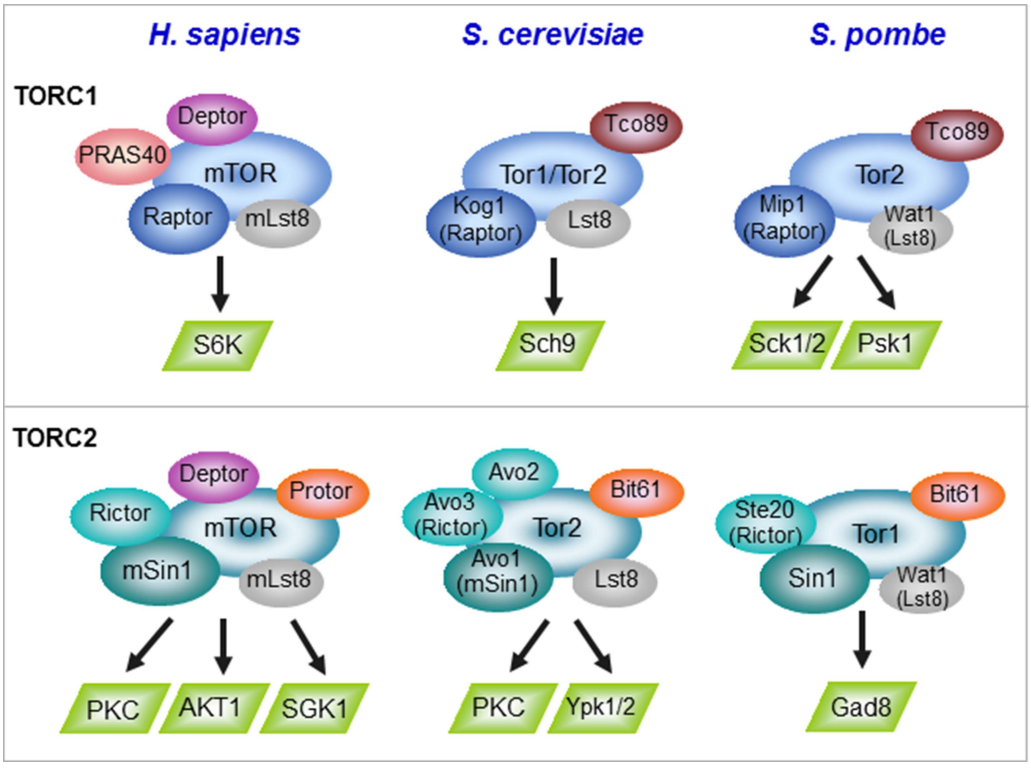
Correspondence: Ronit Weisman, ronitwe@openu.ac.il

Rapamycin induces cell growth arrest by mimicking a starvation-like response, which is characterized, among other features, by the induction of autophagy. This feature of rapamycin and its derivatives, or second-generation ATP-competitive TOR-specific inhibitors, may prove useful for the treatment of neurodegenerative and metabolic diseases (7, 8). Rapamycin, somewhat similar to calorie restriction, extends the life span in several model systems, including yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice, making it the first drug with the potential to treat aging (7, 8).

TOR proteins can be found in two distinct evolutionarily conserved complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (see Fig. 1). TOR provides the catalytic subunit in these complexes and is accompanied by TORC1-specific and TORC2-specific subunits, as well as protein subunits that are shared by the two complexes. TORC1 and TORC2 are controlled by distinct upstream and downstream regulators but share common features, such as the phosphorylation and activation of AGC kinases (a family of protein kinases named after protein kinases A, G, and C) at their C terminal hydrophobic and turn motifs. Since distinct AGC kinases are phosphorylated and activated by TORC1 and TORC2, monitoring the phosphorylation status of these kinases at specific sites is most useful for determining TORC1- or TORC2-specific activation (1).

In higher eukaryotes (nematodes, flies, mice, and humans) there is a single gene for TOR. In contrast, two TOR genes are found in several fungal groups as the result of independent events of whole genome or segmental duplications (9). The two best-studied unicellular organisms for TOR signaling are the budding yeast *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, which contain two TOR genes. In *S. cerevisiae*, TORC1 (*Sc*TORC1) mainly contains *Sc*Tor1 as the catalytic subunit, but a minor *Sc*TORC1 variant that contains *Sc*Tor2 also exists; ScTORC2 contains *Sc*Tor2 as the catalytic subunit (10). In *S. pombe*, *Sp*Tor2 mainly acts as the catalytic subunit of *S. pombe* TORC1 (*Sp*TORC1), while *Sp*Tor1 mainly acts as the catalytic subunit of *Sp*TORC2 (11, 12). This awkward naming

FIGURE 1 TORC1 and TORC2 subunits and downstream AGC kinases in mammalian, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* cells. TORC1 and TORC2 have shared and unique components. The human protein Raptor is TORC1-specific and is conserved in both yeast species. The human proteins Rictor and Sin1 are TORC2-specific subunits and are conserved in both yeast species. The target kinases of TORC1 and TORC2 are shown as green parallelograms.



of the *S. pombe* TOR genes is the result of their identification prior to the isolation of the TOR complexes in *S. cerevisiae* (13) and reflects the lack of known specific sequences for the TOR catalytic subunit that can predict its association with TORC1 or TORC2 subunits. A single TOR gene is found in *Candida albicans* and filamentous fungi, and TOR inhibition plays an important role in the pathogenicity of these organisms (14–16) (see further discussion below).

TORC1: A MASTER REGULATOR OF GROWTH AND STARVATION RESPONSES

TORC1 Architecture, Rapamycin Sensitivity, and Downstream Signaling

TORC1 is defined by the essential subunit known as Raptor in metazoans, Kog1 in *S. cerevisiae*, and Mip1 in *S. pombe*. A highly conserved but nonspecific subunit is Lst8 (also known as GβL in metazoans and Wat1 or Pop3 in *S. pombe*), which is found both in TORC1 and TORC2. Other components of TORC1 are not as highly conserved; Tco89 is found in *S. cerevisiae*, *S. pombe*, and *C. albicans*, but no homologues have been identified in higher eukaryotes. In contrast, PRAS40 and Deptor are found in higher eukaryotes, but no homologues have been identified in fungi (1) (see Fig. 1).

The TOR catalytic subunit in TORC1 binds rapamycin when the drug is in complex with the prolyl isomerase FKBP12. The FKBP12-rapamycin complex (hereafter referred to simply as rapamycin) binds the FKBP12-rapamycin binding (FRB) domain in TOR, which is located in close proximity to the kinase domain. As a result, most (but not all) TORC1-dependent activities are inhibited. Studies of *S. cerevisiae* played a central role in understanding the mode of action of rapamycin, including the identification of FKBP12 and the FKBP12-rapamycin binding (FKBP12/rapamycin-binding) domain and the characterization of the differential sensitivity to rapamycin of TORC1 and TORC2 (1, 4–6, 10). Still, the mechanism by which rapamycin inhibits TOR signaling and the determinants that establish rapamycin sensitivity are not fully understood. Several mechanisms of action have been suggested, including rapamycin-mediated disruption of TOR complexes and inhibition of the access of specific substrates to the kinase domain of TOR (reviewed in 17). TORC2 is less sensitive to inhibition by rapamycin compared with TORC1 or is not sensitive at all. Recent structural studies of *Sc*TORC2 suggest that the *Sc*TORC2-specific subunit Avo3 (Rictor in metazoans) masks the rapamycin-FKBP12 binding site of Tor2, thus preventing the inhibition of *Sc*TORC2 signaling (18).

The specificity of rapamycin for TORC1 proved most useful in deciphering cellular functions of this complex. Disruption of TORC1 in *S. cerevisiae*, either through genetic manipulations or inhibition by rapamycin, results in a phenotype that resembles starved cells, including arrest at the G1 phase of the cell cycle, G0-like cellular morphology and physiology, rapid drop in protein synthesis, repression of ribosomal gene expression, induction of stress- and nutrient-starvation genes, and stimulation of autophagy (19–22). Further studies demonstrated a conserved role of TORC1 in growth regulation through stimulating nutrient uptake, ribosome biogenesis, and the synthesis of proteins, lipids, and nucleotides (23–25; reviewed in 1, 26). To methodically screen for conditions that activate *Sc*TORC1, the phosphorylation status of Sch9, the AGC kinase downstream of *Sc*TORC1, was monitored. These studies demonstrated that Sch9 becomes dephosphorylated and inactivated in response to nitrogen, carbon, or phosphate starvation; high temperature; salt or redox conditions (27); and amino acid starvation or low quality of the nitrogen source (28). However, the signal or signals that are transduced to *Sc*TORC1 under these conditions are only partially understood.

Somewhat curiously, the growth of wild-type *S. pombe* cells is resistant to treatment with rapamycin (29). Yet rapamycin inhibits the phosphorylation of Psk1, the AGC kinase that lies downstream of *Sp*TORC1, or the phosphorylation of the Rps6 protein (S6 in human), the substrate of Psk1 (30, 31). S6 is a well-established target for mTORC1-S6K in humans and is often used to screen for hyperactivation of mTOR in cancer cells (32). Since rapamycin does not inhibit the essential function of *Sp*TORC1, conditional mutants of the catalytic subunit of *Sp*TORC1, *tor2*, or *mip1* (Raptor homologue) are used to study the cellular functions of *Sp*TORC1. Disruption of *Sp*TORC1 resulted in a phenotype that specifically resembles nitrogen-starved cells: growth arrest at the G1 phase of the cell cycle (unlike growth arrest at the G2 phase, characteristic for carbon starvation in *S. pombe*), small and rounded cell morphology, induction of nitrogen-starvation-induced genes, and activation of the sexual development pathway (11, 12, 33, 34). Complete withdrawal of the nitrogen source from the medium or a shift to a poor-quality nitrogen source (proline instead of ammonium) resulted in downregulation of *Sp*TOC1 activity as measured by decreased Rps6 phosphorylation (30, 35), leading to division at a reduced size and

advancement into mitosis (36, 37). In *C. albicans*, rapamycin inhibits growth via inhibition of the single TOR gene, *Tor1*, while nitrogen and carbon starvation both result in reduced *Ca*S6 phosphorylation (15, 38), suggesting that similar to *S. cerevisiae*, *Ca*TORC1 is sensitive to rapamycin and responds to both carbon and nitrogen limitation.

At the transcriptional level, inhibition of *Sc*TORC1 or rapamycin treatment in *S. cerevisiae* (20, 21, 39, 40), disruption of *Sp*TORC1 (12, 33), or rapamycin treatment in *C. albicans* (41) led to the reduction of ribosomal gene expression and induction of starvation- or stress-responsive transcription. Of note is the regulation of the response to changes in quantity or quality of the nitrogen source by the conserved TORC1-protein phosphatase 2A (PP2A) module, which regulates the activity of GATA-transcription factors (a family of transcription factors that are characterized by their ability to bind DNA sequences that contain the GATA motif). Early studies identified the zinc-finger GATA transcription factors Gln3 and Gat1 as targets for *Sc*TORC1 (20, 21, 40). Further studies unraveled a pathway in which *Sc*TORC1 regulates the activity of PP2A and PP2A-like phosphatases such as Sit4 by regulating their association with the inhibitory regulatory subunit Tap42. Upon reduction of the quantity or quality of the nitrogen source, *Sc*TORC1 is inactivated, promoting the disassociation of Tap42 and the activation of the phosphatases. In turn, the Ure2 protein that sequesters Gln3 and Gat1 is dephosphorylated, and Gln3 and Gat1 are dephosphorylated and translocate into the nucleus (20, 21, 42–44). Gln3 and Gat1 are required for transcriptional activation of genes that are normally repressed in the presence of high quantity and quality of the nitrogen source and are critical regulators of nitrogen catabolite repression, the processes by which high-quality nitrogen sources are imported and assimilated in preference to poor-quality nitrogen sources.

The *C. albicans GLN3* and *GAT1* homologues are also required for regulation of nitrogen metabolism, and their disruption resulted in rapamycin resistance, similar to the effect of disrupting *GLN3* and *GAT1* in *S. cerevisiae* (45). More recently, *Sp*TORC1 was demonstrated to regulate the phosphorylation and nuclear localization of the GATA transcription factor Gaf1. Gaf1 becomes dephosphorylated and inactivated in a manner dependent on the PP2A phosphatase Ppe1 in response to nitrogen (but not carbon) starvation (46, 47). The conservation of the TORC1-PP2A-GATA-transcription signaling in the distantly related *S. cerevisiae* and *S. pombe*, two yeasts that diverged in evolution more than 300 million years ago, suggests that this pathway may also be conserved in higher eukaryotes.

Early studies showed that treatment of *S. cerevisiae* with rapamycin led to a rapid drop in protein synthesis (19). Further studies revealed that *Sc*TORC1-Sch9 and *Sc*TORC1-Tap42-PP2A play prominent roles in the regulation of ribosome biogenesis, one of the most energetically demanding processes and thus a focal point for the coordination of growth with nutritional changes. *Sc*TORC1 regulates ribosome biogenesis at many levels, including transcriptional induction of rRNA, ribosomal proteins, and the ribosome biogenesis (Ribi) regulon. Several transcription factors were identified as targets of *Sc*TORC1 in the regulation of ribosome biogenesis, including Maf1, the repressor of RNA pol III (23, 48), Sfp1 (49), the forkhead transcription factor Fhl1 (50), and the transcriptional repressors Stb3, Dot6, and Tod6 (24). *Sc*TORC1 also directly binds 35S rDNA promoters to promote rRNA synthesis (51). Similar to *S. cerevisiae*, *Sp*TORC1 also regulates transcription of ribosomal proteins (33) and phosphorylates the repressor of RNA pol III Maf1 (52). At the posttranscriptional level, *Sc*TORC1 regulates protein synthesis through regulation of the translation-initiation factor eIF2α, in a manner that is at least partially conserved in *S. pombe* (53, 54). In both *S. cerevisiae* and *S. pombe* eIF2α is phosphorylated in response to amino acid starvation or rapamycin treatment. The phosphorylation of eIF2α is mediated via the *S. cerevisiae* or *S. pombe* Gcn2 kinases; however, *Sc*TORC1 regulates the phosphorylation of Gcn2 at sites that are not conserved in *S. pombe*.

Autophagy is a general name for catabolic processes mediated by membrane trafficking pathways that lead cytoplasmic material from the cytosol into the lumen of the lysosome or vacuole, where massive degradation takes place. *Sc*TORC1 inhibits autophagy under favorable growth conditions through the phosphorylation of Atg13 that inhibits the assembly of the Atg1 protein kinase complex (55). Autophagy has been linked to several human pathologies, including cancer and neurodegenerative diseases and is also implicated in the process of aging. Most interestingly, rapamycin treatment or reduced TORC1 activity in *S. cerevisiae*, in *S. pombe*, or in higher eukaryotes extended the life span (56–58). TORC1 controls aging through Sch9 in *S. cerevisiae* (56), in a process that involves the induction of autophagy and stress genes, as reviewed in more detail in reference 8. Additional downstream effects of TORC1 have been studied in detail, including amino acid uptake, regulation of cell size, and developmental responses, including entrance into quiescence or regu-

lation of filamentous growth. These are not detailed here due to limited space but are reviewed elsewhere (1, 59).

TORC1 Upstream Signaling and the Response to Nitrogen Quantity or Quality or the Presence of Amino Acids

In yeast cells, the activity of TORC1 is sensitive to nutritional starvation and a variety of stresses, while mTORC1 is also sensitive to energy levels (ATP) and growth factors (3). The direct signals or mechanisms for activation of TORC1 are as yet unknown; however, a large body of evidence suggests that the nitrogen source and/or amino acids (which may also serve as a nitrogen source) play a key role in TORC1 activation.

Nitrogen is an essential element required for synthesis of amino acids, nucleotides, and other cellular components. Yeast cells can sense, take up, and assimilate several nitrogen sources. A high-quality nitrogen source (glutamine or ammonium) is defined by its ability to promote rapid growth and suppress the nitrogen catabolite repression genes (60). As described above, *Sc*TORC1 and *Sp*TORC1 are major regulators of the transcriptional control in response to the quality and quantity of the nitrogen source, and the activity of these complexes drops in response to low levels or poor quality of the nitrogen source (28, 30, 35). *Sc*TORC1 and *Sp*TORC1 are also activated in response to specific amino acids such as leucine, glutamine, asparagine, arginine, aspartate, methionine, and cysteine (28, 61–64), raising the intriguing possibility that the nitrogenous compounds critical for TORC1 activation are amino acids. This possibility is particularly tempting in view that in higher eukaryotes, amino acids (particularly leucine, glutamine, and arginine) are potent activators of mTOR signaling (65–67). Whether amino acid levels are directly sensed by TORC1 and, if so, by what mechanisms is still an open question. Moreover, the mechanism of activation of TORC1 by the quantity and quality of the nitrogen source appears distinct from the mechanism of response to amino acids (28, 36). Moreover, different amino acids are thought to activate TORC1 through different mechanisms, including glutaminolysis and leucyl-tRNA synthetase (61, 68).

TORC1 and several remarkably conserved upstream regulators of TORC1 are localized to the vacuole in yeast cells and to its equivalent compartment, the lysosome, in higher eukaryotes (reviewed in 17). At the vacuolar or lysosomal surface TORC1 is activated by two distinct guanosine triphosphate GTPases: the Gtr (Rag in higher eukaryotes) and the Rhb1 (Rheb in higher eukaryotes). The Rag/Gtr-TORC1 axis is conserved in *S. cerevisiae* and *S. pombe*, while the Rhb1/Rheb-TORC1 axis is conserved in *S. pombe* but is absent in *S. cerevisiae* (see Fig. 2). The vacuole and lysosome act as major sites for protein degradation and reservoirs of amino acids; however, whether TORC1 responds to amino acids in the vacuole/lysosome or in the cytoplasm is not known yet.

In *S. cerevisiae*, the Gtr complex is composed of Gtr1 and Gtr2. Gtr1 is analogous to the mammalian RagA or RagB, while Gtr2 is analogous to RagC or RagD. Only when Gtr1 or RagA/B is bound to GTP and Gtr2 or RagC/D is bound to GDP is the heterodimer active and able to lead to activation of TORC1 (69–71). The Gtr1/2 GTPases associate with the EGO complex, which contains Ego1, Ego2, and Ego3 to form a complex that tethers Gtr1/2 and *Sc*TORC1 to the vacuolar membrane (71–73). The EGO equivalent complex in higher eukaryotes is the pentameric Ragulator complex, which is required for lysosmal localization and also acts as a guanine exchange factor toward RagA/B. In *S. cerevisiae* the guanine exchange factor activity toward Gtr1 is provided by Vam6 (vacuolar morphogenesis protein), which apparently has no mammalian equivalent (reviewed in 17). The vacuolar Vam6-Gtr1/2 module is conserved in *S. pombe* and activates *Sp*TORC1 in response to the presence of amino acids (62). The interaction and activation of either *Sc*TORC1 or *Sp*TORC1 by the Vam6-Gtr1/2 module are sensitive to the presence of amino acids, but *Sc*TORC1 and *Sp*TORC1 are localized to the vacuole irrespective of the nutritional status (62, 71). In contrast, mTORC1 dissociates from the lysosome upon amino acid starvation (69, 70). The Gtr and Rag complexes are negatively regulated by conserved GAP (GTPase activating protein) complexes that are named SEACIT and GATOR1 in *S. cerevisiae* and humans, respectively (Fig. 2). SEACIT and GATOR1 are in turn subjected to negative regulation by additional conserved complexes, SEACAT and GATOR2 (74; reviewed in 17) (Fig. 2). More recently, Lst4-Lst7 was identified as the complex that acts as a GAP toward Gtr2, promoting the binding of GDP to Gtr2 and thus activating *Sc*TORC1 (64), similar to the FNIP-folliculin complex in mammalian cells (reviewed in 17).

The *S. cerevisiae* Gtr1/2 GTPases regulate TORC1 activity but are not essential genes, suggesting that they play only a limited role in activation of *Sc*TORC1. Similarly, disruption of *gtr1/2* in *S. pombe* leads to a reduced level of *Sp*TORC1 activity and a hyper-mating phenotype, characteristic of conditional loss-of-function *Sp*TORC1 mutants; still, *S. pombe* cells lacking Gtr1/2 or Vam6 can respond to changes in the nitrogen quan-

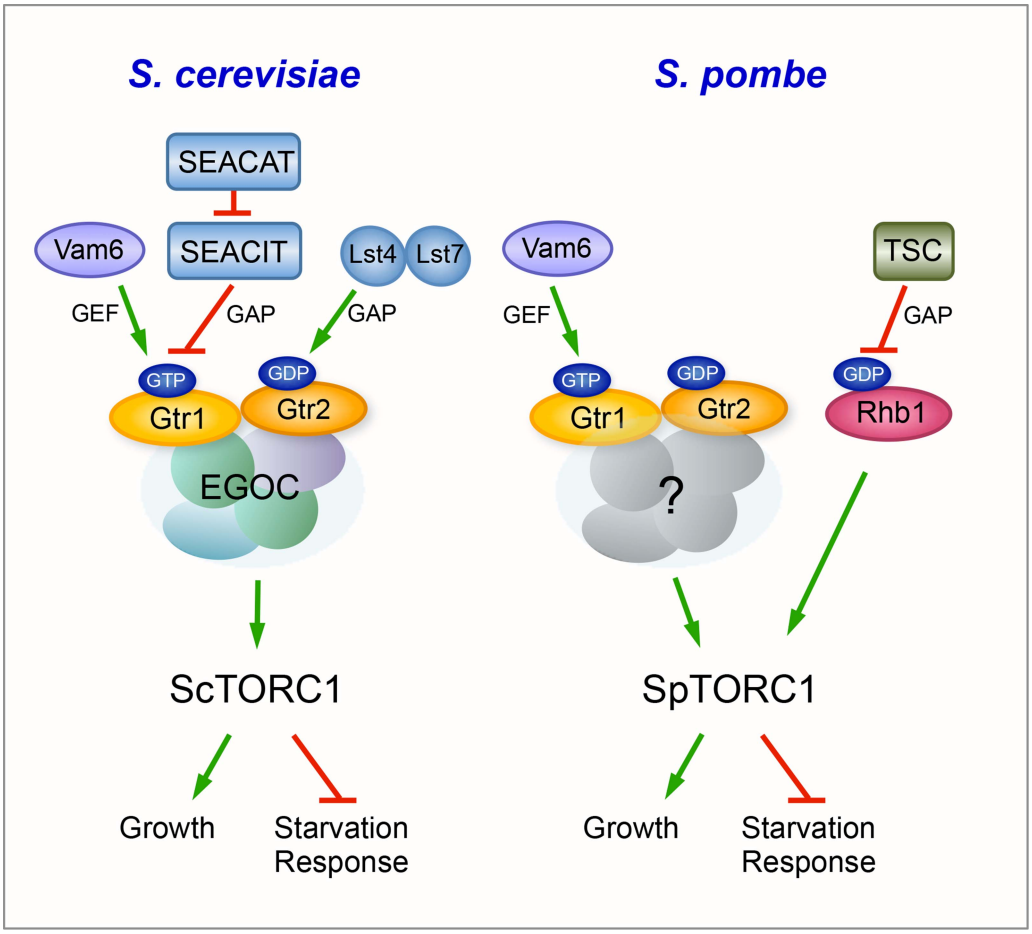


FIGURE 2 TORC1 is activated by GTPases to promote growth and inhibit starvation responses. *Saccharomyces cerevisiae* TORC1 (*Sc*TORC1) and *Schizosaccharomyces pombe* TORC1 (*Sp*TORC1) are activated by the GTPase complex Gtr1/Gtr2. The complex is active when Gtr1 is bound to GTP and Gtr2 is bound to GDP. Vam6 is a guanine exchange factor (GEF) for Gtr1 that is conserved between *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, the Gtr1/Gtr2 complex is associated with the EGO complex and is controlled by the SEACIT (which acts as GTPase activating protein, GAP), SEACAT, and Lst4-Lst7 complexes. These complexes have as yet unidentified equivalents in *S. pombe*. *Sp*TORC1 is also regulated by the Rhb1 GTPase (Rheb in mammals) and the TSC (tuberous sclerosis complex, a tumor suppressor complex in mammals), which acts as a GAP towards Rhb1.

tity or quality. These findings argue that the Gtr-TOR axis plays only a relatively limited role in activation of TORC1 (62).

While the Gtr1/2 complex is dispensable for activation of TORC1 in yeast cells, the *S. pombe* Rhb1 GTPase (Rheb in human) is essential for *Sp*TORC1 activation (75–78). Accordingly, loss of function of Rhb1 results in a phenotype that mimics nitrogen starvation and is highly similar to disruption of *Sp*TORC1, including the G1 arrest and activation of nitrogen-starvation gene expression. Rhb1 is negatively regulated by the TSC1-TSC2 complex, which is conserved in humans and functions as a GAP toward Rhb1. The TSC complex functions as a tumor suppressor complex, and mutations in either TSC1 or TSC2 can lead to the tuberous sclerosis syndrome, which is characterized by rapid development of tumors and severe neurological defects (79). Disruption of the TSC complex in *S. pombe* results in prolonged phosphorylation of ribosomal S6 under nitrogen starvation (31), reduced transcription of nitrogen-starvation-induced amino acid permeases, and reduced amino acid uptake (80, 81). These findings suggest that deletion of TSC in *S. pombe* leads to hyperactivation of *Sp*TORC1 that shuts down transcriptional programs dedicated to scavenging for nitrogen sources. *C. albicans* homologues for Rheb and TSC2 were also identified.

Disruption of *Ca*Rhb1 resulted in hypersensitivity to rapamycin, while *Ca*Rhb1 overexpression and *Ca*Tsc2 deletion are defective in filamentous growth under low or poor nitrogen, suggesting that Rhb1 activates *Ca*TORC1 and prevents normal responses to nitrogen starvation (82).

The TSC-Rheb axis acts as a hub for several signaling pathways, among them the AMPK, a serine/threonine kinase that coordinates cell growth and metabolism with available energy resources (reviewed in 83). AMPK is a heterotrimeric complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits. In humans, the tumor suppressors LKB1 and CaMKK (calcium/calmodulin-dependent protein kinase kinase) activate mammalian AMPK, which in turn directly phosphorylates TSC2 and activates its GAP activity, leading to conversion of Rheb to its inactive form and inhibition of mTORC1 (84; reviewed in reference 83). It was recently demonstrated that in *S. pombe*, the α subunit of AMPK is required to respond to changes in the quality of the nitrogen source. Under nitrogen stress conditions (shift from a high-quality to poor-quality nitrogen source), a specific homologue of CaMKK (Ppk34) stimulates *Sp*AMPK activity, which leads to downregulation of *Sp*TORC1 in a manner dependent on the TSC-Rhb1 (36). How TORC1 may integrate the complex signals from different pathways and coordinate these with the nutritional status awaits further studies.

TORC2: ROLES IN CELLULAR METABOLISM, GROWTH, AND SURVIVAL

TORC2 Architecture and Downstream Signaling

TORC2 contains the TOR catalytic subunit together with two additional highly conserved proteins that are essential for the function of the complex: Rictor in metazoans (Avo3 in *S. cerevisiae* and Ste20 in *S. pombe*) and mSin1 in metazoans (Avo1 in *S. cerevisiae* and Sin1 in *S. pombe*) (see Fig. 1). TORC2 contains the Lst8 subunit, which is also found in TORC1. The Protor subunit in mammals has orthologues in *S. cerevisiae* and *S. pombe* (Bit61). Avo2 is specific for *S. cerevisiae* and *C. albicans*, while Deptor (which is also part of mTORC1) has been identified only in mammals (reviewed in 1).

The cellular functions of TORC2 are less well understood compared with TORC1. This is partly due to the lack of TORC2-specific inhibitors. In addition, in contrast to the starvation-like phenotypes observed upon disruption of TORC1, the loss of TORC2 results in diverse effects (reviewed in 85), making it difficult to attribute a primary cellular function to TORC2. Recent findings suggest that TORC2 plays significant roles in cellular metabolism, including cancer metabolism, growth control, and survival, encouraging further research of TORC2 and the search for TORC2-specific inhibitors (85, 86). As in TORC1 signaling, AGC kinases downstream of TORC2 play important roles in mediating downstream effects (see Fig. 1). The best-studied AGC kinases downstream of *Sc*TORC2 are Ypk1 and Ypk2 (87), while most of *Sp*TORC2 functions are mediated via Gad8 (88).

*Sc*TORC2 is essential for growth, while *Sp*TORC2 becomes essential only under starvation and a variety of other stress conditions. So far, only a limited overlap exists between the functions attributed to *Sc*TORC2 and *Sp*TORC2. It is not clear whether this reflects a true divergence in function or our incomplete understanding of the cellular roles of TORC2. *Sc*TORC2 was first implicated in the regulation of the polarization of the actin cytoskeleton, suggesting that it mainly regulates spatial aspects of cell growth (89, 90). Further studies revealed that *Sc*TORC2-Ypk1/2 also controls endocytosis (91) and sphingolipid biosynthesis (92–95), in a manner that is antagonized by the calcium- and calmodulin-dependent protein phosphatase calcineurin, which acts either directly downstream of *Sc*TORC2 or in a parallel pathway (92, 93). *Sc*TORC2 regulates actin polarization and endocytosis via flipase protein kinases (96–99), in a manner that was also suggested to be linked to the control of reactive oxygen species accumulation (97, 98). Interestingly, the level of reactive oxygen species is regulated by *Sc*TORC2 and can act as a signal to activate *Sc*TORC2 (97, 98). The Slm proteins, which were first identified downstream of *Sc*TORC2, can also function upstream of *Sc*TORC2 to promote activation of this complex in response to plasma membrane stress, resulting in a homeostatic loop in which *Sc*TORC2 regulates several aspects of membrane biogenesis and is also affected by changes in the membrane (100). Recently, it was demonstrated that *Sc*TORC2-Ypk1 positively regulates autophagy in response to amino acid starvation (101). This finding is interesting because it suggests that *Sc*TORC1 and *Sc*TORC2 oppositely regulate autophagy, albeit by different mechanisms. It may also suggest that *Sc*TORC2 is linked to nutritional changes more closely than has previously been recognized.

The most pronounced effects of loss of function of *Sp*TORC2-Gad8 become apparent under starvation and stress conditions. *Sp*TORC2 is essential to execute the two main responses to starvation: sexual development

and entrance into stationary phase. Thus, cells that lack *Sp*TORC2 or Gad8 are highly sterile and quickly die once they exit the logarithmic phase (13, 102). Loss of *Sp*TORC2-Gad8 also renders cells sensitive to a variety of stress conditions, including low or high temperature, osmotic or oxidative stress, and DNA damage or replication stress conditions (13, 103, 104). Under normal growth conditions, *Sp*TORC2-Gad8 is required for the G2/M transition, and cells disrupted for *Sp*TORC2 show an elongated phenotype characteristic of a delay in entrance into mitosis (13, 103, 105, 106). *Sp*TORC2 is also required for amino acid uptake (107) and for the localization of Ght5, a high-affinity glucose transporter, to the cell surface under low-glucose conditions (108). An abnormal distribution of actin cortical dots and excess actin polymerization at the cell equator occur in cells lacking functional *Sp*TORC2 (106), as do defects in reorganizing the actin observed during new end take-off, the phase during which the new cell end starts to grow (109).

Unexpectedly, transcriptional profiles of *S. pombe* cells lacking the catalytic subunit of *Sp*TORC2 resembled those of cells lacking histone deacetylases or chromatin remodeling subunits (104). This finding led to the identification of additional defective phenotypes in *Sp*TORC2 mutant cells, including elongation of telomeres and loss of gene silencing (104). Of interest are the similarities observed upon loss of function of *Sc*TORC2 or *Sp*TORC2 with respect to DNA damage sensitivity (104, 110). In both cases, TORC2 plays a role in survival of DNA damage in a manner that is independent of DNA checkpoint activation but involves suppression of accumulation of DNA damage sites. However, in *S. cerevisiae*, elevated levels of DNA damage are thought to be the result of the defect in the actin cytoskeleton, whereas in *S. pombe* no such link is known (85). Interestingly, the inability of cells that lack *Sp*TORC2 or Gad8 to execute nitrogen starvation responses is opposite to the "always starved for nitrogen" phenotype of cells lacking *Sp*TORC1 (35, 106). This is somewhat reminiscent of the opposite effects of *Sc*TORC1 and *Sc*TORC2 in the regulation of autophagy and may suggest regulatory links between *Sp*TORC1 and *Sp*TORC2.

TORC2 Upstream Signaling and the Response to Glucose Availability

The upstream regulation of TORC2 is poorly characterized. *Sc*TORC2 has been localized to membrane structure organelles, primarily to the plasma membrane, but has also been detected throughout the cytoplasm (111, 112). Visualization of GFP tagged *Sp*TORC2 components also indicated cytoplasmic as well as cortical localization (113), but a biochemical approach has suggested that Tor1, the catalytic subunit of *Sp*TORC2, and Gad8 are also found in the nucleus (114). *Sc*TORC2 is activated by direct association with the ribosome (115, 116), in a manner that is conserved in mammals and that may link TORC2 to growth capacity. In accord with the localization of *Sc*TORC2 to the plasma membrane, *Sc*TORC2 is activated by plasma membrane stress, which stems from cell surface expansion or stress on the plasma membrane (100). This mode of activation is thought to reflect a feedback loop mechanism in which *Sc*TORC2 regulates membrane biosynthesis but is also affected by membrane growth (17). In *S. pombe*, glucose but not nitrogen is required for activation of *Sp*TORC2 (117, 118). The activity of *Sp*TORC2-Gad8 in response to glucose requires the activation of Rhy1, a Rab family GTPase that lies upstream of *Sp*TORC2-Gad8 (118). The regulation of *Sp*TORC2-Gad8 in response to glucose availability is fast and does not require protein translation, suggesting a close link between a nutritional change and TORC2 activation, although the nature of the glucose signal that is sensed by *Sp*TORC2 is as yet unknown.

TOR IN PATHOGENIC FUNGI

Virulence in plant or human pathogenic fungi requires several cellular traits, including invasive growth, morphogenetic yeast-to-hyphal transition (e.g., in *C. albicans*), vegetative hyphal fusion, and expression of adhesion and stress response genes. As discussed above, TOR signaling regulates many aspects of the stress response as well as the switch from vegetative growth to developmental programs in response to environmental changes. Accordingly, accumulation of data indicates that TOR signaling is critical for virulence in several pathogenic fungi and may thus serve as a target for antifungal treatment. Early studies demonstrated that rapamycin and several less immunosuppressive analogues inhibited growth in *S. cerevisiae*, *C. albicans*, and *Cryptococcus neoformans*, suggesting that the use of rapamycin analogues may be beneficial as antifungal agents (15; reviewed in 16). Further support of the role of TOR in *C. albicans* pathogenicity is evident from studies that demonstrate the role of *Ca*Tor1 in regulating the expression of cell wall- and hyphal-specific genes, including adhesins (41). Moreover, genetic manipulation of *Ca*TOR signaling, including disruption of *Ca*Sch9 (119), *Ca*Rhb1 or *Ca*Tsc2 (82), or *Ca*Sit4 (120), supports a role for *Ca*Tor1 in promoting hyphal formation and virulence in response to nutritional changes, as well as in response to changes in pH (121).

Interestingly, reduced *Ca*Tor1 signaling during hyphal initiation leads to the expression of the GATA transcription factor Brg1, which competes for promoter binding with Nrg1, the major transcription repressor of hyphal development (122). Thus, similar to *S. cerevisiae* or *S. pombe*, TOR is found to regulate a family member of the GATA transcription factors in *C. albicans*, but unlike *S. cerevisiae* or *S. pombe*, *Ca*Tor1 regulates the level of Brg1 indirectly via the Hog1 MAP kinase pathway (122). A study of fluconazole-sensitive mutants in *C. neoformans* also suggests that TORC2 signaling is conserved and may play an important role in recurrent infections of *C. neoformans* (123). Thus, for example, similar to equivalent mutations in *S. cerevisiae*, the deletion of *C. neoformans* Sin1 or Ypk1 resulted in hypersensitivity to inhibition of sphingolipid synthesis, and the mutant cells contained low levels of complex sphingolipids (123).

Rapamycin also induced growth inhibition in filamentous fungi, including the human pathogens *Aspergillus fumigatus* (124) and *Aspergillus nidulans* (125). The single gene encoding the TOR kinase in *A. fumigatus* (*Af*Tor1) is essential for growth. However, the use of a conditionally repressible version of *Af*Tor1 in combination with proteomic analysis identified potential protein targets of *Af*Tor1 that are involved in cell cycle regulation, nutrient sensing, and stress response (124). *Af*Tor1 also regulates siderophore biosynthesis and the adaptation to iron starvation, a response that is critical for the virulence of *A. fumigatus* (124). TOR signaling may play an essential role in *Aspergillus* growth via its function as part of TORC2. Thus, for example, the Sin1 homologue in *Aspergillus niger*, RmsA, is required for hyphal elongation and branching (126) and for viability and stress responses (127), reminiscent of the roles identified for TORC2 in cell polarity and stress response in yeasts.

Conservation in the architecture of TOR signaling and the response to rapamycin also exists in phytopathogenic fungi. Rapamycin inhibits the growth and virulence of phytopathogenic fungi, including several species of *Fusarium* (128, 129). Moreover, it was demonstrated that TOR participates in nitrogen metabolite repression (e.g., in *Fusarium fujikuroi* [128]). In addition, the Tap42-type 2A phosphatase module (129) and Sch9 homologues (130) were identified as downstream targets of TOR signaling in *Fusarium graminearum*. Still, better understanding of the response of pathogenic and filamentous fungi to rapamycin or other TOR inhibitors awaits further identification of TOR components, especially the characterization of the distinct cellular roles of TORC1 and TORC2 and the identification of additional components of these pathways.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Since its early discovery as the target for action of rapamycin, TOR has emerged as a central signaling pathway that regulates many different aspects of growth in response to environmental changes, in particular nutritional changes. Regulation of TORC1 in response to nitrogen availability and/or amino acids is the most well-established nutritional response for the TOR pathway. TORC2 may also respond to nutritional changes, but the mechanisms involved are far less well understood. How TOR signaling mediates response to stress conditions, other than nutritional stress, also remains to be determined. The finding that TOR complexes often act as part of feedback loops has suggested that TOR may function primarily as a mediator of cellular homeostasis (17).

The identification of the two TOR complexes marked a breakthrough in our understanding of TOR signaling pathways and rapamycin response. TORC1 and TORC2 are structurally and functionally distinct, and the search for specific TORC1 and TORC2 downstream and upstream effectors is still the focus of many studies. More recent studies suggest that understanding the regulatory links between TORC1 and TORC2 is also critical for understanding the role of TOR in growth control. *Sp*TORC1 and *Sp*TORC2 oppositely regulate several responses to nitrogen starvation including sexual development and transcriptional reprogramming (131). mTORC1 negatively regulates mTORC2 through phosphorylation of several mTORC2-specific components, while mTORC1 and mTORC2 also converge to control certain aspects of metabolism (86). Future studies are expected to unravel the links between TORC1 and TORC2 in more detail.

Finally, *S. cerevisiae* has played a key role in deciphering the mode of action of rapamycin, a drug that has already proved most valuable for the treatment of several human pathologies. The powerful tools of yeast genetics are expected to contribute to further understanding of the mode of action of rapamycin and second-generation ATP-competitive TOR inhibitors. TORC2-specific inhibitors have been much sought after. Whether yeast will also contribute to finding such valuable research tools and possible drugs is an exciting option that at present remains an open question.

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TOR Controls Translation Initiation and Early G1 Progression in Yeast

Nik C. Barbet,* Ulrich Schneider,* Stephen B. Helliwell,* Ian Stansfield,† Michael F. Tuite,† and Michael N. Hall*‡

*Department of Biochemistry, Biozentrum, University of Basel, CH-4056 Basel, Switzerland; and †Research School of Biosciences, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, United Kingdom

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Saccharomyces cerevisiae cells treated with the immunosuppressant rapamycin or depleted for the targets of rapamycin TOR1 and TOR2 arrest growth in the early G1 phase of the cell cycle. Loss of TOR function also causes an early inhibition of translation initiation and induces several other physiological changes characteristic of starved cells entering stationary phase (G0). A G1 cyclin mRNA whose translational control is altered by substitution of the *UBI4* 5′ leader region (UBI4 is normally translated under starvation conditions) suppresses the rapamycin-induced G1 arrest and confers starvation sensitivity. These results suggest that the block in translation initiation is a direct consequence of loss of TOR function and the cause of the G1 arrest. We propose that the TORs, two related phosphatidylinositol kinase homologues, are part of a novel signaling pathway that activates eIF-4E-dependent protein synthesis and, thereby, G1 progression in response to nutrient availability. Such a pathway may constitute a checkpoint that prevents early G1 progression and growth in the absence of nutrients.

INTRODUCTION

The immunosuppressant rapamycin and the related compound FK506 exert their immunosuppressive effects by inhibiting intermediate steps in signal transduction that lead to T cell activation and proliferation (Heitman *et al.*, 1991; Schreiber and Crabtree, 1992; Sigal and Dumont, 1992; Liu, 1993; Fruman *et al.*, 1994). FK506 in complex with its intracellular receptor FKBP inhibits the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. As a downstream effector of the T cell receptor (TCR), calcineurin normally triggers nuclear import of a subunit of the transcription factor NF-AT which, in turn, activates 50–100 genes, including the gene encoding the lymphokine interleukin-2 (IL-2) (Weiss and Littman, 1994). Rapamycin also forms a toxic complex with FKBP, but instead of inhibiting the TCR signaling pathway, inhibits a subsequent signal transduction cascade that is stimulated by IL-2 (Bierer *et al.*, 1990; Dumont *et al.*, 1990). The IL-2 signaling pathway mediates G1 progression (proliferation) of a T cell. Rapamycin prevents the phosphorylation and activation of p70 S6 kinase, a downstream effector of IL-2 and several other growth factors, including insulin, EGF, PDGF, IL-3, and erythropoietin (Calvo *et al.*, 1992, 1994; Chung *et al.*, 1992; Kuo *et al.*, 1992; Price *et al.*, 1992; Terada *et al.*, 1992; Ferrari *et al.*, 1993; Lane *et al.*, 1993). Although best known for its inhibition of IL-2–dependent p70 S6 kinase activation, rapamycin also inhibits p70 S6 kinase activation in response to these other mitogens (Calvo *et al.*, 1992; Chung *et al.*, 1992, 1994; Price *et al.*, 1992). The p70 S6 kinase phosphorylates the ribosomal protein S6 which, in turn, leads to the activation of translation initiation (Kuo *et al.*, 1992; Thomas, 1992; Jefferies *et al.*, 1994; Terada *et al.*, 1994). The p70 S6 kinase thus links mitogenic stimulation and the initiation of protein synthesis. A homologue of the yeast TOR proteins (FRAP/RAFT1/RAPT1/mTOR) (see below) has recently been identified in mammalian cells as a direct target of the rapamycin-FKBP complex (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995), suggesting that FRAP/RAFT1/RAPT1/mTOR is required for p70 S6 kinase activation and is part of a general mitogenic signaling

‡ Corresponding author.

pathway (for a figure that summarizes the pathway, see Downward, 1994).

In the yeast *Saccharomyces cerevisiae*, rapamycin-FKBP inhibits the *TOR1* and *TOR2* gene products and arrests cells with an unbudded morphology, indicative of a G1 cell cycle arrest similar to that observed in T cells (Heitman *et al.*, 1991; Cafferkey *et al.*, 1993; Kunz and Hall, 1993; Kunz *et al.*, 1993; Helliwell *et al.*, 1994; Stan *et al.*, 1994; Zheng *et al.*, 1995). A dominant point mutation in either *TOR* gene renders a cell resistant to rapamycin, whereas disruption of both genes results in an unbudded morphology, similar to that seen when wild-type cells are treated with rapamycin, and a 1n DNA content (Heitman *et al.*, 1991; Cafferkey *et al.*, 1993; Kunz *et al.*, 1993; Helliwell *et al.*, 1994). TOR1 and TOR2 are large (~280 kDa), functionally homologous proteins that are structurally related to phosphatidylinositol kinases (PI kinases) (Kunz *et al.*, 1993; Garcia-Bustos *et al.*, 1994; Helliwell *et al.*, 1994; Yoshida *et al.*, 1994). PI kinases are required for production of phosphatidylinositol-derived second messengers (Carpenter and Cantley, 1990). Thus, TOR1 and TOR2, like FRAP/RAFT1/RAPT1/mTOR, may be components of a rapamycin-sensitive signaling pathway required for cell cycle progression in G1.

In *S. cerevisiae*, the decision to commit to a cell cycle is made at a point in late G1 termed START. Traversal of START and entry into S phase is regulated by the activity of the cyclin-dependent kinase encoded by *CDC28* (Reed, 1992; Nasmyth, 1993). Three G1-specific cyclin genes were originally identified and named *CLN1*, *CLN2*, and *CLN3* (Cross, 1988; Nash *et al.*, 1988; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990); additional candidate G1 cyclin genes have subsequently been identified and named *HCS26*, *ORFD*, *CLB5*, and *CLB6* (Frohlich *et al.*, 1991; Ogas *et al.*, 1991; Epstein and Cross, 1992; Kuehne and Linder, 1993; Schwob and Nasmyth, 1993). All except *CLN3* are transcribed only in late G1 with *CLN1*, *CLN2*, and *HCS26*, and possibly *ORFD*, under control of the transcription factor SBF. CLN3, whose transcript is present throughout the cell cycle, is regulated post-transcriptionally and acts as an upstream activator of other G1 cyclins (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991; Tyers *et al.*, 1992, 1993; Cvrckova and Nasmyth, 1993).

When nutrients are limiting, haploid yeast cells do not proceed to START in late G1, but instead exit the mitotic cell cycle in early G1 and enter a stationary or G0 phase (for review see Werner-Washburne *et al.*, 1993). Stationary phase enables a cell to maintain viability for long periods when nutrients are not available, and is characterized by several physiological properties including 1n DNA content, failure to reach START, reduced protein synthesis, accumulation of glycogen, acquisition of thermotolerance, and changes in the pattern of transcription (Werner-Washburne *et al.*, 1993). Nutrient sensing and the regulation of entry into stationary phase are poorly understood, but are generally thought to involve the RAS/cAMP pathway (Broach, 1991; Thevelein, 1994). However, this is not the sole nutrient-sensing pathway, as mutants in the RAS/cAMP pathway have been isolated that exhibit a normal response to starvation independently of intracellular cAMP levels (Cameron *et al.*, 1988).

Here we report that cells lacking TOR function (cells treated with rapamycin or depleted of TOR) arrest growth and rapidly exhibit, by all criteria examined, properties diagnostic of G0 or stationary phase, including a reduction in translation initiation. The cell cycle arrest upon loss of TOR function is suppressed by altering the translational control of the G1 cyclin CLN3. Our results and analogy with mammalian cells suggest that TOR is part of a novel signal transduction pathway required for translation initiation and G1 progression, perhaps in response to nutrients.

MATERIALS AND METHODS

Strains, Plasmids, and Media

The parental strain in this study was JK9–3da (*MATa leu2–3, 112 ura3–52 trp1 his4 rme1 HMLa*). Isogenic derivatives with only the changes indicated are shown in Table 1. The composition of rich medium (YPD), synthetic galactose/glycerol medium (SGal/Gly), and synthetic glucose medium (SD) supplemented with the appropriate nutrients was as described (Sherman, 1991). All cultures were incubated at 30°C unless otherwise indicated. Rapamycin (provided by Sandoz Pharma, Basel, Switzerland) was added to the medium to a final concentration of 0.2 μg/ml. Rapamycin was diluted into media from a stock solution of 1 mg/ml in 10% Tween-20/90% ethanol (Heitman *et al.*, 1993). Plasmid pJK5 contains the entire *TOR2* gene under control of the *GAL1* promoter (Kunz *et al.*, 1993). YEplac181::*tor2–61*^{ts} (amp^{r} 2 μ *LEU2*) contains the entire *TOR2* gene and was isolated by hydroxylamine mutagenesis as a temperature-sensitive *TOR2* allele (Barbet and Hall, unpublished data). The *RAS2*^{val19} allele on plasmid YEp213 (amp^{r} 2 μ *URA3*) (Broek *et al.*, 1987) was transformed into JK9–3da. YCplac111 is amp^{r} *CEN4 LEU2* (Gietz and Sugino, 1988). The *BCY1* gene was disrupted (*bcy1::URA3*) as described using the one-step gene replacement technique (Toda *et al.*, 1987; Rothstein, 1991). Integration of *ADH-*

Table 1. Strains used in this study

Strain	Genotype
JK9-3da	*MATa leu2-3,112 ura3-42 trp1 his4 rme1 HMLa*
JK350-21a	JK9-3da *tor1:LEU2-4 tor2::ADE2-3*/pJK5
JH11-1c	JK9-3da *TOR1-1*
JH12-17b	JK9-3da *TOR2-1*
NB17-3d	JK9-3da *his3 HIS4 tor1::HIS3*
NB30	JK9-3da *bcy1::URA3*
NB32	JK9-3da *ura3::[URA3 ADH-CLN2]*
NB33	JK9-3da *ura3::[URA3 CLN3-1]*
NB34	JK9-3da/YEp213::*RAS2*^{val19}
NB35	JK9-3da *ade2 his3 HIS4 tor1::HIS3 tor2::ADE2*//YEplac181::*tor2-61*^{ts}
NB36	JK9-3da/YCplac111::*UB14*$^{5'}$*-CLN3*
NB37	JK9-3da/YCplac111::*UB14*$^{5'}$
NB38	JK9-3da/YEpURA::*CLN3*

CLN2 (Nasmyth and Dirick, 1991) at the *ura3* locus was achieved by linearizing the plasmid containing the *CLN2* construct with *Eco*RV. Disruptions and integrations were confirmed by Southern blot analysis. All transformations were performed using the lithium acetate procedure (Ito *et al.*, 1983).

Flow Cytometry

Overnight cultures of yeast in SD complete medium were diluted to OD_{600} <0.05 and allowed to grow before the addition of rapamycin at OD_{600} = 0.2. Three hundred-microliter samples were taken from these cultures at hourly intervals, sonicated for 2 min, and immediately fixed by addition of 700 μl absolute ethanol. Samples were incubated overnight at 4°C, washed, and resuspended in 50 mM sodium citrate, pH 7.4, and treated with RNAse (0.25 mg/ml) for 1 h at 37°C. DNA was stained by the addition of 500 μl citrate buffer containing 16 μg/ml propidium iodide. For each timepoint taken, 10,000 events were analyzed for DNA content using a Becton Dickinson FACScan (Mountain View, CA) and data was processed using Lysys II software (Lincoln Park, NJ).

Order-of-Function Mapping

A reciprocal shift experiment was performed with the temperature-sensitive *tor2* strain NB35 and α-factor as described (Hereford and Hartwell, 1974) and also with the modification of a 1.5-h overlap in which both blocks were imposed. Because the effects of rapamycin are irreversible (presumably because the drug cannot be washed out) we were unable to perform a standard reciprocal shift experiment with α-factor and rapamycin. To circumvent this problem, we performed a double block experiment and an α-factor to rapamycin shift experiment. For the double block experiment, logarithmically growing cultures of JK9–3da in SD medium were treated with nocodazole (10 μg/ml; Sigma, St. Louis, MO) for 2.5 h to arrest the cells in mitosis. Cells were harvested by filtration and nocodazole was washed out of the cells with 10 volumes of sterile water followed by 10 volumes of SD medium. Cells were then incubated in fresh SD medium either with no addition, with 10 μg/ml mating pheromone (α-factor), with 0.2 μg/ml rapamycin, or with both mating pheromone and rapamycin. Samples were taken at 30-min intervals, sonicated for 2 min to separate cells, and scored for emergence of buds and appearance of the shmoo phenotype (Sprague, 1991). For the α-factor to rapamycin shift experiment, logarithmically growing cultures of JK9–3da in SD medium were treated with 10 μg/ml α-factor for 2.5 h. Cells were harvested by filtration and washed with 10 volumes of sterile water followed by 10 volumes of SD medium. The culture was then split; one half of the culture received 0.2 μg/ml rapamycin and the other half received drug vehicle alone. Samples were removed at 30 min intervals, sonicated for 2 min, and scored for the emergence of buds.

Northern Analysis

Extraction of total cellular RNA was performed as previously described (Jensen *et al.*, 1983). For Northern analysis, 10 μg of total RNA was separated on 1% agarose gels containing 6% formaldehyde, and transferred overnight to Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL) in 20× SSC. The *HCS26*, *ORFD*, *CTT1*, *SSA3*, *UBI4*, *CLB5*, and *CLN2* DNA probes were amplified from genomic DNA by the polymerase chain reaction (PCR). The primers used for PCR were as follows, with the 5′ primer listed first and the fragment size generated given in parentheses: *HCS26*, 5′-ATGTGTGAATACAGCAAG-3′ and 5′-AAACCCATGTTGACTCAT-3′ (963 bp); *ORFD*, 5′-ATGTCAAACTACGAAGCC-3′ and 5′-CCTGTGTTCTTCCGCCTT-3′ (998 bp); *CTT1*, 5′-ATGAACGTGTTCGGTAAA-3′ and 5′-TGGCACTTGCAATGGACC-3′ (1686 bp); *SSA3*, 5′-ATGTCTAGAGCAGTTGGT-3′ and 5′-ATCAACCTCTTCCACTGT-3′ (1947 bp); *UBI4*, 5′-ATGCAGATTTTCGTCAAG-3′ and 5′-GTTACCACCCCTCAACCT-3′ (1142 bp); *CLB5*, 5′-ATGGGAGAGAACCACGAC-3′ and 5′-TGCTATGCATTTCGGATG-3′ (1278 bp); and *CLN2*, 5′-ATGGCTAGTGCTGAACCA-3′ and 5′-TATTACTTGGGTATTGCC-3′ (1634 bp). The *SWI4* probe was a 2.2-kbp *Bam*HI fragment from the plasmid YCplac33::SWI4 (gift of K. Nasmyth). The *SWI6* probe was a 2.2-kbp *Xho*I/*Cla*I fragment from 1941 (gift of K. Nasmyth). The *CLN3* probe was a 500-bp *Hin*dIII/*Eco*RI fragment from pBF30 (Nash *et al.*, 1988). The probe for *CLN1* was a 2-kbp *Hin*dIII fragment from pcln1::URA3 (Hadwiger *et al.*, 1989). The probe for *CDC28* was a 1.2-kbp *Xho*I/*Xba*I fragment from YEp13::CDC28 (gift of K. Nasmyth). The probe for *TOR1* was a 4.3-kbp *Hin*dIII fragment from pPW20 (Helliwell *et al.*, 1994). The *TOR2* probe was a 5.3-kbp *Bgl*II fragment from pJK3–3 (Kunz *et al.*, 1993). The *HSP26* probe was a 800-bp *Bgl*II/*Nde*I fragment from pHSP26 (gift of S. Lindquist). The probe for *SSB1* was a 2.2-kbp *Hin*dIII fragment from pFKR15. The probe for *SSA1* was a 2-kbp *Sal*I fragment from EC551 (gift of E. Craig). The *ACT1* probe was a 1-kbp *Eco*RI/*Pst*I fragment from pUC18::*ACT1* (gift of P. Linder). *SSA1* and *SSA2* transcripts are indistinguishable, as are *SSB1* and *SSB2* transcripts, because the DNA sequences of these pairs of genes are 97% and 94% identical, respectively (Werner-Washburne *et al.*, 1989). Probes were labeled with [^{32}P]dATP using the random-primed DNA labeling kit (United States Biochemical, Cleveland, OH). Filters were exposed to x-ray film (Kodak X-OMAT) AR at −70°C with intensifying screens (Dupont Cronex). Signals were quantitated by scanning appropriately exposed films using a Molecular Dynamics densitometer (Sunnyvale, CA). In the experiment shown in Figure 7B, the total cellular RNAs of strains NB36 and NB38 were prepared identically, run on the same gel, transferred to the same filter, and hybridized to the same probe at the same time.

Incorporation of [^{35}S]Methionine into Total Yeast Protein

For analysis of gross protein synthesis, tricholoracetic acid (TCA)-precipitable counts were quantitated from pulse-labeled cultures at the indicated times after treatment. For rapamycin treatment, exponentially growing cultures of JK9–3da in SD medium minus methionine were treated with 0.2 μg/ml rapamycin, 100 μg/ml cycloheximide, or with drug vehicle alone (10% Tween-20/90% ethanol). For TOR depletion, exponentially growing cultures of the *tor*^{ts} strain NB35 and the control strain NB17–3d in SD minus methionine were resuspended in prewarmed medium at 37°C. For each timepoint, 0.01 OD_{600} equivalents were removed and labeled at 30°C for 7 min with 2 μCi [^{35}S]methionine (Amersham). Aliquots of the pulse-labeled cells were lysed on Whatman filters presoaked in 50% TCA, and deacylated by boiling for 10 min in 5% TCA. Filters were washed in acetone, air dried, and TCA-precipitable counts were quantitated by scintillation counting using a Canberra Packard 1900TR liquid scintillation analyzer.

Polysome Gradient Analysis

Strains JK9–3da and NB35 were grown in YPD to a cell density of 10^7 cells ml^{-1}. Following harvesting, polysomes were prepared as described (Stansfield *et al.*, 1992), except that polysomes were resolved on a 15–50% w/v sucrose gradient by centrifuging for 2.1 h at 17,000 × *g* using a Beckman SW40 Ti rotor. Cycloheximide (200 μg/ml) and rapamycin (0.2 μg/ml) were added to cultures at the indicated times before harvest. Drugs used in this way to inhibit yeast cultures were also included at the same concentration in the lysis buffer (Stansfield *et al.*, 1992).

Glycogen Staining

Logarithmically growing cultures in SD medium were treated with 0.2 μg/ml rapamycin and incubated at 30°C in the presence of the drug. At hourly intervals up to 5 h after rapamycin addition, 5 OD equivalents of cells were harvested onto Millipore HA filters (Bed-

ford, MA), placed upon a solid agar matrix, and exposed to iodine vapor for 1 min.

Construction and Analysis of the UBI4-CLN3 Fusion

The 5′ region (containing the untranslated leader and promoter sequences) of the *UBI4* polyubiquitin gene and a sequence containing the open reading frame of the *CLN3* gene were amplified from *S. cerevisiae* genomic DNA using the polymerase chain reaction. Oligonucleotides were designed to produce a 752-bp *UBI4* 5′ region fragment flanked by a 5′ *Hin*dIII and a 3′ *Sal*I restriction site, and a 1821-bp *CLN3* fragment flanked by 5′ *Sal*I and 3′ *Sma*I sites. The oligonucleotides were as follows: *UBI4* 5′ end, 5′-GCAAAGCTTC-CCACCACCAGCACTAGCTTAGAT-3′; *UBI4* 3′ end, 5′-AATGTC-GACCTATTAGTTAAAGTAAAGTGGGTG-3′; *CLN3* 5′ end, 5′-TACGTCGACTGTACGATGGCCATATTGAAGGAT-3′; and *CLN3* 3′ end, 5′-GTACCCGGGACGTATTTGCTTTGCAAATTTTA-3′. The *UBI4-CLN3* construct was obtained by first introducing the *Hin*dIII/*Sal*I-cut *UBI4* 5′ region fragment into a *Hin*dIII/*Sal*I cut YCplac111 vector (*CEN4 LEU2*). Following transformation and amplification in *E. coli*, this "parent plasmid" was digested with *Sal*I and *Sma*I and the *Sal*I/*Sma*I-cut *CLN3* fragment was introduced. The *UBI4* 5′ region was fused 7 bp upstream of the *CLN3* start codon. The resultant plasmid (YCplac111::UBI4$^{5'}$-CLN3) and its parent plasmid (YCplac111::UBI4$^{5'}$) were transformed into the wild-type haploid yeast strain JK9–3da to yield strains NB36 and NB37, respectively. Strain NB38 is JK9–3da containing the plasmid YEpURA::CLN3 (gift of K. Nasmyth), which consists of a 7-kb genomic *Bgl*II fragment containing the *CLN3* gene inserted into YEp352. For the asynchronous flow cytometry experiments, strains were grown in SD medium minus leucine to early log phase, and treated with 0.2 μg/ml rapamycin. Cell number and DNA content were analyzed hourly for 5 h following rapamycin treatment. For the synchrony experiments, NB36 and NB37 were grown to early log phase, then treated with 10 μg/ml α-factor for 2.5 h to arrest cells at start. α-Factor was removed by filtration and washing with water, followed by SD medium minus leucine, and cells were resuspended in fresh SD medium minus leucine. Samples were removed at 20-min intervals, washed, sonicated to separate cells, and assessed for emergence of buds and DNA content (flow cytometry). At maximal budding (generally 60 min after release from α-factor), the cultures were split; half received 0.2 μg/ml rapamycin, the remaining half received drug vehicle alone. Flow cytometry was performed as described above.

Assay of Starvation Sensitivity

Strain NB36 containing the *UBI4-CLN3* fusion and control strain NB37 containing the *UBI4* 5′ region without the *CLN3* open reading frame, on a *LEU2* plasmid, were grown in SD medium minus leucine for 6 days. Samples were removed daily and assessed for cell number/milliliter of culture, cell viability, and percentage of budded cells. For viability determination, 10^3 cells were plated on rich medium (YPD) in duplicate, and the number of cells able to form colonies was determined as a percentage of total number of cells plated. Replica plating to SD medium minus leucine showed that over 80% of the cells retained their respective plasmid, even after prolonged incubation.

RESULTS

Rapamycin Blocks G1 Progression

We have shown previously that rapamycin treatment causes yeast cells to arrest with an unbudded morphology (Heitman *et al.*, 1991; Kunz *et al.*, 1993). Such a phenotype, although suggestive of, is not necessarily indicative of a G1 arrest, as mutants have been isolated that are perturbed in budding but not in the onset of DNA synthesis (Adams *et al.*, 1990; Johnson and Pringle, 1990; Bender and Pringle, 1991; Cvrckova and Nasmyth, 1993). We therefore examined whether yeast cells treated with rapamycin arrest with a 1n DNA content, and are thus indeed impaired in G1 progression. An exponentially growing asynchronous culture of the haploid strain JK9–3da was treated with 0.2 μg/ml rapamycin, and at hourly intervals samples were removed for flow cytometry. As shown in Figure 1, a shift to a 1n DNA content was observed after 1 h of rapamycin treatment, and after 2–3 h, ~85% of the cells contained a 1n DNA complement (Figure 1D). The shift to 1n DNA content paralleled growth arrest; rapamycin-treated cells never completed more than one doubling, as determined by direct counting of the cells in the treated culture at the different time intervals. A control culture treated with the drug vehicle alone (10% Tween/90% ethanol) continued to grow normally, doubling in cell number every 125 min for the duration of the experiment. Thus, rapamycin causes a G1 arrest within one generation. As shown previously, TOR depletion also causes cells to arrest growth with a 1n DNA content (Helliwell *et al.*, 1994).

When the size distribution of cells was analyzed, we observed two subpopulations in the rapamycin-treated cells (Figure 1E). The major subpopulation of cells increased in size throughout the experiment, whereas the minor subpopulation of cells appeared to remain as small cells. Although the two subpopulations became more evident at later time points as the larger cells continued to increase in volume, two discrete populations could already be discerned after 2 h. The small cells most likely represent newly formed, starved daughter cells (see below) (Johnston *et al.*, 1977). The increased size of the larger cells can be accounted for by the observation that they contain an exceptionally large vacuole (Heitman *et al.*, 1991). Because an enlarged vacuole is also symptomatic of starvation (Granot and Snyder, 1991), these cells might also be starved (in G0) despite the presence of nutrients. The reason for the biphasic size distribution is unclear.

The TOR Restriction Point Is in Early G1 Before START

To determine the TOR restriction point within G1, we performed an order-of-function (reciprocal shift) analysis using a temperature-sensitive *tor* mutant and the mating pheromone α-factor (Hereford and Hartwell, 1974). This maps the TOR restriction point relative to START, the α-factor arrest point. The mutant strain (NB35) used in this experiment contained a temperature-sensitive *tor2* allele on a plasmid and chromosomal disruptions of both *TOR1* and *TOR2*. NB35

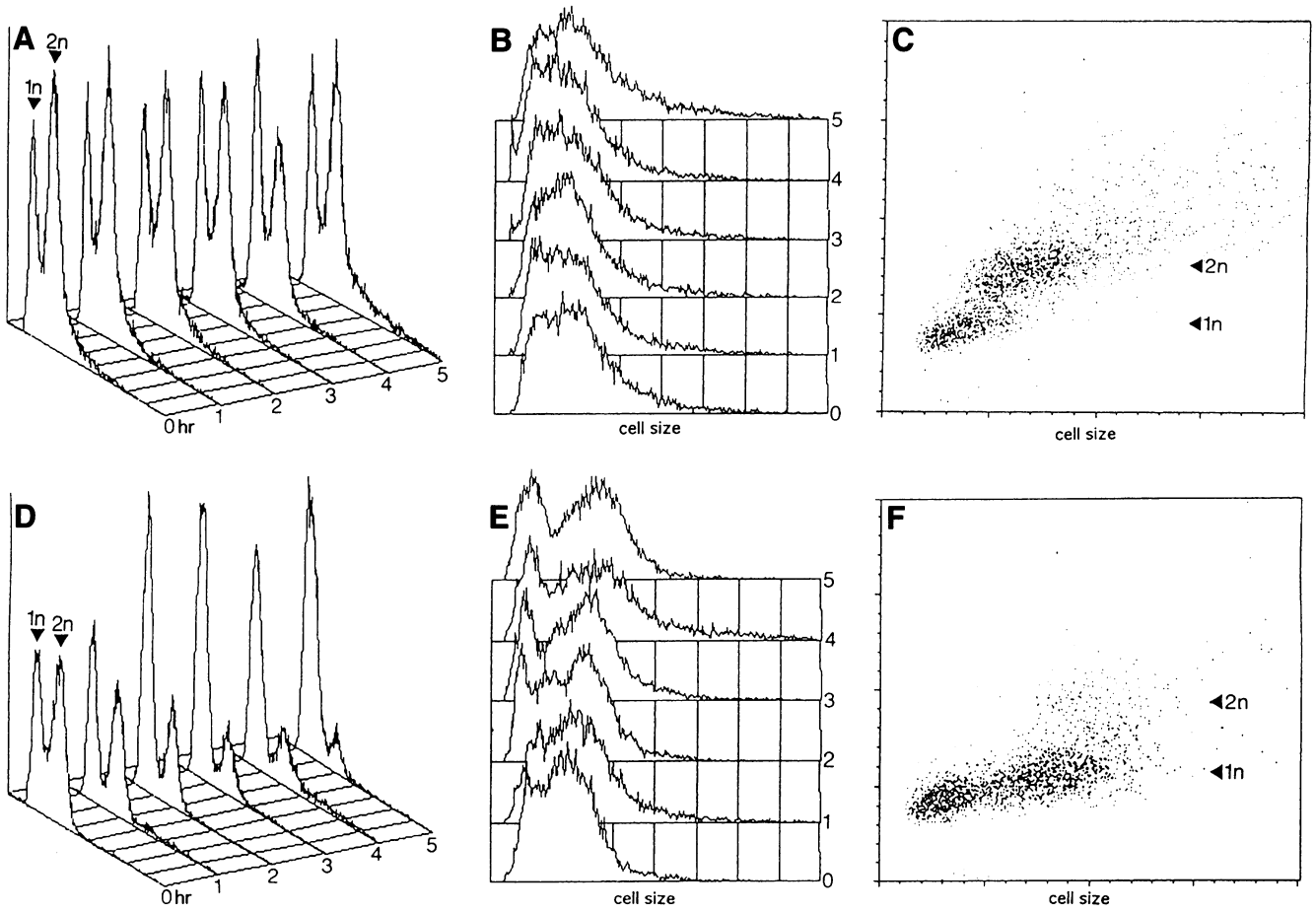


Figure 1. Rapamycin causes wild-type yeast cells (JK9–3da) to arrest with a 1n DNA content. Exponentially growing cells were treated with 0.2 μg/ml rapamycin and sampled for flow cytometry at hourly intervals up to 5 h. (A and D) DNA content, (B and E) cell size, and (C and F) a two-dimensional plot of cell size distribution (x-axis) versus DNA content (y-axis) for rapamycin-untreated (A–C) and -treated cells (D–F). The two-dimensional plot corresponds to the 5-h time point. 1n and 2n refer to DNA content.

(*tor*^{ts}) arrests growth with a 1n DNA content after shift to the nonpermissive temperature, and resumes growth upon return to the permissive temperature. The growth arrest of NB35 (*tor*^{ts}) occurs within one generation; this strain fails to complete more than one doubling after shift to the nonpermissive temperature, as determined by cell counting. Following release from a mating pheromone block and a simultaneous shift from the permissive temperature (24°C) to the nonpermissive temperature (37°C), cells synchronously entered S phase as determined by emergence of new buds (Figure 2A); cells maintained at 24°C behaved similarly. In contrast, when cells were arrested at the TOR restriction point, then released by resuspending in fresh medium at 24°C and treated with mating pheromone, they formed shmoos and did not initiate a new round of budding for the duration of the experiment (Figure 2B). Budding after shift from α-factor to the restrictive temperature was not due to a slow inactivation of temperature-sensitive TOR. First, NB35 (*tor*^{ts}) arrests within one generation. Second, shifting cells to the nonpermissive temperature 1.5 h before release from the α-factor block did not prevent budding (Figure 2C). Third, wild-type cells released from an α-factor block into medium containing rapamycin also resumed budding (Figure 2D). The results of a double block experiment performed with α-factor and rapamycin (see MATERIALS AND METHODS) were also consistent with a TOR restriction point in early G1; rapamycin prevented nocodazole-synchronized cells from forming shmoos in response to α-factor (our unpublished results). Thus, the TOR restriction point is in early G1 before START.

As further evidence that loss of TOR function causes an early G1 arrest, we observed that rapamycin-treated cells lack START-specific transcripts encoding the G1 cyclins (Figure 3) (see below), and that providing *CLN2* under control of the rapamycin-unresponsive, constitutive *Schizosaccharomyces*

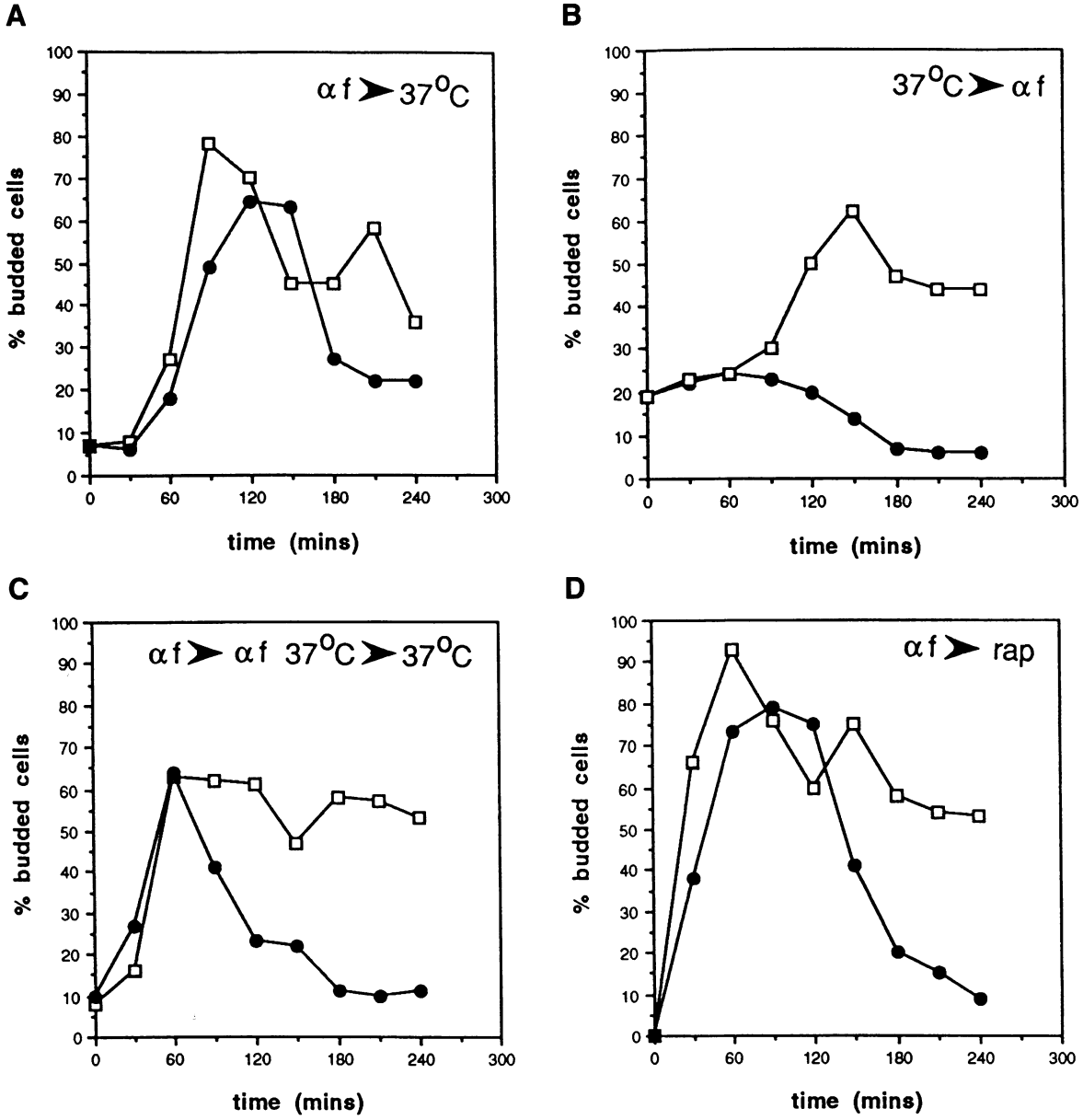


Figure 2. TOR depletion arrests cells in early G1 before START. (A–C) Order-of-function determination by an α-factor (αf) and *tor*^{ts} reciprocal shift. The percentage of budded cells was monitored at the indicated time points after release from the first block. (A) Strain NB35 (*tor*^{ts}) was arrested by pretreatment with α-factor at the permissive temperature, washed, and resuspended in fresh medium without α-factor at 24°C (open squares) or 37°C (closed circles). (B) Strain NB35 (*tor*^{ts}) was arrested by preincubation for 5 h at the nonpermissive temperature (37°C), washed, and resuspended in fresh medium at the permissive temperature (24°C) containing vehicle alone (open squares) or α-factor (closed circles). (C) Strain NB35 (*tor*^{ts}) was treated as in panel A with the modification that cells were shifted to the nonpermissive temperature 1.5 h before release from the α-factor block. (D) Order-of-function determination by an α-factor to rapamycin shift. Wild-type strain JK9–3da was arrested at START by α-factor treatment, washed, and resuspended in medium containing vehicle alone (open squares) or 0.2 μg/ml rapamycin (closed circles). The percentage of budded cells was determined at the indicated times after release from α-factor.

pombe ADH promoter (Nasmyth and Dirick, 1991) does not abrogate the rapamycin-induced cell cycle arrest (our unpublished results). Thus, TOR is not directly (or solely) required for *CLN* gene transcription, and the loss of START-specific transcripts is a downstream effect rather than the direct cause of the cell cycle arrest. A constitutively expressed *CLN2* transcript does not suppress the rapamycin-induced cell cycle arrest presumably because it is not translated (see below).

The effects of rapamycin treatment on START-specific transcripts were as follows. The mRNAs for *CLN1*

and *CLN2* (Figure 3 and our unpublished results for *CLN2*) were no longer detectable after 2 h of rapamycin treatment. Surprisingly, the normally constitutively expressed *CLN3* transcript was also reduced with similar kinetics as seen for *CLN1* and *CLN2*, but was not completely eliminated. As determined by densitometry of appropriately exposed autoradiographs and normalization to *ACT1* transcript levels, the *CLN3* mRNA level was maximally reduced by ~60%. The mRNAs for the three additional genes, *HCS26*, *ORFD*, and *CLB5*, which bear limited homology to the *CLN* genes and are also expressed only in late G1 also disappeared upon rapamycin treatment, with kinetics identical to those seen for the *CLN1* and *CLN2* transcripts (Figure 3 for *HCS26* and *ORFD*).

Expression of the *CLN1*, *CLN2*, and *HCS26* genes (and possibly *ORFD*) is under control of the transcription factor SBF, which is composed of the DNA binding moiety SWI4 and its regulatory subunit SWI6 (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). We therefore assessed the levels of *SWI4* and *SWI6* transcripts in rapamycin-treated cells. Normally, the mRNA for SWI6 is constitutively expressed whereas the mRNA for SWI4 oscillates, peaking in late G1 and falling to a low but detectable basal level elsewhere in the cell cycle (Breeden and Mikesell, 1991). Like the *CLN3* transcript, the mRNA for SWI6 was depleted by ~60% (Figure 3). The transcript for SWI4 fell to basal levels 2 h after rapamycin treatment, thus behaving like other START-specific mRNAs.

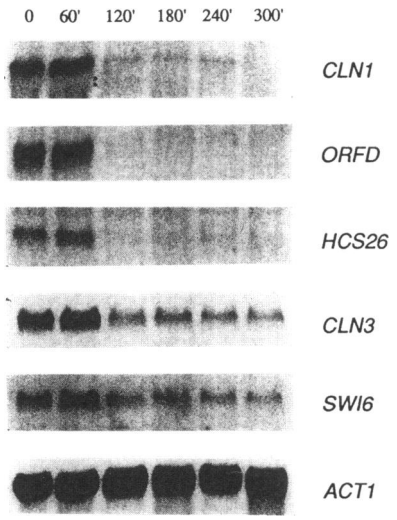


Figure 3. START-specific transcripts are depleted upon rapamycin treatment. Northern blot analysis of RNA isolated from cells (JK9–3da) treated with 0.2 μg/ml rapamycin for 0, 1, 2, 3, 4, and 5 h (indicated in minutes). The mRNAs for *CLN1*, *ORFD*, and *HCS26* are abolished, and mRNAs for *CLN3* and *SWI6* are reduced by ~60% relative to *ACT1* levels. *ACT1* encodes actin and is a control for a message that is not START specific. The level of *ACT1* message is not affected by rapamycin. See text for additional information.

These observations are not due to a global repression of transcription as the transcripts for actin (*ACT1*) and *CDC28* and also the previously identified targets of rapamycin *TOR1* and *TOR2* were not depleted throughout the time course of these experiments (Figure 3 and our unpublished results). Furthermore, some transcripts are actually induced upon rapamycin treatment (see below). As mentioned above, the absence of START-specific transcripts upon rapamycin treatment is presumably an indirect consequence of a cell cycle arrest before START (Hubler *et al.*, 1993). The reduction in the normally constitutive messages could reflect the inherent instability of untranslated (see below) mRNAs.

TOR Is Required for Translation Initiation

Because rapamycin blocks activation of protein synthesis in mammalian cells (Jefferies *et al.*, 1994; Terada *et al.*, 1994) and because inhibition of protein synthesis in yeast causes an early G1 arrest (Hartwell and Unger, 1977; Pringle and Hartwell, 1981; Brenner *et al.*, 1988), we investigated whether rapamycin blocks protein synthesis in yeast by assaying incorporation of [^{35}S]methionine at intervals after addition of rapamycin. We observed an early decrease in incorporation upon rapamycin treatment (Figure 4A). Protein synthesis fell to a low (~10% of normal levels) but detectable level after 120 min, and remained at this low level throughout the course of the experiment. The low level of protein synthesis was greater than that observed in cells treated with cycloheximide (100 μg/ml), which reduced protein synthesis to undetectable levels. Up to 100-fold higher concentrations of rapamycin did not have a more severe effect on incorporation. Protein synthesis was not affected in a rapamycin-resistant *TOR1–1* (JH11–1c) or *TOR2–1* (JH12–17b) mutant, as assayed by [^{35}S]methionine incorporation in the presence of rapamycin. Thus, rapamycin is an effective inhibitor of protein synthesis acting through TOR.

To confirm that TOR is required for protein synthesis, as suggested by the above observation, we examined the effect of TOR depletion on protein synthesis. The *tor*^{ts} strain NB35 was shifted to the nonpermissive temperature and levels of protein synthesis were determined at time intervals after the temperature shift. At the nonpermissive temperature, we observed a progressive decrease in the levels of [^{35}S]methionine incorporation (Figure 4B). Incorporation levels fell to a minimum of ~10% after 6 h of incubation at the nonpermissive temperature. Levels of incorporation in NB35 (*tor*^{ts}) at the permissive temperature were less than those in wild type, indicating that there is a protein synthesis defect in this mutant even at the permissive temperature. Thus, TOR is required for protein synthesis. Furthermore, because an inhibi-

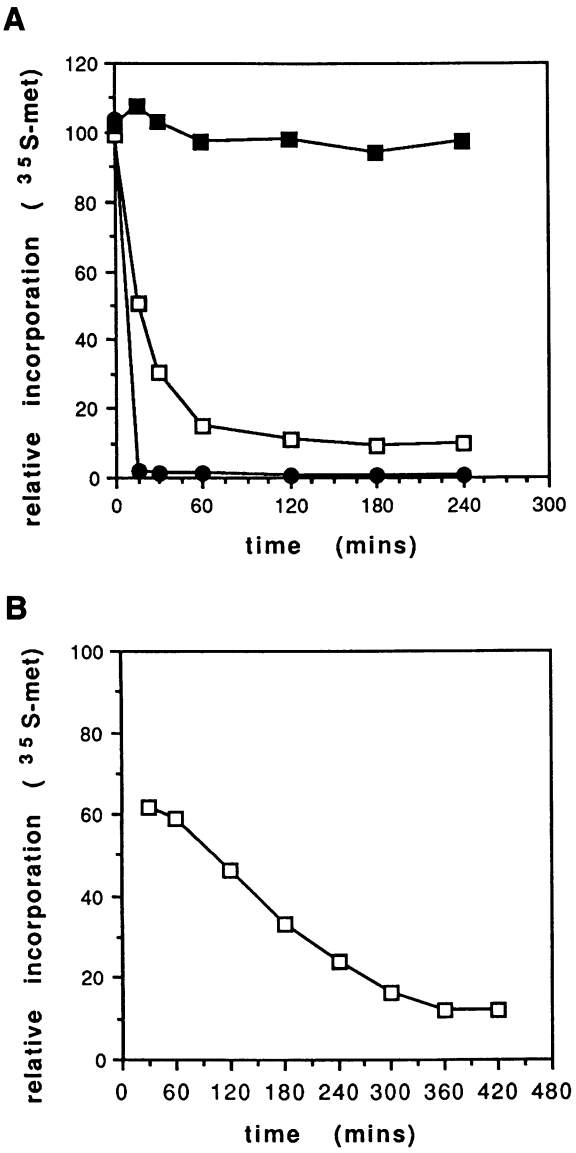


Figure 4. Rapamycin treatment inhibits protein synthesis. (A) Cells were assessed for incorporation of [^{35}S]methionine by labeling for 7 min at intervals (0, 15, 30, 60, 120, 180, and 240 min) after addition of 0.2 μg/ml rapamycin. Cells treated were wild-type JK9–3da (open squares) and rapamycin-resistant *TOR1–1* mutant JH11–1c (closed squares). Also plotted is JK9–3da treated with 100 μg/ml cycloheximide (closed circles). Incorporation (relative incorporation) is plotted as a percentage of the control, wild-type strain JK9–3da treated with drug vehicle alone. (B) Inhibition of protein synthesis upon TOR depletion. Strain NB35 (*tor*^{ts}) was incubated at the restrictive temperature, and samples were removed at the indicated time intervals for determination of [^{35}S]methionine incorporation. Values are plotted as a percentage of [^{35}S]methionine incorporation in NB17–3d at the restrictive temperature. An early time point is not included because a reliable value could not be obtained for either the temperature-sensitive mutant NB35 or NB17–3d immediately after shift to the nonpermissive temperature. Shown (A and B) are representative curves of three or more independent experiments.

tion of protein synthesis causes an early G1 arrest (Hartwell and Unger, 1977; Pringle and Hartwell, 1981; Brenner *et al.*, 1988), the protein synthesis defect may be the cause of the cell cycle arrest; the relatively slow inhibition of incorporation in NB35 (*tor*^{ts}) at the nonpermissive temperature, compared with rapamycin-treated cells at the permissive temperature, is not necessarily inconsistent with the first cycle arrest of NB35 (*tor*^{ts}) because this strain has a translation defect even at the permissive temperature and because cells have a longer cell cycle at the higher temperature.

To determine whether the inhibition of protein synthesis was at the level of initiation or elongation, polysome profiles of wild-type cells treated with rapamycin for 1 and 2 h were analyzed. This experiment was performed in the absence of the translation elongation inhibitor cycloheximide so that a block in elongation, if imposed, could be observed. Such a block is characterized by an accumulation of polysomes. No polysomes were present in either extract, only a single peak corresponding to 80S monosomes and ribosomes (our unpublished results). Rapamycin does not, therefore, cause a translation elongation block; however, a mild defect in the rate of elongation that is not sufficiently stringent to prevent ribosome "run-off" during the time needed to harvest, wash, and lyse cells in preparation for sucrose gradients would not be detected. To investigate whether rapamycin causes a block in translation initiation, wild-type cells were treated with drug vehicle alone or with rapamycin for 1 and 2 h followed by a 10-min treatment with cycloheximide to prevent run-off of any polysomes present (Figure 5, A and B; our unpublished result for 1-h timepoint). Rapamycin treatment caused a progressive decay of polysomes with a coincident increase in the 80S peak, indicating an initiation block. The apparent discrepancy between the observed inhibition of [^{35}S]methionine incorporation (~90%) and the inhibition of polysomes (~60%) after 2 h of rapamycin treatment may reflect a difference in the sensitivities of the two assays or a mild elongation defect in addition to a block in initiation.

We next examined the polysome profiles of TOR-depleted cells using the *tor*^{ts} strain NB35. Again, a severe reduction in the number of polysomes and a coincident increase in the 80S peak were evident after incubation for 5 h at the nonpermissive temperature (Figure 5C). A similar but less pronounced effect was observed after 3 h at the nonpermissive temperature. Thus, TOR is required for translation initiation.

Loss of TOR Causes a Starvation Response, but TOR Is Not Part of the RAS/cAMP Pathway

Starved yeast cells exit the cell cycle (stop dividing) and enter G0. Cells entering G0 are characterized by

several distinct properties (Werner-Washburne *et al.*, 1993) including 1n DNA content, failure to reach START (Pringle and Hartwell, 1981), a reduction in protein synthesis to ~10% of normal levels, down-regulation of *CLN3* message (Hubler *et al.*, 1993), and enlargement of the vacuole (Granot and Snyder, 1991). As described above, rapamycin-treated or TOR-depleted cells display all these characteristics. Additionally, rapamycin-treated or TOR-depleted cells are still alive (metabolically active) despite the observed reduction in protein synthesis; rapamycin-treated cells exclude the vital dye phloxin B even 24 h after treatment, and all temperature-sensitive *tor2* alleles isolated to date are reversible (Barbet and Hall, unpublished data). This led us to consider that rapamycin might be causing a starvation response despite the presence of nutrients, and inducing cells to enter G0. To test this, we examined by Northern analysis the effect of rapamycin on the transcription of genes whose mRNA levels are known to change upon entry into G0. The heat shock genes *SSA3* and *HSP26* and the ubiquitin gene *UBI4* are transcriptionally induced upon entry into G0 (Werner-Washburne *et al.*, 1993). The catalase T gene *CTT1* is also transcriptionally induced upon entry into G0, with enzymatic activity peaking and then declining 3 h after cells enter stationary phase (Werner-Washburne *et al.*, 1993). In contrast, the mRNA level of the heat shock genes *SSA1* and *SSA2* (*SSA1/2*) fluctuates in different ways depending on the starvation regimen but can remain largely unchanged, and transcription of the cold-inducible "heat shock" genes *SSB1* and *SSB2* (*SSB1/2*) is severely repressed upon entry into G0 (Werner-Washburne *et al.*, 1993). As shown in Figure 6A, we observed these same changes in transcription upon rapamycin treatment. The mRNAs for *SSA3*, *HSP26*, and *UBI4* were induced upon rapamycin treatment; maximal induction occurred 2 h after rapamycin addition for *SSA3* and *HSP26*, and after 30 min for *UBI4*. The *CTT1* transcript was also induced upon rapamycin treatment, and transcript levels remained high for 2 h before falling. In contrast, the *SSB1/2* transcripts decreased to almost undetectable levels within 1 h of treatment. The level of *SSA1/2* transcripts fluctuated but remained largely unchanged. Thus, it appears that rapamycin causes a starvation response and induces entry into G0.

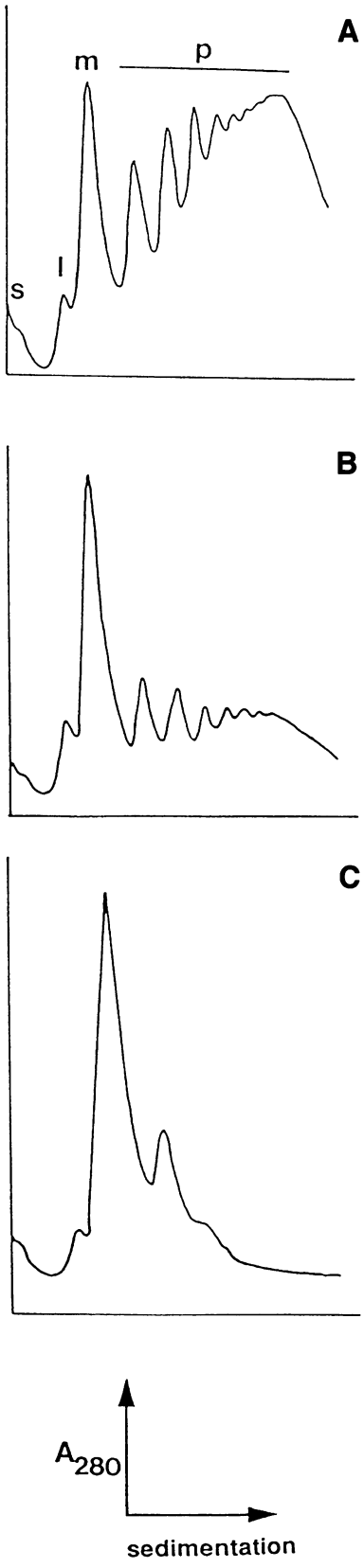
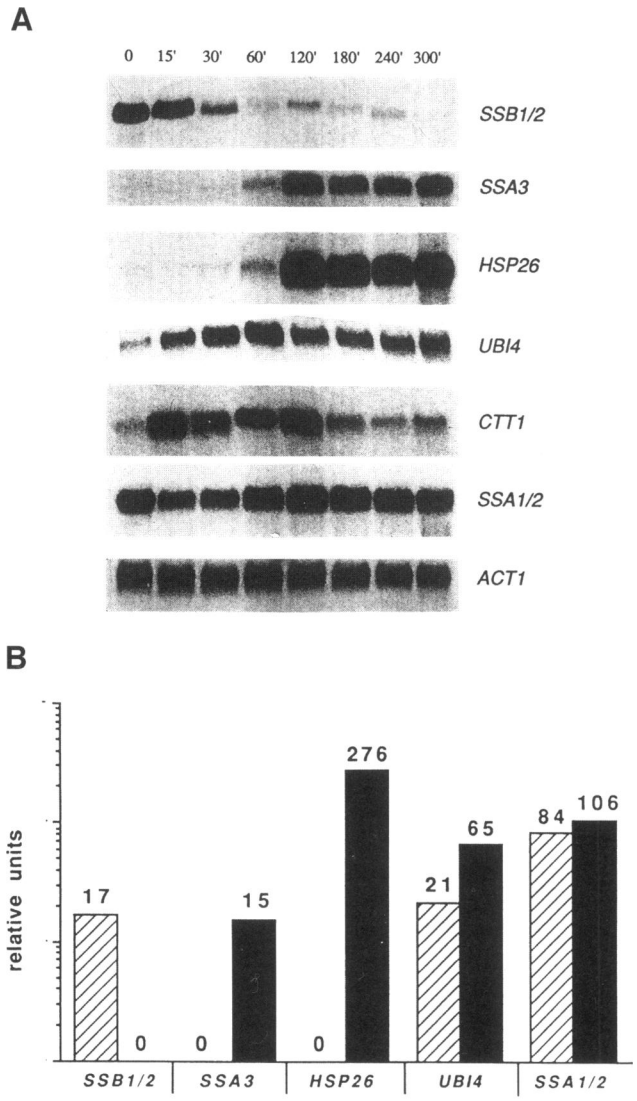


Figure 5. Rapamycin treatment or TOR depletion blocks translation initiation. (A and B) Polysome profiles of wild-type cells (JK9–3da) treated with (A) vehicle alone, and (B) 0.2 μg/ml rapamycin for 2 h. (C) Polysome profile of the *tor*^{ts} strain NB35 after 5 h at the nonpermissive temperature (37°C). In all the above cases, cycloheximide was added 10 min before harvest, to prevent "run off." Wild-type strain JK9–3da grown at the nonpermissive temperature is slightly stimulated for polysome accumulation. The positions of 40S ribosomal subunits (s), 60S ribosomal subunits (l), 80S monosomes (m), and polysomal ribosomes (p) are indicated.



We next examined whether TOR depletion elicits the same starvation-induced changes in transcript levels. We could not utilize the *tor*^{ts} strain for these experiments, because many of the same changes in transcript levels occur normally at high temperature (the nonpermissive temperature of our *tor*^{ts} mutant) independently of starvation. Therefore, we used a strain containing *TOR2* under control of the regulatable *GAL1* promoter and chromosomal disruptions of both *TOR1* and *TOR2* (JK350–21a) to deplete the cells of TOR (Kunz *et al.*, 1993; Helliwell *et al.*, 1994). After shifting from galactose- (SGal/Gly) to glucose-containing (SD) medium (TOR-depletion conditions), we observed changes in the pattern of transcription similar to those seen when wild-type cells are treated with rapamycin (Figure 6B). Thus, TOR depletion also induces a starvation response.

Additional indicators of stationary phase are the accumulation of the storage carbohydrate glycogen and acquisition of thermotolerance. We examined whether cells treated with rapamycin accumulate glycogen. Cultures were treated for 5 h with rapamycin, harvested by filtration at hourly intervals, and stained for glycogen using iodine vapor, which stains glycogen-containing cells dark brown (Chester, 1968). As shown in Figure 6C for the 5-h time point, cells treated with rapamycin did indeed stain darkly when exposed to iodine. Accumulation of glycogen was weakly detectable after 1 h of treatment. Also confirming that loss of TOR function induces a starvation response, we observed that cells depleted for TOR exhibit increased resistance to the killing effects of high temperatures when compared with wild-type cells (our unpublished results).

The RAS/cAMP signal transduction pathway acts in early G1 (before the mating pheromone arrest point) and may be involved in the controlled entry into G0 (Broach, 1991; Thevelein, 1994). To investigate

Figure 6. Rapamycin treatment or TOR depletion induces a starvation response. (A) RNA was isolated from cells (JK9–3da) treated with 0.2 μg/ml rapamycin for 0, 15, 30, 60, 120, 180, 240, and 300 min, and probed by Northern analysis with the indicated genes (see MATERIALS AND METHODS). *SSA1/2* refers to *SSA1* and *SSA2*. *SSB1/2* refers to *SSB1* and *SSB2*. The observed changes in transcript levels are characteristic of cells entering G0. (B) Histogram showing level changes for the indicated transcripts upon depletion of TOR by galactose to glucose shift. Conditions of the galactose to glucose shift were as described (Helliwell *et al.*, 1994). Hatched bars correspond to a wild-type (JK9–3da) strain; solid bars correspond to a TOR-depleted strain (JK350–21a). Transcript levels were normalized to *ACT1* mRNA levels. Transcript level values in relative units are given above each bar. A value of 0 indicates an undetectable mRNA level. (C) Rapamycin treatment causes accumulation of glycogen. (Top left) Rapamycin-resistant strain JH12–17b treated with drug vehicle alone. (Top right) JH12–17b treated for 5 h with 0.2 μg/ml rapamycin. (Bottom left) Wild-type strain JK9–3da treated with drug vehicle alone. (Bottom right) JK9–3da treated for 5 h with 0.2 μg/ml rapamycin. Filters were exposed to iodine vapor for 1 min to stain for glycogen.

whether loss of TOR function induces entry into G0 by inhibiting the RAS/cAMP cascade, we constitutively activated this pathway, and then tested for abrogation of the rapamycin-induced cell cycle arrest. Two methods were used to constitutively activate the pathway. First, we disrupted the *BCY1* gene (Toda *et al.*, 1987). A *BCY1* disruption activates the RAS/cAMP pathway by eliminating the negative regulatory subunit of the cAMP-dependent protein kinase A (Cannon and Tatchell, 1987; Toda *et al.*, 1987). Second, we introduced the dominant, activated *RAS2* allele $RAS2^{val19}$. The $RAS2^{val19}$ mutation hyperactivates the RAS/cAMP pathway by maintaining RAS2 in its active, GTP-bound state (Kataoka *et al.*, 1984). Both *bcy1* (NB30) and $RAS2^{val19}$ (NB34) cells were as sensitive as wild-type cells to rapamycin, based upon growth arrest in the presence of drug. Flow cytometry on these strains indicated that greater than 85% of the cells arrested with a 1n DNA content after 3 h of rapamycin treatment, as observed with wild-type cells (see Figure 1 for wild-type cells). Rapamycin-treated *bcy1* and $RAS2^{val19}$ cells also accumulated glycogen, as determined by iodine staining. Therefore, activation of the RAS/cAMP pathway does not abrogate the rapamycin-induced cell cycle arrest, indicating that TOR is not part of the RAS/cAMP pathway.

Our data do not rule out the possibility that TOR lies in the RAS/cAMP pathway downstream of BCY1, but we consider this very unlikely. First, subcellular localization studies (Kunz, Stevenson, Schneider, and Hall, unpublished data) and their homology to lipid kinases indicate that the TORs are membrane-associated proteins, whereas BCY1 is a membrane-distal component of the RAS/cAMP pathway. Second, diploid cells lacking TOR function arrest in G1 (2n DNA content) but do not sporulate, whereas diploids compromised in the RAS/cAMP pathway do sporulate. Third, activation of p70 S6 kinase, a presumed downstream component of TOR in mammalian cells, is independent of $p21^{ras}$ (Downward, 1994; Ming *et al.*, 1994). Fourth, there is no example of, or need for, a lipid kinase in a signaling pathway that utilizes cAMP as a second messenger; the lipid kinases mediate production of the fundamentally different, phosphatidylinositol-derived second messengers. Thus, TOR1 and TOR2 appear to define a novel nutrient-related process mediating progression through early G1. This would be in agreement with the observations of Cameron *et al.* (1988), who described mutants that express low-level, constitutive cAMP-dependent protein kinase A activity but that still respond appropriately to nutrient conditions, even in the absence of essential upstream components of the RAS/cAMP pathway.

Expression of CLN3 under Altered Translational Control Confers TOR-independent G1 Progression

The finding that loss of TOR function causes an early reduction in protein synthesis and a G1 arrest within one generation suggested that TOR might be controlling translation of an unstable protein(s) required for G1 progression. Good candidates for such proteins were the G1 cyclins, as these proteins are unstable and limiting for G1 progression (Cross, 1988; Nash *et al.*, 1988; Hubler *et al.*, 1993; Tyers *et al.*, 1993). To test whether cells lacking TOR function arrest in early G1 (G0) because they do not synthesize G1 cyclins, we devised a situation in which one of these, CLN3, would be synthesized upon rapamycin treatment, and asked whether this would be sufficient to drive rapamycin-treated cells through G1. CLN3 was chosen because the transcript for this cyclin is normally present under conditions of rapamycin treatment (Figure 3). We fused the *CLN3* open reading frame to the 5′ region (untranslated leader and promoter) of the *UBI4* gene. The *UBI4* 5′ region was chosen because it is both transcriptionally and translationally active in G0 and would therefore express CLN3 upon rapamycin treatment (Finley *et al.*, 1987; Brenner *et al.*, 1988; Werner-Washburne *et al.*, 1993) (Figure 6A). We then examined whether the *UBI4-CLN3* fusion suppresses the rapamycin-induced cell cycle arrest.

An asynchronously growing wild-type yeast strain containing the *UBI4-CLN3* fusion on a centromeric plasmid (NB36) was treated with rapamycin, and at hourly intervals the DNA content of the cells was analyzed by flow cytometry. Like a control strain (NB37) containing a plasmid-borne *UBI4* 5′ region without the *CLN3* open reading frame, NB36 cells arrested growth after approximately 2 h of rapamycin treatment. This was expected because rapamycin causes a general inhibition of protein synthesis (Figure 4), and TOR has an essential non-cell cycle function in addition to its essential role in G1 (Kunz *et al.*, 1993). Analysis of DNA content of the arrested cells, however, indicated that NB36 (*UBI4-CLN3*) arrested throughout the cell cycle, whereas the control strain arrested in G1 (Figure 7). Thus, cells containing the *UBI4-CLN3* fusion no longer arrest in G1 upon rapamycin treatment.

Northern analysis of the strain (NB36) containing the *UBI4-CLN3* fusion indicated that it produces approximately 20-fold more *CLN3* mRNA upon rapamycin treatment than an isogenic strain lacking the fusion. To determine whether the suppression of the cell cycle arrest in strain NB36 was due to altered control of CLN3 translation or merely to the increased dosage of the *CLN3* transcript, we examined whether cells containing the wild-type *CLN3* gene on a high-copy-number plasmid (NB38) also arrested outside of G1 upon rapamycin treatment. After 2 h of treatment,

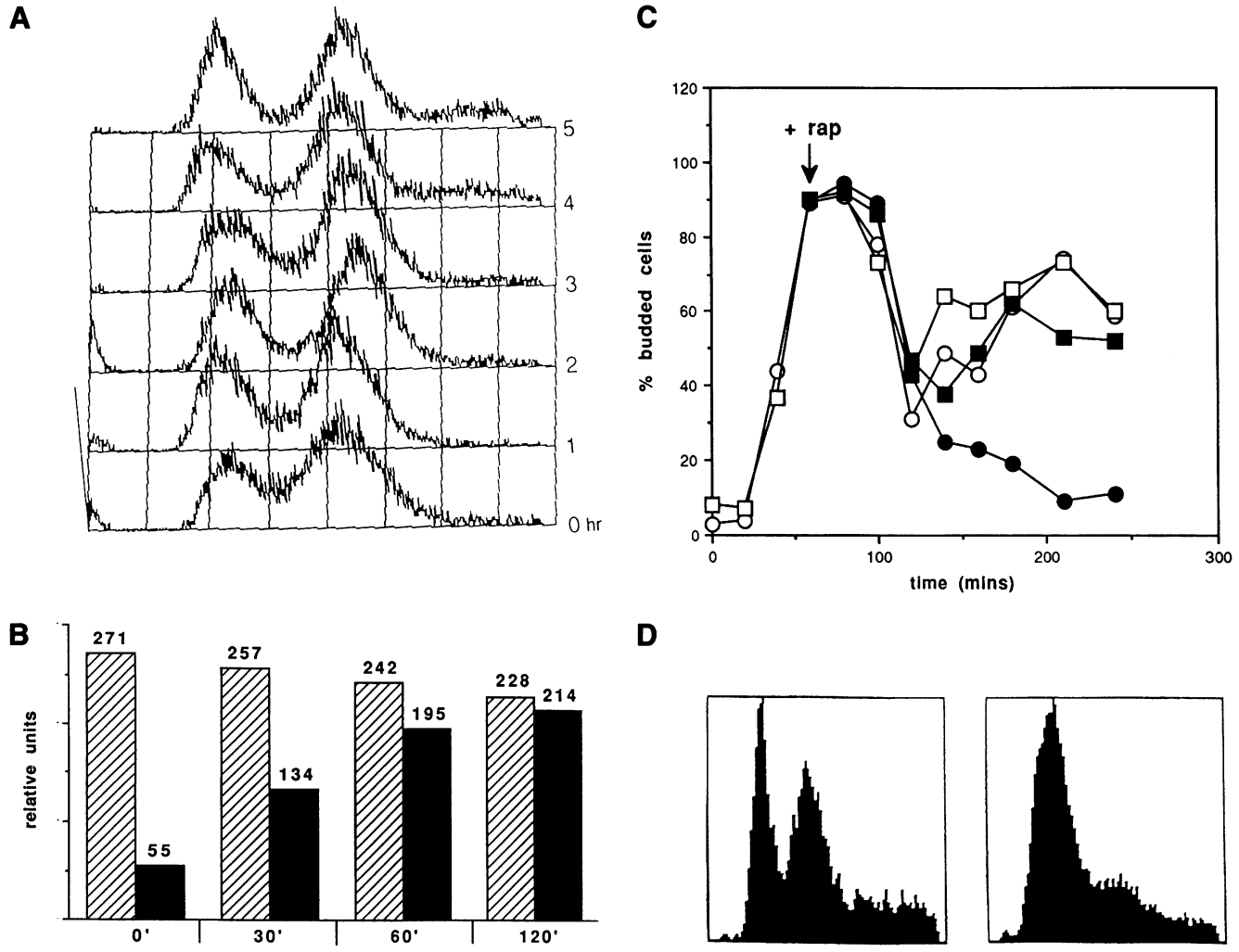


Figure 7. Altered translational control of CLN3 suppresses the cell cycle–specific arrest of rapamycin-treated cells. (A) Exponentially growing cells containing the *UBI4-CLN3* fusion (NB36) were treated with 0.2 μg/ml rapamycin and sampled for flow cytometry at hourly intervals up to 5 h. Rapamycin-treated NB36 arrested throughout the cell cycle, as indicated by a roughly even distribution of cells with a 1n and 2n DNA content. Results of flow cytometry on control strain NB37 treated with rapamycin were indistinguishable from the results in Figure 1D. (B) Northern analysis of strains treated with rapamycin and assessed for levels of *CLN3* transcript. Shown are the levels of *CLN3* transcript in strain NB38 (hatched bars) containing the wild-type *CLN3* gene in a high copy number plasmid and strain NB36 (solid bars) containing the *UBI4-CLN3* fusion, at the indicated times (in minutes) following rapamycin treatment. All values were normalized to the levels of actin transcript. At 120 min following treatment, cells had arrested growth. (C and D) A *UBI4-CLN3* strain treated with rapamycin is able to traverse G1. (C) Percentage of budded cells of rapamycin-treated (closed squares) or -untreated (open squares) NB36 (*UBI4-CLN3*) compared with rapamycin-treated (closed circles) or -untreated (open circles) control strain NB37. Percentage of budded cells was determined at 20-min intervals following release from the α-factor block at START. Rapamycin (rap) was added 60 min following release from α-factor. (D) Flow cytometry of NB36 (*UBI4-CLN3*) and NB37 control cells released from an α-factor block at time 0 and treated with rapamycin 60 min following release from α-factor. Shown is the DNA content of the rapamycin-treated cells at the end of the experiment (240 min). The rapamycin-treated cells arrested growth approximately 150 min after rapamycin addition. The left panel shows the DNA content for strain NB36, the right panel shows the DNA content for NB37 (G1 arrest). DNA content of untreated cells at 240 min was indistinguishable from that shown in the left panel. Data shown is representative of three independently performed experiments.

NB38 cells arrested growth, with ~85% of cells containing a 1n DNA content. Northern analysis of the rapamycin-treated NB36 (*UBI4-CLN3*) and NB38 (high copy *CLN3*) cells indicated that the level of CLN3 transcripts in NB38 was greater than that in NB36 (Figure 7B). In addition, high level overexpression of *CLN3* from the inducible *GAL1* promoter was also unable to overcome a rapamycin-induced G1 arrest, and plasmid-borne *UBI4-CLN3* still caused a random arrest despite disruption of the chromosomal copy of

CLN3 (our unpublished results). Furthermore, an integrated copy of the *CLN3–1* allele (strain NB33), which bears a mutation that stabilizes the CLN3 protein but does not otherwise affect its cyclin function (Cross, 1988; Nash *et al.*, 1988), had the same effect as *UBI4-CLN3* in causing a random arrest upon rapamycin treatment. This confirms that the *UBI4-CLN3* fusion does not promote G1 progression simply because of an elevated level of *CLN3* transcripts.

To determine more directly whether rapamycin-treated cells containing the *UBI4-CLN3* fusion are able to traverse the G1 phase of the cell cycle, we examined the effect of rapamycin on synchronized cells. Strain NB36 (*UBI4-CLN3*) and the control strain NB37 were synchronized at START by addition of α-factor. Following release from the pheromone block, the cultures were split into two and rapamycin was added to one half, the remaining halves receiving drug vehicle alone. As shown in Figure 7C, NB37 control cells treated with rapamycin entered G1 and arrested as unbudded cells. Rapamycin-treated NB36 (*UBI4-CLN3*), however, entered G1 but then began to produce new buds before arresting growth, indicating that cells were traversing G1 and beginning a new cycle. Analysis of the arrested cells by flow cytometry confirmed that the NB36 (*UBI4-CLN3*) cells had traversed G1 whereas the NB37 control cells had not (Figure 7D). Thus, *UBI4* leader-dependent expression of CLN3 causes rapamycin-treated cells to traverse G1.

Expression of UBI4-CLN3 Confers Starvation Sensitivity

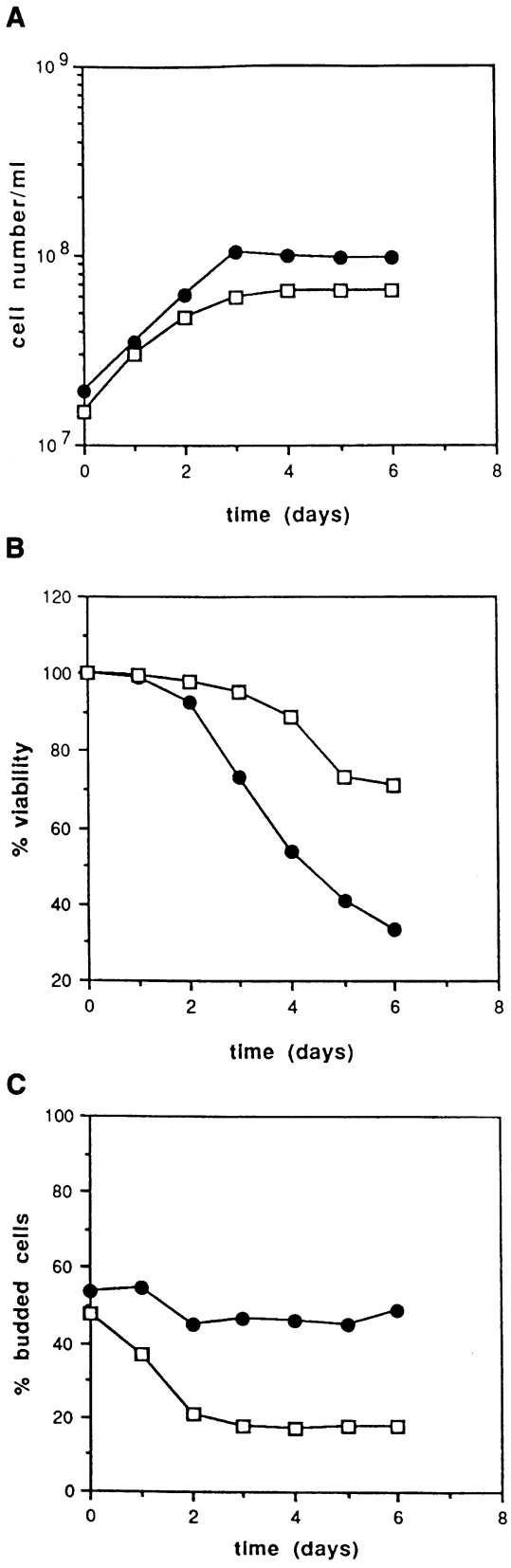
The finding that TOR may normally modulate synthesis of CLN3 (among other proteins) as part of a starvation response suggested that cells containing the *UBI4-CLN3* fusion might be sensitive to starvation. To test this suggestion, *UBI4-CLN3* strain NB36 and control strain NB37 were grown to stationary phase (starved) and samples were removed daily for assessment of cell viability and the percentage of budded cells. As NB36 (*UBI4-CLN3*) cells entered stationary phase (cell number no longer increased) (Figure 8A), their ability to form colonies on rich medium rapidly decreased (Figure 8B). In contrast, starved NB37 control cells retained high viability for the duration of the experiment. The starvation sensitivity of NB36 (*UBI4-CLN3*) was most likely due to this strain's inability to arrest in G1 (G0), as suggested by the observations that it stopped dividing at a higher cell density (~1.5-fold) than the control strain (Figure 8A) and with a high percentage of budded cells (Figure 8C). Strain NB38 containing the wild-type *CLN3* gene in high dosage behaved in this experiment like control strain NB37. These findings support the involvement of TOR in nutrient sensing, and also confirm that modulating the level of translation is part of the regulated entry into stationary phase (G0).

DISCUSSION

We have shown that loss of TOR function (rapamycin treatment or TOR depletion) causes yeast cells to arrest in early G1 and to exhibit, by all criteria examined, characteristics of starved cells entering stationary phase, or G0. We have also demonstrated that loss of TOR function causes a general inhibition of translation initiation. Providing the transcript for the G1 cyclin CLN3 under the translational control of the *UBI4* 5′ region suppresses the rapamycin-induced G1 arrest and confers starvation sensitivity. These results suggest the following model for the role of TOR in cell cycle control (Figure 9). In response to nutrient availability, TOR stimulates general translation initiation, including translation of G1-regulatory transcripts such as those for CLN3 and other G1 cyclins. This then drives cells through G1 and into S phase. In the converse situation, the absence of nutrients causes inactivation of TOR, which leads to loss of translation and a subsequent early G1 arrest and entry into G0. It is important to emphasize that TOR is required for general translation and that the role of TOR in cell cycle control, as proposed here, is just part of a greater role in general growth control. We would also like to stress that, although it accounts for all our data, the model is largely speculative and is intended only as a framework to bring together our and other findings.

Several lines of evidence suggest that TOR is part of a signaling pathway. First, the TORs are homologous to PI kinases, enzymes implicated in signaling. Second, because loss of TOR rapidly causes a starvation response, TOR is likely involved in sensing and relaying the availability of nutrients. Indeed, constitutively activating the proposed pathway by providing CLN3 independently of upstream components (*CLN3* under the translational control of the *UBI4* untranslated leader) causes starvation sensitivity. Third, the mammalian counterpart of TOR (FRAP/RAFT1/RAPT1/mTOR) appears to mediate an intermediate step in a defined, rapamycin-sensitive signal transduction pathway required for cell proliferation (Brown *et al.*, 1994; Chiu *et al.*, 1994; Downward, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). The putative TOR pathway is novel because it acts in early G1, and TOR is not part of the RAS/cAMP pathway.

The observed inhibition of translation initiation is likely a direct consequence of loss of TOR function and the cause (rather than an effect) of the cell cycle arrest, for the following reasons. First, the reduction in translation is the earliest effect observed upon loss of TOR function. Second, a specific block in translation initiation, either by mutation of an initiation factor or by treatment with a low concentration of cyclo-



heximide, causes yeast cells to arrest in early G1 (Hartwell and Unger, 1977; Johnston *et al.*, 1977; Pringle and Hartwell, 1981; Hanic-Joyce *et al.*, 1987; Brenner *et al.*, 1988; Hubler *et al.*, 1993; Barnes *et al.*, 1995). Third, and most important, allowing translation initiation of an appropriate, cell cycle–controlling transcript is sufficient to suppress the rapamycin-induced G1 arrest. Fourth, TOR in mammalian cells probably activates translation initiation and G1 progression in response to mitogens (Downward, 1994; see INTRODUCTION). Thus, the TOR pathway in yeast appears to control translation initiation and, thereby, early G1 progression.

The observation that phosphorylation of the yeast equivalent of S6 (S10) is not important for growth (Zinker and Warner, 1976; Kruse *et al.*, 1985; Johnson and Warner, 1987) suggests that TOR is not regulating translation initiation in yeast through S6 (see INTRODUCTION). One alternative possibility is that the TOR pathway controls translation initiation through the initiation factor eIF-4E (or an associated subunit). eIF-4E is the cap-binding subunit of the eIF-4F complex, which also contains eIF-4A, an RNA helicase, and eIF-4γ, a protein of unknown function (Rhoads, 1988; Lanker *et al.*, 1992; Linder, 1992; Goyer *et al.*, 1993; Redpath and Proud, 1994). eIF-4F binds to the 5′ cap structure of mRNA and promotes unwinding of 5′ secondary structure, facilitating binding of the 43S ribosomal preinitiation complex to the mRNA. Several observations suggest that TOR could control eIF-4E. First, analyses of *CDC33* (encodes eIF-4E) and *TOR* mutants indicate that eIF-4E and TOR have remarkably similar roles. Both have essential functions required for general translation initiation (Altmann *et al.*, 1989; Kunz *et al.*, 1993; see RESULTS). Furthermore, both have an early G1-specific function and an essential function that is not G1 specific (Johnston *et al.*, 1977; Pringle and Hartwell, 1981; Brenner *et al.*, 1988; Kunz *et al.*, 1993); protein synthesis is required at several points in the cell cycle but is most limiting in G1 (Burke and Church, 1991). Second, in mammalian cells, eIF-4E is the rate-limiting protein in translation (Duncan *et al.*, 1987) and a target for regulation. Growth factors activate protein synthesis by triggering the phosphorylation and release of the eIF-4E-

Figure 8. The *UBI4-CLN3* fusion confers starvation sensitivity and an inability to arrest in G0. (A) Growth curve of NB36 cells (closed circles) expressing *UBI4-CLN3* and NB37 cells (open squares) expressing the *UBI4* 5′ region alone. (B) Viability curve of NB36 (closed circles) and NB37 (open squares) strains. Cells reached stationary phase after 3 days of growth. Strains were grown in SD medium minus leucine for the indicated times. Viability was assessed by plating 10^3 cells on YPD medium and counting colony-forming units. (C) Percentage of budded cells in cultures of NB36 (closed circles) and NB37 (open squares).

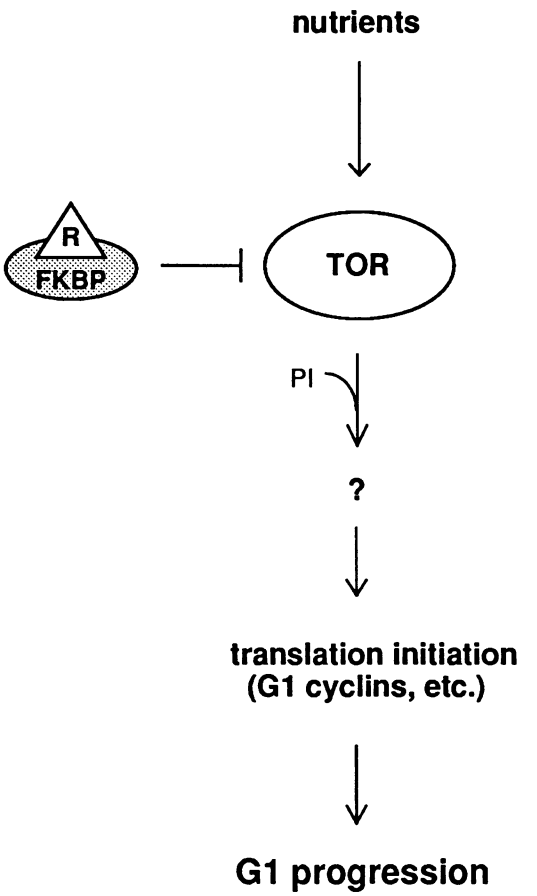


Figure 9. Model of the TOR pathway in cell cycle control. Rapamycin (R) forms a complex with FKBP to inhibit TOR (Heitman *et al.*, 1993; Kunz *et al.*, 1993). TOR is TOR1 and TOR2. PI is phosphatidylinositol. See DISCUSSION for further details. Because TOR is required for general translation (see RESULTS), the role of TOR in cell cycle control is just part of a greater role in general growth control; the model proposed here focuses exclusively on that part of TOR's role in general growth control that affects progression through the G1 phase of the cell cycle.

inhibiting factor 4E-BP1/PHAS-I (Haystead *et al.*, 1994; Hu *et al.*, 1994; Lin *et al.*, 1994; Pause *et al.*, 1994). Importantly, rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation (Beretta *et al.*, 1996). Third, in proliferating yeast and mammalian cells, eIF-4E and an associated subunit are phosphorylated and therefore potentially subject to regulation by this type of modification (Duncan *et al.*, 1987; Joshi-Barve *et al.*, 1990; Morley *et al.*, 1991; Rhoads *et al.*, 1993; Redpath and Proud, 1994; Zanchin *et al.*, 1994). Fourth, translation of UBI4 appears to have, at least, reduced dependence on eIF-4E (Brenner *et al.*, 1988). Thus, the block in translation initiation caused by loss of TOR function may be due to a down regulation of eIF-4E.

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Target of Rapamycin (TOR) in Nutrient Signaling and Growth Control

Robbie Loewith*,[1] and Michael N. Hall†,[1]
*Department of Molecular Biology and National Centers of Competence in Research and Frontiers in Genetics and Chemical Biology, University of Geneva, Geneva, CH-1211, Switzerland, and †Biozentrum, University of Basel, Basel CH-4056, Switzerland

ABSTRACT TOR (**T**arget **O**f **R**apamycin) is a highly conserved protein kinase that is important in both fundamental and clinical biology. In fundamental biology, TOR is a nutrient-sensitive, central controller of cell growth and aging. In clinical biology, TOR is implicated in many diseases and is the target of the drug rapamycin used in three different therapeutic areas. The yeast *Saccharomyces cerevisiae* has played a prominent role in both the discovery of TOR and the elucidation of its function. Here we review the TOR signaling network in *S. cerevisiae*.

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Available freely online through the author-supported open access option.
[1]Corresponding authors: Biozentrum, University of Basel, Klingelbergstrasse 70, Basel CH-4056, Switzerland. E-mail: m.hall@unibas.ch; and Department of Molecular Biology, NCCRs Chemical Biology and Frontiers in Genetics, University of Geneva, 30 quai Ernest Ansermet Geneva CH-1211, Switzerland. E-mail: Robbie.Loewith@unige.ch

THE contributors to this GENETICS set of reviews were asked to focus on the developments in their field since 1991, the year the last yeast monographs were published. Coincidentally, **T**arget **O**f **R**apamycin (TOR) was discovered in 1991. We thus have the whole TOR story to tell, from the beginning, in a review that marks the 20th anniversary of TOR. As we review TOR signaling in *Saccharomyces cerevisiae*, the reader is referred to other reviews for descriptions of TOR in other organisms (Wullschleger *et al.* 2006; Polak and Hall 2009; Soulard *et al.* 2009; Caron *et al.* 2010; Kim and Guan 2011; Zoncu *et al.* 2011).

The story of the TOR-signaling network begins with a remarkable drug, rapamycin (Abraham and Wiederrecht 1996; Benjamin *et al*. 2011). Rapamycin is a lipophilic macrolide and a natural secondary metabolite produced by *Streptomyces hygroscopicus*, a bacterium isolated from a soil sample collected in Rapa-Nui (Easter Island) in 1965—hence the name rapamycin. Rapamycin was originally purified in the early 1970s as an antifungal agent. Although it effectively inhibits fungi, it was discarded as an antifungal agent because of its then undesirable immunosuppressive side effects. Years later, it was "rediscovered" as a T-cell inhibitor and as an immunosuppressant for the treatment of allograft rejection. Preclinical studies subsequently showed that rapamycin and its derivatives, CCI-779 (Wyeth-Ayerst) and RAD001 (Novartis), also strongly inhibit the proliferation of tumor cells. Rapamycin received clinical approval in 1999 for use in the prevention of organ rejection in kidney transplant patients, and additional applications as an immunosuppressive agent have since been developed. CCI-779 (Torisel) and RAD001 (Afinitor) were approved in 2007 and 2009, respectively, for treatment of advanced kidney cancer. Rapamycin is effective against tumors because it blocks the growth of tumor cells directly and because of the indirect effect of preventing the growth of new blood vessels (angiogenesis) that supply oxygen and nutrients to the tumor cells (Guba *et al.* 2002). Finally, rapamycin-eluting stents prevent restenosis after angioplasty. Thus, rapamycin has clinical applications in three major therapeutic areas: organ transplantation, cancer, and coronary artery disease. What do fungi and the seemingly very different conditions of transplant rejection, cancer, and restenosis have in common in their underlying biology such that all can be treated with the same drug? All three conditions (and the spread of pathogenic fungi) are due to ectopic or otherwise undesirable cell growth, suggesting that the molecular target of rapamycin is a central controller of cell growth. TOR is indeed dedicated to controlling cell growth, but what is this target and how does it control cell growth?

The Early Days

Studies to identify the cellular target of rapamycin and to elucidate the drug's mode of action were initiated in the late 1980s by several groups working with yeast (Heitman *et al.* 1991a; Cafferkey *et al.* 1993; Kunz *et al.* 1993) and mammalian cells (Brown *et al.* 1994; Chiu *et al.* 1994; Sabatini *et al.* 1994; Sabers *et al.* 1995). At that time, rapamycin was known to inhibit the vertebrate immune system by blocking a signaling pathway in helper T cells that mediates cell cycle (G1) progression in response to the lymphokine IL-2. However, the molecular mode of action of the drug was not known other than it possibly involved binding and inhibiting the cytosolic peptidyl-prolyl *cis-trans* isomerase FKBP12 (FK506-binding protein 12), also known as an immunophilin (Schreiber 1991). Furthermore, the observation that rapamycin inhibited cell cycle progression in yeast as in mammalian cells suggested that the molecular target was conserved from yeast to vertebrates and that yeast cells could thus be exploited to identify the target of rapamycin (Heitman *et al.* 1991a). It should be noted that the early

researchers were interested not only in understanding rapamycin's mechanism of action, but also in using rapamycin as a probe to identify novel proliferation-controlling signaling pathways (Kunz and Hall 1993). In the late 1980s, significantly less was known about signaling pathways than today; indeed, few and only incomplete pathways were known.

The early studies in yeast first focused on identifying an FKBP (FK506-binding protein) (Heitman *et al.* 1991b; Koltin *et al.* 1991; Tanida *et al.* 1991; Wiederrecht *et al.* 1991). FKBP12 had previously been identified in mammalian cell extracts as a rapamycin (and FK506)-binding protein. Yeast FKBP was purified to homogeneity using an FK506 column and partially sequenced. The protein sequence information was used to design degenerate oligonucleotides that were then used to isolate the FKBP-encoding gene *FPR1* (Heitman *et al.* 1991b). The predicted amino acid sequence of yeast Fpr1 was 54% identical to that of the concurrently characterized human FKBP12, providing further support that the mode of action of rapamycin was conserved from yeast to humans. Curiously, disruption of the FKBP gene in yeast (*FPR1*) revealed that FKBP is not essential for growth (Heitman *et al.* 1991b; Koltin *et al.* 1991; Tanida *et al.* 1991; Wiederrecht *et al.* 1991). Additional FKBPs and cyclophilins (also an immunophilin and proline isomerase) were subsequently discovered and cloned, and again single and multiple disruptions were constructed without consequential loss of viability (Heitman *et al.* 1991b, 1992; Davis *et al.* 1992; Kunz and Hall 1993; Dolinski *et al.* 1997). The finding that *FPR1* disruption did not affect viability was paradoxical because FKBP was believed to be the *in vivo* binding protein/target for the toxic effect of rapamycin. Why did inhibition of FKBP by rapamycin block growth whereas inhibition of FKBP by disruption of the *FPR1* gene have no effect on growth? The subsequent finding that an *FPR1* disruption confers rapamycin resistance (Heitman *et al.* 1991a,b), combined with the observation that some drug analogs are not immunosuppressive despite being able to bind and inhibit FKBP12 proline isomerase (Schreiber 1991), provided the answer to the above question and led to the well-established model of immunosuppressive drug action: an immunophilin-drug complex (*e.g.*, FKBP-rapamycin) gains a new toxic activity that acts on another target. In other words, FKBP is only a cofactor or receptor required by the drug to act on something else; FKBP itself is not the target required for viability. This mode of drug action also applies to the well-known immunosuppressants cyclosporin A and FK506 (from cyclophilin–cyclosporin A and FKBP–FK506 complexes) and is conserved from yeast to humans (Schreiber 1991). These early studies in yeast were the first of many that have since contributed to an understanding of rapamycin action and TOR signaling even in mammalian cells (Crespo and Hall 2002), illustrating that a model organism such as yeast is valuable in both basic and biomedical research.

To identify the target of the FKBP–rapamycin complex, rapamycin-resistant yeast mutants were selected (Heitman *et al.* 1991a; Cafferkey *et al.* 1993). As expected, *fpr1* mutants defective in FKBP were recovered, but also obtained were mutants altered in either one of two novel genes termed *TOR1* and *TOR2*. The *fpr1* mutations were common and recessive. Interestingly, the *TOR1* and *TOR2* mutations were rare and dominant. The *TOR1* and *TOR2* genes were cloned, on the basis of the dominant rapamycin-resistance phenotype of the mutant alleles, and sequenced (Cafferkey *et al.* 1993; Kunz *et al.* 1993; Helliwell *et al.* 1994). Both TOR1 and TOR2 proteins are 282 kDa in size (2470 and 2474 amino acids, respectively) and 67% identical. TOR1 and TOR2 are also the founding members of the PI kinase-related protein kinase (PIKK) family of atypical Ser/Thr-specific kinases (other members include TEL1, ATM, DNA-PK, and MEC1) (Keith and Schreiber 1995). Although the catalytic domain of all members of this protein kinase family resembles the catalytic domain of lipid kinases (PI3K and PI4K), no PIKK family member has lipid kinase activity, and the significance of the resemblance to lipid kinases is unknown. Two reports in 1995—before TOR was shown to be a protein kinase—claimed that yeast and mammalian TOR had lipid kinase (PI4K) activity, but these findings were never confirmed and are now thought to have been due to a contaminating PI4K. Disruption of *TOR1* and *TOR2* in combination caused a growth arrest similar to that caused by rapamycin treatment, suggesting that TOR1 and TOR2 are indeed the targets of FKBP–rapamycin and that the FKBP–rapamycin complex inhibits TOR activity (Kunz *et al.* 1993). It was subsequently demonstrated that the FKBP–rapamycin complex binds directly to TOR1 and TOR2 (Stan *et al.* 1994; Lorenz and Heitman 1995; Zheng *et al.* 1995) and that TOR is widely conserved both structurally and as the target of FKBP–rapamycin (Schmelzle and Hall 2000). However, *S. cerevisiae* is unusual in having two *TOR* genes whereas almost all other eukaryotes, including plants, worms, flies, and mammals, have a single *TOR* gene. As described below, this additional complexity in *S. cerevisiae* helped the analysis of TOR signaling because it allowed differentiating two functionally different signaling branches on the basis of different requirements for the two TORs.

It should be noted that there is no evidence to indicate that FKBP has a role in normal TOR signaling, *i.e.*, in the absence of rapamycin. Rapamycin hijacks or corrupts FKBP to interact with TOR. In addition, some have speculated that rapamycin mimics an endogenous metabolite that normally regulates TOR with or without FKBP. Although this would provide an explanation for the evolution of the mechanism of action of rapamycin, no evidence has been reported for an endogenous rapamycin-like compound or for such a mode of TOR regulation.

All TORs have a similar domain structure (Figure 1A). The domains found in TOR—in order from the N to the C terminus of TOR—compose the so-called HEAT repeats, the FAT domain, the FRB domain, the kinase domain, and the FATC domain (Schmelzle *et al.* 2002). The HEAT repeats (originally found in **h**untingtin, **e**longation factor 3, the **A** subunit of PP2A, and **T**OR1) consist of ~20 HEAT motifs,

each of which is ~40 residues that form a pair of interacting antiparallel α-helices (Andrade and Bork 1995; Perry and Kleckner 2003). The HEAT repeats occupy the N-terminal half of TOR and are the binding region for subunits of the TOR complexes (Wullschleger *et al.* 2005) (see below). The central FAT domain (~500 residues) and the extreme C-terminal FATC domain (~35 residues), flanking the FRB and kinase domains, are always paired and found in all PIKK family members (Alarcon *et al.* 1999; Bosotti *et al.* 2000; Dames *et al.* 2005). The FRB domain (~100 residues) is the FKBP–rapamycin-binding region. All rapamycin resistance-conferring *TOR* mutations fall within the FRB domain, thereby directly preventing the binding of FKBP–rapamycin without otherwise affecting TOR activity (Heitman *et al.* 1991a; Cafferkey *et al.* 1993; Helliwell *et al.* 1994; Stan *et al.* 1994; Chen *et al.* 1995; Lorenz and Heitman 1995; Choi *et al.* 1996). Interestingly, all the original rapamycin-resistance conferring mutations in *TOR1* and *TOR2* are missense mutations confined to a single, equivalent codon encoding a critical serine residue (Ser1972Arg or Ser1972Asn in TOR1 and Ser1975Ile in TOR2) (Cafferkey *et al.* 1993; Helliwell *et al.* 1994), which explains why the rapamycin-resistance *TOR* mutations were rare. Recreating an equivalent mutation (Ser2035Ile) in mammalian TOR (mTOR) was instrumental in demonstrating that mTOR is the target of FKBP–rapamycin in mammalian cells (Brown *et al.* 1995). Thus, the early rapamycin-resistant yeast mutants turned out to be very informative. They not only identified TOR, but also identified the FKBP–rapamycin-binding site in TOR and contributed to elucidating the mechanism of action of rapamycin. The kinase domain is the catalytic domain and resembles the kinase domain of PI3K and PI4K lipid kinases. Despite high interest in a structure of the kinase domain, no such structure exists for any TOR, which is likely due to technical difficulties in expressing this domain for structural studies. In the absence of a true model, a homology model based on the crystal structure of related PI3K has been elaborated (Sturgill and Hall 2009). A number of groups have identified activating, missense mutations in *S. cerevisiae* and *Schizosaccharomyces pombe* TORs (Reinke *et al.* 2006; Urano *et al.* 2007; Ohne *et al.* 2008). These mutations fall within the FAT, FRB, and kinase domains, and, interestingly, one of the hotspots in the kinase domain corresponds to a region for oncogenic mutations in PI3K (Sturgill and Hall 2009; Hardt *et al.* 2011).

In the mid-1990s, research in the TOR field focused on elucidating the cellular roles of TOR1 and TOR2. It was found that TOR1 and TOR2 play a central role in controlling cell growth as part of two separate signaling branches. Although structurally similar, TOR1 and TOR2 are not functionally identical (Kunz *et al.* 1993; Helliwell *et al.* 1994). Combined disruption of *TOR1* and *TOR2*, or rapamycin treatment, mimics nutrient deprivation including causing a G0 growth arrest within one generation (Barbet *et al.* 1996). Disruption of *TOR1* alone has little-to-no effect, and disruption of *TOR2* alone causes cells to arrest growth

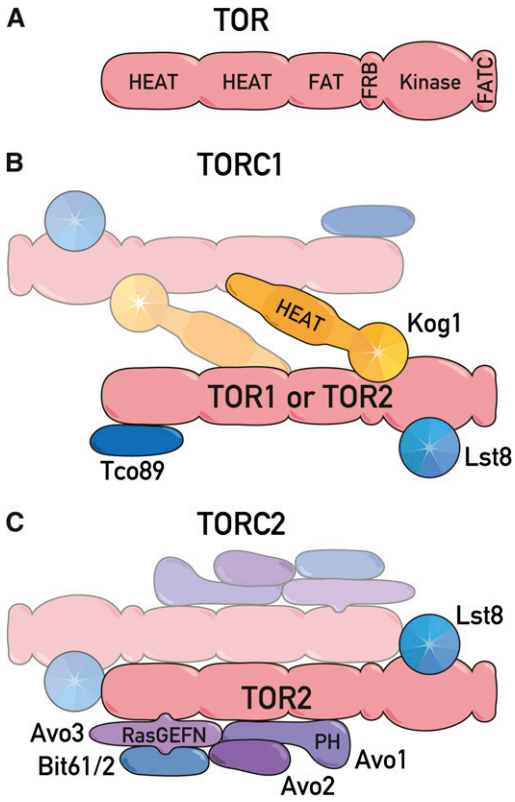

Figure 1 (A) Conserved domain structure of TOR. The N-terminal half of TOR is composed of two blocks of ~20 HEAT repeats, 40 aa that form pairs of interacting antiparallel α-helices. The ~500-aa FAT (FRAP-ATM-TRRAP) domain contains modified HEAT repeats. Missense mutations in the ~100-aa FRB (FKBP12-rapamycin-binding) domain confer complete resistance to rapamycin. The kinase domain phosphorylates Ser/Thr residues in protein substrates, but at the sequence level resembles the catalytic domain of phosphatidylinositol kinases. The ~35-aa FATC domain is always found C-terminal to the FAT domain and is essential for kinase activity. (B) Composition of TOR complex 1. TORC1 is ~2 MDa in size and contains Kog1, Tco89, Lst8, and either TOR1 or TOR2. The HEAT repeats found in Kog1 and the seven-bladed propellers of the WD-40 repeats found in Kog1 and Lst8 are depicted. The binding of Kog1 to TOR is complex, involving multiple domains on each protein. Lst8 binds to the kinase domain of TOR. Each component is likely present in two copies. (C) Composition of TOR complex 2. TORC2 is ~2 MDa in size and contains Avo1, Avo2, Avo3, Bit61, and/or its paralog Bit2, Lst8, and TOR2 but not TOR1. The RasGEFN domain of Avo3 and the PH domain of Avo1 are indicated. Each component is likely present in two copies.

within a few generations as small-budded cells in the G2/M phase of the cell cycle and with a randomized actin cytoskeleton (Kunz *et al.* 1993; Helliwell *et al.* 1994, 1998a; Schmidt *et al.* 1996). These and other findings led to the model that TOR2 has two essential functions: one function is redundant with TOR1 (TOR shared) and the other function is unique to TOR2 (TOR2 unique) (Hall 1996; Helliwell *et al.* 1998a). As described below, these two TOR2 functions turned out to be two separate signaling branches (each corresponding to a structurally and functionally distinct TOR complex) that control cell growth in different ways (Barbet *et al.* 1996; Schmidt *et al.* 1997, 1998; Bickle *et al.* 1998; Helliwell *et al.* 1998a; Loewith *et al.* 2002; Loewith and Hall

2004; De Virgilio and Loewith 2006; Breitkreutz *et al.* 2010; Kaizu *et al.* 2010).

The early characterization of *TOR* disruptions and rapamycin treatment led to two more important conclusions. First, as described in more detail below, the finding that TOR inhibition mimics starvation led to the notion that TOR controls cell growth in response to nutrients (Barbet *et al.* 1996; Rohde *et al.* 2001). Subsequent studies confirmed this notion and demonstrated that TOR in higher eukaryotes also controls cell growth in response to nutrients; *i.e.*, TOR is conserved in structure and function (Thomas and Hall 1997; Hara *et al.* 1998; Schmelzle and Hall 2000). Second, the observation that inhibition specifically of the TOR-shared signaling branch (disruption of both *TOR*s but not of *TOR2* alone) or rapamycin treatment mimics starvation suggested that only the TOR-shared pathway is nutrient responsive and rapamycin sensitive (Zheng *et al.* 1995; Barbet *et al.* 1996; Schmidt *et al.* 1996; Rohde *et al.* 2001). The molecular basis of these findings would remain a mystery until the discovery of the two structurally and functionally distinct TOR complexes (see below).

The realization that TOR controls growth (increase in cell size or mass) was a particularly important development (Barbet *et al.* 1996; Thomas and Hall 1997; Schmelzle *et al.* 2002). Rapamycin or loss of *TOR* function causes a cell cycle arrest, and TOR was thus originally thought to be a controller of cell division (increase in cell number). Furthermore, at that time, growth was largely thought to be controlled passively: *i.e.*, the simple availability of nutrients (building blocks) led to cell growth. As described below, the realization that TOR controls many cellular processes that collectively determine mass accumulation, combined with the fact that there was no direct role for TOR in the cell cycle machinery then being characterized, led to the notions that TOR controls growth and that growth is thus actively controlled. The originally confusing defect in cell cycle progression observed upon TOR inhibition is in fact an indirect effect of growth inhibition: a growth defect is dominant over cell cycle progression.

Since the late 1990s, many groups have been characterizing the two separate TOR2-signaling branches. It was found that the TOR-shared signaling branch is composed of various effector pathways that control a wide variety of readouts that collectively determine the mass of the cell. The readouts controlled by this branch include protein synthesis and degradation, mRNA synthesis and degradation, ribosome biogenesis, nutrient transport, and autophagy (Schmelzle and Hall 2000). This branch is viewed as mediating temporal control of cell growth. The TOR2-unique branch controls the polarized organization of the actin cytoskeleton, endocytosis, and sphingolipid synthesis. This second branch is viewed as mediating spatial control of cell growth, on the basis largely of early work showing that it controls the actin cytoskeleton. Thus, the logic of the two branches appears to be to integrate temporal and spatial control of cell growth (Loewith and Hall 2004). However, this way of thinking about the two branches has subsided in recent years as the TOR2-unique pathway was shown to control sphingolipid synthesis and endocytosis in addition to the actin cytoskeleton (Powers *et al.* 2010).

Another major breakthrough in the TOR field occurred in 2002: the identification of the two multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 (Loewith *et al.* 2002; Wedaman *et al.* 2003; Reinke *et al.* 2004; Wullschleger *et al.* 2006). The two structurally and functionally distinct TOR complexes were biochemically purified from yeast cells and subsequently shown to correspond to the two genetically defined TOR-signaling branches. TORC1, which contains either TOR1 or TOR2 and is rapamycin sensitive, mediates the TOR-shared pathway. TORC2, which specifically contains TOR2 and is rapamycin insensitive, mediates the TOR2-unique pathway. The TORCs were a major breakthrough because they provided a molecular basis for the functional complexity and selective rapamycin sensitivity of TOR signaling. The biochemical identification of the TORCs and the genetic definition of the two signaling branches also, gratifyingly, cross-validated each other such that there is a high level of confidence in the current "two branch-two complex" model of TOR signaling. The subsequent identification of TORCs in other eukaryotes, including plants, worms, flies, and mammals (Table 1), showed that the two complexes, like TOR itself, are conserved and gave further support to the above model (Hara *et al.* 2002; Kim *et al.* 2002; Loewith *et al.* 2002; Jacinto *et al.* 2004; Sarbassov *et al.* 2004). Below we focus on the structure, function, and regulation of the two TOR complexes. We discuss some downstream readouts of the TORCs that were originally described before the discovery of the TORCs but are now retroactively attributed to TORC1 or TORC2 on the basis of their TOR requirement or rapamycin sensitivity.

TOR Complex 1

Composition of TOR complex 1

TORC1 consists of Kog1, Lst8, Tco89, and either TOR1 or TOR2 (Figure 1B) (Loewith *et al.* 2002; Wedaman *et al.* 2003; Reinke *et al.* 2004). Gel filtration chromatography (R. Loewith, W. Oppliger, and M. Hall, unpublished results) indicated that TORC1 has a size of ~2 MDa, suggesting that the entire complex is likely dimeric. This would be consistent with the dimeric structures proposed for TORC2 (Wullschleger *et al.* 2005) and mTORC1 (Yip *et al.* 2010). The names of mammalian and invertebrate orthologs of TORC1 subunits and the salient features of *S. cerevisiae* TORC1 subunits are summarized in Table 1 and Table 2, respectively. Although all subunits are thought to act positively with TOR1/2 *in vivo*, by and large their functions await characterization. In the presence of rapamycin, all components of TORC1 can be coprecipitated with FKBP12 (Loewith *et al.* 2002), demonstrating that, unlike mammalian TORC1 (Yip *et al.* 2010), the structural integrity

Table 1 TORC1, TORC2, and EGO complex orthologs in various genera

S. cerevisiae	*S. pombe*	*C. albicans*	*D. discoideum*	*A. thaliana*	*C. elegans*	*D. melanogaster*	Mammals
				TORC1			
TOR1 or TOR2	Tor1 or Tor2	Tor1	Tor	TOR	TOR/let-363	TOR	mTOR
Kog1/Las24	Mip1	Kog1	Raptor	RAPTOR1A and RAPTOR1B	daf-15	Raptor	Raptor
Lst8	Wat1/Pop3	Orf19.3862	lst8?	*AT2G22040* *AT3G18140*	lst-8?	*CG3004*	mLST8
Tco89	Tco89	Tco89	pcr25kl1p3887	—	—	—	—
—	Toc1	—	—	—	—	—	—
—	—	—	—	—	—	—	PRAS40
—	—	—	—	—	—	—	DEPTOR
				TORC2			
TOR2	Tor1 or Tor2	Tor1	tor	TOR	TOR/let-363	TOR	mTOR
Avo1	Sin1	orf19.5221	piaA		sinh-1	Sin1	mSIN1
Avo2	—	Avo2		—	—	—	—
Avo3/Tsc11	Ste20	Tsc11	rip3		rict-1	Rictor	Rictor
Lst8	Wat1/Pop3	Orf19.3862	lst8	*AT2G22040* *AT3G18140*	lst-8	*CG3004*	mLST8
Bit61	Bit61	—	—	—	—	—	PRR5/Protor
—	—	—	—	—	—	—	DEPTOR
				EGO complex			
Gtr1	Gtr1	Gtr1	ragA	—	raga-1	RagA	RagA,B
Gtr2	Gtr2	Gtr2	ragC	—	ragc-1	RagC	RagC,D
Ego1/Meh1/Gse2	—	—	—	—	—	*CG14184*	LAMTOR1/p18
Ego3/Slm4/Nir1/Gse1	—	—	—	—	lamtor-2, ?	*CG5189*, *CG5110*	LAMTOR2/p14, LAMTOR3/ MP1

Orthologs listed are from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *Dictyostelium discoideum*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Dictyostelium melanogaster*, and mammals. P-POD: Princeton Protein Orthology Database/BLAST. We note that TORC2 appears to be absent in plants, *e.g., A. thaliana*. —, no demonstrated/obvious ortholog.

Table 2 Salient features of TORC1 components

Protein	Size	Motifs/domains	Potential function
TOR1	2470 aa	HEAT repeats, FAT domain, FRB domain, kinase domain, and FATC domain	Protein kinase, scaffold
TOR2	2474 aa	HEAT repeats, FAT domain, FRB domain, kinase domain, and FATC domain	Protein kinase, scaffold
Kog1	1557 aa	An N-terminal conserved region 4, HEAT repeats, 7 C-terminal WD-40 repeats	Present substrate to TOR
Tco89	799 aa	None obvious	Receive signals from EGO complex
Lst8	303 aa	7 WD-40 repeats	Stabilize kinase domain

of yeast TORC1 is not compromised by this macrolide. Despite recent molecular reconstructions from low resolution (25 Å) electron microscopy of a TOR1–Kog1 subcomplex (Adami *et al.* 2007), the molecular mechanism by which binding of FKBP-rapamycin inhibits TORC1 activity is enigmatic and remains a fascinating question.

Localization of TORC1

Tagging of Kog1, Tco89, Lst8, and TOR1 with GFP demonstrates that TORC1 is concentrated on the limiting membrane of the vacuole (Urban *et al.* 2007; Sturgill *et al.* 2008; Berchtold and Walther 2009; Binda *et al.* 2009). These observations are consistent with previous studies that localized TORC1 via immunogold electron microscopy and cellular fractionation (Chen and Kaiser 2003; Reinke *et al.* 2004). Artificial tethering of a TORC1 peptide substrate to the vacuole demonstrates that vacuole-localized TORC1 is catalytically competent (Urban *et al.* 2007). This localization appears to be constitutive (Binda *et al.* 2009), suggesting that changes in "geography" play no obvious role in regulating yeast TORC1-signaling output. The yeast vacuole is a major nutrient reservoir and TORC1 signaling is responsive to nutrient cues (see below). Thus, vacuolar localization of TORC1 seems logical. Although convincing, these studies do not exclude the possibility that a fraction of TORC1 may also be active elsewhere in the cell. Li *et al.* (2006), for example, have reported that TOR1 dynamically associates with the rDNA locus to regulate 35S rRNA transcription.

Upstream of TORC1

Physiological regulators (carbon, nitrogen, phosphate, stress, caffeine): A major breakthrough in the TOR field came with the observation that rapamycin treatment alters yeast physiology in much the same way as nutrient starvation (Barbet *et al.* 1996). Like starvation, exposure of yeast cells to rapamycin results in a dramatic drop in protein synthesis, induction of autophagy, and exit from the cell cycle and entrance into a quiescent G0 state. This was the first indication that TOR, actually TORC1, might regulate growth downstream of nutrient cues. This hypothesis was strengthened when TORC1, in response to nitrogen and carbon cues, was found to promote the sequestration of several nutrient-responsive transcription factors in the cytoplasm (Beck and Hall 1999). Consistently, transcriptome profiling demonstrated a highly similar transcriptional response of yeast cells exposed to rapamycin, nutrient starvation, or noxious stressors (Cardenas *et al.* 1999; Hardwick *et al.* 1999; Komeili *et al.* 2000; Shamji *et al.* 2000; Gasch and Werner-Washburne 2002). Although suggestive, these observations provided only correlative evidence that TORC1 activity is regulated in response to environmental cues. Characterization of a *bona fide* substrate of TORC1 allowed this model to be tested directly.

As detailed below, Sch9 presently remains the best-characterized substrate of TORC1, and monitoring its phosphorylation by Western blotting serves as a convenient proxy for TORC1 activity. In addition to exposure to rapamycin, Sch9 is rapidly dephosphorylated in cells experiencing acute starvation of carbon, nitrogen, phosphate, or amino acids (Urban *et al.* 2007; Binda *et al.* 2009). These and other observations confirm that TORC1 is responsive to both the abundance and the quality of nutrients in the environment; but, with few exceptions (see *The EGO complex*), how nutrient cues are sensed and how this information is transduced to TORC1 remain unknown.

TORC1 activity is also regulated in response to noxious stressors. When cells are subjected to various stress conditions, including high salt, redox stress, a shift to a higher temperature, or caffeine, Sch9 phosphorylation is reduced dramatically (Kuranda *et al.* 2006; Urban *et al.* 2007). With the exception of caffeine, which directly inhibits TORC1

Table 3 Salient features of EGO Complex components

Protein	Size	Motifs/domains	Potential function
Gtr1	310 aa	Ras-family GTPase	GTP-bound form activates TORC1
Gtr2	341 aa	Ras-family GTPase	GDP-bound form activates TORC1
Ego1/Meh1/ Gse2	184 aa	N-terminal palmitoylation/myristolation[a]	Vacuolar recruitment
Ego3/Slm4/Nir1/Gse1	162 aa	Transmembrane domain, PtdIns(3,5)P2 binding[a]	Vacuolar recruitment

[a] Described in Dubouloz *et al.* (2005), Hou *et al.* (2005), and references therein.

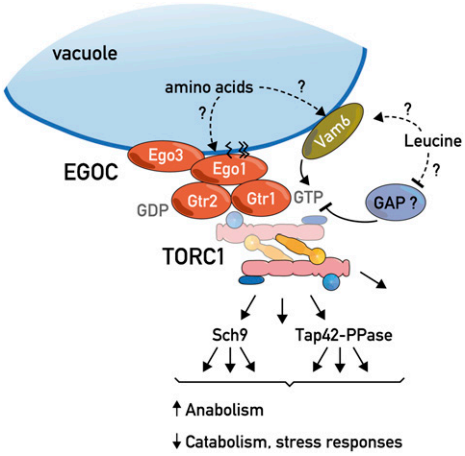

Figure 2 The EGO complex is a major regulator of TORC1. The EGO complex (EGOC) is composed of four proteins: the palmitoylated and myristolated protein Ego1, the transmembrane protein Ego3, and two Ras-family GTPases, Gtr1 and Gtr2. Like TORC1, the EGO complex is localized to the vacuolar membrane where it appears to sense/respond to intracellular leucine levels and potentially to intravacuolar amino acid levels. Vam6 has been identified as a guanine nucleotide exchange factor for Gtr1 but no other GEFs or GAPS for this GTPase system have been reported. In the $Gtr1^{GTP}$ and $Gtr2^{GDP}$ configuration, the EGO complex somehow activates TORC1; the reverse conformation inactivates TORC1. Activated TORC1, via its two main effector branches, the AGC kinase Sch9 and the Tap42-PP2a and PP2a-like protein phosphatases, stimulates growth by favoring anabolic processes and by antagonizing catabolic processes and stress-response programs.

kinase activity (Kuranda *et al.* 2006; Reinke *et al.* 2006; Wanke *et al.* 2008), how environmental stress signals are transduced to TORC1 is also unclear.

The EGO complex: When environmental conditions are inappropriate for growth, cells stop dividing, slow their metabolism, induce the expression of stress-responsive proteins, and accumulate energy stores. This nondividing but metabolically active state is known as quiescence (G0). How cells enter into quiescence is relatively well characterized. In contrast—and despite its medical relevance (inappropriate exit from quiescence can lead to cancer or reactivation of a latent infection)—how quiescent cells reinitiate growth is poorly understood. To shed light on this process, a clever screen was performed to identify mutants that are unable to **e**scape from rapamycin-induced **gro**wth arrest (*EGO*) mutants (Dubouloz *et al.* 2005). This and a follow-up study (Binda *et al.* 2009) identified the EGO complex as an important regulator of TORC1.

The EGO complex is composed of four proteins: Ego1, Ego3, Gtr1, and Gtr2 (Table 3 and Figure 2). Gtr1 and Gtr2 are Ras-family GTPases and orthologs of the metazoan Rag GTPases (Kim *et al.* 2008; Sancak *et al.* 2008) (Table 1). Although they lack obvious sequence homologies, Ego1 and Ego3 are likely the functional homologs of vertebrate p18 (LAMTOR1) and p14 + MP1 (LAMTOR2 + LAMTOR3), respectively, which function together as the "Ragulator" complex (Kogan *et al.* 2010; Sancak *et al.* 2010). Ragulator and the Rags mediate amino acid sufficiency signals to mTORC1 (reviewed in Kim and Guan 2011). Like its mammalian counterpart, the EGO complex resides on the vacuolar/lysosomal membrane and is thought to couple amino acid signals to TORC1 (Binda *et al.* 2009). Curiously, the $Gtr1^{GTP}$ $Gtr2^{GDP}$ combination activates TORC1 with the nucleotide-binding status of Gtr1 seemingly dominant over the nucleotide-binding status of Gtr2.

TORC1 activity in both metazoans and yeast appears to be particularly responsive to glutamine (Crespo *et al.* 2002) and the branched-chain amino acid leucine (Binda *et al.* 2009; Cohen and Hall 2009). In yeast, leucine starvation destabilizes Gtr1-TORC1 association and causes a reduction in Sch9 phosphorylation whereas GTP-locked $Gtr1^{Q65L}$ remains bound to TORC1 and Sch9 dephosphorylation is delayed in cells expressing this mutant (Binda *et al.* 2009). Loss of Gtr1 results in reduced Sch9 phosphorylation and slow growth whereas GDP-locked $Gtr1^{S20L}$ is dominant negative. When $Gtr1^{S20L}$ is expressed as the sole version of Gtr1, cells are extremely sick. This near inviability is suppressed by deletion of the *TCO89* gene encoding the TORC1 subunit Tco89. Collectively, these observations suggest that the EGO complex can both positively and negatively regulate TORC1 activity via Tco89. The fact that the EGO complex can negatively regulate TORC1 activity seems to be at odds with the current metazoan model according to which the EGO complex counterpart serves only to localize TORC1 to the vacuole. Indeed, in contrast to the results obtained in metazoans, in yeast, TORC1 appears to stably localize to the vacuolar membrane regardless of nutrient conditions. Thus, how the EGO complex influences TORC1 activity remains a mystery although the crystal structure of the Gtr1–Gtr2 complex, reported very recently, provides some mechanistic insights (Gong *et al.* 2011).

Also mysterious are the mechanisms by which amino acid sufficiency modulates Gtr1/2 guanine nucleotide loading. Given its localization, it is tempting to postulate that the EGO complex responds to levels of intravacuolar amino acids, possibly via the recently described Gtr1 guanine–nucleotide exchange factor (GEF) Vam6/Vps39 (Binda *et al.* 2009). It is equally plausible, however, that this signal is mediated by an as-yet-unidentified GTPase-activating protein (GAP) activity. Consistent with the conserved function of the EGO/Ragulator complex, and like its yeast ortholog, hVPS39 has been found to function positively upstream of mTORC1 (Flinn *et al.* 2010).

Feedback loop/ribosome biogenesis homeostasis: Although most recognized as a target of signals emanating from extracellular nutrients and noxious stresses, it is becoming increasingly apparent that TORC1 also responds to intracellular cues. In addition to the sensing of intracellular amino acids as described above, outputs from distal effectors also regulate TORC1 in apparent feedback loops. For example, in both yeast and mammalian cells, it is well documented that TORC1 activity stimulates translation initiation

(Wullschleger *et al.* 2006). Interestingly, inhibition of translation with cycloheximide causes a pronounced increase in (m)TORC1 activity presumably by triggering an increase in the concentration of free amino acids in the cytoplasm (Beugnet *et al.* 2003; Urban *et al.* 2007; Binda *et al.* 2009). Ribosome biogenesis (described in more detail below) is a second example. TORC1 regulates ribosome biogenesis in part via two substrates, Sch9 and the transcription factor Sfp1. Reduced ribosome biogenesis resulting from deletion of *SCH9* or *SFP1* results in a dramatic increase in TORC1 activity (Lempiainen *et al.* 2009). It is possible that blocking ribosome biogenesis, like translation inhibition, causes an increase in free amino acids that subsequently activates TORC1. Alternatively, other mechanisms could be at play. Regardless of mechanism, such feedback loops provide an elegant means by which growth homeostasis can be maintained by TORC1.

Downstream of TORC1

In general terms, when growth conditions permit, TORC1 is active and its signals promote the accumulation of cellular mass. However, as both proximal and distal TORC1 effectors continue to be described, the extent of this temporal regulation of growth control is only starting to be appreciated.

Proximal TORC1 effectors: *Characterization of Sch9 as a TORC1 substrate:* Arguably, the best-characterized substrates of both yeast and metazoan TOR complexes are the AGC family kinases. This rather well-studied family of kinases is so named on the basis of its mammalian members PKA, PKG, and PKC (Pearce *et al.* 2010). Typically, activation of AGC family kinases requires phosphorylation of two conserved regulatory motifs, the "T," or "activation," loop located in the catalytic domain and the "hydrophobic" motif found toward the C terminus. Phosphorylation of these motifs helps stabilize the kinase domain in an active conformation. Several AGC family kinases additionally contain a "turn" motif located between the kinase domain and the hydrophobic motif, phosphorylation of which is thought to promote protein stability. While the T loop is phosphorylated by PDK1 or its ortholog Pkh in mammalian or yeast cells, respectively, phosphorylation of the hydrophobic and possibly the turn motifs is often mediated by TORC1 or TORC2.

Analogous to S6K for mTORC1, the AGC kinase Sch9 was recently found to be a direct substrate for yeast TORC1 (Powers 2007). Six target sites in the C terminus of Sch9 are phosphorylated by TORC1: Thr737 found in a classical hydrophobic motif; Thr723 and Ser726, Ser/Thr-Pro sites found in what appears to be a turn motif; Ser758 and Ser765 found in sequences that resemble the hydrophobic motif; and Ser711 in a region that partially resembles a hydrophobic motif. TORC1-mediated phosphorylation is necessary for Sch9 activity. Replacing the target amino acids with alanine yields a nonfunctional Sch9, whereas replacing them with a phosphomimetic residue confers constitutive kinase activity, *i.e.*, activity even in the absence of TORC1 (Urban *et al.* 2007). Presumably, phosphorylation of the turn motif helps to stabilize Sch9 while phosphorylation of the hydrophobic motif stabilizes Sch9 in an active conformation. Curiously, although their *in vivo* functions are unknown, *in vitro* TORC1 preferentially phosphorylates Ser758 and Ser765 within the hydrophobic-like motifs (R. Loewith, unpublished results). That TORC1 can phosphorylate amino acids found within such diverse sequence contexts, which is rather atypical for protein kinases, is also curious.

Characterization of Tap42-PP2A as a TORC1 effector: In addition to Sch9, TORC1 also regulates type 2A (Pph21, Pph22, and Pph3—generically PP2Ac) and 2A-related phosphatases (Sit4, Ppg1). These partially redundant yet pleiotropic enzymes are notoriously difficult to study. Analysis of Sit4 function, and therefore of TORC1 function, is further complicated by strain-dependent polymorphisms at the *SSD1* (**S**uppressor of ***SIT4*** **D**eletion) locus (Reinke *et al.* 2004).

A role for these phosphatases downstream of TORC1 was first described by the Arndt lab (Di Como and Arndt 1996). In this work, a subpopulation of these enzymes was found to interact in a TORC1-dependent manner with a regulatory protein known as Tap42. Rrd1 and Rrd2, two peptidyl-prolyl *cis/trans* isomerases, were subsequently also found to be present in these Tap42 complexes (Zheng and Jiang 2005; Jordens *et al.* 2006). Work, done in large part by the Jiang group, posits that when TORC1 is active, Tap42 is phosphorylated and bound tightly to the phosphatase–Rrd complex (Di Como and Arndt 1996; Jiang and Broach 1999; Zheng and Jiang 2005). Inactivation of TORC1 results in Tap42 dephosphorylation and a weakened association with phosphatases that presumably results in their activation and/or change in substrate preference (Duvel *et al.* 2003; Yan *et al.* 2006). How TORC1 maintains Tap42 phosphorylation is mechanistically unclear. It may phosphorylate Tap42 directly (Jiang and Broach 1999), or it may act via the **T**ap42 **i**nteracting **p**hosphoprotein Tip41 (Jacinto *et al.* 2001). Interestingly, Tip41 has been proposed to both antagonize and cooperate with Tap42 in controlling TORC1 signaling (Jacinto *et al.* 2001; Kuepfer *et al.* 2007).

Although the mechanisms coupling TORC1 to Tap42–PPase complexes remain to be elucidated, genetic arguments clearly position Tap42 as a prominent effector of TORC1. Specifically, several alleles of *TAP42* (*e.g.*, *TAP42-11*) that confer strong resistance to rapamycin by blocking a subset of rapamycin-induced readouts have been identified (Di Como and Arndt 1996; Duvel *et al.* 2003).

Curiously, *TAP42-11* does not provide rapamycin resistance in all strain backgrounds. However, co-expression of genetically activated Sch9 (described above) in rapamycin-sensitive *TAP42-11* backgrounds results in a very strong synthetic resistance to rapamycin (Urban *et al.* 2007). From this observation, it appears that Sch9 and Tap42-PPase complexes are major effector branches downstream of TORC1 with each branch, at least in some backgrounds, performing

one or more essential function. The readouts mediated by these two TORC1 branches are discussed below.

Other TORC1 substrates: In addition to the regulation of these two major effector branches, TORC1 has been reported to directly phosphorylate other substrates including Sfp1(Lempiainen *et al.* 2009), Gln3 (Bertram *et al.* 2000), and Atg13 (Kamada *et al.* 2010). The roles that these proteins play downstream of TORC1 are discussed below.

Tyers and colleagues have recently defined a global protein kinase and phosphatase interaction network in yeast (Breitkreutz *et al.* 2010). This study, consisting of affinity purification followed by mass spectrometry, included TOR1 and TOR2. They found and confirmed that TORC1 physically interacts with the following proteins: Mks1, a protein involved in retrograde (RTG) mitochondria-to-nucleus signaling (see below); curiously, FMP48, an uncharacterized protein presumed to localize to the mitochondria (Reinders *et al.* 2006); Npr1, a protein kinase involved in the intracellular sorting of nutrient permeases (see below); Ksp1, a protein kinase involved in nutrient-regulated haploid filamentous growth (Bharucha *et al.* 2008); Nap1, a chromatin assembly factor and a mitotic factor involved in regulation of bud formation (Calvert *et al.* 2008); Nnk1, the **n**itrogen **n**etwork **k**inase presumably involved in intermediate nitrogen metabolism (Breitkreutz *et al.* 2010); Sky1, an Ser/Arg domain kinase involved in pre-mRNA splicing (Shen and Green 2006); and Bck1 and Kdx1, which are involved in MAPK signaling (Breitkreutz *et al.* 2010). Given their physical interaction with TORC1, all of these proteins, in addition to multiple other, as-yet-unconfirmed interactors, are potential substrates (or regulators) of TORC1. These results underscore the central role that TORC1 plays in cell growth.

Distal readouts downstream of TORC1: *TORC1 promotes cell growth:* When environmental conditions are favorable, TORC1 coordinates the production and accumulation of cellular mass, *i.e.*, growth, via regulation of several processes.

Protein synthesis: The first realization that TORC1 serves to couple environmental cues to the cell growth machinery came with the observation that rapamycin treatment elicits a marked drop in protein synthesis by blocking translation initiation (Barbet *et al.* 1996). A major target for this regulation appears to be the translation initiation factor eIF2. Upon amino acid starvation or rapamycin treatment, the α-subunit of eIF2 is phosphorylated and this dominantly interferes with 5′CAP-dependent mRNA translation (reviewed in Hinnebusch 2005). TORC1 signals to eIF2α via both the Sch9 and Tap42-PPase branches. The sole eIF2α kinase is the conserved Gcn2 protein. Gcn2 binds and is activated by uncharged tRNAs that accumulate when cells are starved for an amino acid (detailed below). Gcn2 activity is also regulated by phosphorylation. Gcn2 phosphorylation on Ser577 reduces tRNA binding and, consequently, kinase activity. Treating cells with rapamycin elicits a rapid, Tap42-PPase-dependent dephosphorylation of Ser577 and, consequently, an increase in Gcn2 activity and a reduction in 5′CAP-dependent translation (Cherkasova and Hinnebusch 2003). It is possible that one or more Tap42-associated phosphatases directly dephosphorylates Ser577, but this has not been formally demonstrated. The nature of the kinase that phosphorylates Gcn2 Ser577 is unknown other than it is not Sch9 (M. Stahl and R. Loewith, unpublished results). Sch9 inhibition, however, also leads to eIF2α phosphorylation via an undefined pathway (Urban *et al.* 2007).

Studies with rapamycin suggest that, in addition to eIF2α, TORC1 may target additional translation factors such as the 5′CAP-binding protein (eIF4E) interacting proteins Eap1 and/or the eIF4G scaffold (Berset *et al.* 1998; Cosentino *et al.* 2000). Finally, recent phosphoproteomics studies (Huber *et al.* 2009; Loewith 2010; Soulard *et al.* 2010) have identified several translation-related proteins whose phosphorylation is altered by rapamycin treatment, suggesting that these factors could also couple TORC1 to protein synthesis.

Ribosome biogenesis: In optimal conditions, yeast cells grow and divide approximately every 100 min. Such rapid growth requires robust protein synthesis, which of course requires ribosomes. Indeed, rapidly growing yeast cells contain ∼200,000 ribosomes, implying that each cell must produce and assemble ∼2000 ribosomes per minute (Warner 1999). This is not a trivial feat as each ribosome contains 78 unique proteins (encoded by 137 *RP* genes) in addition to four rRNA molecules, three derived from the RNA Pol I-transcribed 35S pre-rRNA and one transcribed by RNA Pol III. Fifty percent of RNA Pol II transcription is devoted to ribosomal proteins. In addition, numerous protein and small RNA *trans*-acting factors, known as **ri**bosome **bi**ogenesis (RiBi) factors, are required for the correct processing, folding, assembly, nuclear export of pre-ribosomal particles to the cytoplasm, and final maturation events into 40S and 60S particles. The production of all these abundant molecules represents a huge energetic investment. Not surprisingly, yeast cells have developed elaborate measures to coordinate the expression of rRNA, tRNA, RPs, and RiBi factors in response to environmental conditions. Much of this regulation is mediated by TORC1 at the level of transcription. As ribosome biogenesis has clear links to diseases such as cancer, anemia, and aging, dissection of its regulation will undoubtedly have clinical ramifications (Lempiainen and Shore 2009).

In *S. cerevisiae*, the rDNA locus consists of ∼150 tandemly repeated transcription units on chromosome XII, and yet rRNA production is still limiting for cell growth (Warner 1999). Each of these rDNA units comprises the RNA polymerase III transcribed 5S rRNA gene, the intergenic spacer region, and the RNA Pol I-transcribed 35S rRNA gene, encoding the 35S precursor of the mature 18S, 5.8S, and 25S rRNAs. RNA Pol III also transcribes tRNA genes as well as several additional genes encoding small noncoding RNAs. In the late 1990s, it was reported that rapamycin results in

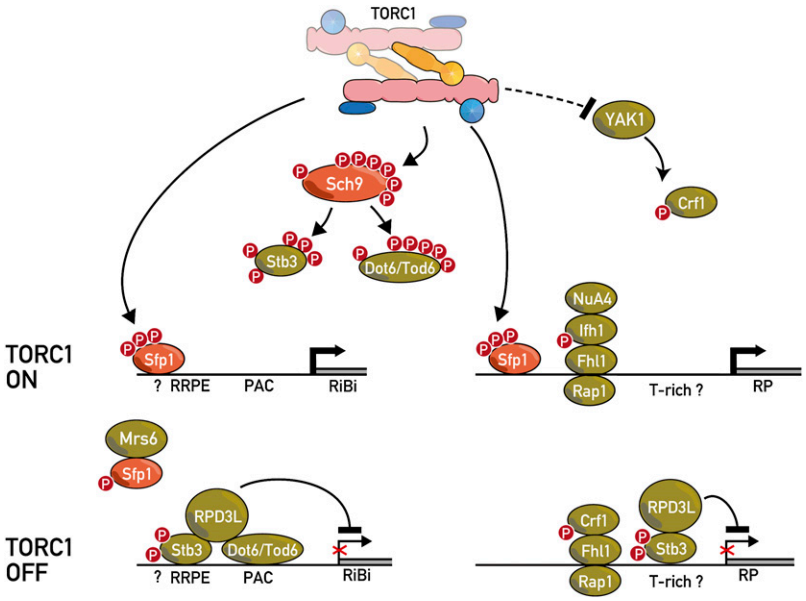

Figure 3 Control of RiBi and RP gene transcription by TORC1. RiBi factors are required for the proper expression, processing, assembly, export, and maturation of rRNA and RPs into ribosomes. This energetically costly procedure is under tight regulation, particularly at the transcription level. TORC1 regulates RiBi and RP gene transcription via multiple pathways: (1) TORC1 directly phosphorylates the Split Zn-finger transcription factor Sfp1, which presumably regulates its nuclear localization and/or binding to RP and possibly RiBi gene promoters to stimulate their expression. (2) Fhl1 and Rap1 bind constitutively to *RP* promoters. When TORC1 is active, phosphorylated Ifh1 binds to Fhl1 to stimulate transcription, possibly by recruiting the NuA4 histone acetyltransferase. When TORC1 is inactive, Yak1 phosphorylates Crf1, which subsequently outcompetes Ifh1 for binding to Fhl1. (3) Sch9 phosphorylates and thus inhibits Stb3 and the paralogs Dot6 and Tod6. Inhibition of TORC1/Sch9 results in the dephosphorylation of these three transcription repressors, which subsequently bind to *RRPE* and *PAC* elements found in RiBi promoters. Stb3 additionally binds RP promoters. Bound to promoters, these repressors recruit the RPD3L histone deacetylase complex to repress transcription.

a rapid and pronounced drop in 5S, 35S, and tRNA production (Zaragoza *et al.* 1998; Powers and Walter 1999). Recently, the relevant signaling pathways in this regulation have become clearer.

TORC1 regulates the accumulation of RNA Pol I transcripts at multiple levels. Processing of the 35S pre-rRNA occurs cotranscriptionally and is dependent on the presence of ribosomal proteins (Tschochner and Hurt 2003). The fast drop in RNA Pol I-dependent transcripts observed upon rapamycin treatment is apparently due to decreased translation (described above) of ribosomal proteins (Reiter *et al.* 2011). The majority of mRNAs being translated in a rapidly growing cell encode ribosomal proteins (Warner 1999), and thus a drop in translation will rapidly reduce the levels of free ribosomal proteins that are themselves needed stoichiometrically for processing of rRNA into pre-ribosome particles. rRNA that is not efficiently processed is immediately degraded, presumably to prevent imbalances in structural components of the ribosome. At later time points following rapamycin treatment, RNA Pol I no longer associates with the rDNA and transcription stops. This late effect could be the result of rapamycin-induced degradation of the essential RNA Pol I transcription factor Rrn3 (Claypool *et al.* 2004; Laferte *et al.* 2006; Reiter *et al.* 2011).

TORC1 regulates RNA Pol III apparently exclusively via Sch9 and a repressor protein named Maf1 (Upadhya *et al.* 2002; Oficjalska-Pham *et al.* 2006; Reina *et al.* 2006; Huber *et al.* 2009; Lee *et al.* 2009). Sch9 directly phosphorylates seven sites in Maf1 that prevent it from interacting with and thus inhibiting RNA Pol III (Vannini *et al.* 2010). Phosphomimetic variants of Maf1 clearly fail to associate with RNA Pol III, but, curiously, Sch9 inhibition still causes a reduction in RNA Pol III activity in these strains but not in *maf1Δ* strains. This and other observations suggest that an additional Sch9 target exists that, when dephosphorylated, represses RNA Pol III in a Maf1-dependent fashion (Huber *et al.* 2009; Michels 2011). Maf1 is conserved and also functions downstream of mTORC1 to regulate RNA Pol III activity. However, in mammalian cells, and perhaps in yeast cells too, Maf1 is directly phosphorylated by mTORC1 rather than by the Sch9 ortholog S6K1 (Wei *et al.* 2009; Wei and Zheng 2010; Michels 2011).

A total of 137 genes encode the 78 proteins that make up a yeast ribosome (most RPs are encoded by two genes yielding nearly identical proteins). TORC1 coordinately regulates the expression of these genes through several mechanisms (Figure 3) (Lempiainen and Shore 2009). A central component of this regulation is the Fhl1 protein (Lee *et al.* 2002; Martin *et al.* 2004; Schawalder *et al.* 2004; Wade *et al.* 2004; Rudra *et al.* 2005). Fhl1 has a forkhead DNA-binding domain, and its constitutive association to ribosomal protein gene (*RP*) promoters is facilitated by the DNA-binding protein Rap1 and the high mobility group protein Hmo1 (Hall *et al.* 2006; Berger *et al.* 2007). TORC1 regulates *RP* transcription by determining the association between Fhl1 and either one of two FHB-containing proteins, Ifh1 and Crf1. Both Ifh1 and Crf1 are phosphoproteins. When cells are growing and TORC1 is active, Ifh1 is phosphorylated and binds to Fhl1 to stimulate *RP* transcription. Conversely, inhibition of TORC1 results in the phosphorylation of Crf1, which displaces Ifh1 to repress *RP* transcription. The signaling events upstream of Ifh1 are not known, whereas TORC1 seems to signal to Crf1 via the Ras/PKA pathway target Yak1 (Martin *et al.* 2004). However, it should be noted that the crosstalk between TORC1 signals and Ras/PKA signals has been debated. While it is clear that hyperactivation of Ras/PKA can suppress many rapamycin-induced phenotypes (Schmelzle *et al.* 2004), suggesting that PKA is downstream of TORC1, it has also been proposed that TORC1 and PKA signal in parallel

pathways that impinge on common targets (Zurita-Martinez and Cardenas 2005; Ramachandran and Herman 2011). Recently, Soulard *et al.* (2010) have provided some clarification of this dilemma by proposing that TORC1 functions upstream of PKA but only for a subset of PKA targets. Thus, TORC1 may be both upstream and parallel to PKA.

TORC1-dependent regulation of *RP* gene transcription still occurs in the absence of the Fhl1/Ifh1/Crf1 system, suggesting the existence of additional regulatory mechanisms. One of these is the split zinc (Zn)-finger protein Sfp1 (Fingerman *et al.* 2003; Jorgensen *et al.* 2004; Marion *et al.* 2004; Lempiainen *et al.* 2009; Singh and Tyers 2009). TORC1 binds and directly phosphorylates Sfp1 to promote its binding to a subset of *RP* gene promoters. Curiously, unlike Sch9, TORC1-mediated Sfp1 phosphorylation appears to be insensitive to osmotic or nutritional stress, suggesting that TORC1 regulates these two substrates via very different mechanisms (Lempiainen *et al.* 2009). Sfp1 also interacts with the conserved Rab escort protein Mrs6, an essential protein functioning in membrane sorting (Lempiainen *et al.* 2009; Singh and Tyers 2009). Sfp1-Mrs6 association is important for the nuclear localization of Sfp1, but its functional implications are otherwise unclear. Intriguingly, this association may underlie the presently unexplained genetic and biochemical interactions between TORC1 and vesicular transport machineries (Aronova *et al.* 2007; Zurita-Martinez *et al.* 2007). Although physical interaction with *RiBi* promoters has not been reported, overexpression of Sfp1 causes a rapid upregulation of most *RiBi* genes, suggesting that Sfp1 also regulates this important regulon (Jorgensen *et al.* 2004). Better understood is the regulation of *RiBi* gene expression downstream of Sch9. *RiBi* promoters typically possess polymerase A and C (*PAC*) and/or **r**ibosomal **R**NA **p**rocessing **e**lement (*RRPE*) elements. *PAC* elements are bound by the myb-family transcription factors Dot6 and Tod6 (Freckleton *et al.* 2009; Zhu *et al.* 2009) whereas *RRPE* elements are bound by Stb3 (Liko *et al.* 2007). Stb3 seems to bind to T-rich elements in *RP* promoters as well (Huber *et al.* 2011). All three transcription factors are phosphorylated by Sch9 and thus are under TORC1 control (Lippman and Broach 2009; Liko *et al.* 2010; Huber *et al.* 2011). When TORC1 is inactivated, Dot6, Tod6, and Stb3 are dephosphorylated, allowing them to bind to their cognate promoter elements and recruit the RPD3L histone acetyltransferase complex to repress transcription.

In summary, TORC1 plays a central role in regulating ribosome biogenesis, particularly at the transcriptional level. However, it is now clear that TORC1 also influences ribosome biogenesis post-transcriptionally. Phosphoproteomics as well as more directed studies suggest that TORC1 regulates various catalytic steps of ribosome assembly *per se* (Honma *et al.* 2006; Huber *et al.* 2009; Loewith 2010). Phosphoproteomics and biochemical studies (Albig and Decker 2001; Grigull *et al.* 2004; Huber *et al.* 2009; Breitkreutz *et al.* 2010; Loewith 2010; Soulard *et al.* 2010) also suggest that TORC1 plays an active role in mRNA stability and, via its potential substrate Sky1, in pre-mRNA splicing. This observation is significant when one considers that 90% of all mRNA splicing events occur on *RP* transcripts (Warner 1999). Thus, TORC1 is well positioned to coordinate multiple aspects of ribosome biogenesis in response to growth stimuli. As introduced above, TORC1 activity is dramatically increased in *sfp1* and *sch9* cells (Lempiainen *et al.* 2009), suggesting that some aspect of ribosome biogenesis must also signal in a feedback loop to TORC1. It will be interesting to see what steps of ribosome biogenesis contribute to TORC1 regulation.

Regulation of cell cycle/cell size: Although distinct processes, cell growth and cell division are often intimately linked. Yeast cells, for example, commit to a new round of cell division only after attaining a critical size. This cell-size threshold is dictated in large part by environmental growth conditions (Cook and Tyers 2007). How cells couple environmental cues to the cell cycle machinery is fascinating but poorly understood. Interestingly, *sfp1* and *sch9* were the top two hits in a systematic search for mutations conferring small cell size (Jorgensen *et al.* 2002, 2004). This and follow-up observations demonstrated that ribosome biogenesis plays a major role in cell-size determination. These results further predict that environmental cues regulate the cell-size threshold via TORC1, *i.e.*, that poor growth conditions reduce the activity of TORC1 and subsequently the activities of Sfp1 and Sch9. Consequently, this would decrease ribosome biogenesis, which, in mysterious ways, would lower the cell-size threshold required for cell division. In contrast, acute inhibition of TORC1 with high concentrations of rapamycin leads to an arrest in G1 due to reduced translation of the cyclin Cln3 (Barbet *et al.* 1996) and a paradoxical increase in cell size. This increase in cell size is actually due to swelling of the vacuole as a consequence of increased autophagy (see below; *sfp1* or *sch9* deletions presumably do not induce autophagy).

Although best appreciated for its role in G1 regulation, TORC1 additionally regulates the transition through other phases of the cell cycle. TORC1 promotes S phase by maintaining deoxynucleoside triphosphate pools. Deoxynucleoside triphosphates are the obligate building blocks for DNA synthesis, and a role for TORC1 in their synthesis becomes apparent under conditions of DNA replication stress or DNA damage when elevated deoxynucleoside triphosphate pools are necessary for error-prone translesion DNA polymerases (Shen *et al.* 2007). Via the Tap42-PPase branch, TORC1 also influences the G2/M transition (Nakashima *et al.* 2008). Specifically, TORC1 regulates the subcellular localization of the polo-like kinase Cdc5. Cdc5 activity destabilizes Swe1, a kinase that phosphorylates and thus inactivates the mitotic cyclin-dependent kinase Cdc28. Inhibition of TORC1 mislocalizes Cdc5, causing an inappropriate stabilization of Swe1 and, consequently, inactivation of Cdc28 and prolonged G2/M. Although TORC1 signals likely impinge upon additional nodes in the cell division cycle (Huber *et al.* 2009; Soulard *et al.* 2010), the above

observations already exemplify the intricate connections between cell growth signals and the cell division cycle. Reciprocal, but less well described, cues and/or outputs from the cell division cycle regulate cell growth, likely in part via TORC1 (Goranov and Amon 2010).

TORC1 inhibits stress responses: In addition to stimulating anabolic processes, TORC1 also promotes growth by suppressing a variety of stress-response programs. Although essential for surviving environmental insults, activation of stress-responsive pathways is incompatible with rapid growth, and constitutive activation of these pathways generally results in cell death. As described below, the best-characterized stress-response programs under the influence of TORC1 are transcriptional in nature. However, it is clear that TORC1 also regulates post-transcriptional aspects of stress responses such as mRNA stability, protein trafficking, and the activities of metabolic enzymes.

Environmental stress response: Exposure of yeast cells to noxious stressors, including nutrient limitation and entry into stationary phase, elicits a stereotypic transcriptional response known as the environmental stress response (ESR) (Gasch and Werner-Washburne 2002). This includes ~300 upregulated genes that encode activities such as protein chaperones and oxygen radical scavengers that help cells endure stressful environments. The central components of this pathway are the Zn-finger transcription factors Msn2/4 and Gis1, the LATS family kinase Rim15, and the α-endosulfine family paralogs Igo1 and Igo2 (De Virgilio 2011). TORC1 via Sch9, and possibly also Tap42-PPase, promotes cytoplasmic anchoring of Rim15 to 14-3-3 proteins by maintaining Rim15 phosphorylated on Ser1061 and Thr1075 (Wanke *et al.* 2005, 2008). Inhibition of TORC1 results in nuclear localization of Rim15, which subsequently triggers the activation, in a poorly understood fashion, of the expression of Msn2/4- and Gis1-dependent ESR genes. However, TORC1 inhibition results in a marked turnover of mRNAs (Albig and Decker 2001), and, as noted above, in a dramatic drop in translation. Thus it would appear that increasing transcription of protein-coding genes in TORC1-inhibited cells would be futile as these mRNA would likely be degraded before ever being translated. This appears not to be the case as Rim15 phosphorylates Igo1 and its paralog Igo2, allowing them to associate with newly transcribed Msn2/4- and Gis1-regulated mRNAs to protect these transcripts from degradation via the 5′-3′ mRNA decay pathway (Talarek *et al.* 2010; Luo *et al.* 2011).

Nutrient uptake and intermediary metabolism: To best compete with other microbes in their environment, yeast have optimized the use of available nutrients to accommodate fast growth (De Virgilio and Loewith 2006). Although a wide variety of compounds can be utilized as carbon or nitrogen sources, yeast cells will exclusively assimilate preferred nutrient sources before using nonpreferred, suboptimal ones. To attain this dietary specificity, and to respond to nutritional stress, yeast cells carefully regulate the expression and sorting of their many (>270) membrane transporters, enabling them to selectively import only the desired nutrients (Van Belle and Andre 2001). In general terms, in good growth conditions, many high-affinity, substrate-selective permeases are expressed and sorted to the plasma membrane to actively pump in nutrients that are used directly in ATP production and/or anabolism of nitrogenous compounds. Shift to poor growth conditions results in the replacement of high-affinity permeases, which are targeted to the vacuole for degradation with few low-affinity, broad-specificity permeases that facilitate uptake of a wide range of carbon and nitrogenous compounds that can be catabolized by the cell. For example, in response to nitrogen starvation, the high-affinity tryptophan-specific permease, Tat2, localized to the plasma membrane, is ubiquitinated, endocytosed, and ultimately degraded. In contrast, the general amino acid permease Gap1 is re-routed to the plasma membrane instead of to the vacuole/endosomes. Although details are still emerging, TORC1 appears to regulate such permease-sorting events primarily via Tap42-PPase and its (potentially direct) effector Npr1 (Schmidt *et al.* 1998; Beck *et al.* 1999; De Craene *et al.* 2001; Jacinto *et al.* 2001; Soetens *et al.* 2001; Breitkreutz *et al.* 2010). Npr1 is a heavily phosphorylated, seemingly fungal-specific, Ser/Thr kinase that upon TORC1 inactivation is rapidly dephosphorylated and activated (Gander *et al.* 2008). Although genetic studies clearly imply a role for Npr1 in protein-sorting events, the mechanisms of this regulation have remained elusive. It is possible that the permeases themselves are Npr1 substrates. Indeed, several nutrient and cation permeases have been identified as rapamycin-sensitive phosphoproteins (Huber *et al.* 2009; Soulard *et al.* 2010). Also identified in these phosphoproteomics studies were several α-arrestin-related proteins. These phosphoproteins function as clathrin adaptor molecules and have been implicated in mediating the sorting fates of a number of different permeases; and, one, Aly2, has recently been reported to be an Npr1 substrate (Lin *et al.* 2008; Nikko *et al.* 2008; Nikko and Pelham 2009; O'Donnell *et al.* 2010). Whether this observation is indicative of a more general trend in Npr1-meditated permease trafficking remains to be seen.

TORC1 regulates permease activity by regulating not only permease localization but also expression. This was shown in early transcriptomics experiments, which clearly demonstrated that TORC1 regulates the expression of a large number of permeases and other factors required for the assimilation of alternative nitrogenous sources (Cardenas *et al.* 1999; Hardwick *et al.* 1999; Komeili *et al.* 2000; Shamji *et al.* 2000). TORC1 regulates the expression of **n**itrogen **c**atabolite **r**epression (NCR)-sensitive genes via the Tap42-PPase branch. The proteins encoded by these genes (*e.g.*, Gap1) enable cells to import and metabolize poor nitrogen sources such as proline and allantoin. In the presence of preferred nitrogen sources such as glutamine, glutamate, or ammonia, active TORC1 promotes the association of the GATA-family transcription factor Gln3 with its cytoplasmic anchor Ure2. Mechanistically, this involves both TORC1-dependent

and TORC1-independent regulation of Gln3, and possibly of Ure2, phosphorylation (Beck and Hall 1999; Cardenas *et al.* 1999; Hardwick *et al.* 1999; Carvalho and Zheng 2003; Georis *et al.* 2009a; Tate *et al.* 2009, 2010). Two other less-characterized GATA factors, Gat1 and Dal81, also have roles in the regulation of NCR-sensitive genes (Georis *et al.* 2009b).

In addition to the NCR pathway, TORC1 also regulates the expression of amino acid permeases by modulating the activity of the SPS-sensing pathway. This pathway consists of a plasma-membrane-localized sensor of external amino acids, **S**sy1, and two downstream factors, **P**tr3 and **S**sy5 (Ljungdahl 2009). Upon activation of the pathway, Ssy5 catalyzes an endoproteolytic processing event that cleaves and releases an N-terminal regulatory domain from two transcription factors, Stp1 and Stp2, the shortened forms of which translocate to the nucleus and activate the transcription of a number of amino acid permease-encoding genes. TORC1 via Tap42-PPase modulates this pathway by promoting the stability of Stp1 and thus the ability of cells to utilize external amino acids (Shin *et al.* 2009).

In contrast to the SPS-sensing pathway that is activated by amino acids, the Gcn4 transcription factor is activated upon amino acid starvation (Hinnebusch 2005). As mentioned above, rapamycin treatment or amino acid starvation results in a rapid decline in translation initiation by triggering phosphorylation of the α-subunit of eIF2. Although eIF2α phosphorylation results in the repression of bulk translation, due to the presence of four short upstream open reading frames in its leader sequence, the mRNA encoding Gcn4 is, in contrast, preferentially translated. Subsequent accumulation of Gcn4 protein leads to the transcriptional induction of nearly all genes encoding amino acid biosynthetic enzymes.

TORC1 also regulates amino acid biosynthesis, in particular glutamine and glutamate homeostasis, via the retrograde response pathway (Komeili *et al.* 2000; Crespo and Hall 2002; Crespo *et al.* 2002; Liu and Butow 2006). This signaling pathway serves to communicate mitochondrial dysfunction to the nucleus to induce an appropriate transcriptional response. In addition to hosting the aerobic energy production machinery, mitochondria are also the sites of amino acid precursor, nucleotide, and lipid production. Signals, possibly changes in glutamate or glutamine levels, emanating from dysfunctional mitochondria impinge upon a cytosolic regulatory protein, Rtg2. Thus activated, Rtg2 antagonizes the ability of Mks1 to sequester the heterodimeric bZip/HLH transcription factor complex composed of Rtg1 and Rtg3 in the cytoplasm. Allowed to enter the nucleus, Rtg1/3 activates genes encoding enzymes required for anaplerotic reactions that resupply tri-carboxylic acid cycle intermediates that have been extracted for biosynthetic reactions. Key among these intermediates is α-ketoglutarate, the precursor of glutamate and glutamine from which all nitrogen-containing metabolites evolve (Magasanik and Kaiser 2002). Both transcriptome-profiling experiments as well as genetic studies have implicated TORC1 as a negative regulator of Rtg1/3-dependent transcription (Komeili *et al.* 2000; Shamji *et al.* 2000; Chen and Kaiser 2003). However, it is presently unclear how TORC1 influences this pathway; TORC1 inhibition could indirectly influence retrograde response signaling via alterations in metabolite levels. Alternatively, the direct association between TORC1 and Mks1 observed by the Tyers group and described above and the fact that Mks1 is a rapamycin-sensitive phosphoprotein instead suggest that TORC1 could play a much more direct role in regulating this pathway (Liu *et al.* 2003; Breitkreutz *et al.* 2010). Finally, phosphoproteomics studies suggest that TORC1 regulates intermediate metabolism by directly altering the activities of metabolic enzymes, particularly those involved in the early steps of glycolysis (Loewith 2011).

Autophagy: As described above, starved cells express a suite of stress-responsive proteins to help them negotiate hostile environmental conditions. This new synthesis requires both energy and amino acids that yeast cells obtain by inducing autophagy. Autophagy refers to a variety of mechanisms by which cytosplasmic material, including proteins and lipids, is translocated to the vacuole and catabolized. Amino acids and fatty acids thus acquired are, respectively, used to synthesize new proteins and oxidized by mitochondria to produce ATP. Mechanistically, there are two different modes of autophagy in yeast. One is microautophagy, which involves the direct transfer of cytoplasm into the vacuole via invaginations of the vacuolar membrane. The other is macroautophagy, which involves the *de novo* formation of double-membrane vesicles called autophagosomes. Autophagosomes encapsulate cytoplasm and then fuse with the vacuole. Both forms of autophagy are regulated by TORC1 (De Virgilio and Loewith 2006) although, mechanistically, macroautophagy is better understood (reviewed in Cebollero and Reggiori 2009; Nakatogawa *et al.* 2009; Inoue and Klionsky 2010).

Autophagy is conserved across eukarya, and there is much interest in understanding how macroautophagy is regulated as it has been linked to several pathologies including cancer, neurological disorders, and longevity (Yang and Klionsky 2010). In yeast, many **au**toph**ag**y-related (*ATG*) genes encode proteins that participate in the induction of autophagy, the nucleation of the autophagosome, elongation and completion of the autophagosome, and, finally, in fusion of the autophagosome with the vacuole to release the autolysosome into the vacuolar lumen (Chen and Klionsky 2011; Reiter *et al.* 2011). TORC1 regulates macroautophagy by signaling to the Atg1 kinase complex that is required for the induction of macroautophagy. Specifically, when TORC1 is active, Atg13 is hyperphosphorylated, presumably directly by TORC1 (although Tap42-PPase has also been implicated in this regulation), and this prevents the association of Atg13 with Atg1, Atg17, Atg31, and Atg29 (Yorimitsu *et al.* 2009; Kamada *et al.* 2010). Inhibition of TORC1 results in dephosphorylation of Atg13, assembly of the Atg1 protein kinase complex, phosphorylation and

activation of Atg1 (Kijanska *et al.* 2010; Yeh *et al.* 2010), and, subsequently, macroautophagy mediated by as-yet-unidentified Atg1 substrates. Although metazoan homologs exist for many of the Atg1 kinase complex components, a unifying model of how TORC1 regulates this complex in different species has yet to emerge (Chen and Klionsky 2011; Reiter *et al.* 2011).

Cell-wall integrity pathway: The cell wall is essential for yeast cells to survive hostile environments and, more importantly, to prevent internal turgor pressure from rupturing the plasma membrane. Although a thickening of the cell wall helps protect stressed or stationary-phase cells, this rigid structure must also be remodelled to accommodate cell growth. Homeostasis of this structure is maintained by the cell-wall integrity (CWI) pathway (Levin 2005). Cell-wall integrity is monitored by WSC (cell-wall integrity and stress response component) family proteins. WSCs, which are integral plasma membrane proteins, function upstream of the Rho1 GTPase by modulating the activity of the GEFs Rom1 and Rom2. $Rho1^{GTP}$ has several effectors including the yeast protein kinase C homolog, Pkc1. The best-characterized Pkc1 effector is a mitogen-activated protein kinase (MAPK) cascade composed of Bck1 (a MAPKKK), Mkk1 and -2 (redundant MAPKKs), and Slt2/Mpk1 (a MAPK). Activation of this pathway leads to the expression of many cell-wall biosynthetic enzymes, which helps to remodel the cell wall both during normal growth and in response to stress.

Both TORC1 and TORC2 (discussed below) appear to impinge upon the CWI pathway. Entry into stationary phase, carbon starvation, nitrogen starvation, and rapamycin treatment all elicit activation of the CWI pathway, demonstrating that TORC1 negatively regulates the CWI pathway (Ai *et al.* 2002; Krause and Gray 2002; Torres *et al.* 2002; Reinke *et al.* 2004; Araki *et al.* 2005; Soulard *et al.* 2010). Furthermore, *pkc1*, *bck1*, and *mpk1* mutants rapidly lose viability upon carbon or nitrogen starvation, demonstrating that the CWI pathway is required for viability in G0. Mechanistically, how TORC1 signals impinge on the CWI pathway is not clear. Soulard *et al.* (2010) have implicated the Sch9 effector branch while Torres *et al.* (2002) have postulated that signals through the Tap42-PPase branch causes membrane stress that, via WSC family members, activates downstream components of the CWI pathway.

TORC1 accelerates aging: Arguably one of the most interesting functions of TORC1 is its involvement in the regulation of life span. It is well established that, in virtually every biological system, aging, *i.e.*, the progressive deterioration of cell, tissue, and organ function, can be delayed through calorie or dietary restriction. Epistasis studies have led many to believe that this is due to reduced TORC1 signaling (reviewed in Weindruch and Walford 1988; Kapahi *et al.* 2010; Zoncu *et al.* 2010, 2011; Kaeberlein and Kennedy 2011). Indeed, genetic or chemical targeting of TORC1 has been demonstrated to increase life span in yeast, worms, flies, and mice (Vellai *et al.* 2003; Jia *et al.* 2004; Kapahi *et al.* 2004; Wanke *et al.* 2008; Harrison *et al.* 2009; Bjedov *et al.* 2010). These observations have created much excitement in that aging is now thought of as a disease, which, like other diseases, can be ameliorated through pharmaceutical intervention. These observations have also raised the important question, what are the downstream function(s) of TORC1 that modulate life span? The answer to this question is presently unclear, and it is very likely that multiple TORC1 effector pathways contribute (Blagosklonny and Hall 2009). Studies in many model systems are presently underway to address this issue. Below are some of the highlights from studies in yeast.

Yeast life span is assayed in one of two ways. Replicative life span (RLS) is a measure of the number of progeny that a single mother cell can produce before senescence. Chronological life span (CLS) is a measure of the length of time a population of yeast cells can remain in stationary phase before they lose the ability to restart growth following reinoculation into fresh media. RLS is thought to be a paradigm for aging of mitotic cells while CLS is thought to be a paradigm for aging of quiescent cells. Consistent with bigger eukaryotes, where newborns are obviously born young, gametogenesis (*i.e.*, cells derived from meiotic cell divisions) resets RLS in yeast (Unal *et al.* 2011).

Kaeberlein *et al.* (2005) have recently attempted labor-intensive approaches to identify genes involved in both replicative and chronological life span. A random screen of 564 yeast strains, each lacking a single nonessential gene, implicated both *TOR1* and *SCH9* in RLS downstream of caloric restriction. Also identified in this screen were a number of genes encoding ribosomal proteins. Further analyses of *RP* genes subsequently demonstrated that specific depletion of 60S ribosomal protein subunits extends RLS (Steffen *et al.* 2008). Curiously, RLS extension observed upon TORC1 inhibition and 60S subunit depletion seems to be mediated by Gcn4, the TORC1-dependent transcription factor that regulates the expression of amino acid biosynthetic genes as described above. The relevant Gcn4 target genes/processes involved in RLS are not yet known, but an interesting candidate could be macroautophagy. Induction of macroautophagy, like TORC1 and Sch9 inhibition, increases both RLS and CLS (Madeo *et al.* 2010a,b; Morselli *et al.* 2011; and see below), and Gcn4 is required for amino acid-starvation-induced macroautophagy (Ecker *et al.* 2010). Furthermore, spermidine, a potent inducer of macroautophagy, potentially via Gcn4 (Teixeira *et al.* 2010), appears to promote longevity not only in yeast but also in several other model organisms (Eisenberg *et al.* 2009). Since TORC1, Sch9, and Gcn4 homologs are found in most eukaryotes, this appears to represent a conserved aging pathway (Kaeberlein and Kennedy 2011).

Sch9 was one of the first genes to be implicated in CLS (Fabrizio *et al.* 2001). A subsequent high-throughput assay involving 4800 viable single-gene yeast mutants further implicated TORC1 in CLS (Powers *et al.* 2006). These and other studies (Wanke *et al.* 2008; Wei *et al.* 2008) provided evidence that reduced TORC1-Sch9-signaling activity promotes life span by increasing the Rim15-dependent expression of environmental

stress-response genes (described above). Later, Burtner *et al.* (2009) demonstrated that acetic acid-induced mortality is the primary mechanism of chronological aging in yeast under standard conditions and that this toxicity is better tolerated when environmental stress-response genes are artificially induced, for example, upon inhibition of TORC1 or Sch9 activities. However, this model is not universally accepted. Pan *et al.* (2011) have proposed that TORC1 inhibition leads to increased mitochondrial function and a consequent increase in reactive oxygen species that elicit a Rim15-independent pro-survival signal. Furthermore, acetic acid accumulation appears not to be a contributing factor in CLS in this study. Given its apparent conservation across eukarya (Blagosklonny and Hall 2009), elucidation of the mechanisms by which TORC1 regulates life span is eagerly awaited.

Less-characterized effectors identified in phosphoproteomic studies: As alluded to above, large-scale mass spectrometry-based phosphoproteomic studies have recently been performed to identify the rapamycin-sensitive phosphoproteome (Huber *et al.* 2009; Soulard *et al.* 2010). The major limitation of these studies was their poor coverage as evidenced by their rather modest overlap, although this could be partly explained by the different growth conditions and technical approaches employed. Rapamycin exposure times were chosen such that layers of signaling events (*e.g.*, kinase/phosphatase cascades) would be observed. These events should have been triggered as a direct consequence of TORC1 inhibition and not as a secondary consequence of cell cycle delays or changes in transcription. Hundreds of rapamycin-sensitive phosphorylation sites were mapped, the majority of which are in proteins not previously implicated in TORC1 signaling. However, as sufficient time elapsed to activate entire signaling cascades, a potential TORC1 consensus target motif was not evident from the data analyses. Still, the data from these studies will be instrumental in both elucidating how TORC1 signals to its known distal readouts and discovering new TORC1 functions.

TOR Complex 2

Composition and localization of TOR complex 2

TOR complex 2 (TORC2) is rapamycin insensitive and consists of TOR2, Avo1, Avo2, Avo3, Bit61 (and/or its paralog Bit2), and Lst8 (Loewith *et al.* 2002; Wedaman *et al.* 2003; Reinke *et al.* 2004; Zinzalla *et al.* 2010) (Figure 1C). The names of mammalian and invertebrate orthologs of TORC2 subunits and the salient features of *S. cerevisiae* TORC2 subunits are summarized in Table 1 and Table 4, respectively. The highly conserved, essential core subunits are TOR2, Avo1, Avo3, and Lst8. Avo1 and Avo3 bind cooperatively to the N-terminal HEAT repeat region in TOR2 and are required for TORC2 integrity (Wullschleger *et al.* 2005). TORC2 autophosphorylates sites in Avo1 and Avo3, but the purpose of this phosphorylation is unknown. Avo1 has a C-terminal PH-like domain that mediates binding to the plasma membrane (Berchtold and Walther 2009). Avo3 has a RasGEFN domain, a subdomain often found in the N-terminal part of a larger GDP/GTP exchange domain of exchange factors for Ras-like GTPases, but the function of the RasGEFN domain is unknown. Lst8 binds to the kinase domain in TOR2 and is required for TOR2 kinase activity (Wullschleger *et al.* 2005). Lst8 is a Gβ-like propeller protein consisting of seven WD40 motifs. TORC2 is rapamycin insensitive whereas TORC1 is rapamycin sensitive because FKBP-rapamycin binds only TORC1 (Loewith *et al.* 2002). This selective FKBP-rapamycin binding is presumably due to Avo1 masking the FRB domain in TOR2 in TORC2. Finally, co-immunoprecipitation and gel filtration experiments suggest that TORC2 is a multimer, likely a TORC2-TORC2 dimer (Wullschleger *et al.* 2005).

The cellular localization of TORC2 has been studied by subcellular fractionation, indirect immunofluorescence, immunogold electron microscopy, and visualization of GFP-tagged TORC2 components (Kunz *et al.* 2000; Wedaman *et al.* 2003; Aronova *et al.* 2007; Sturgill *et al.* 2008; Berchtold and Walther 2009). In considering these studies, it is important to realize that the vast majority of TOR2 (~90%) is in TORC2 (*vs.* TORC1), and thus TOR2 localization studies presumably detect mainly, if not exclusively, TORC2. All studies indicate that TORC2 is at or near the plasma membrane. Berchtold and Walther (2009) suggest that TORC2 is dynamically localized to a previously unrecognized plasma membrane domain termed the MCT (**m**embrane **c**ompartment containing **T**ORC2). Furthermore, they conclude that TORC2 plasma membrane localization is essential for viability and is mediated by the C-terminal PH domain in Avo1. Most of the localization studies have found that TORC2 is also at another, ill-defined cellular location(s). For example, Kunz *et al.*

Table 4 Salient features of TORC2 components

Protein	Size	Motifs/domains	Potential function
Tor2	2470 aa	HEAT repeats, FAT domain, FRB domain, kinase domain, and FATC domain	Protein kinase, scaffold
Avo1	1176 aa	PH	Recruit TORC2 to plasma membrane
Avo2	426 aa	None obvious	Unknown
Avo3/Tsc11	1430 aa	RasGEFN	Scaffold
Bit61	543 aa	None obvious	Paralogs with unknown function
Bit2	545 aa	None obvious	Paralogs with unknown function
Lst8	303 aa	7 WD-40 repeats	Stabilize kinase domain

Data for this table were obtained from Cybulski and Hall (2009).

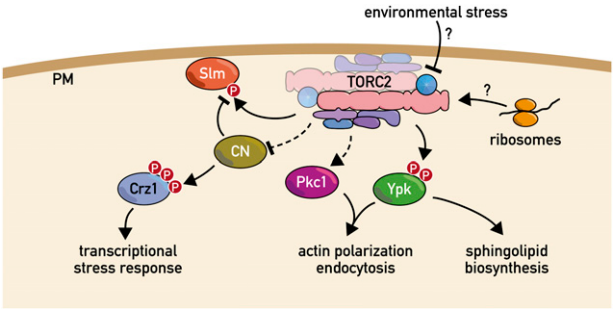

Figure 4 Signaling by TORC2. TORC2 directly phosphorylates the AGC kinase family member Ypk (Ypk1 and 2) and the PH domain containing protein Slm (Slm1 and -2). Downstream effectors include the phosphatase calcineurin, the transcription factor Crz1, and Pkc1. TORC2 controls organization of the actin cytoskeleton, endocytosis, sphingolipid biosynthesis, and stress-related transcription. The effector pathways by which TORC2 controls these processes are incompletely understood (see *Distal readouts downstream of TORC2* for further details).

(2000) report that part of TOR2 is also in an unknown subcellular membrane fraction distinct from Golgi, vacuoles, mitochondria, and the nucleus. Wedaman *et al.* (2003) showed that TOR2 can be in the cell interior often in association with membrane tracks. Sturgill *et al.* (2008) detected a cytoplasmic fluorescent signal in cells expressing GFP-tagged TOR2. In conclusion, TORC2 appears to be at multiple cellular locations, the plasma membrane, and one or possibly more other sites. A plasma membrane location is consistent with the role of TORC2 in controlling the actin cytoskeleton and endocytosis (see below).

Upstream of TORC2

The upstream regulation of TORC2 is poorly characterized (Cybulski and Hall 2009). Several lines of evidence in many different organisms indicate that nutrients regulate TORC1 (see above). On the other hand, there is no reported evidence supporting the notion that TORC2 is controlled by nutrients. Knockout of TORC2 does not confer a starvation-like phenotype, and the nutrient-sensitive EGO complex appears not to be upstream of TORC2. Zinzalla *et al.* (2011) recently devised a "reverse" suppressor screen to identify upstream regulators of TORC2. This screen was based on the observation that constitutively active Ypk2 (Ypk2*) suppresses the loss of viability due to a TORC2 defect. Ypk2 is a protein kinase normally phosphorylated and activated by TORC2 (see below). Zinzalla *et al.* (2011) screened for mutants that require Ypk2* for viability. As predicted, this screen isolated several mutants defective in genes encoding essential TORC2 components, but also in the gene *NIP7*. Subsequent experiments confirmed that Nip7, a ribosome maturation factor, is required for TORC2 kinase activity. The role of Nip7 in the activation of yeast TORC2 has so far not been pursued further, but experiments in mammalian cells suggest that mNip7 is required for mTORC2 activation indirectly via its role in ribosome maturation. In mammalian cells, and presumably also in yeast cells, TORC2 is activated by direct association with the ribosome. As ribosomes determine the growth capacity of a cell, this mechanism ensures that TORC2 is active only in growing cells.

There are also indications that environmental stress inhibits TORC2 signaling, possibly to prevent growth in unfavorable conditions. The mechanism of this regulation and the level at which it intersects with the TORC2 pathway are poorly defined, but it may involve the Slm proteins (see below) and the stress-activated phosphatase calcineurin (Bultynck *et al.* 2006; Mulet *et al.* 2006).

TORC2 substrates

The best-characterized and possibly the major TORC2 substrate is the protein kinase Ypk. Ypk1 and Ypk2 are an essential pair of homologous kinases and members of the AGC kinase family (Roelants *et al.* 2004) (Figure 4). Kamada *et al.* (2005) linked Ypk to TORC2 signaling upon isolating *YPK2* as a multicopy suppressor of a TORC2 defect. They then showed that immunopurified TOR2 directly phosphorylates Ypk2 at Ser641 in the turn motif and Thr659 in the hydrophobic motif. TORC2 phosphorylates and activates Gad8 and SGK1, the *S. pombe* and mammalian orthologs of Ypk, respectively, in a similar manner (Matsuo *et al.* 2003; Garcia-Martinez and Alessi 2008). It is well established that TORC1 or TORC2 phosphorylates the turn and hydrophobic motifs in several kinases as a conserved mechanism of activation of AGC kinase family members (see above) (Jacinto and Lorberg 2008). Ypk/Gad8/SGK1 appears to be a major TORC2 substrate as an *ypk*, *gad8*, or *sgk1* mutation phenocopies a TORC2 defect, and overexpression of Ypk2, Gad8, or SGK1 is sufficient to suppress a TORC2 defect in *S. cerevisiae*, *S. pombe*, or *Caenorhabditis elegans*, respectively (Matsuo *et al.* 2003; Kamada *et al.* 2005; Jones *et al.* 2009; Soukas *et al.* 2009). The two homologous, TORC2- and phosphoinositide (PI4,5P_2)-binding proteins Slm1 and Slm2 have also been reported to be phosphorylated in a TORC2-dependent manner both *in vivo* and *in vitro* (Audhya *et al.* 2004; Fadri *et al.* 2005). However, the physiological relevance of Slm phosphorylation is unknown other than that it appears to be required for localization of Slm to the plasma membrane (Audhya *et al.* 2004; Fadri *et al.* 2005).

Distal readouts downstream of TORC2

The first described and best-characterized TORC2 readout is the actin cytoskeleton (Figure 4). TORC2 controls the cell cycle-dependent polarization of the actin cytoskeleton. As the polarized actin cytoskeleton directs the secretory pathway and thereby newly made protein and lipid to the growing daughter bud, this is a mechanism by which TORC2 mediates spatial control of cell growth. The first indication that TOR2 is linked to the actin cytoskeleton came from the isolation of *TCP20*, which encodes an actin-specific chaperone, as a dosage suppressor of a dominant-negative *TOR2* "kinase-dead" mutation (Schmidt *et al.* 1996). This, in turn, led to the discovery that *tor2* mutants display an actin

organization defect (Schmidt *et al.* 1996). The subsequent isolation of *sac7*, which encodes a Rho-GAP (GTPase-activating protein), as a second-site suppressor of a *tor2*-temperature-sensitive (ts) mutation suggested that TOR2 is linked to the actin cytoskeleton via a signaling pathway containing a Rho GTPase. It was later demonstrated that Sac7 is indeed a GAP for Rho1 and that TOR2 activates the Rho1 GTPase switch via the Rho1-GEF Rom2 (Schmidt *et al.* 1997; Bickle *et al.* 1998). Rom2 GEF activity is reduced in extracts from a *tor2*-ts mutant (Schmidt *et al.* 1997; Bickle *et al.* 1998). The finding that overexpression of Rom2 suppresses a *tor2*-ts mutation, whereas overexpression of catalytically active Rom2 lacking its lipid-binding PH domain does not suppress, suggested that TOR2 signals to Rom2 via the PH domain. It was subsequently shown that TOR2 signals to the actin cytoskeleton mainly, if not exclusively, via the Rho1 effector Pkc1 (protein kinase C) and the Pkc1-controlled cell-wall integrity MAP kinase cascade (Helliwell *et al.* 1998b).

How might TORC2 signal to Rom2 to activate the Rho1 GTPase switch? The PH domain in Rom2 suggests that it may involve a lipid intermediate. This possibility is supported by the observation that overexpression of the PI-4-P 5-kinase Mss4 suppresses a *tor2*-ts mutation (Desrivieres *et al.* 1998; Helliwell *et al.* 1998a) and that $PI4,5P_2$ at the plasma membrane is required to recruit/activate Rom2 (Audhya and Emr 2002). The mechanism by which TORC2 may activate $PI4,5P_2$ signaling or possibly a parallel pathway converging on the cell-wall integrity pathway is unknown, but likely involves the well-established TORC2 substrate Ypk (Roelants *et al.* 2002; Schmelzle *et al.* 2002; Kamada *et al.* 2005; Mulet *et al.* 2006). The phosphoinositide-binding Slm proteins and sphingolipids may also be functionally related to TORC2-mediated control of the actin cytoskeleton (Sun *et al.* 2000; Friant *et al.* 2001; Roelants *et al.* 2002; Audhya *et al.* 2004; Fadri *et al.* 2005; Liu *et al.* 2005; Tabuchi *et al.* 2006; Daquinag *et al.* 2007).

A second downstream process controlled by TORC2 is endocytosis. Efficient internalization of cell-surface components is an important aspect of cell growth control. deHart *et al.* (2003) identified a *tor2* mutation in a screen for mutants defective in ligand-stimulated internalization of a cell-surface receptor. TORC2 appears to control endocytosis via Rho1, Ypk1, and possibly the Slm proteins, but how Rho1, Ypk1, and the Slm proteins are functionally related in mediating TORC2-controlled endocytosis is unknown (deHart *et al.* 2002, 2003; Bultynck *et al.* 2006).

A third TORC2-regulated process is sphingolipid biosynthesis (Powers *et al.* 2010). Sphingolipids serve as essential structural components in lipid bilayers and as signaling molecules. The first indication that TORC2 controls sphingolipid synthesis was the finding that overexpression of *SUR1* suppresses a temperature-sensitive *tor2* mutation (Helliwell *et al.* 1998a). In a parallel study, Beeler *et al.* (1998) reported that a mutation in *TOR2* or *AVO3* (also known as TSC11), or mutations in genes encoding components of the sphingolipid biosynthetic pathway, suppress a *csg2* mutation. Sur1/Csg1 and Csg2 are subunits, probably the catalytic and regulatory subunits, respectively, of mannosylinositol phosphorylceramide synthase that mediates a late step in sphingolipid biosynthesis. The Slm proteins were subsequently also linked to sphingolipid metabolism (Tabuchi *et al.* 2006; Daquinag *et al.* 2007). Most recently, Aronova *et al.* (2008) profiled sphingolipids in a conditional *avo3* mutant and thereby confirmed that TORC2 plays a positive role in sphingolipid biosynthesis. Aronova *et al.* (2008) also investigated the molecular mechanism by which TORC2 controls sphingolipids. They found that TORC2 regulates sphingolipid production via Ypk2 and suggest a model wherein TORC2 signaling is coupled to sphingoid long-chain bases (early intermediates in sphingolipid synthesis) to control Ypk2 and late steps in sphingolipid synthesis. Furthermore, the biosynthetic step controlled by TORC2 and Ypk2 is antagonized by the phosphatase calcineurin that is functionally linked to the Slm proteins (Bultynck *et al.* 2006; Mulet *et al.* 2006; Aronova *et al.* 2008). Another potential target for the regulation of sphingolipid biosynthesis by TOR are the Orm1 and Orm2 proteins. The conserved Orm proteins, identified as a potential risk factor for childhood asthma, form a complex that negatively regulates the first and rate-limiting step in sphingolipid biosynthesis (Breslow *et al.* 2010; Han *et al.* 2010). Both Orm1 and Orm2 are phosphoproteins and at least Orm1 phosphorylation changes upon rapamycin treatment (Huber *et al.* 2009; Soulard *et al.* 2010). Furthermore, loss of Orm2 suppresses a Ypk deficiency (Roelants *et al.* 2002; Schmelzle *et al.* 2002; Kamada *et al.* 2005; Mulet *et al.* 2006). These findings suggest that both TORC1 and TORC2 may control sphingolipid synthesis via Orm proteins.

Future Directions

What is upstream of the two complexes?

How TORC activities are altered in response to environmental cues remains a major void in our understanding of the TOR-signaling network. The TOR complexes are regulated by nutrients, stress, or ribosomes, but the mechanisms by which these inputs are sensed and how this information is transduced, with the notable exceptions discussed above, to ultimately regulate kinase activity remain largely unknown. Genetic screens, such as the reverse suppressor screen described above, should help to further elucidate these signaling pathways. Unlike growth factor-signaling pathways, which are present only in metazoans, nutrient and stress-responsive pathways are found in all eukaryotic cells, and thus their characterization in model organisms would have far-reaching implications.

What is downstream of the TORCs?

The TORCs play a central role in the regulation of cell growth by signaling to a staggering number of distal

downstream processes. Recent phosphoproteomics studies have begun to illuminate the relevant phosphorylation cascades and, in addition, have suggested the existence of novel growth-related effectors downstream of TORC1. Similar studies describing the TORC2-dependent phosphoproteome are eagerly anticipated. Elucidating these downstream signaling events is both academically interesting and medically important; cell growth, like cell birth (division) and cell death, is a fundamental aspect of life, and pathological or pharmaceutical dysregulation of TOR pathways is clinically relevant. For example, unbridled ribosome biogenesis has been strongly implicated in cancer, and the motivation to understand the TORC1 effectors that modulate longevity is obvious. Thus, characterization of TOR pathways in yeast and mammals will identify potentially druggable factors whose targeting could yield therapeutic gain in any of several pathologies.

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JB Review

Evolutionarily conserved regulation of TOR signalling

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Terunao Takahara[1,2] and Tatsuya Maeda[1,*]

[1]Institute of Molecular and Cellular Biosciences, The University of Tokyo; and [2]Graduate School of Bioagricultural Sciences, Nagoya University

*Tatsuya Maeda, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Tel: +81-3-5841-7820, Fax: +81-3-5841-7899, email: maeda@iam.u-tokyo.ac.jp

The target of rapamycin (TOR) is an evolutionarily conserved protein kinase that regulates cell growth in response to various environmental as well as intracellular cues through the formation of 2 distinct TOR complexes (TORC), TORC1 and TORC2. Dysregulation of TORC1 and TORC2 activity is closely associated with various diseases, including diabetes, cancer and neurodegenerative disorders. Over the past few years, new regulatory mechanisms of TORC1 and TORC2 activity have been elucidated. Furthermore, recent advances in the study of TOR inhibitors have revealed previously unrecognized cellular functions of TORC1. In this review, we briefly summarize the current understanding of the evolutionarily conserved TOR signalling from upstream regulators to downstream events.

Keywords: amino acid/cell growth/stress granule/target of rapamycin/TOR complex.

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; CaM, calmodulin; Deptor, the DEP domain-containing mTOR-interacting protein; FKBP12, 12-kDa FK506-binding protein; FRB, FKBP12-rapamycin binding; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; hVps34, human vacuolar protein sorting 34 homologue; α-KG, α-ketogluatrate; LRS, leucyl-tRNA synthetase; mTOR, mammalian (or mechanistic) target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PIKK, phosphatidylinositol 3-kinase-related kinase; PKC, protein kinase C; PLD1, phospholipase D1; PRAS40, proline-rich Akt substrate of 40-kDa; Protor1/2, protein observed with Rictor 1 and 2; Raptor, the regulatory-associated protein of mTOR; Rictor, the rapamycin-insensitive companion of mTOR; SH3BP4, SH3-domain binding protein 4; SL1, selectivity factor 1; S6K1, p70 ribosomal protein S6 kinase 1; TIF-IA, transcription initiation factor 1A; TOP, terminal oligopyrimidine tract; TOR, target of rapamycin; TORC, target of rapamycin complex; TSC, the tuberous sclerosis complex; UBF, upstream binding factor; v-ATPase, vacuolar H^+-ATPase; 4E-BP1, eIF4E-binding protein 1.

The Structure and Complex Formation of TOR

The target of rapamycin (TOR) was originally identified as the cellular target of the immunosuppressant and anti-cancer drug rapamycin. TOR is a conserved serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase (PIKK) family, which includes ATR, ATM and DNA-PKcs. Structurally, TOR possesses HEAT repeats at the N-terminal region and the FAT, kinase catalytic and FATC domains at the C-terminal region (Fig. 1A). Rapamycin, together with its cellular receptor 12-kDa FK506-binding protein (FKBP12), binds to the FKBP12-rapamycin binding (FRB) domain located between the FAT and kinase catalytic domains on TOR and thus allosterically inhibits the activity of TOR (*1*) (Fig. 1A). The precise mechanism of this inhibition remains unclear.

TOR exerts its function by forming two functionally and structurally distinct complexes, called TOR complex 1 (TORC1) and TORC2. Mammalian TORC1 (mTORC1) comprises of mammalian (or mechanistic) TOR (mTOR), the regulatory-associated protein of mTOR (Raptor), mammalian LST8 (mLst8, also known as GβL), the DEP domain-containing mTOR-interacting protein (Deptor), and proline-rich Akt substrate of 40-kDa (PRAS40). On the other hand, mTORC2 contains mTOR, the rapamycin-insensitive companion of mTOR (Rictor), mLst8, mSin1, Deptor and protein observed with Rictor 1 and 2 (Protor1/2) (Fig. 1B). Both mTORC1 and mTORC2, and most likely TORC1 and TORC2 in general, function as multimers (*1*). The structural components of both these TORCs are highly conserved from yeast to mammals (Table I). A prominent difference between these two complexes is their rapamycin sensitivity. TORC1 is sensitive to rapamycin treatment, whereas TORC2 is resistant to rapamycin treatment (*1*). However, mTORC2 is sensitive to long-term (>24 h) rapamycin treatment, and this sensitivity is most likely achieved through inhibition of nascent mTOR molecules from assembling with mTORC2 components such as Rictor and mSin1 (*2*). The rapamycin sensitivity of mTORC2 is cell-type dependent, and the factors that bring about this difference are unknown.

Although it was believed that rapamycin inhibits TORC1 activity completely, development of newer ATP-competitive mTOR inhibitors (Torin1, Ku-0063794, PP242 *etc.*), which inhibit mTOR kinase activity by competing with ATP for binding to the kinase catalytic site (Fig. 1A), revealed that rapamycin only partially inhibits mTORC1 activity (*3*). In fact, even

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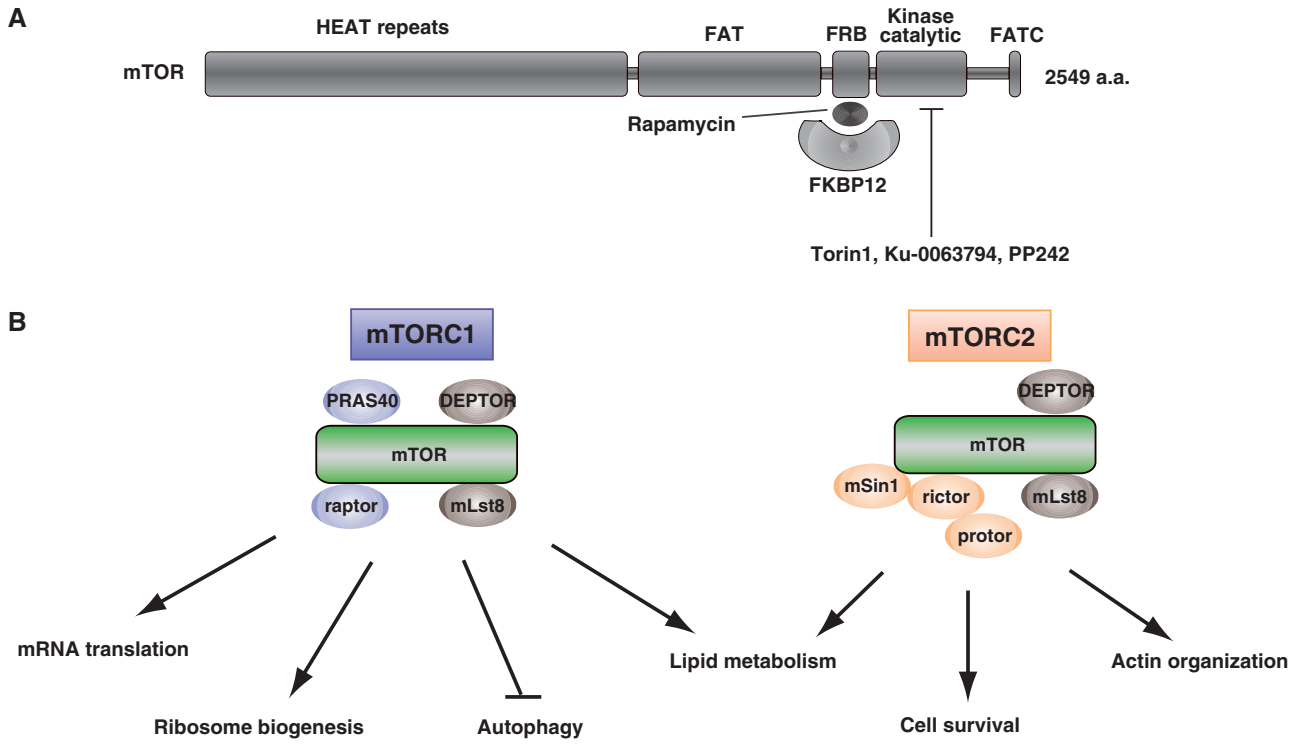

Fig. 1 Schematic representation of the structure and complex formation of mTOR. (**A**) The domain structure of mTOR. Rapamycin, together with FKBP12, binds to the FRB domain of mTOR, and inhibits mTOR (mTORC1) alosterically. ATP-competitive inhibitors (Torin1, Ku-0063794, PP242) directly inhibit kinase activity. FAT and FATC domains are conserved in PIKK family members. (**B**) Two distinct mTOR complexes (mTORC1 and mTORC2) and their functions. Specific components of mTORC1 and mTORC2 are shown in colour.

Table I. The components of TORC1 and TORC2.

	H. sapiens	*S. cerevisiae*	*S. pombe*	*D. melanogaster*	*C. elegans*
TORC1	mTOR	Tor1 (Tor2)	Tor2 (Tor1)	TOR1	TOR
	Raptor	Kog1	Mip1	Raptor	daf-15
	mLst8	Lst8	Wat1/Pop3	Lst8	lst-8
	PRAS40			Lobe	
	DEPTOR				
		Tco89	Tco89		
			Tco1		
TORC2	mTOR	Tor2	Tor1	TOR	TOR
	Rictor	Avo3/Tsc11	Ste20	Rictor	rict-1
	mSin1	Avo1	Sin1	Sin1	sinh-1
	mLst8	Lst8	Wat1/Pop3	Lst8	lst-8
	Protor/PRR5	Bit61, Bit2	Bit61		
	DEPTOR				
		Avo2			

the phosphorylation status of eIF4E-binding protein 1 (4E-BP1), one of the best-characterized substrates of mTORC1, is refractory to inhibition by rapamycin, but is sensitive to the ATP-competitive inhibitors of mTOR, indicating that mTORC1 regulates far more events than previously believed (*3*). Discovery of the two TOR complexes (TORC) and rapamycin-insensitive TORC1 activity expands the list of known cellular functions of TOR.

Regulation of TORC1 Activity

Multiple inputs such as nutrients, energy, growth factors and stress converge on TORC1 to coordinately regulate cellular responses to these environmental cues (*1*, *4*) (Fig. 2). Among these, nutrients, especially amino acids (or nitrogen source for yeast), are the most fundamental inputs affecting TORC1 activity throughout evolution. In multicellular organisms, growth factors further fine-tune the regulation of TORC1 activity to achieve appropriate activation status according to the demand of cell growth and overall body growth.

The mechanism of how growth factors (e.g. insulin) signal mTORC1 regulation has been studied extensively. There are at least two mechanisms leading to activation of mTORC1: the tuberous sclerosis complex (TSC)-dependent and TSC-independent pathways (*4*). TSC1 (also known as hamartin) and TSC2 (also

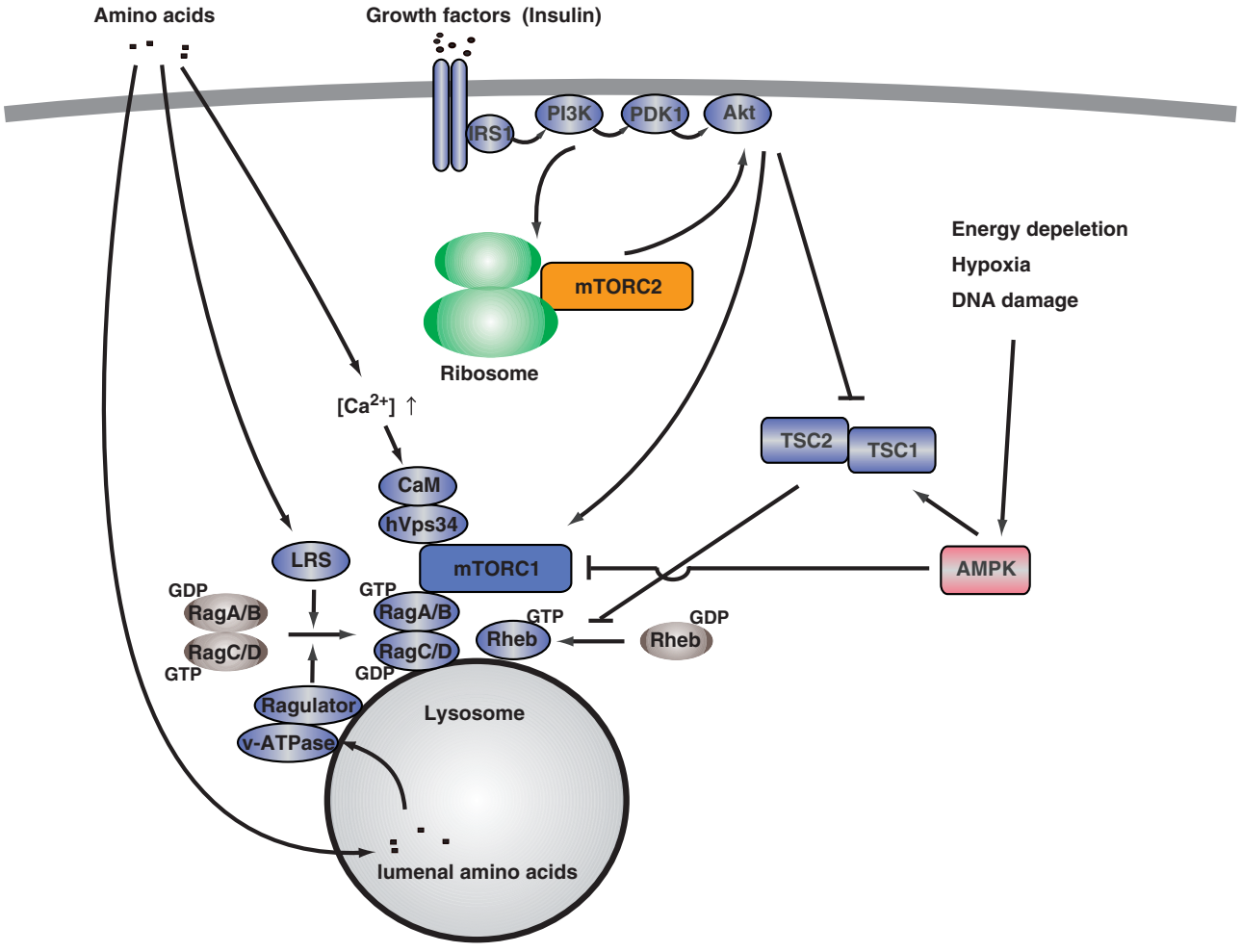

Fig. 2 mTOR signalling pathway. mTORC1 is activated by growth factors and amino acids and repressed by stressful conditions such as energy depletion, hypoxia and DNA damage. mTORC2 is activated by growth factors through association with ribosomes. Only key upstream regulators of mTORC1 and mTORC2 are shown.

known as tuberin) are gene products whose inactivation causes tuberous sclerosis. The TSC1/TSC2 complex acts as a GTPase activating protein (GAP) for the small GTPase Rheb (*1*, *4*). The active GTP-bound Rheb directly binds to mTORC1 and enhances its kinase activity. Rheb also acts as a scaffold and mediates the binding of mTORC1 to its substrates (*5*). Stimulation by growth factors such as insulin leads to activation of the PI3K-Akt pathway, and then activated Akt phosphorylates and inhibits the GAP activity of TSC2, leading to activation of Rheb. Akt phosphorylation of TSC2 also causes sequestration of TSC2 through binding to 14-3-3 protein. In the TSC1/2-independent pathway of mTORC1 activation, upon stimulation by growth factors, Akt phosphorylates and inactivates PRAS40, the inhibitory component of mTORC1, leading to mTORC1 activation (*6*).

Amino acids (especially leucine) or nitrogen sources are essential activators of the TORC1 pathway throughout evolution. However, how this amino acid signal activates TORC1 was a long-standing mystery in the TOR field. Over the past few years, there have been considerable advances in our understanding of the amino acid-induced activation of mTORC1. The identification of Rag, a family of small GTPases, was a great step towards understanding regulation of mTORC1 activation by the amino acids (*7*). The Rag GTPase family consists of four proteins Rag A, B, C and D. They have a unique property, in that they function as heterodimers composed of two highly similar groups of proteins: RagA or RagB forms heterodimers with either RagC or RagD. Activation of mTORC1 by amino acids is regulated by GTP–GDP exchange of these proteins in a reciprocal manner (Fig. 2). The presence of amino acids leads to heterodimer formation between GTP-bound RagA/B and GDP-bound RagC/D, whereas deprivation of amino acids results in the formation of GDP-bound RagA/B and GTP-bound RagC/D (*7*). Rag GTPases localize to the surface of the lysosome through association with a heteropenta protein complex called Ragulator (*8*). The essential role of the Rag GTPases is to recruit mTORC1 to the surface of lysosome through their ability to bind to Raptor when they are in the activated heterodimer form, *i.e.* GTP-bound RagA/B-GDP-bound RagC/D (Fig. 2). Upon being recruited to the surface of the lysosome, mTORC1 is proposed to be activated by association with Rheb that resides on the lysosome (*7*). Thus, amino acid signalling mainly operates through the spatial regulation of mTORC1 in

the cells. The importance of Rag GTPases in the TORC1 activation in response to amino acids seems essentially conserved throughout evolution (*9*, *10*), although the amino acid-dependent redistribution of yeast TORC1 has not been observed.

Recent reports by several groups suggest that multiple factors and pathways participate in the amino acid-induced activation of Rag GTPases. Sabatini's group proposed that the amino acid content inside the lysosome is the primary stimulus for activation of mTORC1 (*11*). In this scenario, the amino acids present in the lumen of the lysosome regulate the association between Rags and Ragulator through an undefined mechanism involving the activity of vacuolar H^+-ATPase (v-ATPase), and Ragulator acts as a guanine nucleotide exchange factor (GEF) for RagA and RagB, leading to activation of Rag heterodimers (Fig. 2). Recent results from both Kim's (in mammalian cells) and Virgilio's (in yeast) groups suggest an amino acid sensing mechanism in the cytoplasm through direct recognition of leucine by leucyl-tRNA synthetase (LRS) (*12*, *13*) (Fig. 2). Mammalian LRS when bound to leucine specifically acts as GAP for RagD, leading to GTP-to-GDP transition of RagD and subsequent mTORC1 activation. In yeast, LRS together with leucine binds to Gtr1, which is the ortholog of mammalian RagA/B, leading to activation of TORC1 through an unknown mechanism. Furthermore, leucine and glutamine are also reported to activate mTORC1 through glutaminolysis (*14*). Glutaminolysis converts glutamine to α-ketogluatrate (α-KG) through two sequential steps, where leucine acts as an allosteric activator in the second step. Elevated α-KG level leads to activation of mTORC1 by enhancing GDP-to-GTP transition of RagB, although the precise mechanism remains unknown. Recent reports have further revealed that SH3-domain binding protein 4 (SH3BP4) acts as an inhibitor of Rag GTPases-mTORC1 association in an amino acid-dependent manner (*15*). Amino acids promote dissociation of SH3BP4 from Rag GTPases, which allows subsequent binding of mTORC1 to Rag GTPases and mTORC1 activation. In addition, MAP4K3 also appears to act upstream of Rag GTPases in the amino acid sensing pathway (*16*).

Although amino acid sensing through the Rag GTPase-mTORC1 axis is a key regulatory mechanism in the mTORC1 pathway not only in cultured cells but also in the physiological setting (*17*), another pathway seems to activate mTORC1 in response to amino acids. It is known that human vacuolar protein sorting 34 homologue (hVps34), a class III PI3K, mediates amino acid signalling through mTORC1 (*18*). Amino acid stimulation elevates intracellular Ca^{2+} levels, which is recognized by calmodulin (CaM). Ca^{2+}-bound CaM binds to hVps34, which in turn directly binds to mTORC1 (Fig. 2). Consistent with this finding, a recent study also demonstrated that increase of intracellular Ca^{2+} level during physical overload activates mTORC1 and promotes protein synthesis, leading to skeletal muscle hypertrophy (*19*). mTORC1 activation by hVps34 requires phosphatidylinositol 3-phosphate (PI3P), the lipid product of hVps34. Interestingly, phospholipase D1 (PLD1) is an effector of PI3P generated by hVps34 (*20*), and has been reported to mediate Rheb action towards mTORC1 (*21*). Therefore, it is possible that amino acid input regulates both Rag GTPases-mediated localization of mTORC1 and hVps34-PLD1-mediated association of Rheb with mTORC1 to fully activate mTORC1 (Fig. 2).

Under many stressful conditions, TORC1 activity is repressed to stop cell growth and to shift cell physiology towards inducing cytoprotective programs (*1*, *22*). Understanding mechanisms leading to TORC1 inactivation are also important because constitutive activation of mTORC1 is implicated in the development of many diseases (*4*). In mammals, adenosine monophosphate-activated protein kinase (AMPK) and TSC1/2 inhibit mTORC1 activity under a broad array of stressful conditions including hypoxia, energy depletion and DNA damage. AMPK is activated by increase in the AMP/ATP ratio under conditions such as energy deprivation and hypoxia. Activated AMPK inhibits mTORC1 via two known mechanisms (*23*). AMPK directly phosphorylates TSC2 at Ser1387, which results in activation of its GAP activity towards Rheb and this in turn represses mTORC1. In the second mechanism, AMPK directly phosphorylates Raptor at Ser922 and Ser972, which promotes association of Raptor with 14-3-3 protein and inactivation of mTORC1 kinase activity (Fig. 2). DNA-damaging agents inhibit mTORC1 through activation of AMPK, in which Sestrin1 and Sestrin2 induced by p53 directly bind and activate AMPK without increase in the AMP/ATP ratio (*24*). p53 may also repress mTORC1 activity through transcriptionally upregulating negative regulators of mTORC1 including PTEN, AMPK and TSC2 (*25*, *26*). Furthermore, DNA damage and hypoxia inhibit mTORC1 by inducing the expression of REDD1 protein that activates TSC1/2 by dissociating 14-3-3 protein from TSC2 (*27*, *28*).

Novel mechanisms of TORC1 regulation under stressful conditions have recently emerged in both yeast and mammals (Fig. 3). Budding yeast (*Saccharomyces cerevisiae*) lacks TSC1/2, but TORC1 activity is still repressed under various stressed conditions such as heat, oxidation and cell wall damage (*29*). Recent studies have uncovered that inactivation of TORC1 under these conditions is at least in part mediated by Rho1 small GTPase (*30*, *31*). Rho1 (RhoA in mammalian cells) directly binds to TORC1 and thereby inhibits the TORC1 kinase activity both in yeast and in mammalian cells. Furthermore, stress granules, cytoplasmic aggregates composed of stalled translation initiation complexes formed under stressful conditions (*32*), play a role in the regulation of TORC1 activity both in yeast and in mammals. Under severe heat stress, yeast TORC1 dissociates from the limiting membrane of the vacuole (the lysosome counterpart in yeast) and is recruited into stress granules (*33*). Spatial sequestration of yeast TORC1 into stress granules maintains yeast TORC1 in an inactive state, which promotes cytoprotective effects. In mammalian cells, mTORC1 is also sequestered into oxidative stress- and osmotic stress-induced stress granules and

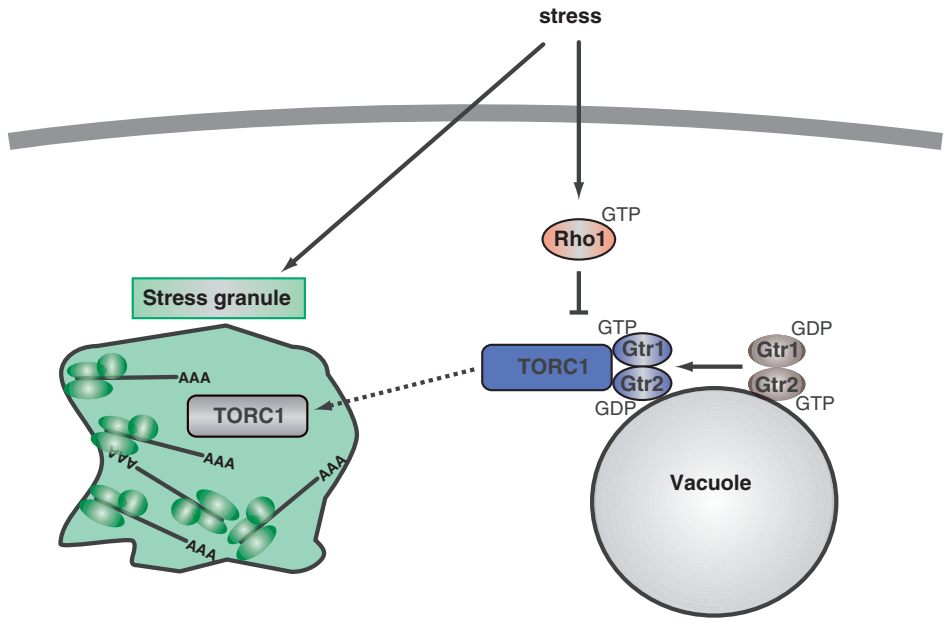

Fig. 3 Stress granule-mediated regulation of TORC1 in yeast. Upon certain stressful conditions (e.g. high temperature), Rho1 is activated, which in turn inhibits TORC1 and releases it from the vacuole. TORC1 is recruited into stress granules and is maintained in an inactive state. Relocalization of TORC1 into stress granules is also conserved in mammals.

inactivated (*34*). Further elucidation of new regulatory mechanisms of TORC1 will lead to a comprehensive understanding of complex regulation of TORC1 under various stressful conditions.

Regulation of TORC2 Activity

Less is known about the regulation of TORC2 than about TORC1 regulation. A well-known signal activating mTORC2 is growth factors such as insulin (*35*). mTORC2 may also be regulated by amino acids, depending on specific substrates, cellular contexts and/or conditions (*30–32*). Although the mechanism of mTORC2 regulation is poorly defined, ribosome has been reported to be a direct activator of mTORC2 in response to insulin (*36*) (Fig. 2).

Insulin promotes binding of the ribosome to mTORC2 and this interaction leads to activation of mTORC2 (*36*). Because mTORC1 is a primary regulator of ribosome biogenesis, activation status of mTORC1 may coordinately control mTORC2 activation to achieve appropriate cell growth. Although yeast lacks a growth factor signalling pathway, the requirement of ribosome for activation of TORC2 is likely conserved (*36*). Thus, ribosome–TORC2 association may be a key step in activation of TORC2 that is conserved throughout evolution. The localization of TORC2 in both mammalian cells and yeast appears to be on the plasma membrane (or at least on membranous structures) (*37*). Interestingly, in endothelial cells, localization of mTORC2 on the plasma membranes is regulated by syndecan-4 in a PKCα-dependent manner, and the localization is required for mTORC2 activation (*38*). It will be interesting to determine whether the plasma membrane-associated pool of ribosomes is specifically involved in the activation of TORC2.

In yeast *S. cerevisiae*, TORC2 is activated by plasma membrane stress, which is encountered during cell growth because of the enlargement of cell surface area (*37*). In this step, the PH-domain-containing plasma membrane proteins Slm1 and Slm2 act as upstream regulators of TORC2. Upon stress on the plasma membrane, such as inhibition of sphingolipid biosynthesis or mechanical stretching of plasma membrane, Slm proteins are relocalized away from specialized compartments within the plasma membrane called eisosomes, and then they associate with TORC2. Slm proteins likely play a role to facilitate TORC2 phosphorylation of Ypk1, a downstream effector, by directly recruiting Ypk1 to proximity of TORC2. The activated Ypk1 in turn promotes biosynthesis of sphingolipids, which eventually limits TORC2 activation, thereby building a feedback loop of regulation of sphingolipids homeostasis (*37*, *39*).

Another upstream regulator of TORC2 is Ryh1 in *Schizosaccharomyces pombe* (also called fission yeast). Although the stimuli that activates TORC2 in *S. pombe* is unclear, some studies have indicated that Ryh1, the ortholog of mammalian Rab6 GTPase, directly binds to TORC2 and regulates TORC2 activity (*40*). Ryh1 has been implicated in regulation of the vesicle transport between endosomes and the Golgi apparatus; although the function of Ryh1 in TORC2 activation is likely distinct from its role in the control of vesicular trafficking.

TORC1 Downstream Events

TORC1 generally regulates anabolic processes such as protein synthesis and catabolic processes such as autophagy to make an appropriate decision of cell growth in response to environmental cues. The well-characterized cellular processes that TORC1 regulates

are mRNA translation, ribosome biogenesis, lipid metabolism and autophagy (Fig. 1B).

mRNA translation

The best-characterized substrates of mTORC1 are 4E-BP1 and p70 ribosomal protein S6 kinase 1 (S6K1), both of which are involved in the regulation of mRNA translation. Very recently, it was reported that inhibition of mTORC1 activity by Torin 1, an ATP-competitive inhibitor, resulted in decrease of translation of almost all mRNAs (99.8% of 4840 transcripts examined) to some extent (median = 60.5%), suggesting the large contribution of mTORC1 in the general control of mRNA translation (*41*).

4E-BP1 is an endogenous inhibitor of eIF4E, a translation initiation factor that binds to the 5′-cap structure of mRNA, which promotes translation initiation. Phosphorylation of 4E-BP1 by mTORC1 prevents its association with eIF4E, thereby allowing cap-dependent translation initiation. The mRNAs that are most suppressed upon mTORC1 inhibition possess 5′-TOP (terminal oligopyrimidine tract), which consists of cytosine at the 5′-terminus followed by a stretch of 4–15 pyrimidines, or 5′-TOP-like sequences (*41*). How translation of these 5′-TOP mRNAs are regulated has been controversial. However, recent studies have clearly demonstrated that translation of the 5′-TOP mRNAs is largely controlled by mTORC1-mediated phosphorylation of 4E-BP1 (*41*, *42*). The 5′-TOP is mainly found in mRNAs that encode ribosomal proteins and other factors responsible for regulation of protein synthesis. Activation of mTORC1 affects overall mRNA translation not only by regulation of translation initiation through relieving eIF4E from inhibition by 4E-BP1, but also by upregulation of the translation capacity.

Phosphorylation of S6K1 by mTORC1 also promotes mRNA translation through various pathways by phosphorylation of multiple downstream effectors of S6K1 involved in mRNA translation and splicing processes, such as eIF4B, PDCD4, eEF2K and SKAR (*43*). Intriguingly, the eukaryotic translation initiation factor eIF3 complex acts as a scaffold for mTORC1 to phosphorylate S6K1 (*43*, *44*). In the absence of nutrients or growth factors, inactive S6K1 is bound to eIF3. Upon activation of mTORC1 by nutrients or growth factors, mTORC1 is recruited to the eIF3 complex and phosphorylates S6K1. Activated S6K1 dissociates from eIF3, and in turn phosphorylates many downstream effectors as mentioned earlier. It will be interesting to determine how and if the activated mTORC1 on the lysosomal surface binds to either the general or a specific pool of eIF3.

Ribosome biogenesis

Ribosome biogenesis is a fundamental process for maintaining cell growth and proliferation. It is a highly energy-consuming task requiring coordinated regulation of all three RNA polymerases (RNA polymerase I, II and III). TORC1 is known to be involved in processes associated with all three RNA polymerases.

rRNA synthesis by RNA polymerase I requires factors such as transcription initiation factor 1A (TIF-IA), selectivity factor 1 (SL1) and upstream binding factor (UBF), which recruit RNA polymerase I onto rDNA promoters. The mTORC1-S6K1 axis activates TIF-IA and UBF, promoting the association of RNA polymerase I with rDNA promoter and enhances rRNA expression (*45*). TORC1 in both yeast and mammals can be recruited to the promoter region of rDNA (*46*, *47*), indicating that mTORC1 may also regulate rRNA transcription in a more direct way.

Regulation of ribosomal protein (RP) transcription by RNA polymerase II has been elucidated in yeast. Ribosome consists of 79 different RPs, and therefore the coordinated expression and assembly of these RPs are required for efficient production of ribosomes. Yeast TORC1 regulates RP transcription and ribosome biogenesis (Ribi) regulon, the latter of which comprises a set of genes involved in ribosome synthesis and maturation, mostly through the activation of the downstream effector Sch9, the yeast ortholog of S6K1 (*48*).

tRNA and 5S RNA are synthesized by RNA polymerase III. TORC1 regulates their transcription through phosphorylation of Maf1, a repressor of RNA polymerase III. In yeast, Maf1 is mainly phosphorylated by Sch9, while it is directly phosphorylated by mTORC1 in mammals (*49*). In both systems, Maf1 phosphorylation blocks its nuclear localization, leading to induction of tRNA and 5S RNA expression.

Lipid biosynthesis

Recent studies have revealed that mTORC1 positively regulates lipid biosynthesis mainly through sterol regulatory element-binding protein-1c (SREBP-1c), a master transcription factor regulating gene required for lipids biosynthesis (*50*). Insulin stimulates nuclear accumulation of SREBP-1c by promoting processing and maturation of it's membrane-bound inactive precursor in the endoplasmic reticulum (ER), thereby leading to transcriptional activation of lipogenic genes. Although the exact mechanism by which mTORC1 could promote SREBP-1c activation has not been well established, it could occur through multiple pathways: mTORC1 activation increases SREBP-1c expression by an unknown mechanism, and promotes SREBP1 processing and nuclear accumulation, in part, through S6K1 (*4*, *50*). Moreover, a recent report has demonstrated that mTORC1 phosphorylation of lipin 1, a phosphatidic acid phosphatase, inhibits its translocation into the nucleus, allowing nuclear accumulation of SREBP-1c through an uncharacterized mechanism (*51*). A recent study has also suggested that mTORC2 activation in addition to mTORC1 activation is required for promotion of SREBP-1c-mediated lipogenesis (*52*).

Autophagy

Autophagy is a major lysosomal degradation process of cytoplasmic components and organelles, which supplies nutrients to cells enabling them to survive under conditions of nutrient deprivation (*53*). In both yeast and mammalian cells, TORC1 regulates the initial step

of autophagy induction, although the precise regulatory mechanism differs between the two systems. In yeast, TORC1 directly phosphorylates and inhibits Atg13, an initiator of autophagy. TORC1 inhibition under nutrient starvation causes dephosphorylation of Atg13, allowing association of Atg13 with the Ser/Thr kinase Atg1 and the subsequent activation of the Atg13–Atg1 kinase complex. The activated Atg13–Atg1 complex phosphorylates downstream effectors to promote formation of autophagosomes. In mammalian cells, mTORC1 regulates the UNC-51-like kinases (ULK1) complex, which consists of ULK1, Atg13, Atg101 and a 200-kDa FAK-family interacting protein (FIP200), of these, ULK1 is the mammalian ortholog of Atg1. In the nutrient-rich condition, mTORC1 directly phosphorylates ULK1 and Atg13 through binding with ULK1, and inactivates the ULK1 complex. Upon mTORC1 inhibition, mTORC1 dissociates from the ULK1 complex, and thereby activates ULK1. Activated ULK1 is thought to phosphorylate Atg13 and FIP200, which in turn induces formation of autophagosomes through unknown mechanisms (*53*).

TORC2 Downstream Events

mTORC2 phosphorylates several AGC family kinases on their hydrophobic motifs, including Akt, SGK1 and protein kinase C (PKC), through which mTORC2 regulates a variety of cellular processes such as cell survival, proliferation and actin reorganization (Fig. 1B).

AGC kinase phosphorylation

mTORC2 phosphorylates Akt1 at Ser473 within the hydrophobic motif, which is required for its full activation (*54*). This phosphorylation appears to occur on the plasma membrane, where mTORC2 resides. Although mTORC2 kinase activity is enhanced in response to growth factors, Akt1 phosphorylation at Ser473 appears to be regulated also through the recruitment of Akt1 to the plasma membrane in response to PI(*3*,*4*,*5*)P_3 production by PI3K. Indeed, hyperactive mTORC2 is unable to maintain Akt1 phosphorylation upon growth factor starvation (*55*). Such a local control of Akt1 phosphorylation, *i.e.* differential phosphorylation of distinct Akt1 fractions in the cells, might affect regulation of downstream effectors. In fact, the need for this Akt1 phosphorylation by mTORC2 differs among the downstream effectors. In mTORC2-deficient cells, where Akt1 Ser473 phosphorylation is completely abolished, FOXO1/3a phosphorylation is impaired, whereas phosphorylation of other downstream effectors such as TSC2 and GSK3β is unaffected (*56*, *57*).

mTORC2 also phosphorylates Akt1 at Thr450 within the turn motif even in the absence of growth factor. This phosphorylation is constitutive and occurs cotranslationally, which contributes to the folding and stability of Akt1 (*58*).

mTORC2 phosphorylates and activates SGK1, a kinase involved in regulating sodium transport and cell survival. SGK1 phosphorylation by mTORC2 is specifically mediated by binding of SGK1 to the mTORC2 component mSin1 and has been shown to be required for activation of sodium transport in epithelial kidney cells (*59*). The yeast counterparts of SGK1, Ypk1 and Ypk2 are also phosphorylated by TORC2 and are involved in controlling sphingolipid biosynthesis and actin organization (*37*, *60*).

Actin organization

The link between TORC2 and actin organization was first shown in yeast and later in mammalian cells (*61*). Depletion of TORC2 causes defects in actin organization. In *S. cerevisiae*, although the precise mechanism by which TORC2 regulates actin organization remains to be elucidated, several effectors such as Rho1 GTPase, Ypk2 and Slm1/2 have been shown to be involved in this process (*48*). Upon activation, Rho1 binds to Pkc1 kinase, which in turn activates the downstream cascade, leading to actin organization in yeast. TORC2 appears to activate Rho1 through its GEF Rom2 by an unknown mechanism. It is possible that changes in the lipid composition of the plasma membrane through the TORC2-Slm1/2-Ypk1/2 axis primarily affects the ability of Rom2 to bind PI(*4*,*5*)P_2 on the plasma membrane, which in turn activates the Rho1-Pkc1 pathway (*62*).

In mammals, mTORC2 appears to regulate actin organization by promoting phosphorylation of PKCα and paxillin and by activation of RhoA and Rac1 (*4*, *35*, *61*). Interestingly, Rac1 also binds to mTOR and regulates subcellular localization of mTORC1 and mTORC2 (*63*), thereby acting both upstream and downstream of mTORC2. It is proposed that Rac1 translocates to the plasma membrane upon growth factor stimulation and recruits mTORC2 and P-Rex1 (a GEF towards Rac1), resulting in local activation of Rac1 and subsequent increase of PI(*3*,*4*,*5*)P_3 synthesis through promotion of PI3K activation (*64*), leading to both actin organization and Akt activation.

Lipid biosynthesis

As described in TORC1-mediated events, lipid biosynthesis is mainly regulated by mTORC1 in mammalian cells. However, recent studies, especially in yeast, suggest that TORC2 plays an important role in the regulation of sphingolipid biosynthesis, building a feedback loop of sphingolipid biosynthesis and TORC2 activation (*37*, *39*).

Although mTORC1 regulates lipogenesis in mammalian cells, activation of mTORC1 alone was not sufficient for activation of SREBP-1c and lipogenesis in TSC1 knockout mice (*65*). Furthermore, a recent study of liver-specific Rictor knockout mice suggests that mTORC2 appears to positively regulate lipogenesis, at least in part, through Akt1-mediated activation of SREBP-1c (*52*). Importantly, SREBP-1c activation and subsequent expression of lipogenic genes are impaired in these mice without affecting mTORC1 activity, suggesting the mTORC2-specific function of lipogenesis. Thus, both mTORC1 and mTORC2 positively activate lipogenesis. Further studies are needed to elucidate the complex metabolic outcomes regulated via TORC1 and TORC2, which will provide valuable

data that can be used to develop targeted therapy for obesity and diabetes.

Conclusion

Much progress has been made in the understanding of TOR signalling during the past years, especially regarding amino acid-induced TORC1 activation. However, only a few of the vast array of functions of TOR are known, given that data revealing new links of TOR to unrecognized cellular activities are constantly emerging. Because TOR is involved in essentially all aspects of cellular activities owing to its fundamental ability to sense and respond to nutrients, the regulatory mechanisms of TOR must be tightly controlled by multiple mechanisms while allowing the necessary sharp and robust responses to even subtle or conflicting environmental conditions. Elucidation of the complex molecular mechanisms of TOR signalling through genetic, pharmacological and biochemical studies on various experimental models ranging from yeast to mammals will certainly provide beneficial knowledge.

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Conflict of Interest

None declared.

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