Characterization of TOR complex 2 (TORC2) in *Saccharomyces cerevisiae*

INAUGURALDISSERTATION

zur Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Stephan Wullschleger

aus Zofingen (AG)

Basel, Juni 2004

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag der Herren Prof. Dr. M. N. Hall und Prof. Dr. M. Spiess.

Basel, den 6. Juli 2004

Prof. Dr. M. Tanner, Dekan

Summary

The TOR proteins play a central role in the control of cell growth. TOR proteins are the founding members of the phosphatidylinositol-related kinase (PIKK) family of protein kinases. In *S. cerevisiae* there are two TOR proteins, TOR1 and TOR2. TOR1 and TOR2 regulate cell growth via a rapamycin-sensitive pathway controlling translation, transcription, nutrient uptake, ribosome biogenesis and autophagy. TOR2 also has a unique, rapamycin-insensitive function, which is the control of the actin cytoskeleton. Recently, it has been found that the TOR proteins exist in two distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2). While TORC1 mediates the rapamycin-sensitive pathway, TORC2 is responsible for the control of the actin cytoskeleton. TORC1 comprises three proteins, TOR1 or TOR2, KOG1 and LST8. TORC2 consists of five proteins, TOR2, AVO1, AVO2, AVO3 and LST8. Thus, these structurally and functionally distinct TOR complexes account for the diversity of TOR signaling in yeast. Here we focused on the characterization of TORC2. Our studies suggest that TORC2 exists in an oligomeric state and that AVO1 and likely AVO3 act as scaffold proteins required for the integrity of TORC2. We also found that AVO1 plays a role as an adaptor protein mediating efficient phosphorylation of substrates. LST8 in turn appears to modulate TOR2 kinase activity possibly by binding directly to the TOR2 kinase domain. LST8 is common to both TORC1 and TORC2 and may therefore be important to respond to upstream signaling factors.

Acknowlegments

I would like to thank Mike Hall for giving me the opportunity to work in his lab.

I would like to thank everybody of our group, Adi, Alex, Andrea, Anja, Débora, Dietmar, Estela, Helena, JM, Karsten, Klaus, Pazit, Pepelu, Ryo, Thierry, Tobias, Véronique, and the sabbatical visitors Kelly, Lucy and Takashi, for their help and the pleasant atmosphere in the lab.
Thanks go also to Paul for his help with proteomics.

Special thanks go to Robbie and Wolfgang for their great support during the last four years and of course the AVO team for stimulating discussions over beer and AVO cigars.

Many thanks go to my parents for their support during all my studies.

Table of contents

Summary

Acknowlegements

1. Introduction 1

1. 1. Rapamycin and the discovery of TOR 1

1. 2. Domain structure of TOR 2

1. 3. TOR signaling in higher eukaryotes 3

1. 3. 1. Downstream effectors of mTOR 3

p70S6K1 3

4E-BP1 4

1. 3. 2. Regulators of mTOR, what is upstream? 6

Growth factors 6

Nutrients 7

Energy 8

1. 3. 3. TOR and control of cell growth 9

1. 4. TOR signaling in *Saccharomyces cerevisiae* 11

1. 4. 1. Temporal control of cell growth 12

Readouts of the rapamycin-sensitive TOR signaling branch 12

Effector pathways of the rapamycin-sensitive TOR signaling branch 16

1. 4. 2. Spatial control of cell growth 18

1. 4. 3. TOR controls cell growth in response to nutrients 21

2. Identification of TOR complexes 24

2. 1. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. 25

Robbie Loewith, Estela Jacinto, Stephan Wullschleger, Anja Lorberg, José L. Crespo, Débora Bonenfant, Wolfgang Oppliger, Paul Jenoe, and Micheal N. Hall

Molecular Cell, 10, 457-468, 2002

2. 2. Additional results 37

2. 3. Discussion 38

3. Characterization of AVO1 and LST8 40
3. 1. Introduction 40
3. 2. Results 41
3. 3. Discussion 49
3. 4. Material and methods 51

4. Functional analysis of TORC2 56
4. 1. Introduction 56
TOR signaling complexes 56
TORC2 in yeast 58
4. 2. Results 61
4. 3. Discussion 74
4. 4. Material and methods 77

5. TOR and its role as protein kinase 82
5.1. Introduction 82
TOR as protein kinase 82
Analysis of phosphorylation sites 83
Identification of substrates for a specific kinase 84
5.2. Results 86
5.3. Discussion 91
5.4. Material and methods 93

6. Perspectives 97

7. Bibliography 98

Curriculum vitae **110**

Publications **111**

Erklärung **112**

1. Introduction

1. 1. Rapamycin and the discovery of TOR

The macrolide rapamycin is produced by a strain of *Streptomyces hygroscopius* which was originally isolated from a soil sample collected from Rapa-Nui (Easter Island). Rapamycin possesses potent antifungal, immunosuppressant, and antitumor properties (Hidalgo and Rowinsky, 2000). Inside a cell rapamycin binds to FKBP12 (FK506-binding protein). FKBP12 is a cytoplasmic petidyl-prolyl cis-trans isomerase that also binds FK506 (a potent immunosuppressant structurally related to rapamycin) (Heitman et al., 1991b; Schreiber, 1991). Disruption of *FPR1*, the gene encoding FKBP12 in yeast, revealed that FKBP12 is not essential for viability, and *frp1* mutant cells were resistant to rapamycin toxicity (Heitman et al., 1991a; Heitman et al., 1991b). In addition, since rapamycin analogues could still bind and inhibit the isomerase activity of FKBP12 but could not act as immunosuppressants lead to the assumption that FKBP12 is not the target through which rapamycin inhibits cell growth (Bierer et al., 1990). Rapamycin rather forms a complex with FKBP12, which then acts on another target essential for growth. To identify this target spontaneous, rapamycin-resistant yeast mutants were selected. Three genes were identified in the screen, *FPR1* and two novel genes that were named *TOR1* and *TOR2* (target of rapamycin) (Heitman et al., 1991a). While the mutations conferring rapamycin resistance in *FPR1* were loss-of-function mutations, the mutations in *TOR1* and *TOR2* were gain-of-function mutations. These dominant *TOR1* and *TOR2* alleles confer complete resistance to the antifungal activity of rapamycin because they are no longer bound by FKBP12-rapamycin (Chen et al., 1995; Choi et al., 1996; Lorenz and Heitman, 1995; Stan et al., 1994).

The TOR proteins are highly conserved through eukaryote evolution. TOR was identified in all eukaryotes examined including *Schizosaccharomyces pombe, Cryptococcus neoformans, Ashbya gossypii, Arabidopsis thaliana, Drosophila melanogaster, Caenorhabditis elegans* and mammals. Interestingly, higher eukaryotes contain only a single *TOR* gene, whereas the fungi, *S. cerevisiae*, *S. pombe* and *C. neoformans*, have two *TOR* genes (Crespo and Hall, 2002).

1. 2. Domain structure of TOR

The highly homologous (67%) TOR1 and TOR2 proteins are large proteins with a molecular mass of 280 kDa. Both proteins contain a region in their C-terminus that is structurally related to lipid kinases, especially to phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4-kinase (Cafferkey et al., 1994; Helliwell et al., 1994; Kunz et al., 1993). Based on this homology TOR has been grouped in a novel class of phosphatidylinositol (PI) kinases, the PI kinase-related kinases (PIKK) (Keith and Schreiber, 1995). The PIKK family includes the yeast MEC1, TEL1 and RAD3 proteins and the mammalian ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia related) and DNA-PK (DNA-dependent protein kinase) (Abraham, 2001). PIKK family members are very large proteins and several members have been implicated to function in the cell cycle checkpoint function. Despite their homology to PI kinases none of its members have been shown to exhibit lipid kinase activity, in fact PIKK are Ser/Thr kinases.

The N-terminus of TOR contains two large blocks of up to 20 tandemly repeated HEAT repeats (named for Huntingtin, elongation factor 3, A subunit of protein phosphatase 2A and TOR1) (Andrade and Bork, 1995). Each HEAT motif comprises approximately 40 amino acids that form antiparallel α-helices (Groves and Barford, 1999; Perry and Kleckner, 2003). This structure provides a large exposed surface with a hydrophobic nature that can mediate protein-protein interaction. It has been demonstrated the HEAT repeats are important for protein-protein interaction and that the HEAT repeats of TOR2 are required for the localization of TOR2 to membranes (Chook and Blobel, 1999; Kim et al., 2002; Kunz et al., 2000). The HEAT domain is followed by a FAT domain. This domain is found in all PIK-related kinases and is accompanied by a small FATC domain, which is located at the very C-terminus of the PIKKs. The FAT and FATC domains may serve as scaffold or as protein-protein interaction domain (Alarcon et al., 1999; Bosotti et al., 2000). Following the FAT domain is the FKBP-rapamycin-binding domain (FRB), which is the direct binding target for the FKBP-rapamycin complex (Fig. 1.1.).

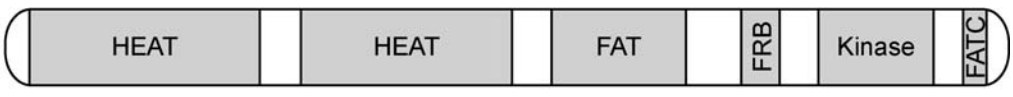


Figure 1.1. Domain structure of TOR

1. 3. TOR signaling in higher eukaryotes

TOR plays a central role in the control of cell growth. Genetic studies of *Drosophila* TOR (dTOR) revealed that cells mutant for *dTOR* were reduced in size at all stages of the cell cycle leading to a fly with a reduced body size (Oldham et al., 2000; Zhang et al., 2000). In addition, *TOR* mutant animals had a growth arrest phenotype similar to flies that have been starved for amino acids suggesting that TOR controls cell growth in response to nutrients.

What are the growth-related readouts of TOR? mTOR has been reported to control various readouts including translation, transcription, PKC signaling and autophagy (Fingar and Blenis, 2004). So far all the growth-related readouts of TOR in higher eukaryotes appear to be sensitive to rapamycin. Nevertheless, a recent report suggests that mTOR controls trafficking of amino acid transporters in a rapamycin-independent manner indicating that mTOR also exerts rapamycin-insensitive functions (Edinger et al., 2003). Currently, the best characterized downstream effectors of mTOR are the translational regulators, p70S6K and 4E-BP1.

1. 3. 1. Downstream effectors of mTOR

p70S6K1

p70S6K1 is activated by various extracellular signals. Activated p70S6K1 phosphorylates the 40S ribosomal protein S6 leading to an upregulation of translation of a subset of mRNA that contain a terminal oligopyrimidine (TOP) tract at their 5'-end (Jefferies et al., 1997). These 5'TOP mRNAs encode components of the translational apparatus, such as ribosomal proteins and elongation factors, which are predicted to account for 15-20% of total cellular mRNA (Meyuhas, 2000). Therefore, it is thought that activation of p70S6K leads to general upregulation of translational capacity.

p70S6K is activated by multiple phosphorylation events that are regulated by mTOR- and growth factor-dependent signaling pathways. At least eight phosphorylation sites have been implicated in the regulation of p70S6K. PDK1 (phospholipid-dependent protein kinase), a downstream kinase of the growth-factor dependent PI3K signaling pathway, phosphorylates Thr229, which is located in the activation loop of the kinase

domain (Alessi et al., 1998). Activation of p70S6K is dependent on the phosphorylation of Thr229 (Dennis et al., 1996). Phosphorylation of two other sites Ser371 and Thr389 are also essential for p70S6K activation and are directly phosphorylated by mTOR in *vitro* (Burnett et al., 1998; Dennis et al., 1996; Pearson et al., 1995; Saitoh et al., 2002). Thr389 as well as Thr229 and Ser404 represent a motif in which the phosphorylated Ser or Thr is followed by a hydrophobic residue whereas Ser371 is flanked by Pro. p70S6K contains an additional four phosphorylation sites at the C-terminus (Ser 411, 418, 421, and Thr424) exhibiting a Ser/Thr-Pro motif. These four sites lie in the autoinhibitory pseudosubstrate domain and they are not essential for kinase activity. In its inactive state the C-terminal pseudosubstrate domain of p70S6K is thought to interact with the N-terminus and thereby inhibit the catalytic activity (Martin and Blenis, 2002). Phosphorylation of the four sites in this domain appears to facilitate the phosphorylation Thr389, the mTOR phosphorylation site (Avruch et al., 2001). Phosphorylation of Thr389 has been proposed to create a binding site for PDK1, thus facilitating in the phosphorylation of Thr229 (Biondi et al., 2001). Therefore, it is thought that prior phosphorylation of p70S6K at the hydrophobic motif (Thr389) promotes phosphorylation of the activation loop (Thr229) by PDK1 required for activation of p70S6K. Treatment with rapamycin results in dephosphorylation and inactivation of p70S6K. Thr389 is the major rapamycin-sensitive phosphorylation site but Thr229 and Ser404 as well as two Ser/Thr-Pro sites (Ser371 and Ser411) are also dephosphorylated upon treatment with rapamycin (Han et al., 1995; Pearson et al., 1995).

Recently, a TOR signaling (TOS) motif has been identified at the N-terminus of p70S6K (Schalm and Blenis, 2002). The TOS motif consists of five residues and is characterized by an aromatic residue followed by alternating hydrophobic and acidic residues. A functional TOS motif is required for efficient phosphorylation and activation of p70S6K as mutation of the TOS motif mimics the effect of rapamycin on p70S6K phosphorylation.

4E-BP1

4E-BP1 (4E-binding protein) inhibits cap-dependent translation by binding to eukaryotic initiation factor 4E (eIF4E). eIF4E is the subunit of the tripartite eukaryotic initiation factor 4 (eIF4F) complex that recognizes the cap, m7GpppX (in which m is a methyl

group and X any nucleotide), which is present at the 5'end of mRNAs. The two other subunits of eIF4F are eIF4G and eIF4A. eIF4A is an RNA helicase which unwind the RNA. eIF4G is a scaffolding protein binding to eIF4E, eIF4A, eIF3, and the poly(A)-binding protein, and thus bridges the 40S ribosome to the mRNA. Cap-dependent translation is regulated by the eIF4E-binding proteins (4E-BPs), which compete with eIF4G for binding to eIF4E. Binding of 4E-BP1 to eIF4E is regulated by phosphorylation. Hypophosphorylated 4E-BP1 binds to eIF4E, whereas hyperphosphorylated 4E-BP1 abolishes this interaction enabling eIF4E to associate with eIF4G. Thus, phosphorylation of 4E-BP1 allows the positioning of the translation machinery on the 5' end of the mRNA (Gingras et al., 2001; Raught et al., 2001).

The TOR signaling pathway, as well as the PI3K pathway are involved in the regulation of the 4E-BP1 phosphorylation (Gingras et al., 2001). Phosphorylation of 4E-BP1 occurs in a hierarchical manner involving at least six phosphorylation sites, four of which are regulated by mTOR signaling and contain a Ser/Thr-Pro motif (Gingras et al., 1999; Mothe-Satney et al., 2000). Phosphorylation of Thr37 and Thr46 is required for the subsequent mitogen-responsive phosphorylation of Thr70 and Ser65 and the subsequent release of eIF4E. While mTOR has been shown to directly phosphorylate Thr37 and Thr46 *in vitro* the kinase regulating Thr70 and Ser65 is unknown (Brunn et al., 1997; Burnett et al., 1998; Gingras et al., 1999). Since phosphorylation of Ser65 and Thr70 is more sensitive to rapamycin than phosphorylation of Thr37 and Thr47 it has been proposed that phosphorylation of Ser65 and Thr70 might be regulated by a mTOR-dependent kinase or a mTOR-inhibited phosphatase (Gingras et al., 2001).

Two domains in 4E-BP1 have been shown to be required for its efficient phosphorylation. In addition to the TOS motif located at the C-terminus (Schalm and Blenis, 2002), 4E-BP1 contains a domain at the N-terminus, that is composed of the four amino acids Arg-Ala-Ile-Pro, called RAIP motif (Tee and Proud, 2002). Recently, it has been reported that raptor, a protein that interacts with mTOR binds 4E-BP1 as well as p70S6K through their TOS motifs bringing these two substrates in proximity of mTOR kinase (Beugnet et al., 2003; Choi et al., 2003; Nojima et al., 2003; Schalm et al., 2003). Since raptor does not appear to mediate interaction of 4E-BP1 via the RAIP motif it has been suggested that by interaction with the RAIP motif other proteins may recruit proximal kinases to 4E-BP1 (Proud, 2004).

1. 3. 2. Regulators of mTOR, what is upstream?

How is mTOR regulated and what is upstream of mTOR? Three major inputs have been implicated in mTOR signaling, all of which converge on the phosphorylation of mTOR target proteins p70S6K and 4E-BP1. First, mTOR activation is dependent on the growth factor signaling pathway. Second, mTOR appears to be regulated by nutrients, especially amino acids. Third, energy levels have been recently shown to be important for regulation of mTOR activity (illustrated in Fig. 1.2.).

Growth factors

The mTOR pathway responds to growth factors via the phophatidylinositol 3-kinase (PI3K) pathway. Binding of insulin or insulin-like growth factors (IGFs) to their receptors leads to receptor dimerimerzation and autophosphorylation which triggers recruitment and phosphorylation of insulin receptor substrates (IRS) to generate a docking site for PI3K at the membrane. PI3K bound to IRS converts phosphatidylinositol-4,5-phosphate (PIP_2) in the cell membrane to phosphatidylinositol-3,4,5-phosphate (PIP_3). The conversion of PIP_2 to PIP_3 is negatively controlled by the lipid phosphatase PTEN. Once generated PIP_3 recruits PDK1 and Akt through their pleckstrin-homology (PH) domain to the membrane. Membrane-bound Akt in turn is phosphorylated and activated by PDK1 (Alessi, 2001).

How does the growth factor pathway regulate mTOR activity? mTOR appears to be controlled by Akt and the Tuberous sclerosis proteins, TSC1 (hamartin) and TSC2 (tuberin). TSC1 contains two coiled-coil domains, whereas TSC2 possesses in addition to a coiled-coil domain a region that is similar to the catalytic domain of GTPase activating proteins (GAP). Through interaction of their coiled-coil domains TSC1 and TSC2 form a complex (van Slegtenhorst et al., 1998). It has been shown that overexpression of TSC1-TSC2 blocks TOR signaling leading to the inhibition of p70S6K and 4E-BP1 phosphorylation. Further, TSC2 is phosphorylated by Akt in response to insulin which destabilizes TSC2 and results in the inactivation of TSC2 and the subsequent disruption of TSC1-TSC2 (Gao et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2003; Tee et al., 2002). Therefore, TSC1-TSC2 appears to function downstream of Akt and upstream of mTOR to negatively regulate p70S6K and 4E-BP1. However, Akt is also able to control mTOR through direct phosphorylation of

mTOR (Nave et al., 1999). The significance of this phosphorylation remains unclear since substitution of the phosphorylated Ser to Ala does not affect the ability of mTOR to activate p70S6K (Sekulic et al., 2000).

How does TSC1-TSC2 regulate mTOR? Recently, it has been found TSC1-TSC2 controls mTOR via Rheb (Ras homolog enriched in brain), a Ras-related small GTPase (Garami et al., 2003; Inoki et al., 2003a; Tee et al., 2003). Overexpression of Rheb enhances mTOR signaling towards p70S6K and 4E-BP1 and activates mTOR even in absence of growth factors or amino acids. On the other hand, the effect of Rheb on p70S6K and 4E-BP1 phosphorylation is blocked by overexpression of TSCs indicating that TSCs are involved in the activation of Rheb (Garami et al., 2003; Inoki et al., 2003a; Tee et al., 2003). It has been demonstrated that TSC2 through its GAP domain stimulates the intrinsic GTPase activity of Rheb resulting in the conversion from active GTP-bound Rheb to inactive GDP-bound Rheb (Inoki et al., 2003a; Tee et al., 2003; Zhang et al., 2003). The activation of Rheb is dependent on insulin as evidenced by the fact that insulin treatment leads to an increase of GTP-bound Rheb likely through activation of the PI3K/Akt signaling pathway that phosphorylates and inhibits TSC2 (Garami et al., 2003). Thus, the growth factor activated PI3K pathway impinges on mTOR via a pathway consisting of the proteins Akt, TSC1-TSC2 and Rheb.

Phosphatidic acid (PA) generated by mitogen-stimulated phospholipase D (PLD) was shown to play a role in mTOR signaling since PA stimulates phosphorylation of p70S6 and 4E-BP1 in an mTOR-dependent but PI3K-independent manner. PA may exert its effects by direct binding to the FRB domain in mTOR (Fang et al., 2001). The significance of PA in mTOR signaling however has to be elucidated.

Nutrients

Nutrients in the form of amino acids have been implicated in the regulation of mTOR signaling. Starvation for amino acids, in particular branched amino acids like leucine, results in a rapid dephosphorylation of p70S6K and 4E-BP1 (Hara et al., 1998; Wang et al., 1998; Xu et al., 1998). Readdition of amino acids restores p70S6K and 4E-BP1 in an mTOR-dependent manner. Abundant leucine increases mTOR activity and the stimulatory effect of leucine on mTOR is not blocked by the PI3K inhibitor wortmannin. Additionally, leucine does not change the activities of PI3K and Akt indicating that leucine signals to mTOR by a mechanism that is independent of the growth factor

induced PI3K signaling pathway. How amino acids are sensed by mTOR is unknown. However, TSC1-TSC2 and Rheb have been implicated in the nutrient sensing process because overexpression of Rheb can bypass the need for amino acids to activate mTOR signaling and loss of TSC1-TSC2 renders cells resistant to amino acid starvation suggesting that mTOR may sense amino acids via TSC1-TSC2/Rheb signaling network (Gao et al., 2002; Saucedo et al., 2003).

Energy

A high rate of translation is coupled to a high energy demand. ATP depletion causes a decrease in p70S6K and 4E-BP1 phosphorylation resulting in the inhibition of translation. It was suggested that mTOR may sense ATP levels since it has a high K_M (1mM) for ATP (Dennis et al., 2001). However, the ATP levels do not change drastically in a cell and the cellular concentration of ATP is higher than the predicted K_M of ATP for mTOR. Therefore, small changes of the ATP concentration could not be sensed by mTOR (Proud, 2002). mTOR may sense the energy status of a cell through the AMP-activated protein kinase (AMPK) (Inoki et al., 2003b). AMPK is activated under low cellular energy conditions (high AMP/ATP ratio). Activated AMPK downregulates high energy demanding processes like protein synthesis and stimulates ATP generation processes, such as glycogenolysis. AMP as well as the drug AICAR, an AMP analogue are able the activate AMPK (Hardie and Hawley, 2001). Activation of AMPK by AICAR has been demonstrated to inhibit phosphorylation of p70S6K and 4E-BP1 via the mTOR pathway (Kimura et al., 2003). Recently, it has been reported that activated AMPK directly phosphorylates TSC2 and that energy starvation-induced dephosphorylation of p70S6K is dependent on TSC2 (Inoki et al., 2003b). mTOR may therefore be regulated by AMP/ATP ratio via AMPK and TSC2.

Another cellular component inorganic polyphosphate (poly P) has been implicated in the regulation of mTOR. Poly P are linear polymers of many hundreds of phosphates present in all organisms from bacteria to animals. In *E. coli* poly P is increased in response to starvation and stimulates degradation of ribosomal proteins by activation of the Lon protease (Kuroda et al., 2001). Poly P has been reported to stimulate mTOR activity *in vitro* and expression of yeast polyphosphatase in human cells leads to inhibition of the insulin or amino acid-stimulated phosphorylation of 4E-BP1 suggesting a role for poly P in mammalian cells (Wang et al., 2003).

1. 3. 3. TOR and control of cell growth

Genetic studies in *Drosophila* revealed that the insulin receptor signaling pathway and the TOR signaling pathway play an important role in the control of cell growth. Loss-of-function mutations of components that act positively in the pathway such as *CHICO* (the insulin receptor substrate homologue), *dPI3K* and *dAkt* results in smaller flies with fewer and smaller cells. Loss-of-function mutations of negative regulators, such as *dPTEN* or *dTSCs* result in enhanced growth and bigger flies. Importantly, mutation of *dTOR* or *dS6K* leads to smaller flies without affecting the cell number indicating that TOR controls the size of individual cells but not proliferation (Saucedo and Edgar, 2002; Stocker and Hafen, 2000).

How is growth coordinated in a multicelluar organism? It has been recently reported that in *Drosophila* the fat body, equivalent to the liver in mammals, plays an important role in responding to nutritional cues. In a genetic screen in *Drosophila* a mutation, *slif* (slim fast) was identified as affecting cell growth (Colombani et al., 2003). *Slif* encodes an amino acid transporter and loss-of-function *slif* mutant flies exhibited a phenotype that is similar to amino acid starved flies. Downregulation of *slif* specifically in the fat body resulted in the reduction of the larvae size suggesting that amino acid deprivation in the fat body alone affects growth of all larval tissues. Therefore, amino acid availability sensed in the fat body appears to trigger a response, which coordinates growth in all different tissues. TOR has been implicated to participate in the amino acid sensing mechanism in the fat body as inhibition of TOR resulted in phenotypes that are similar to those induced by downregulation of *slif*. In contrast, the PI3K signaling pathway was not involved in the amino acid sensing in the fat body. However, PI3K activity was inhibited in peripheral tissues in response to activation of the amino acid sensor mechanism in the fat body triggered by loss of slif function or inhibition of the TOR pathway in the fat body. How the fat body signals to other tissues is not known. Thus, nutritional conditions sensed in the fat body appear to trigger a humoral signal that regulates global growth of an organism. Since TOR is involved in the amino acid sensing mechanism in the fat body TOR does not only control cell-autonomous growth but also participates in the systemic growth control.

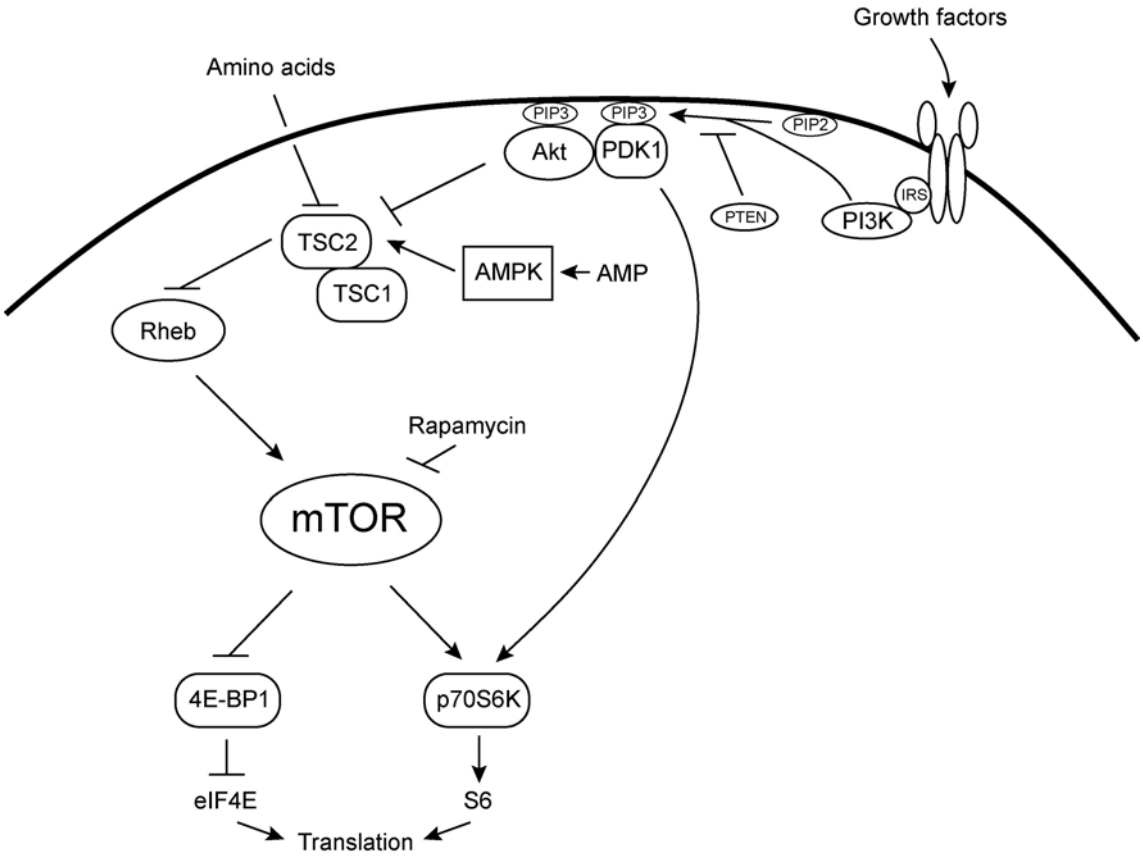


Figure 1.2. mTOR effectors and signaling pathways

1. 4. TOR signaling in *Saccharomyces cerevisiae*

Similar to TOR signaling in higher eukaryotes, the two TOR proteins in yeast, TOR1 and TOR2 regulate cell growth in response to nutrients. Evidence that TOR in yeast integrates nutrient-derived signals will be discussed in this section.

TOR has two functions

Disruption of *TOR1* and *TOR2* individually or in combination results in different phenotypes. *TOR1* disruption results in a slightly reduced growth rate at normal temperature but is lethal at high temperature (39°C) and sensitive to salt (Crespo et al., 2001; Heitman et al., 1991a; Helliwell et al., 1994; Kunz et al., 1993). In contrast, depletion of TOR2 is lethal. Cells lacking TOR2 undergo a few cell divisions before arresting randomly in the cell cycle (Helliwell et al., 1998a; Kunz et al., 1993). The lethality of a *TOR2* disruption cannot be suppressed by overexpression of *TOR1*. Cells lacking both TOR1 and TOR2 exhibit G1 cell cycle arrest like rapamycin-treated cells. These observations lead to the suggestion that TOR performs two functions. TOR1 and TOR2 perform a redundant, rapamycin-sensitive function in G1 progression, and in addition, TOR2 has an essential, unique function that TOR1 cannot perform. This unique function of TOR2 appears to be insensitive to rapamycin since rapamycin resistant *TOR1* mutant alleles can support growth in the presence of rapamycin (Helliwell et al., 1998a; Kunz et al., 1993; Zheng et al., 1995). The functional difference between TOR1 and TOR2 was suggested to involve the HEAT repeats as the HEAT repeats of TOR1 and TOR2 are not interchangeable, whereas the catalytic domains of TOR1 and TOR2 are interchangeable (Helliwell et al., 1994).

Below, the two TOR signaling branches in yeast are discussed. The rapamycin-sensitive signaling branch accounts for the temporal control of cell growth, whereas the TOR2-unique signaling is rapamycin-insensitive and responsible for spatial control of cell growth. Within this thesis (Part 2) we show that these two signaling branches are controlled by two distinct TOR complexes. While TOR complex 1 (TORC1) mediates temporal control of cell growth via the rapamycin-sensitive branch, spatial control of cell growth is mediated by TOR complex 2 (TORC2) (Loewith et al., 2002) (illustrated in Fig. 1.3.).

1. 4. 1. Temporal control of cell growth

Growth of *S. cerevisiae* cells is temporally controlled. Synthesis of macromolecules occurs when nutrients are available. TOR signaling links nutrient availability to macromolecular synthesis and therefore mediates temporal control of cell growth. TOR positively regulates anabolic processes such as translation initiation, ribosome biogenesis and negatively regulates catabolic processes such as RNA degradation, autophagy and other degradative pathways (Schmelzle and Hall, 2000). Thus, TOR signaling ensures that under good nutrient conditions a high rate of translation is maintained, whereas under poor nutrients conditions cell growth arrests.

Readouts of the rapamycin-sensitive TOR signaling branch

Translation initiation

Rapamycin treatment or TOR depletion causes a severe decrease in translation initiation and an arrest in the early G1 phase of the cell cycle (Barbet et al., 1996). The G1 arrest observed upon rapamycin treatment appeared to be a consequence of the translation defect since cap-independent translation of the G1 cyclin *CLN3* suppresses the rapamycin-induced G1 arrest. TOR1 and TOR2 seem to activate translation initiation via the initiation factor eIF4E, the cap-binding subunit of the heterotrimeric initiation factor 4F (eIF4F) (Barbet et al., 1996; Danaie et al., 1999). Similar to the regulation of cap-dependent translation in mammals EAP1 blocks cap-dependent translation via competition with eIF4G thereby inhibiting recruitment of eIF4G to eIF4E. In addition, disruption of *EAP1* confers partial resistance to rapamycin underscoring a role for EAP1 in TOR signaling (Cosentino et al., 2000).

TOR also regulates translation initiation through GCN2 (Cherkasova and Hinnebusch, 2003). In starved cells GCN2 is active and phosphorylates the α-subunit of translation initiation factor 2 (eIF2α) resulting in a reduction of general protein synthesis (Hinnebusch and Natarajan, 2002). However, through a specialized reinitiation mechanism involving short open reading frames (uORF) in the *GCN4* mRNA leader, *GCN4* is derepressed in response to starvation and eIF2α phosphorylation. GCN4 is a transcription factor regulating genes involved in amino acid biosynthesis. GCN2, the eIF2α kinase is activated by uncharged tRNAs that accumulate during amino acid

starvation and bind to a histidyl-tRNA synthetase-related (HisRS) domain in GCN2. Under favourable growth conditions kinase activation is prevented by intramolecular interactions between the kinase domain and the HisRS domain of GCN2. Activation of GCN2 requires release of the autoinhibitory structure by tRNA leading to autophosphorylation of the kinase (Hinnebusch and Natarajan, 2002). It has been recently reported that TOR controls phosphorylation of Ser577, a phosphorylation site that is affecting the activity of GCN2 (Cherkasova and Hinnebusch, 2003). Inactivation of TOR by rapamycin results in the dephosphorylation and activation of GCN2 leading to downregulation of general translation and activation of GCN4.

Ribosome biogenesis

Ribosome biogenesis is a highly energy-demanding process and therefore has to be regulated according to the growth conditions and availability of nutrients (Warner, 1999). TOR controls ribosome biogenesis at both a transcriptional and a translational level (Powers and Walter, 1999). Inhibition of TOR by rapamycin or nutrient starvation results in a downregulation of RNAII-dependent transcription of ribosome protein genes as well as transcription of rRNA and tRNA by polymerase I and polymerase III. In addition, TOR also regulates processing of the 35S precursor rRNA.

Protein traffic and degradation

Yeast cells can use a variety of compounds as nitrogen or carbon sources. Transport of these nutrients across the cell membrane is essential for cell growth and viability. Amino acids are essential for cell growth since they constitute the building blocks for protein synthesis. Yeast contain two classes of amino acid permeases which are regulated according to the available nitrogen source (Sophianopoulou and Diallinas, 1995). One class consists of the low affinity, broad specificity permeases, including the general amino permease GAP1. The other class contains high affinity, amino acid specific permeases, such as the tryptophan transporter TAT2. These two classes of permeases are regulated in an inverse manner: under starvation conditions GAP1 is upregulated and routed to the plasma membrane, while specific permeases like TAT2 are degraded. TOR has been implicated in the turnover of amino acid permease as inhibition of TOR by rapamycin or nitrogen starvation leads to ubiquitinylation, vacuolar sorting and degradation of TAT2 (Schmidt et al., 1998). TOR controls the stability of

TAT2 via the protein kinase NPR1. NPR1 is a phosphoprotein whose phosphorylation is regulated in a TOR-dependent manner. Under favourable conditions TOR keeps NPR1 in a highly phosphorylated and inactive state. Upon TOR inhibition by rapamycin or nitrogen starvation NPR1 is rapidly dephosphorylated and activated. Active NPR1 leads to the subsequent ubiquitinylation and degradation of TAT2 (Schmidt et al., 1998). However, the substrate of NPR1 is not known. On the other hand, TOR regulates GAP1 inversely to TAT2. Upon TOR inactivation by rapamycin GAP1 is transcriptionally upregulated (Beck and Hall, 1999). GAP1 is also posttranslationally controlled by NPR1 as NPR1 is required for sorting to and stabilization of GAP1 at the plasma membrane under starvation conditions (De Craene et al., 2001; Vandenbol et al., 1990). It was proposed that TOR may negatively control sorting of GAP1 by keeping NPR1 in its phosphorylated and inactive state (Beck et al., 1999).

Autophagy

When starved yeast cells degrade their cytosol and organelles to ensure survival under these unfavourable growth conditions. This catabolic process is mediated by autophagy involving the enclosure of cytoplasm by a double membrane structure (autophagosome) and the subsequent delivery to the vacuole (Klionsky and Ohsumi, 1999). TOR has been implicated in autophagy as inhibition of TOR by rapamycin induces autophagy (Noda and Ohsumi, 1998). TOR controls autophagy via APG13 (Kamada et al., 2000). APG13 forms a complex with the protein kinase APG1 and formation of APG13-APG1 complex is required for autophagy. Under good nutrient conditions TOR maintains APG13 in a hyperphosphorylated form preventing the association with APG1 and thereby also autophagy. Starvation or treatment with rapamycin leads to a dephosphorylation of APG13, association of APG1 with APG13 and induction of autophagy.

Transcription

TOR positively and negatively regulates transcription. Under favourable nutrient conditions TOR activates transcription of ribosomal protein, rRNA and tRNA genes (Powers and Walter, 1999). TOR also inhibits transcription of many nutrient or stress-regulated genes mainly by maintaining several nutrient and stress-responsive

transcription factors in the cytoplasm (Beck and Hall, 1999; Cardenas et al., 1999; Hardwick et al., 1999; Komeili et al., 2000).

GATA transcription factors GLN3 and GAT1

GLN3 and GAT1 control genes which are involved in the uptake and the assimilation of alternative nitrogen sources (Magasanik and Kaiser, 2002). GLN3 and GAT1 reside in the cytoplasm in cells, which are grown in the presence of a good nitrogen source (glutamine, glutamate or ammonia). Upon shift to a poor nitrogen source (urea or proline) or upon TOR inactivation by rapamycin, GLN3 and its cytoplasmic anchor URE2 are dephosphorylated resulting in a dissociation of GLN3 from URE2. Subsequently, GLN3 translocates to the nucleus and induces the expression of its target genes (Beck and Hall, 1999).

RTG1 and RTG3

RTG1 and RTG3 regulate expression of tricarboxylic acid and glyoxylate cycle genes. Expression of these genes primarily leads to the synthesis of α-ketoglutarate, which is required for the de novo synthesis of amino acids such as glutamate and glutamine. Nitrogen starvation or inhibition of TOR by rapamycin leads to the nuclear translocation of RTG1/3 and the activation of their target genes (Komeili et al., 2000). TOR negatively controls the activity of RTG1 and RTG3 through the regulatory proteins RTG2 and MKS1 (Dilova et al., 2002; Liu et al., 2003).

Zn-finger transcription factor MSN2 and MSN4

MSN2 and MSN4 are redundant transcription factors which activate a large number of genes in response to many types of stress including carbon starvation (Gorner et al., 1998; Smith et al., 1998). Additionally, MSN2/4 are negatively controlled by TOR. Under optimal growth conditions MSN2/4 are associated with the 14-3-3 proteins BHM1 and BMH2 and localized in the cytoplasm. Upon carbon-starvation or rapamycin treatment MSN2/4 are released from BMH1/2 and translocate to the nucleus (Beck and Hall, 1999).

Effector pathways of the rapamycin-sensitive TOR signaling branch

The TOR effector pathways of the rapamycin-sensitive signaling branch, which mediates the temporal control of cell growth, are not known in all cases. Some of the readouts of the rapamycin-sensitive signaling branch involve the negative regulation of type 2A phosphatases by TOR.

TOR regulates protein phosphatases

Type 2A phosphatases (PP2A) are composed of a catalytic (C) and two regulatory subunits (A and B). While the catalytic subunit is broadly active the regulatory subunits determine the specificity towards a target protein as well as the subcellular localization of the phosphatase complex (Goldberg, 1999). The PPH21 and PPH22 catalytic subunits associate with the regulatory A subunit, TPD3, and one of two B subunits, CDC55 or RTS1 (Zabrocki et al., 2002). The PP2A-related phosphatase is composed of the catalytic subunit SIT4 which associates with one of four regulatory proteins, SAP4, SAP155, SAP185, SAP190 (Luke et al., 1996). TOR negatively controls SIT4 and PP2A by promoting the association of the catalytic subunits PPH21/22 and SIT4 to TAP42 (Di Como and Arndt, 1996). Binding of the catalytic subunits to TAP42 prevents the association of the catalytic subunits with regulatory subunits leading to the inhibition of SIT4 and PP2A. TOR might regulate the association of TAP42 with PPH21/22 by phosphorylation of TAP42 (Jiang and Broach, 1999), whereas the binding of TAP42 to SIT4 seems to be indirectly controlled by TOR through the TAP42-interacting protein TIP41 (Jacinto et al., 2001). TOR maintains TIP41 in a phosphorylated form and thereby promotes the binding of TAP42 to SIT4. Upon TOR inactivation by rapamycin or under starvation conditions TIP41 is dephosphorylated and binds TAP42. As a consequence SIT4 is released from TAP42 and is activated. Activated SIT4 is able to dephosphorylate more TIP41 resulting in the amplification of the phosphatase activity.

TOR controls several readouts via the TAP42-SIT4 effector pathway. The phosphorylation status and therefore also the activity of the transcription factors GLN3 and GAT1, the protein kinase NPR1 and eIF2α kinase GCN2 are regulated in a SIT4-dependent manner (Loewith and Hall, 2004).

TOR and RAS signaling pathway

Similar to the TOR pathway the RAS/cyclic AMP (cAMP) pathway plays a major role in the control of growth in response to nutrients. The RAS signaling pathway consists of two redundant small GTPases RAS1 and RAS2 (RAS), which are activated by the guanine nucleotide exchange factor CDC25. In its activated state RAS activates adenylate cyclase (encoded by *CDC35*) leading to the production of cAMP. Protein kinase A (PKA) is composed of a catalytic subunit (redundantly encoded by *TPK1, TPK2* and *TPK3*) and the regulatory subunit (encoded by *BCY1*). cAMP causes a release of TPK from BCY1 which activates the kinase. Similar to nutrient-depleted cells, inactivation of PKA causes a cell cycle arrest in G1, an accumulation of storage carbohydrates (glycogen and trehalose) and a downregulation of ribosome biogenesis. Downstream targets of PKA include enzymes involved in intermediary carbon metabolism, the transcription factors MSN2 and MSN4 and RIM15, a protein kinase involved in stationary phase induction (Thevelein and de Winde, 1999).

Recently, the RAS/cAMP signaling pathway have been proposed to act as an effector pathway of the TOR signaling pathway (Schmelzle et al., 2004). It has been shown that activation of the RAS/cAMP pathway renders cells resistant to rapamycin-induced, SIT4-independent processes, such as nuclear translocation of MSN2, accumulation of glycogen, and downregulation of ribosome biogenesis. On the other hand, activation of the RAS/cAMP pathway did not affect rapamycin-induced processes regulated by SIT4, like the nuclear transport of GLN3 and the phosphorylation of NPR1. Thus, TOR appears to control rapamycin-sensitive readouts that are independent of SIT4 through the RAS/cAMP pathway. TOR may control the RAS/cAMP pathway via regulating the localization of the PKA catalytic subunits as TOR inactivation by rapamycin leads to the nuclear translocation and presumably inactivation of TPK1. However, the mechanism by which TOR regulates the RAS/cAMP pathway remains unclear as it has been also suggested that MSN2 is controlled in parallel by TOR and the RAS/cAMP pathway (Gorner et al., 1998; Gorner et al., 2002).

1. 4. 2. Spatial control of cell growth

TOR does not only mediate the temporal control of cell growth but also the spatial control of cell growth. Growth of a daughter cell in yeast occurs at a discrete site (the bud site) on the surface of a mother cell. TOR2 regulates spatial control of cell growth via the organization of the actin cytoskeleton. Polarization of the actin cytoskeleton orients the secretory pathway to the bud and therefore ensures that newly synthesized proteins and other cellular constituents are targeted to the growth site.

Organization of the actin cytoskeleton

In yeast cells growth is polarized, occurring at a defined position at the cell surface. The structural basis for cell polarity is provided by the actin cytoskeleton. The yeast actin cytoskeleton is organized into at least three morphologically distinct structures: cortical patches, actin cables and a cytokinetic ring. Cortical patches are discrete cytoskeletal bodies whereas actin cables are long bundles of actin filaments. The distribution of actin patches and cables is polarized in a cell-cycle dependent manner. The polarity is established by actin cables forming a network that polarizes the cell towards the bud. Cortical patches are thought to maintain polarity by recycling components such as enzymes necessary for cell wall synthesis ensuring a proper cell wall assembly when growth is redirected through the cell cycle. The function of the actin cytoskeleton is to provide cell polarity that guides a variety of events, such as transport of vesicles from the Golgi and endosomal elements from the mother cell into the bud, organelle movement and positioning, mRNA anchorage, endocytosis and mitotic spindle orientation (Pruyne and Bretscher, 2000a; Pruyne and Bretscher, 2000b). The actin cytoskeleton is also required to respond to environmental signals. The organization of the actin cytoskeleton is altered in response to nutrient availability, pheromone or change in osmolarity or temperature (Beck et al., 2001).

The actin cytoskeleton is organized in a cell cycle dependent manner. During early G1 actin cables and patches are randomly distributed in the cytoplasm whereas late in G1 when the cell initiates a new cell cycle the actin cytoskeleton polarizes. Actin patches concentrate at the preselected bud site and actin cables orients towards the bud site. During bud emergence and maturation actin patches are located in the bud while actin cables orient from the mother cells into the bud of the daughter cell. At the end of bud

growth the polarization is lost and the actin patches and cable redistribute randomly in the mother and daughter cell. Prior to cytokinesis a ring consisting of F-actin is assembled at the bud neck, then contracts and disassembles after mitosis. Following cytokinesis the actin cytoskeleton is repolarized. Actin patches concentrate at the bud neck while actin cables orient towards the bud neck directing synthesis of new cell walls. The cell-cycle dependent polarization of the actin cytoskeleton is established by RHO-type GTPases (Beck et al., 2001; Schmidt and Hall, 1998).

RHO-GTPases

RHO-GTPases play a pivotal role in the organization of the actin cytoskeleton not only in *S. cerevisiae* but also in higher eukaryotes (Etienne-Manneville and Hall, 2002). Like all small G-proteins, RHO-GTPases cycle between an active, GTP-bound, and an inactive, GDP-bound, conformation. In its GTP-bound form the GTPase is able to recognize target proteins and thereby exert a signaling response. Active GTPase returns to its inactive state through hydrolysis of GTP. The cycle between GTP-bound and GDP-bound form is regulated by three classes of proteins: GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating proteins) and GDIs (guanine nucleotide exchange inhibitors). The GEFs activate the GTPase by catalyzing the nucleotide exchange whereas the GAPs stimulate the hydrolysis of GTP leading to the inactivation of the GTPase.

There are six RHO-GTPases in *S. cerevisiae*, CDC42, RHO1, RHO2, RHO3, RHO4 and RHO5 (Chant, 1999). CDC42 plays a central role in controlling the polarization of the yeast cell during budding and mating. RHO1 and RHO2 are important for maintenance of the actin cytoskeleton polarization and cell wall activities during bud growth. Bud growth is also controlled by RHO3 and RHO4.

TOR2 controls the organization of the actin cytoskeleton

Cells disrupted for *TOR2* exhibit a defect in the organization of the actin cytoskeleton and it has been reported that TOR2 signals to the actin cytoskeleton through activation of the small GTPase RHO1 (Schmidt et al., 1997; Schmidt et al., 1996). Deletion of *SAC7*, a GAP for RHO1, suppresses the growth defect of a *tor2* mutant but not of a *tor1 tor2* double mutant indicating that deletion of *SAC7* only suppresses a defect in the

signaling branch that is specifically controlled by TOR2. Conversely, overexpression of *ROM2*, a GEF for RHO1, or overexpression of the small GTPases *RHO1* and *RHO2* are able to restore the growth defect of *tor2* mutant cells. TOR2 appears to regulate the GTPase switch via ROM2 as the GDP/GTP exchange activity of ROM2 toward RHO1 is drastically reduced in a *tor2* mutant (Schmidt et al., 1997). Thus, TOR2 activates RHO1 and RHO2 but the mechanism by which TOR2 controls the activity of ROM2 is not known.

Activated RHO1 in turn regulates different effectors including PKC1, the 1,3-β-glucan synthase FKS1 and FKS2, the formin-family protein BNI1, the two-component signaling protein SKN7, and SEC7, a spatial landmark for exocytosis (Alberts et al., 1998; Guo et al., 2001; Schmidt and Hall, 1998). Suppressor studies in *tor2* mutant strains using different RHO1 effectors indicated that TOR2 signals to the actin cytoskeleton via the RHO1 effector PKC1 (Helliwell et al., 1998b). PKC1 controls a mitogen-activated protein kinase (MAPK) cascade comprising BCK1 (MEKK), the redundant MKK1 and MKK2 (MEK), and MPK1 (MAPK). Activation of the pathway leads to the upregulation of genes that are involved in cell wall biogenesis (Heinisch et al., 1999). Further the PKC1-MAP kinase pathway have been implicated in the organization of the actin cytoskeleton (Helliwell et al., 1998b). First, *rho1* mutant cells display an actin defect that is suppressed by overexpression of PKC1. Second, overexpression of components of the PKC1-MAP kinase cascade restored the actin defect of *tor2* mutant cells. Thus, by controlling cell wall biosynthesis and the actin cytoskeleton the RHO1-PKC1-MAP kinase pathway maintains cell shape and integrity and is therefore also referred as cell integrity pathway. The cell integrity pathway is controlled by a variety of signaling events including cell wall sensing mechanisms via WSC1 and MID2, lipid signaling mediated by MSS4, and actin depolarization (Audhya and Emr, 2002; Harrison et al., 2001; Heinisch et al., 1999). It has to be noted that the PKC1-MAP kinase pathway is required for entry of starved cells into stationary phase and viability in G_0, a process that includes remodelling of the cell wall and that is also controlled by the rapamycin-sensitive TOR signaling branch (Krause and Gray, 2002; Torres et al., 2002).

1. 4. 3. TOR controls cell growth in response to nutrients

Several lines of evidence indicate that TOR controls cell growth in response to nutrients. First evidence that TOR may respond to nutrients came from the observation that inactivation of TOR by rapamycin results in an early G1 arrest, reduced protein synthesis and accumulation storage carbohydrates (glycogen), a phenotype characteristic for starved cells. Thus, inactivation of TOR appears to induce a starvation response (Barbet et al., 1996). The role of TOR in nutrient sensing was further supported by the findings that TOR controls autophagy, ribosome biogenesis as well as the activity of amino acid permeases via NPR1, all of which are regulated in response to nutrients (Noda and Ohsumi, 1998; Powers and Walter, 1999; Schmidt et al., 1998). Analysis of the genome-wide transcription program in rapamycin treated cells revealed that TOR controls transcription of nutrient-regulated genes (Cardenas et al., 1999; Hardwick et al., 1999). TOR regulates these genes through several transcription factors, including GLN3, GAT1, RTG1/RTG3 and MSN2/MSN4 (Beck and Hall, 1999; Komeili et al., 2000). Since GLN3, GAT1 and RTG1/RTG3 are regulated in response to the nitrogen source and MSN2/MSN4 in response to the carbon source it has been proposed that TOR signaling might respond to nitrogen and possibly carbon. How might TOR sense these nutrients? In particular the nitrogen source appears to play an important role in the TOR signaling as genes required for the import and metabolism of alternative nitrogen sources are strongly upregulated upon rapamycin treatment (Cardenas et al., 1999; Hardwick et al., 1999; Shamji et al., 2000). Yeast cells can use a variety of nitrogen sources. In order to use a compound as nitrogen source yeast cells have to convert this compound into glutamate or glutamine which serve as nitrogen donors. α-ketoglutarate derived from the TCA cycle is the precursor for glutamate while glutamine, the preferred nitrogen source, is synthesized out of ammonium and glutamate by glutamine synthetase (GS), encoded by *GLN1*(ter Schure et al., 2000). It has been reported that glutamine controls a subset of TOR-regulated readouts (Crespo et al., 2002). Chemical inhibition of GS leads to the depletion of glutamine and results in the activation of the TOR-controlled transcription factors GLN3, RTG1 and RTG3, whereas other TOR-regulated transcription factors, GAT1, MSN2, MSN4 and expression of ribosomal genes are not affected by glutamine levels. It was proposed that TOR might sense a variety of nutrients and accordingly trigger different responses (Kuruvilla et al., 2001; Shamji et al., 2000). Glutamine might be one

of these nutrients serving as an indicator of the nutritional status of a cell. How TOR senses glutamine or other nutrients remains unknown.

The TOR signaling pathway has been recently linked to the RAS/cAMP pathway underscoring a role of TOR in control of cell growth in response to nutrients (Schmelzle et al., 2004). Similar to the TOR signaling pathway the RAS/cAMP pathway is controlled by nutrient availability. While TOR is likely regulated in response to nitrogen, the RAS/cAMP pathway is controlled in particular by the presence of glucose which is sensed by G-protein-coupled receptor system consisting of GPR1 and its Gα protein GPA2 (Thevelein and de Winde, 1999). It was therefore suggested the RAS/cAMP pathway is controlled by different inputs: TOR may mediate the nitrogen-responsive input, whereas the glucose-responsive input is sensed by the GPR1/GPR2 system.

Thus, similar to TOR in higher eukaryotes TOR in yeast controls cell growth in response to nutrients, but the mechanism by which nutrient levels or the quality of nutrients are sensed by TOR is not understood. Unlike higher organisms where TOR is controlled in addition to nutrients by growth factors, yeast cells lack a growth factor signaling pathway. It is thought that the TOR signaling pathway may represent an ancestral pathway developed in unicellular organisms that controls cell-autonomous growth in response to nutrients; the growth factor signaling pathway evolved in higher eukaryotes impinges on TOR to coordinate growth of different tissues (Jacinto and Hall, 2003).

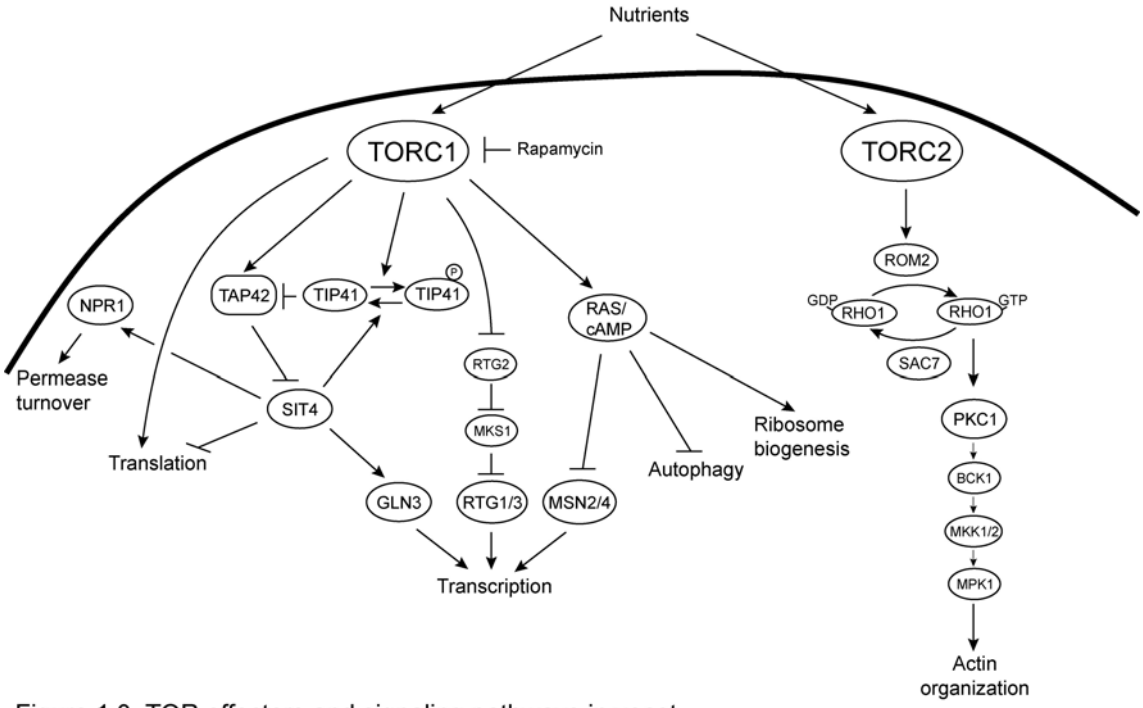


Figure 1.3. TOR effectors and signaling pathways in yeast

2. Identification of TOR complexes

TOR controls two signaling branches that can be genetically and functionally discriminated. TOR1 and TOR2 redundantly regulate translation, transcription and ribosome biogenesis in a rapamycin-sensitive fashion. TOR2 but not TOR1 regulates polarization of the actin cytoskeleton in a rapamycin-insensitive fashion. How specificity is achieved in the TOR signaling network was not understood. Since TOR is composed of multiple domains such as the HEAT repeats which could serve as potential protein-protein interaction domains it was speculated that TOR might interact with other, so far unknown proteins. The biochemical purification of both TOR1 and TOR2 presented in the following article lead to the identification of two structurally and functionally distinct TOR complexes which account for the specificity of TOR signaling. My contribution to this work was the characterization of AVO1.

Molecular Cell, Vol. 10, 457–468, September, 2002, Copyright ©2002 by Cell Press

Two TOR Complexes, Only One of which Is Rapamycin Sensitive, Have Distinct Roles in Cell Growth Control

Robbie Loewith, Estela Jacinto, Stephan Wullschleger, Anja Lorberg, José L. Crespo, Débora Bonenfant, Wolfgang Oppliger, Paul Jenoe, and Michael N. Hall[1]
Division of Biochemistry
Biozentrum, University of Basel
Klingelbergstrasse 70
CH-4056 Basel
Switzerland

Summary

The target of rapamycin (TOR) proteins in *Saccharomyces cerevisiae*, TOR1 and TOR2, redundantly regulate growth in a rapamycin-sensitive manner. TOR2 additionally regulates polarization of the actin cytoskeleton in a rapamycin-insensitive manner. We describe two functionally distinct TOR complexes. TOR Complex 1 (TORC1) contains TOR1 or TOR2, KOG1 (YHR186c), and LST8. TORC2 contains TOR2, AVO1 (YOL078w), AVO2 (YMR068w), AVO3 (YER093c), and LST8. FKBP-rapamycin binds TORC1, and TORC1 disruption mimics rapamycin treatment, suggesting that TORC1 mediates the rapamycin-sensitive, TOR-shared pathway. FKBP-rapamycin fails to bind TORC2, and TORC2 disruption causes an actin defect, suggesting that TORC2 mediates the rapamycin-insensitive, TOR2-unique pathway. Thus, the distinct TOR complexes account for the diversity, specificity, and selective rapamycin inhibition of TOR signaling. TORC1 and possibly TORC2 are conserved from yeast to man.

Introduction

TOR, the target of rapamycin, is a phosphatidylinositol kinase-related protein kinase (PIKK) that controls cell growth in response to nutrients (Schmelzle and Hall, 2000). TOR, originally identified by mutations in yeast that confer resistance to the growth inhibitory properties of rapamycin (Heitman et al., 1991a), is structurally and functionally conserved in all eukaryotes examined, including fungi, worms, flies, plants, and mammals. Mammalian TOR, mTOR (also known as FRAP, RAFT1, or RAPT), controls translation and other growth-related processes in response to amino acids and in conjunction with a growth factor signal via PI 3-kinase and PDK1 (Schmelzle and Hall, 2000; Gingras et al., 2001). mTOR controls translation by activating p70 S6 kinase and inhibiting the eIF4E inhibitor 4E-BP1 (Dennis et al., 1999; Gingras et al., 2001). The mechanism by which TOR senses amino acids is unknown, as is the precise nature of the link between mTOR and PI 3-kinase signaling. In budding yeast, two TORs, TOR1 and TOR2, control many growth-related processes in response to nutrients and favorable environmental conditions (Cardenas et al., 1999; Hardwick et al., 1999; Komeili et al., 2000; Schmelzle and Hall, 2000; Crespo et al., 2002). Rapamycin-treated or TOR-depleted yeast cells display several physiological properties characteristic of starved or otherwise stressed cells, including an altered cell morphology, a downregulation of translation, an inhibition of ribosome biogenesis, specific changes in transcription, sorting and turnover of nutrient permeases, accumulation of the storage carbohydrate glycogen, and an induction of autophagy (Schmelzle and Hall, 2000). Thus, TOR1 and TOR2 are functionally redundant in a rapamycin-sensitive pathway(s) that signals both positively and negatively to control cell growth. The yeast TORs control many of the above processes via a phosphatase switch composed of the type 2A-related phosphatase SIT4, TAP42, and TIP41 (Di Como and Arndt, 1996; Jacinto et al., 2001; Jiang and Broach, 1999). Under good nutrient conditions, TOR promotes binding of SIT4 to TAP42 and thereby maintains SIT4 inactive. Upon nutrient limitation or rapamycin treatment, conditions that inactivate TOR, SIT4 dissociates from its inhibitor TAP42. Liberated and activated SIT4 in turn dephosphorylates and activates targets such as the transcription factor GLN3, the kinase NPR1, and TIP41 (Beck and Hall, 1999; Jacinto et al., 2001; Schmidt et al., 1998). GLN3 and NPR1 are involved in scavenging or synthesizing alternative nutrient sources. Dephosphorylated TIP41, as part of a feedback loop, binds and inhibits TAP42, and thereby further amplifies SIT4 activity (Jacinto et al., 2001). Thus, TOR negatively regulates GLN3, NPR1, and TIP41 by inhibiting SIT4. TOR also negatively regulates the transcription factors RTG1/3 and MSN2/4, but independently of SIT4 (Beck and Hall, 1999; Komeili et al., 2000; Liu et al., 2001). The mechanisms and effectors by which TOR signals positively to promote, for example, translation and ribosome biogenesis are unknown. The mechanisms by which the TORs sense nutrients and environmental stress are also unknown.

In addition to its redundant function with TOR1 in a rapamycin-sensitive signaling pathway, TOR2 also has a rapamycin-insensitive function that TOR1 is unable to perform (Helliwell et al., 1994; Kunz et al., 1993; Zheng et al., 1995). TOR depletion studies have shown that this TOR2-unique function mediates the cell cycle-dependent polarization of the actin cytoskeleton (Schmidt et al., 1996). A polarized actin cytoskeleton orients the secretory pathway toward a discrete growth site (the bud) and thereby determines the spatial growth pattern of yeast cells. Rapamycin-insensitive TOR signaling has not been detected in mammalian cells, possibly because studies on TOR signaling in mammalian cells have relied exclusively on the use of rapamycin to inhibit mTOR. TOR2 signals to the actin cytoskeleton by activating a Rho-type GTPase switch. More specifically, TOR2 activates the GDP/GTP exchange factor (GEF) ROM2 (Schmidt et al., 1997). ROM2 in turn converts RHO1 and RHO2 to an active, GTP-bound state. GTP-bound RHO binds and activates PKC1 which then signals to the actin cytoskeleton via a MAP kinase cascade composed of

[1]Correspondence: m.hall@unibas.ch

BCK1, MKK1, and MPK1/SLT2 (Helliwell et al., 1998a, 1998b; Schmidt et al., 1997). Thus, TOR, via the TOR-shared and TOR2-unique signaling pathways, appears to integrate the temporal and spatial control of cell growth (Schmelzle and Hall, 2000). The upstream regulators and direct effectors of TOR in both the TOR-shared and the TOR2-unique pathways are unknown. In other words, the molecular determinants of the diversity and specificity of TOR signaling are not known.

Rapamycin acts by forming a toxic complex with its intracellular receptor FKBP12 (Schreiber, 1991). The FKBP-rapamycin complex binds and inhibits TOR directly (Schmelzle and Hall, 2000). Mutations in *TOR* that confer rapamycin resistance prevent the binding of FKBP-rapamycin and do not otherwise affect TOR function. The mechanism by which FKBP-rapamycin bound to TOR inhibits TOR function is not known. Also unknown is the molecular basis for the observation that rapamycin selectively inhibits TOR2 in the TOR-shared pathway; as described above, rapamycin does not inhibit TOR2 in the TOR2-unique pathway (Zheng et al., 1995; Schmidt et al., 1996).

The TORs are large, ~280 kDa, proteins that contain a number of domains. An N-terminal domain comprises up to 20 tandemly repeated HEAT motifs (Andrade and Bork, 1995). HEAT motifs, each an approximately 40 amino acid sequence that forms antiparallel α helices, mediate protein-protein interactions in multiprotein complexes (Groves and Barford, 1999) and are required for the proper subcellular localization of TOR in yeast (Kunz et al., 2000). Between the HEAT repeats and a C-terminal catalytic domain is a so-called FAT domain (Alarcon et al., 1999; Bosotti et al., 2000). The FAT domain is found in all PIKKs and is accompanied by a small FATC domain at the extreme C terminus of these kinases. The FAT domain is also believed to mediate protein-protein interactions or to serve as a scaffold. The abundance of protein-protein interaction domains in TOR suggests that the TORs associate with several other proteins; however, confirmed TOR partner proteins are yet to be described.

Here we describe the purification and characterization of two distinct TOR complexes from yeast cells. We demonstrate that TOR1 or TOR2 forms a complex, TORC1, with LST8 and KOG1. Consistent with the presence of either TOR1 or TOR2 in this complex, TORC1 mediates the TOR-shared signaling function and is bound by rapamycin. TOR2 additionally and uniquely forms a distinct complex, TORC2, with AVO1, AVO2, AVO3, and LST8. Consistent with the absence of TOR1 in this complex, TORC2 mediates the TOR2-unique signaling function and is rapamycin insensitive. Furthermore, TORC1 and possibly TORC2 are conserved from yeast to man. These findings account for the diversity, specificity, and differential rapamycin sensitivity of TOR signaling.

Results

TOR1 and TOR2 Stably Associate with Several Other Proteins

The protein-protein interaction domains in TOR suggest that the TORs interact with other proteins, possibly as members of one or more complexes. By gel filtration chromatography, TOR1 and TOR2 have an apparent molecular weight of approximately 2 MDa (data not shown), indicating that the TORs are in large complexes. To identify TOR1- and TOR2-associated proteins, we developed a procedure to isolate TOR1- and TOR2-containing complexes. The procedure relied on functional, epitope-tagged versions of TOR1 (2myc-TOR1) and TOR2 (3HA-TOR2) expressed at endogenous levels, and involved ion exchange chromatography followed by immunoprecipitation with anti-myc (TOR1) or anti-HA (TOR2) antibody (see Experimental Procedures). To identify proteins that specifically copurify with the TORs, a mock purification of untagged TOR was also performed. Two proteins copurified with TOR1, and four proteins copurified with TOR2 (Figures 1A and 1B). The interactions were all confirmed by coimmunoprecipitation, as described below. To determine the identity of the copurifying proteins, silver-stained gel bands from the TOR purifications were excised and analyzed by MALDI-TOF mass spectrometry. TOR1 and TOR2 did not copurify in either the TOR1 or the TOR2 purification.

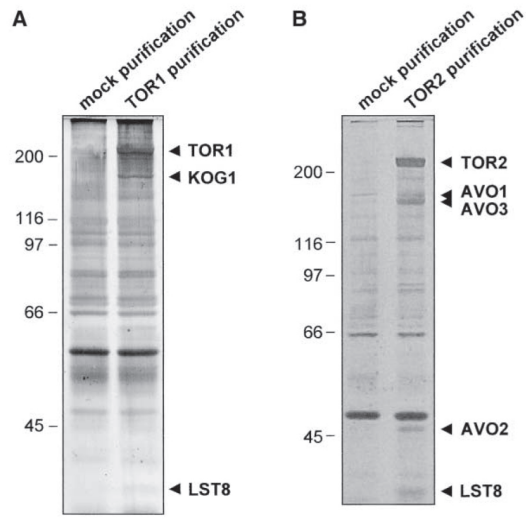


Figure 1. TOR1 and TOR2 Copurify with Several Other Proteins

(A) Silver-stained gel of TOR1 purification (see Experimental Procedures) from RL60a cells (myc-TOR1) and TB50a cells (mock purification).

(B) Silver-stained gel of TOR2 purification (see Experimental Procedures) from SF43-1c cells (HA-TOR2) and TB50a cells (mock purification). Protein bands unique to the TOR purifications are indicated. These bands were excised and identified by mass spectroscopy (see Experimental Procedures).

The two proteins that copurified with TOR1 were LST8 and an uncharacterized protein encoded by the open reading frame YHR186c. We named the uncharacterized protein KOG1 (kontroller of growth 1). KOG1 is essential, as revealed by disruption of the *KOG1* gene (data not shown). The KOG1 protein has a MW of 176 kDa and contains four internal HEAT repeats (http://dove.embl-heidelberg.de/andrade/rep/get_info.pl?HEAT; R. Latek and D. Sabatini, personal communication) and seven C-terminal WD-40 repeats (Shinozaki-Yabana et al.,

2000). KOG1 sequence and domain organization are conserved from yeast to man (27% sequence identity). LST8 (lethal with sec thirteen) is an essential protein of unknown function originally identified genetically as a mutation, *lst8-1*, that is synthetically lethal with *sec13-1,* a mutation causing a sorting defect in the secretory pathway (Roberg et al., 1997). *lst8* mutations were also identified in a screen for negative regulators of the RTG1/3 transcription factor (Liu et al., 2001). Interestingly, TOR1 and TOR2 have also been shown to control RTG1/3 and protein sorting in the secretory pathway (Beck et al., 1999; Komeili et al., 2000; Schmidt et al., 1998). The 34 kDa LST8 protein is composed essentially entirely of seven WD-40 repeats (Ochotorena et al., 2001). Furthermore, LST8, like KOG1, is conserved from yeast to man, displaying 28% sequence identity with the human ortholog.

The four proteins that copurified with TOR2 were LST8, AVO3, and two uncharacterized proteins encoded by YOL078w and YMR068w. We named the two uncharacterized proteins AVO1 (YOL078w) and AVO2 (YMR068w) (adheres voraciously to TOR2). AVO3, formerly TSC11, was renamed AVO3 to avoid confusion with the unrelated mTOR-interacting proteins TSC1 and TSC2. The noteworthy features of LST8, a protein that copurified with both TOR1 and TOR2, are described above. AVO3 (formerly TSC11) is an essential protein of unknown function previously identified genetically as a temperature-sensitive suppressor of *csg2*, a mutation causing a defect in sphingolipid synthesis (Beeler et al., 1998). Interestingly, Beeler et al. (1998) also identified a *tor2* mutation as a suppressor of *csg2*. The 164 kDa AVO3 protein contains a RasGEFN domain between amino acids 991 and 1047 (Yaffe et al., 2001). This domain is found in exchange factors and activating proteins for Ras-like small GTPases. In guanine nucleotide exchange factors, it is found N-terminal to the catalytic RasGEF domain, hence the name RasGEFN. AVO3 orthologs are found in fungi but are not evident in metazoans. AVO1 is essential, as revealed by disruption of the *AVO1* gene (data not shown). The AVO1 protein has a predicted MW of 131 kDa (but an apparent MW of 165 kDa), and contains a domain (amino acids 840–933) weakly similar to a Ras association domain found in Ras target proteins (Yaffe et al., 2001). Interestingly, the orthologs of AVO1 and AVO3 in *Dictyostelium discoideum*, RIP3 and pianissimo, respectively, are functionally related in mediating Ras signaling (Chen et al., 1997; Lee et al., 1999). A deletion of *Sin1*, the *AVO1* ortholog in *Schizosaccharomyces pombe*, is complemented by the human *SIN1* gene, *hSIN1*, suggesting that AVO1 is conserved from yeast to man (Wilkinson et al., 1999) (see Discussion). The AVO2 protein is nonessential, has a MW of 47 kDa, contains five ankyrin repeats in its N-terminal half (Yaffe et al., 2001), and appears to be unique to *S. cerevisiae*. Thus, all five TOR partner proteins are of unknown function, but, like TOR, most are essential (KOG1, AVO1, AVO3, and LST8) and widely conserved (KOG1, AVO1, and LST8).

The interactions between TOR1 or TOR2 and the copurifying proteins were confirmed by coimmunoprecipitation experiments. Strains coexpressing a functional, tagged KOG1, LST8, AVO1, AVO2, or AVO3 and a differently tagged TOR1 or TOR2 were constructed. The putative partner proteins were immunoprecipitated, and the precipitates were then examined for the presence of TOR1 or TOR2. TOR1 efficiently immunoprecipitated with both KOG1 and LST8, but not with the TOR2 partners AVO1, AVO2, or AVO3 (Figure 2A). TOR2 efficiently immunoprecipitated with AVO1, AVO2, AVO3, and LST8 (Figure 2B). Unexpectedly, we also detected a weak, but reproducible, interaction between TOR2 and KOG1. The weakness of this interaction and the similar apparent MWs of KOG1, AVO1, and AVO3 complicating protein identification by mass spectrometry may account for the failure to detect KOG1 in the TOR2 purification described above. Finally, we did not detect an interaction between TOR1 and TOR2 (Figures 2A and 2B), consistent with the above observation that TOR1 and TOR2 do not copurify. Thus, TOR1 and TOR2 independently associate with KOG1 and LST8. TOR2 also associates with AVO1, AVO2, and AVO3.

Two Distinct TOR Complexes: TORC1 and TORC2

Do TOR1 and TOR2 associate with their partner proteins individually to form several dimeric complexes, or do the TORs associate with several partners simultaneously to form a few higher order complexes? Gel filtration experiments suggest that both TOR1 and TOR2 are in large and therefore higher order complexes (see above; data not shown). To determine the number and composition of TOR complex species, coimmunoprecipitations were performed with the TOR partner proteins in all possible pairwise combinations. LST8 immunoprecipitated with all other TOR partner proteins (Figures 2C–2E). AVO1, AVO2, and AVO3 all immunoprecipitated with each other, but none of these proteins immunoprecipitated with KOG1 (Figures 2C–2E). These findings combined with the above finding that TOR1 and TOR2 independently associate with KOG1 and LST8 suggest that there are two species of TOR complexes (Figure 3). TOR complex 1 (TORC1) contains TOR1 (TORC1-A) or TOR2 (TORC1-B), KOG1, and LST8. TOR complex 2 (TORC2) contains TOR2, AVO1, AVO2, AVO3, and LST8. The above data do not demonstrate that LST8 is in TORC1-B in addition to being in TORC2, but evidence for this is provided below. Thus, there are two distinct, higher order TOR complexes.

Rapamycin Treatment or Starvation Does Not Affect the Integrity of TORC1 or TORC2

Rapamycin treatment and starvation inhibit TOR signaling; however, the mechanism by which either condition inhibits TOR is unknown. Does rapamycin treatment or starvation inhibit TOR by disrupting TORC1 or TORC2? To assay the effect of rapamycin treatment or starvation on the TOR complexes, we immunoprecipitated tagged partner proteins from rapamycin-treated or nitrogen-starved cells and examined the immunoprecipitates for the presence of TOR1 and TOR2. Cells were treated with rapamycin (200 nM) for 30 min or starved for a nitrogen source for 60 min—both conditions under which TOR signaling is inhibited (Beck et al., 1999; Schmidt et al., 1998). The association of TOR1 with KOG1 and LST8, and the association of TOR2 with KOG1, AVO1, AVO2, AVO3, and LST8 were unaffected by either treatment (data not shown). Thus, rapamycin

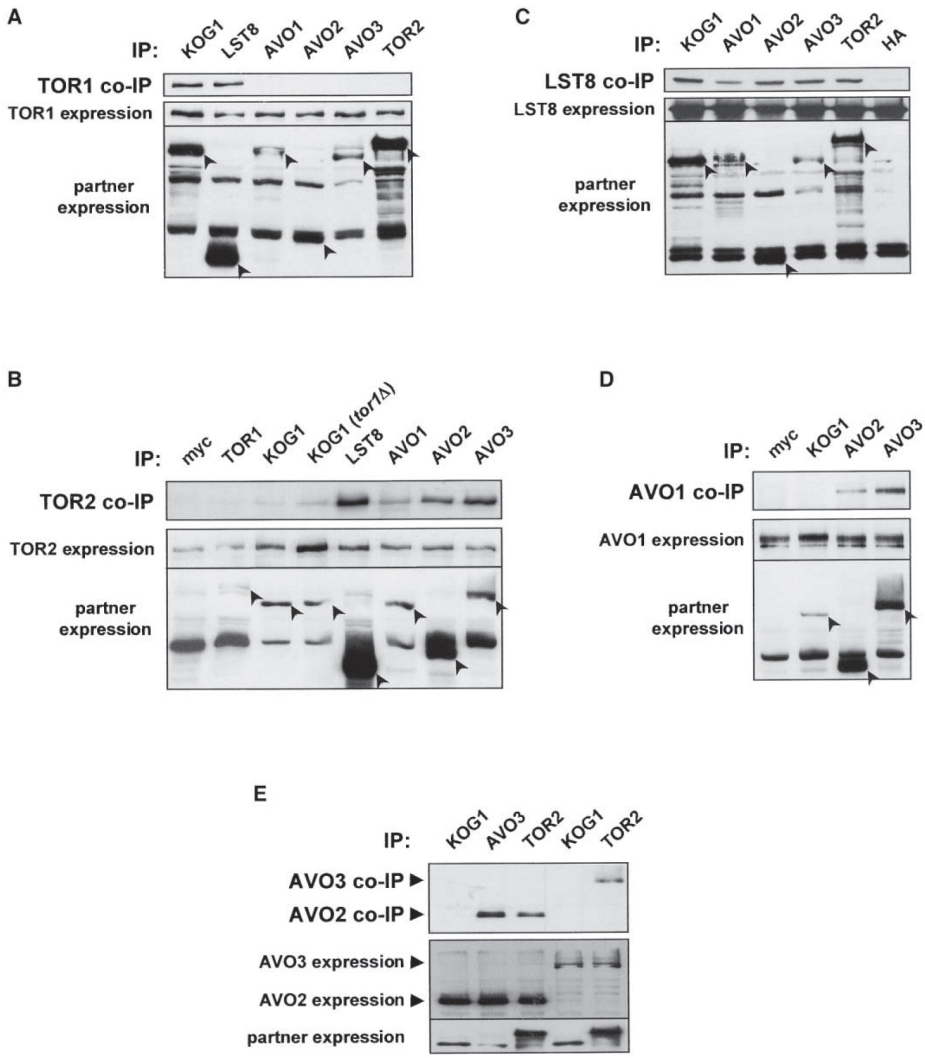


Figure 2. Identification of Two Distinct TOR Complexes

(A) TOR1 coimmunoprecipitates with KOG1 and LST8 but not with AVO1, AVO2, AVO3, or TOR2. Lysates from cells coexpressing myc-TOR1 and KOG1-HA (RL72-1a), LST8-HA (RL83-6a), AVO1-HA (SW21-3c), AVO2-HA (RL70-2a), AVO3-HA (RL71-1b), or HA-TOR2 (RL67-2c) were subjected to immunoprecipitation with anti-HA. Immunoprecipitates were probed with anti-myc to detect coimmunoprecipitated TOR1 (top panel).

(B) TOR2 coimmunoprecipitates with KOG1, LST8, AVO1, AVO2, and AVO3 but not with TOR1. Lysates from cells expressing HA-TOR2 (SF43-1c, negative control, labeled myc) or coexpressing HA-TOR2 and myc-TOR1 (RL67-2c), myc-KOG1 (AN31-1a), myc-KOG1 in a *tor1* background (AN32-3a), LST8-myc (RL68-1b), myc-AVO1 (SW22-4d), AVO2-myc (RL50-7b), or AVO3-myc (RL51-11a) were subjected to immunoprecipitation with anti-myc. Immunoprecipitates were probed with anti-HA to detect coimmunoprecipitated TOR2 (top panel).

(C) LST8 coimmunoprecipitates with KOG1, AVO1, AVO2, and AVO3. Lysates from cells expressing LST8-myc (RL59-2d, negative control, labeled HA) or coexpressing LST8-myc and KOG1-HA (RL89-1a), AVO1-HA (SW31-5d), AVO2-HA (RL82-1d), AVO3-HA (RL81-3a), or HA-TOR2 (RL68-1b, positive control) were subjected to immunoprecipitation with anti-HA. Immunoprecipitates were probed with anti-myc antibody to detect coimmunoprecipitated LST8 (top panel).

(D) AVO1 coimmunoprecipitates with AVO2 and AVO3 but not with KOG1. Lysates from cells expressing AVO1-HA (RL69-1c, negative control, labeled myc) or coexpressing AVO1-HA and myc-KOG1 (RL119-5c), AVO2-myc (RL120-1b), or AVO3-myc (RL121-2b) were subjected to immunoprecipitation with anti-myc. Immunoprecipitates were probed with anti-HA antibody to detect coimmunoprecipitated AVO1 (top panel).

(E) Lanes 1–3: AVO2 coimmunoprecipitates with AVO3 but not with KOG1. Lysates from cells coexpressing AVO2-myc and KOG1-HA (RL107-4b), AVO3-HA (RL79-6a), or HA-TOR2 (RL50-7b, positive control) were subjected to immunoprecipitation with anti-HA. Immunoprecipitates were probed with anti-myc antibody to detect coimmunoprecipitated AVO2 (top panel). Lanes 4 and 5: AVO3 does not coimmunoprecipitate with KOG1. Lysates from cells coexpressing AVO3-myc and KOG1-HA (RL108-1b), or HA-TOR2 (RL51-11a, positive control) were subjected to immunoprecipitation with anti-HA. Immunoprecipitates were probed with anti-myc to detect coimmunoprecipitated AVO3 (top panel).

(A–E) HA precipitates and myc precipitates were probed with anti-HA and anti-myc, respectively, to control for expression levels of coimmunoprecipitated proteins (middle panels) and immunoprecipitated partners proteins (bottom panels).

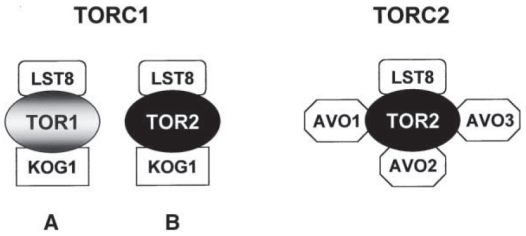


Figure 3. Composition of Two Distinct TOR Complexes: TORC1 (A and B) and TORC2

Model does not necessarily reflect the stoichiometries and interactions of subunits within each complex.

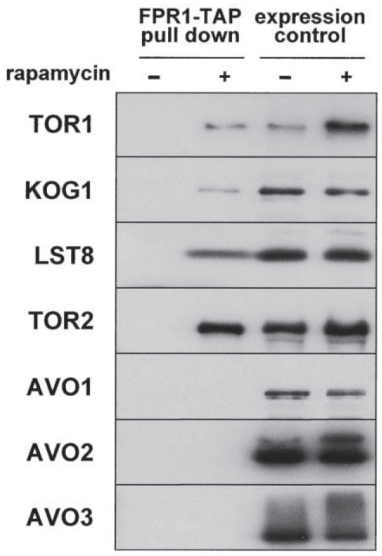


Figure 4. FPR1-Rapamycin Binds TORC1 but Not TORC2

Cells coexpressing FPR1-TAP (a TAP-tagged version of the yeast FKBP12 protein) and myc-TOR1 (RL90-2d), KOG1-HA (RL87-2d), LST8-HA (RL103-5b), HA-TOR2 (RL7-1b), AVO1-HA (RL102-1d), AVO2-myc (RL111-7a), or AVO3-myc (RL110-4c) were treated with rapamycin or vehicle alone for 30 min prior to harvesting. Lysates were subjected to IgG pull-down (FPR1-TAP pull-down) or immunoprecipitation with anti-HA or anti-myc as appropriate (expression control). Precipitates resolved by SDS-PAGE were blotted to detect the indicated proteins.

treatment or starvation does not inhibit TOR by altering the integrity of TORC1 or TORC2.

FKBP-Rapamycin Binds TORC1 but Not TORC2

The above results indicate that rapamycin does not disrupt either TORC1 or TORC2. Is either TORC1 or TORC2 a target of rapamycin? Rapamycin acts by forming a toxic complex with its intracellular receptor FKBP12, and this complex then binds and inhibits TOR directly. In *S. cerevisiae*, FKBP12 is encoded by the *FPR1* gene, and FPR1-rapamycin specifically binds and thereby inhibits TOR1 and TOR2 (Heitman et al., 1991a, 1991b; Kunz et al., 1993; Lorenz and Heitman, 1995; Stan et al., 1994). To determine which TOR complex, if any, is bound by FPR1-rapamycin, we pulled down affinity-tagged FPR1 (FPR1-TAP) from rapamycin-treated cells and examined the pull-downs for the presence of TOR1, TOR2, and the TOR partner proteins. The FPR1-TAP strains, expressing FPR1-TAP as the sole cellular copy of FPR1, exhibited normal sensitivity to rapamycin, indicating that the tagged FPR1 was fully functional in mediating rapamycin toxicity. FPR1-TAP pulled down TOR1, TOR2, KOG1, and LST8 specifically from rapamycin-treated cells (Figure 4). In contrast, FPR1-TAP failed to pull down AVO1, AVO2, or AVO3 (Figure 4). Failure to pull down AVO1, AVO2, or AVO3 was not due to rapamycin dissociating TORC2 because, as described above, rapamycin does not affect the integrity of TORC2. Furthermore, the pull-down quantitatively depleted the abundant FPR1-TAP from the cell extract but did not quantitatively deplete the significantly less abundant TOR2 (data not shown), suggesting that there is a pool of TOR2 that is inaccessible to FKBP-rapamycin. Thus, FKBP-rapamycin binds TORC1 (A and B) but not TORC2 in vivo, suggesting that rapamycin inhibits only TORC1 signaling.

Importantly, FPR1-TAP pulled down LST8 from a rapamycin-treated *tor1* deletion strain (data not shown). This finding, alluded to above, indicates that LST8 is in TORC1-B.

TORC1 Mediates Rapamycin-Sensitive, TOR-Shared Signaling

The TORs mediate two general signaling branches, the rapamycin-sensitive, TOR-shared pathway(s) and the rapamycin-insensitive, TOR2-unique pathway (see Introduction). How do TORC1 and TORC2 correspond functionally to these TOR signaling pathways? The findings that TORC1 binds rapamycin and contains either TOR1 or TOR2 suggest that TORC1 mediates the rapamycin-sensitive, TOR-shared signaling. To confirm this, we investigated whether disruption of TORC1 but not TORC2 mimics rapamycin treatment. To disrupt the TORCs, we created strains with the essential *KOG1*, *AVO1*, or *LST8* gene under control of the glucose-repressible and galactose-inducible *GAL1* promoter. These strains allowed disruption of TORC1, TORC2, or both TORC1 and TORC2, respectively, simply by shifting cells from galactose-containing medium to glucose-containing medium (see Experimental Procedures). The strains were depleted of KOG1, AVO1, or LST8 protein (as determined by immunoblotting) and arrested growth within 12–15 hr after a shift from galactose medium to glucose medium (data not shown). On galactose medium, the growth of the strains was indistinguishable from that of a wild-type strain (Figure 6B; data not shown), indicating that overexpression of the TOR partners from the strong galactose promoter does not significantly affect TOR signaling.

Inhibition of the TOR-shared pathway(s) by rapamycin treatment causes several starvation-like phenotypes, including an altered cell morphology, a reduction in protein synthesis, specific changes in transcription, and glycogen accumulation (see Introduction). To determine if disruption of either TOR complex mimics rapamycin treatment, we investigated all of the above phenotypes in cells lacking either one or both of the TORCs (Figure

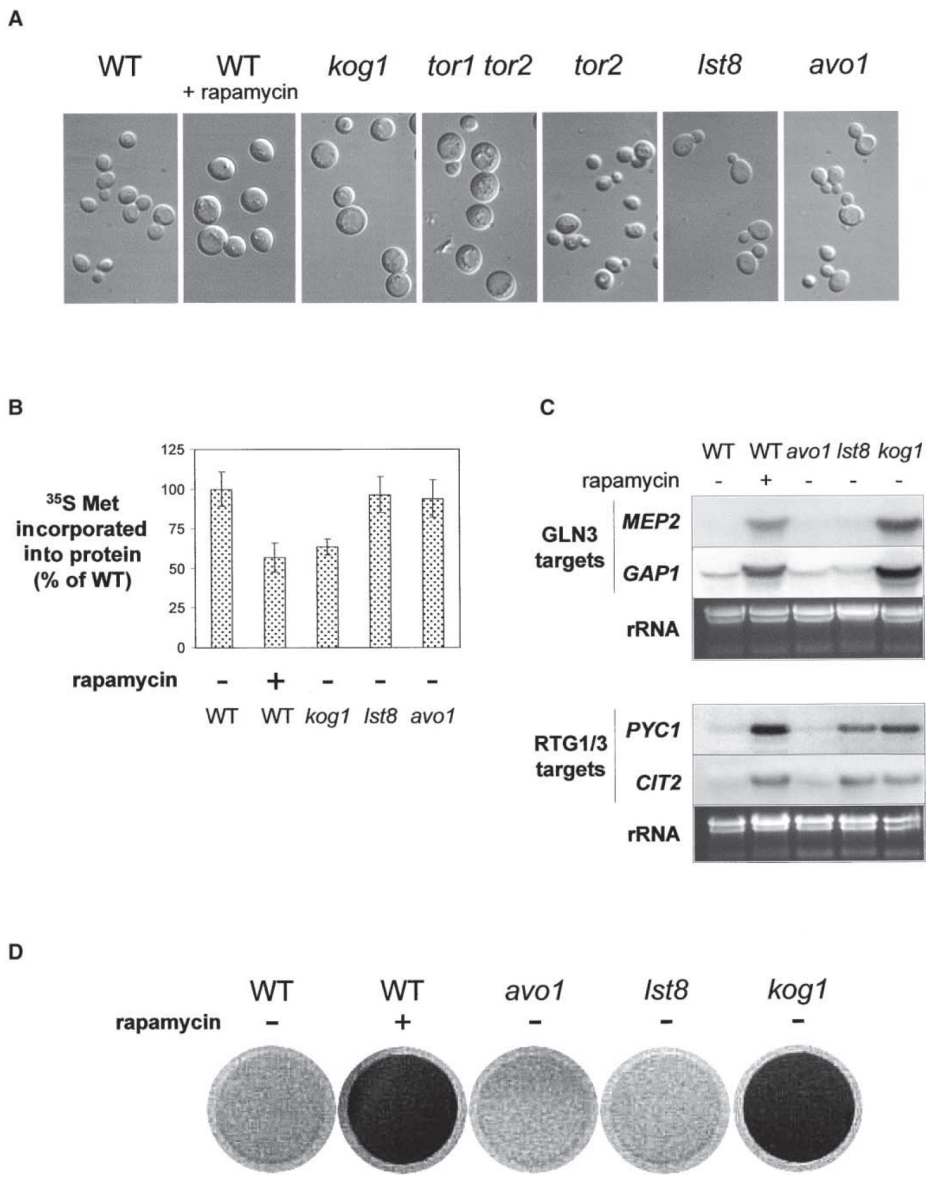


Figure 5. TORC1 Disruption Mimics Rapamycin Treatment

(A) Cells treated with rapamycin or depleted of TOR (TOR1 and TOR2) or KOG1 become swollen. Wild-type cells (WT, TB50a) or cells expressing *KOG1* (RL93a), *TOR2* in a *tor1* background (JK350-21a), *TOR2* (JK350-18a), *LST8* (RL57-2d), or *AVO1* (RL23-1c) from the glucose-repressible and galactose-inducible *GAL1* promoter were examined after 15 hr in glucose medium. Cells were examined microscopically using Nomarski optics. Wild-type cells were treated with rapamycin for 3 hr and similarly examined. All images are at the same magnification.

(B) Cells treated with rapamycin or depleted of KOG1 exhibit reduced protein synthesis. Wild-type cells grown in glucose medium were treated with vehicle or rapamycin for 30 min. *GAL1* promoter-*KOG1*, -*LST8*, and -*AVO1* containing cells were grown for 15 hr in glucose medium. Aliquots of cells were labeled with ^{35}S methionine, and the percentage of total imported label incorporated into protein was calculated and normalized to vehicle-treated wild-type controls. Means ± the standard deviations of the mean of at least three assays were plotted. See (A) for strain names.

(C) Cells treated with rapamycin or depleted of KOG1 or LST8 have altered transcription profiles. Wild-type cells and *GAL1* promoter-*KOG1*, -*LST8*, and -*AVO1* containing cells were grown for 15 hr in SD medium (GLN3 targets) or in SD medium supplemented with 3% L-glutamine (RTG1/3 targets). Wild-type cells were treated with drug vehicle or rapamycin for 30 min. Total RNA was probed with ^{32}P-labeled DNA probes specific for the GLN3 transcription factor targets *MEP2* and *GAP1*, or the RTG1/3 transcription factor targets *PYC1* and *CIT2* (Experimental Procedures). Ethidium bromide-stained rRNA is included as a loading control. See (A) for strain names.

(D) Cells treated with rapamycin or depleted of KOG1 accumulate glycogen. Wild-type cells were grown in glucose medium and treated for 3 hr with drug vehicle or rapamycin. *GAL1* promoter-*AVO1*, -*LST8*, and -*KOG1* containing cells were grown in glucose medium for 15 hr. Seven OD_{600} equivalents were collected onto nitrocellulose filters and exposed to iodine vapor for 1 min to stain glycogen. See (A) for strain names. These results indicate that TORC1 mediates the rapamycin-sensitive, TOR-shared signaling.

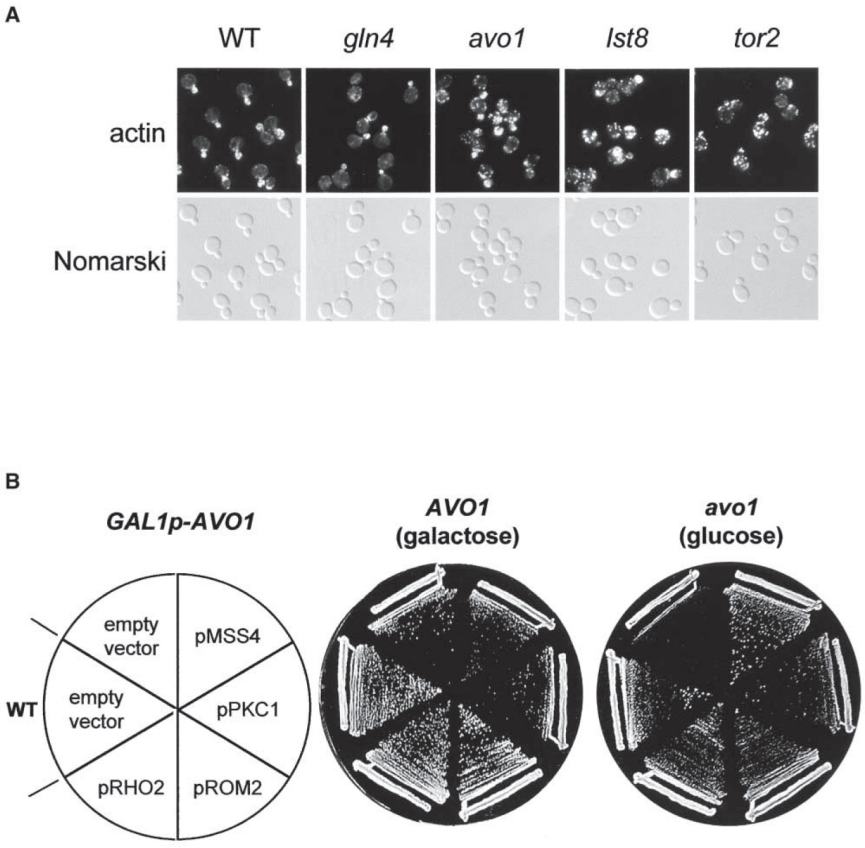


Figure 6. TORC2 Disruption Mimics TOR2 Depletion

(A) AVO1-, LST8-, and TOR2-depleted cells exhibit a depolarized actin cytoskeleton. Wild-type cells (WT), *GAL1* promoter-*GLN4*, -*AVO1*, -*LST8*, and -*TOR2* containing cells were grown in glucose medium for 15 hr. Cells were fixed and treated with rhodamine-phalloidin to stain actin (Experimental Procedures). Actin was visualized by fluoresence microscopy using TRITC filters. Cells were visualized by Nomarski optics. The *GAL1* promoter-*GLN4* strain is named JC26-4a. See Figure 5A for other strain names.

(B) Multicopy *MSS4*, *PKC1*, *ROM2*, or *RHO2* restores growth in AVO1-depleted cells. Wild-type cells (WT, TB50a) containing empty vector (YEplac195), and *GAL1* promoter-*AVO1* cells (RL23-1c) containing empty vector or multicopy *MSS4* (pSH22), *PKC1* (pSH24), *ROM2* (pAS30), or *RHO2* (pC-186) were incubated on an SGal/Gly-uracil plate (AVO1-expressed) or on an SD-uracil (AVO1-depleted) for 2 days. These results indicate that TORC2 mediates the rapamycin-insensitive, TOR2-unique signaling.

5). Like rapamycin-treated cells, *kog1* cells (TORC1 disrupted) appeared swollen with an expanded vacuole, exhibited a decrease in the incorporation of ^{35}S-labeled methionine into protein, expressed GLN3 and RTG1/3 target genes, and accumulated glycogen. In contrast, *avo1* cells (TORC2 disrupted) did not display any of these phenotypes. *lst8* cells (both TORC1 and TORC2 disrupted) expressed RTG1/3 target genes but otherwise resembled *avo1* cells. Thus, disruption specifically of TORC1 mimics rapamycin treatment, indicating that TORC1 but not TORC2 indeed mediates the rapamycin-sensitive, TOR-shared signaling.

TORC2 Mediates Rapamycin-Insensitive, TOR2-Unique Signaling

The findings that TORC2 does not bind rapamycin and contains TOR2 (but not TOR1) suggest that TORC2 mediates the rapamycin-insensitive, TOR2-unique signaling to the actin cytoskeleton. To confirm this, we investigated whether disruption of TORC2, like TOR2 depletion, causes a depolarization of the actin cytoskeleton. *tor2* cells fail to concentrate actin patches in the bud and instead exhibit actin patches randomly distributed throughout the mother cell and the emerging bud (Figure 6A). As a control, we examined the actin cytoskeleton in cells depleted of the essential but unrelated protein GLN4, a glutaminyl-tRNA synthetase. 75%, 60%, 78%, 2%, and 2% of *avo1*, *lst8*, *tor2*, *gln4*, and wild-type cells, respectively, were defective in polarization of the actin cytoskeleton. Thus, *avo1* and *lst8* cells, like *tor2* cells, are defective in polarization of the actin cytoskeleton (Figure 6A). *kog1* cells were omitted from this analysis because the expanded vacuole in these cells interfered with the actin cytoskeleton. To investigate further the in vivo function of TORC2, we examined whether previously identified suppressors of a *tor2* mutation also suppress *kog1*, *avo1*, or *lst8*. Overexpression of *ROM2*,

RHO2, *PKC1*, *BCK1*, or *MKK1*, encoding components of the pathway by which TOR2 signals to the actin cytoskeleton, suppresses the growth defect of a *tor2* mutant (see Introduction). Similarly, overexpression of *ROM2*, *RHO2*, or *PKC1*, or expression of an activated allele of *BCK1* or *MKK1* suppressed the growth and actin defects of *avo1* cells (Figure 6B; data not shown). Overexpression of *MSS4*, encoding a PI-4-P 5-kinase and another multicopy suppressor of *tor2* (Helliwell et al., 1998a), also suppressed the *avo1* mutation. In contrast, *kog1* and *lst8* were not suppressed by any of the above suppressors (data not shown). Thus, disruption specifically of TORC2 mimics TOR2 depletion, confirming that TORC2 mediates the rapamycin-insensitive, TOR2-unique signaling to the actin cytoskeleton.

TORC1 and Possibly TORC2 Are Evolutionarily Conserved

KOG1, LST8, and possibly AVO1 are conserved from yeast to man (see above). Are TORC1 and TORC2 also conserved? To investigate this, we first determined the tissue expression profiles of the human genes encoding the mammalian counterparts of the KOG1, LST8, and AVO1 proteins. We refer to the mammalian orthologs as raptor (mKOG1) (accession number BAA92541), mLST8 (accession number AAH01313), and hSIN1 (mAVO1) (accession number Q9BPZ7), respectively. In parallel with this study, Kim et al. (2002) and Hara et al. (2002) also reported the identification of mKOG1 and named it raptor. hSIN1 (human SIN1) was previously named based on its homology to Sin1p, the AVO1 ortholog in *S. pombe* (Wilkinson et al., 1999). The *raptor* (*mKOG*), *mLST8*, *hSIN1* (*mAVO1*), and *mTOR* genes displayed a remarkably similar expression pattern in adult human tissues (Figure 7A). All were expressed in all tissues examined, including brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, and leukocytes, with particularly high expression in skeletal muscle and kidney. Interestingly, *SIN1* also has a similar expression profile with *mTOR* in rhombomeres 3 and 5 of the developing hindbrain (Christiansen et al., 2001; Hentges et al., 2001). Thus, *mTOR*, *raptor*, *mLST8*, and *hSIN1* are coexpressed, consistent with the possibility that mTOR, raptor, mLST8, and hSIN1 interact. We next investigated directly, by coimmunoprecipitation, whether mTOR interacts with raptor, mLST8, or hSIN1. myc-tagged raptor, mLST8, and hSIN1 were immunoprecipitated from HEK293 cells transiently coexpressing HA-tagged mTOR, and the immunoprecipitates were probed for the presence of HA-mTOR (see Experimental Procedures). mTOR efficiently immunoprecipitated with raptor and mLST8 (Figure 7B). These interactions, as observed previously for the interactions of the yeast orthologs, were not significantly affected by prior treatment (30 and 60 min) of the cells with rapamycin (100 nM) (data not shown). We failed to detect an interaction between mTOR and hSIN1(data not shown). We also investigated whether raptor interacts with mLST8. raptor and mLST8 coimmunoprecipitated (Figure 7C). Thus, mTOR, raptor (mKOG1), and mLST8 interact in vivo, suggesting that TORC1 is conserved (mTORC1). Although we did not detect an interaction between mTOR and hSIN1 (mAVO1), possibly because the tagged hSIN1 may not be functional, the similar tissue expression profile of mTOR and hSIN1 and the interaction between mTOR and mLST8 suggest the possibility that TORC2 is also conserved (mTORC2).

Discussion

Here we describe the identification and characterization of two structurally and functionally distinct TOR complexes, TORC1 and TORC2. TORC1 contains TOR1 (TORC1-A) or TOR2 (TORC1-B), KOG1, and LST8, and mediates rapamycin-sensitive, TOR-shared signaling to the translation machinery, the transcription apparatus, and other targets (see Introduction). TORC1 signals to these targets via the SIT4 phosphatase switch and the GLN3 and RTG1/3 transcription factors, among other effectors. TORC2 contains TOR2 (but not TOR1), AVO1, AVO2, AVO3, and LST8, and mediates rapamycin-insensitive, TOR2-unique signaling to the actin cytoskeleton. TORC2 signals to the actin cytoskeleton via the effector pathway comprising ROM2, RHO1, PKC1, and the PKC1-controlled MAP kinase cascade. Our findings provide a molecular basis for a number of important open issues concerning TOR signaling. First, the observation that the TORs are found in two functionally distinct complexes accounts for the diversity of TOR signaling. The two distinct complexes are in different signaling pathways each with distinct targets. Second, the observation that TOR2 is in both complexes, whereas TOR1 is in only one complex, accounts for the broader specificity of TOR2 signaling. The molecular promiscuity of TOR2 (but not TOR1) explains the earlier findings that TOR2 is essential and in both TOR signaling branches, whereas TOR1 is nonessential and in only one signaling branch. Third, the observation that FKBP-rapamycin binds TORC1 but not TORC2 accounts for the earlier finding that rapamycin selectively inhibits TOR-shared signaling (Zheng et al., 1995; Schmidt et al., 1996). We do not yet know why FKBP-rapamycin fails to bind TOR2 in TORC2, but a reasonable possibility is that one of the components unique to TORC2 (AVO1, AVO2, or AVO3) binds near the FKBP-rapamycin binding site in TOR2 and thereby prevents FKBP-rapamycin binding.

TORC1 and possibly TORC2 are conserved from yeast to man (see Results). The possibility that TORC2 is conserved in mammalian cells has interesting implications. First, this suggests that the single TOR ortholog in mammalian cells is more functionally related to TOR2 than to TOR1. Second, this suggests that there may be rapamycin-insensitive TOR signaling in mammalian cells. Rapamycin-insensitive signaling in mammalian cells may have remained undetected because studies on mTOR signaling have relied exclusively on rapamycin to inhibit mTOR function. However, it remains to be determined that TORC2 is indeed conserved.

What are the individual molecular functions of KOG1, AVO1, AVO2, AVO3, and LST8 in TOR signaling? The findings that depletion of KOG1, AVO1, AVO3, or LST8 mimics a TOR deficiency (unpublished for AVO3) and overexpression does not inhibit growth suggest that all these proteins have an essential, positive role in TOR signaling. AVO2, the sole nonessential protein, may perform a peripheral role. Previous findings on the TOR

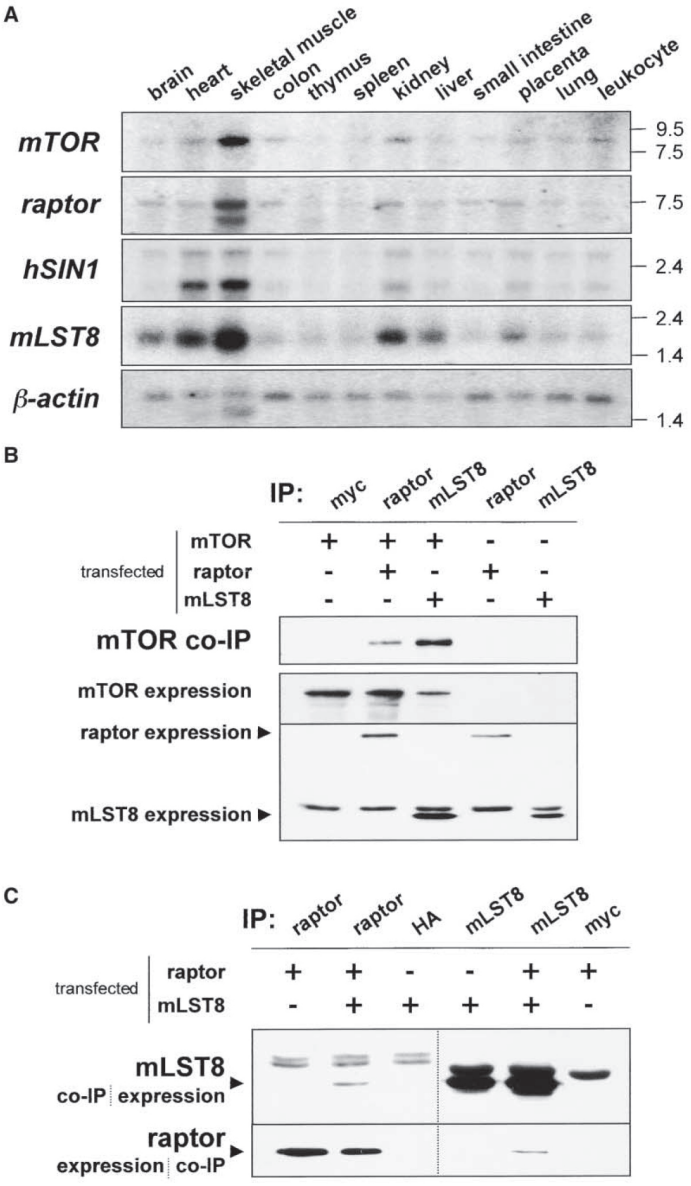


Figure 7. TORC1 and Possibly TORC2 Are Evolutionarily Conserved

(A) *mTOR*, *raptor* (*mKOG1*), *hSIN1*, and *mLST8* have similar tissue expression profiles. PCR products corresponding to *mTOR*, *raptor*, *hSIN1*, *mLST8*, or β-actin were labeled with ^{32}P and used to probe RNA prepared from the indicated human tissues (Experimental Procedures). Numbers on the right indicate the size standards in kilobases.

(B) mTOR coimmunoprecipitates with raptor (mKOG1) and mLST8. HEK293 cells were transfected with plasmids expressing HA-mTOR (pHA-mTOR [WT]), myc-raptor (myc-prk5-raptor), and/or myc-mLST8 (pCIM-mLST8) as indicated. Protein lysates were prepared and subjected to immunoprecipitation with anti-myc. Immunoprecipitates resolved by SDS-PAGE were blotted with anti-HA to detect coimmunoprecipitated mTOR (top panel). Identical protein lysates subjected to immunoprecipitation with anti-HA and anti-myc and subsequently probed with anti-HA and anti-myc are shown to control for mTOR expression (middle panel), and raptor and mLST8 expression (bottom panel).

(C) raptor (mKOG1) and mLST8 coimmunoprecipitate. HEK293 cells were transfected with plasmids expressing HA-raptor (HA-prk5-raptor) and/or myc-LST8 (pCIM-mLST8) as indicated. Protein lysates were prepared and immunoprecipitated with anti-HA antibody (lanes 1–3) or anti-myc antibody (lanes 4–6). Precipitates were resolved by SDS-PAGE and blotted with anti-myc antibody (top panels) or anti-HA antibody (bottom panels) to control for protein expression and to detect coimmunoprecipitated protein.

partner proteins or their orthologs provide further hints on individual function. The highly conserved KOG1 protein contains four internal HEAT repeats and seven C-terminal WD-40 repeats. The KOG1 ortholog in *S. pombe*, Mip1p, is an essential protein that binds the RNA binding protein Mei2p and the transcription factor Ste11p and, like SpTor1p, is involved in nutrient-responsive sexual development (Kawai et al., 2001; Shinozaki-Yabana et al., 2000; Weisman and Choder, 2001). Thus, KOG1 may serve as a scaffold protein to couple TOR to its effectors.

LST8 consists essentially of seven WD-40 repeats (Ochotorena et al., 2001). Like TOR, LST8 has previously been implicated in nutrient-sensitive permease sorting and regulation of the RTG1/3 transcription factor (see Introduction). The findings that LST8 is common to both TORC1 and TORC2 and that both complexes may respond to common upstream cues but have different downstream targets suggest that LST8 acts upstream of TOR, possibly in nutrient sensing. *S. pombe* mutants defective in the LST8 ortholog Wat1p have multiple, seemingly unrelated phenotypes, including a defect in actin organization (Kemp et al., 1997; Ochotorena et al., 2001). The complexity of the *wat1/lst8* phenotype may reflect multiple functions of the Wat1p/LST8 protein, including a TOR-independent function. Interestingly, LST8 is more abundant than TOR, and a pool of LST8 elutes from a gel filtration column separately from TOR

(unpublished data). Multiple functions of LST8 may account for our observation that LST8 depletion only partly mimics rapamycin treatment.

AVO1 has a Ras association domain, also known as a Raf-like Ras binding domain. The AVO1 ortholog in *D. discoideum*, RIP3, contains a similar Ras association domain and interacts with Ras (Lee et al., 1999). hSIN1 (mAVO1), although it does not contain an evident Ras association domain, was originally isolated in a screen for human cDNAs that interfere with RAS signaling in yeast (Colicelli et al., 1991). Thus, the function of AVO1 may be to couple TORC2 to Ras, a coupling that has not been reported previously.

AVO3 contains a RasGEFN domain, a domain found in exchange factors and activating proteins for Ras-like small GTPases. The AVO3 ortholog in *D. discoideum*, pianissimo, appears to function with the AVO1 ortholog RIP3 to mediate Ras signaling. Thus, AVO3 and AVO1 are functionally related in *D. discoideum*, as in yeast. AVO3 may act with AVO1 to couple TORC2 to RAS. Alternatively, AVO3 may be part of the so far unknown mechanism by which TOR2 (TORC2) activates ROM2 and the Ras-like small GTPase RHO1. AVO3 was originally identified in *S. cerevisiae* as a mutation that suppresses *csg2*. Interestingly, mutations in *TOR2* and *MSS4*, a multicopy suppressor of *tor2*, were also identified in this screen (Beeler et al., 1998). *csg2* mutants accumulate the sphingolipid inositolphosphorylceramide (IPC) (Beeler et al., 1998). Sphingolipids, like TORC2, may be required upstream of RHO1 for PKC1-mediated organization of the actin cytoskeleton (Schmelzle et al., 2002). It will be of interest to determine whether sphingolipids signal to TORC2, or vice versa, to control RHO1 and ultimately the actin cytoskeleton.

In conclusion, we have identified several TOR partner proteins that play an essential, positive role in TOR signaling. Some of these proteins are most likely the long sought upstream regulators and direct effectors of TOR. Future work will further elucidate the molecular functions of the partner proteins in TOR signaling and may reveal new drug targets.

Experimental Procedures

Strains, Plasmids, Media, and Reagents

The *S. cerevisiae* strains used in this study are listed in Supplemental Table S1 at http://www.molecule.org/cgi/content/full/10/3/457/DC1. All strains are isogenic derivatives of JK9-3da. Plasmids used in this study are described in Supplemental Table S2 at http://www.molecule.org/cgi/content/full/10/3/457/DC1. PCR cassettes were used to generate gene deletions and modifications, as described (Longtine et al., 1998; Puig et al., 2001). A functional myc-*TOR1* from the plasmid pAN42 was integrated into the genome of AN9-2a by selecting for resistance to 3 mM caffeine and screening for the loss of the *kan*MX4 marker originally in the chromosomal *TOR1* locus to generate strain RL60a. Rich media, YPD or YPGal/Gly, and synthetic complete media, SD or SGal/Gly, were as described previously (Beck et al., 1999; Sherman, 1991). Nitrogen starvation experiments were performed with synthetic media as previously described (Schmidt et al., 1998). All cultures were grown at 30°C. The lysis buffer used to prepare cell extracts was 1× PBS, 10% (w/v) glycerol, 0.5% (v/v) tween 20, and the inhibitors used were 10 mM NaF, 10 mM NaN_3, 10 mM p-nitrophenylphosphate, 10 mM sodiumpyrophosphate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail (Roche). For TOR2-, KOG1-, AVO1-, or LST8-depletion experiments, cells from logarithmically growing SGal/Gly or YPGal/Gly cultures were inoculated into SD or YPD medium, respectively. Glucose cultures were shaken for 15 hr before cells were harvested for analysis. Final OD_{600} values were between 0.1 and 0.8. Rapamycin was used at a final concentration of 200 nM from a 1 mg/ml stock in 90% ethanol/10% Tween 20.

Gel Filtration

A 500 ml culture of logarithmically growing cells expressing tagged TOR1, TOR2, or LST8 was chilled, harvested by centrifugation, and washed with cold water before being resuspended in 2 ml lysis buffer. Cells were lysed by vortexing 6 × 30 s with glass beads, using a FastPrep machine (Savant Instruments). Crude extracts were cleared with a 5 min, 500 g spin, and the supernatant was passed through a 0.22 μm filter. Five milligrams of protein (in a volume of less than 0.5 ml) was loaded onto a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech). The flow rate was adjusted to 0.2 ml/minute, and 1 ml fractions were collected. Elution profiles of tagged proteins were analyzed by immunoprecipitation/Western analysis and compared to the elution profile of known standards.

TOR1 and TOR2 Purifications

Cells expressing a tagged or untagged (mock purification) version of TOR were grown in five liters of YPD to late log phase and harvested as described above. Cells were lysed in 30 ml lysis buffer plus inhibitors with glass beads using a Biospecs Beadbeater. Cell lysates typically circa 700 mg were cleared with a 5 min, 500× g spin, diluted with lysis buffer to 5 mg/ml, and subsequently passed over an ion exchange resin—20 ml packed column volume Q-sepharose (myc-TOR1) or 20 ml packed column volume SP-sepharose (HA-TOR2). The resins were washed with lysis buffer (2.5 column volumes) and then lysis buffer containing 50 mM potassium acetate (2.5 column volumes). Bound proteins were eluted with 45 ml lysis buffer containing 600 mM potassium acetate. The eluate was precleared over a Protein A-sepharose (Amersham Pharmacia Biotech) column (300 μl packed volume) prior to the addition of anti-myc or anti-HA crosslinked to Protein A-sepharose beads (150 μl). After extensive washing with lysis buffer, immunoprecipitated proteins were solubilized in SDS-PAGE sample buffer, separated by electrophoresis, and visualized by silver staining (Shevchenko et al., 1996).

Mass Spectrometric Analysis of Protein Bands

Silver-stained proteins separated by 1D gel electrophoresis were digested as described (Perrot et al., 1999). Peptides for MALDI-TOF analysis were desalted on C18 ZipTips (P10 tip size, Millipore) with 1.5 microliters 80% AcCN, 0.1% TFA, containing 1 μg/μl α-cyano-4-hydroxycinnamic acid (CHCA, Aldrich Chemical Co.). Five hundred nanoliters of the eluate was deposited onto anchor spots of a Scout 400 m/36 sample support (Bruker Daltonik). Mass spectra were recorded on a Bruker Scout 26 Reflex III instrument (Bruker Daltonik). For database analysis, all spectra recorded on tryptic peptides were compared to the *S. cerevisae* protein database using the Mascot search program (www.matrixscience.com).

Immunoprecipitations and FPR1-TAP Pull-Downs

Yeast extracts from cells (typically a 250 ml culture in YPD grown to midlog phase) expressing the appropriately tagged proteins were prepared in lysis buffer plus inhibitors, and cleared as described above. An aliquot of extract containing 3 mg of protein was adjusted to 0.75 ml with lysis buffer plus inhibitors. For immunoprecipitations, 1 μl of either concentrated 12CA5 (anti-HA) or 9E10 (anti-myc) tissue culture supernatant was added, tubes were rotated for 1.5 hr at 4°C, 20 μl protein G-sepharose slurry (Sigma) was added, and tubes were rotated for an additional 2 hr at 4°C. For FPR1-TAP pull-downs, 20 μl IgG Sepharose 6 fast flow (Amersham Pharmacia Biotech) was added, and tubes were rotated for 2 hr at 4°C. For both immunoprecipitations and pull-downs, beads were collected by centrifugation, washed three times with 1 ml lysis buffer, and resuspended in 2× SDS-PAGE sample buffer for electrophoresis. After SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes, blocked in 5% milk in 1× PBS 0.1% tween 20, and incubated with primary antibody (12CA5 or 9E10 1:10000 in blocking solution). The membranes were subsequently washed, and tagged proteins were de-

tected with horseradish peroxidase-conjugated anti-mouse secondary antibodies and ECL reagents (Amersham Pharmacia Biotech).

RNA Isolation and Northern Blot Analysis

RNA from cells grown for 15 hr in SD medium containing drug vehicle alone or rapamycin for 30 min was analyzed. Total RNA preparation and Northern blot analysis were performed according to standard protocols (Collart and Oliviero, 1993). Human RNA blots were obtained commercially (Multiple Tissue Northern Blot, CLONTECH) and were probed according to the manufacturer's instructions. Yeast DNA probes were generated by PCR using genomic DNA from yeast strain JK9-3da as template and oligonucleotide primers specific for the genes to be analyzed. Human DNA probes were generated by PCR using cloned cDNAs as templates. PCR products were labeled using a DecaLabel DNA labeling kit (MPI Fermentas).

Actin Staining

Cells from 15 hr YPD cultures were fixed in formaldehyde (3.7%) and potassium phosphate buffer (100 mM [pH 6.5]) and stained with tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin (Sigma) to visualize actin, as described previously (Benedetti et al., 1994). Actin cytoskeleton organization was assessed in several hundred cells, as described (Helliwell et al., 1998a).

^{35}S-methionine Incorporation

To analyze protein synthesis, 0.02 OD_{600} equivalents of cells from 15 hr SD cultures were adjusted to a final volume of 100 ul with 5 μCi Easy Tag EXPRESS protein Labeling Mix [^{35}S] (NEN) and SD medium. Rapamycin treatment was for 30 min preceding the incorporation assay. Cells were labeled for 10 min at 30°C after which a 10 μl aliquot was removed and diluted into 1 ml of cold 1 mM NaF/NaN_3. These cells were collected on glass fiber filters, and were washed with 10 ml cold water. Counts from these filters were used to calculate total methionine uptake. To the remaining 90 μl of cells, 210 μl of 10% TCA was added. These samples were boiled to deacylate tRNAs, and precipitated material was collected on glass fiber filters and washed twice with 5 ml 10% TCA and once with 5 ml acetone. Counts from these filters were used to calculate the amount of methionine incorporated into protein. Typically, approximately 45% of methionine imported into untreated wild-type cells was incorporated into protein. This value was set to 100%, and incorporation/uptake ratios for rapamycin-treated or mutant cells are expressed relative to rapamycin-untreated wild-type cells.

Glycogen Staining

Seven OD_{600} equivalents from 15 hr YPD cultures (OD_{600} 0.1–0.3) or YPD cultures treated with rapamycin or drug vehicle alone for 3 hr were harvested onto Millipore HA filters. Filters were subsequently placed on a solid agar matrix and exposed to iodine vapor for 1 min as described (Barbet et al., 1996).

Mammalian Tissue Culture, Transfections, and Lysate Preparation

HEK293 cells were maintained in DMEM (Sigma) + 10% fetal bovine serum. Cells, 50%–60% confluent, in 35 mm dishes were transfected with 1 μg of DNA using jetPEI Transfection Reagent (Qbiogene) following the manufacturer's instructions for transfection of adherent cells. Fifteen to twenty hours later, cells were washed with 500 μl 1× PBS and scraped into 200 μl lysis buffer plus inhibitors. Cells were incubated on ice for 10 min, and lysates were cleared with a 5 min, 500× g spin. The total cleared lysate was used for immunoprecipitation with 0.5 μl of appropriate antibody as otherwise described above.

Acknowledgments

We thank D. Sabatini for communicating unpublished results and for providing plasmids encoding full-length, tagged versions of raptor (mKOG1). We also thank K. Yonezawa for communicating unpublished results, and G. Thomas for providing a plasmid-encoding HA-tagged mTOR. We thank T. Schmelzle and S. Helliwell for helpful comments and critical reading of the manuscript. R.L. is a recipient of a European Molecular Biology Organization (EMBO) Long-Term Fellowship. E.J. is a recipient of a fellowship from the Cancer Research Institute. A.L. and J.L.C. are recipients of fellowships from the Federation of European Biochemical Societies (FEBS). This work was supported by grants from the Canton of Basel and the Swiss National Science Foundation (M.N.H.).

Received: July 8, 2002
Revised: August 20, 2002

References

Alarcon, C.M., Heitman, J., and Cardenas, M.E. (1999). Protein kinase activity and identification of a toxic effector domain of the target of rapamycin TOR proteins in yeast. Mol. Biol. Cell *10*, 2531–2546.

Andrade, M.A., and Bork, P. (1995). HEAT repeats in the Huntington's disease protein. Nat. Genet. *11*, 115–116.

Barbet, N.C., Schneider, U., Helliwell, S.B., Stansfield, I., Tuite, M.F., and Hall, M.N. (1996). TOR controls translation initiation and early G1 progression in yeast. Mol. Biol. Cell *7*, 25–42.

Beck, T., and Hall, M.N. (1999). The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature *402*, 689–692.

Beck, T., Schmidt, A., and Hall, M.N. (1999). Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. J. Cell Biol. *146*, 1227–1238.

Beeler, T., Bacikova, D., Gable, K., Hopkins, L., Johnson, C., Slife, H., and Dunn, T. (1998). The Saccharomyces cerevisiae TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca2+-sensitive csg2Δ mutant. J. Biol. Chem. *273*, 30688–30694.

Benedetti, H., Raths, S., Crausaz, F., and Riezman, H. (1994). The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. Mol. Biol. Cell *5*, 1023–1037.

Bosotti, R., Isacchi, A., and Sonnhammer, E.L. (2000). FAT: a novel domain in PIK-related kinases. Trends Biochem. Sci. *25*, 225–227.

Cardenas, M.E., Cutler, N.S., Lorenz, M.C., Di Como, C.J., and Heitman, J. (1999). The TOR signaling cascade regulates gene expression in response to nutrients. Genes Dev. *13*, 3271–3279.

Chen, M.Y., Long, Y., and Devreotes, P.N. (1997). A novel cytosolic regulator, Pianissimo, is required for chemoattractant receptor and G protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in Dictyostelium. Genes Dev. *11*, 3218–3231.

Christiansen, J.H., Coles, E.G., Robinson, V., Pasini, A., and Wilkinson, D.G. (2001). Screening from a subtracted embryonic chick hindbrain cDNA library: identification of genes expressed during hindbrain, midbrain and cranial neural crest development. Mech. Dev. *102*, 119–133.

Colicelli, J., Nicolette, C., Birchmeier, C., Rodgers, L., Riggs, M., and Wigler, M. (1991). Expression of three mammalian cDNAs that interfere with RAS function in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *88*, 2913–2917.

Collart, M.A., and Oliviero, S. (1993). Preparation of yeast RNA. In Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, R.E. Kinston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds. (New York: John Wiley & Sons), pp. 13.12.1–13.12.5.

Crespo, J.L., Powers, T., Fowler, B., and Hall, M.N. (2002). The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. Proc. Natl. Acad. Sci. USA *99*, 6784–6789.

Dennis, P.B., Fumagalli, S., and Thomas, G. (1999). Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. Curr. Opin. Genet. Dev. *9*, 49–54.

Di Como, C.J., and Arndt, K.T. (1996). Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev. *10*, 1904–1916.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.

Groves, M.R., and Barford, D. (1999). Topological characteristics of helical repeat proteins. Curr. Opin. Struct. Biol. *9*, 383–389.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N. Hidyaat, S., Tokunaga, K., Avruch, J., and Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell *110*, 177–189.

Hardwick, J.S., Kuruvilla, F.G., Tong, J.K., Shamji, A.F., and Schreiber, S.L. (1999). Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. Proc. Natl. Acad. Sci. USA *96*, 14866–14870.

Heitman, J., Movva, N.R., and Hall, M.N. (1991a). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science *253*, 905–909.

Heitman, J., Movva, N.R., Hiestand, P.C., and Hall, M.N. (1991b). FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA *88*, 1948–1952.

Helliwell, S.B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R., and Hall, M.N. (1994). TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. Mol. Biol. Cell *5*, 105–118.

Helliwell, S.B., Howald, I., Barbet, N., and Hall, M.N. (1998a). TOR2 is part of two related signaling pathways coordinating cell growth in Saccharomyces cerevisiae. Genetics *148*, 99–112.

Helliwell, S.B., Schmidt, A., Ohya, Y., and Hall, M.N. (1998b). The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. Curr. Biol. *8*, 1211–1214.

Hentges, K.E., Sirry, B., Gingeras, A.C., Sarbassov, D., Sonenberg, N., Sabatini, D., and Peterson, A.S. (2001). FRAP/mTOR is required for proliferation and patterning during embryonic development in the mouse. Proc. Natl. Acad. Sci. USA *98*, 13796–13801.

Jacinto, E., Guo, B., Arndt, K.T., Schmelzle, T., and Hall, M.N. (2001). TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. Mol. Cell *8*, 1017–1026.

Jiang, Y., and Broach, J.R. (1999). Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. EMBO J. *18*, 2782–2792.

Kawai, M., Nakashima, A., Ueno, M., Ushimaru, T., Aiba, K., Doi, H., and Uritani, M. (2001). Fission yeast tor1 functions in response to various stresses including nitrogen starvation, high osmolarity, and high temperature. Curr. Genet. *39*, 166–174.

Kemp, J.T., Balasubramanian, M.K., and Gould, K.L. (1997). A wat1 mutant of fission yeast is defective in cell morphology. Mol. Gen. Genet. *254*, 127–138.

Kim, D.-H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163–175.

Komeili, A., Wedaman, K.P., O'Shea, E.K., and Powers, T. (2000). Mechanism of metabolic control: target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. J. Cell Biol. *151*, 863–878.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R., and Hall, M.N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell *73*, 585–596.

Kunz, J., Schneider, U., Howald, I., Schmidt, A., and Hall, M.N. (2000). HEAT repeats mediate plasma membrane localization of Tor2p in yeast. J. Biol. Chem. *275*, 37011–37020.

Lee, S., Parent, C.A., Insall, R., and Firtel, R.A. (1999). A novel Ras-interacting protein required for chemotaxis and cyclic adenosine monophosphate signal relay in Dictyostelium. Mol. Biol. Cell *10*, 2829–2845.

Liu, Z., Sekito, T., Epstein, C.B., and Butow, R.A. (2001). RTG-dependent mitochondria to nucleus signaling is negatively regulated by the seven WD-repeat protein Lst8p. EMBO J. *20*, 7209–7219.

Longtine, M.S., McKenzie, A., III, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953–961.

Lorenz, M.C., and Heitman, J. (1995). TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. J. Biol. Chem. *270*, 27531–27537.

Ochotorena, I.L., Hirata, D., Kominami, K., Potashkin, J., Sahin, F., Wentz-Hunter, K., Gould, K.L., Sato, K., Yoshida, Y., Vardy, L., and Toda, T. (2001). Conserved Wat1/Pop3 WD-repeat protein of fission yeast secures genome stability through microtubule integrity and may be involved in mRNA maturation. J. Cell Sci. *114*, 2911–2920.

Perrot, M., Sagliocco, F., Mini, T., Monribot, C., Schneider, U., Shevchenko, A., Mann, M., Jenoe, P., and Boucherie, H. (1999). Two-dimensional gel protein database of *Saccharomyces cerevisiae*. Electrophoresis *20*, 2280–2298.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods *24*, 218–229.

Roberg, K.J., Bickel, S., Rowley, N., and Kaiser, C.A. (1997). Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae by SEC13, LST4, LST7 and LST8. Genetics *147*, 1569–1584.

Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. Cell *103*, 253–262.

Schmelzle, T., Helliwell, S.B., and Hall, M.N. (2002). Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast. Mol. Cell. Biol. *22*, 1329–1339.

Schmidt, A., Kunz, J., and Hall, M.N. (1996). TOR2 is required for organization of the actin cytoskeleton in yeast. Proc. Natl. Acad. Sci. USA *93*, 13780–13785.

Schmidt, A., Bickle, M., Beck, T., and Hall, M.N. (1997). The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. Cell *88*, 531–542.

Schmidt, A., Beck, T., Koller, A., Kunz, J., and Hall, M.N. (1998). The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. EMBO J. *17*, 6924–6931.

Schreiber, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science *251*, 283–287.

Sherman, F. (1991). Getting started with yeast. Methods Enzymol. *194*, 3–21.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. *68*, 850–858.

Shinozaki-Yabana, S., Watanabe, Y., and Yamamoto, M. (2000). Novel WD-repeat protein Mip1p facilitates function of the meiotic regulator Mei2p in fission yeast. Mol. Cell. Biol. *20*, 1234–1242.

Stan, R., McLaughlin, M.M., Cafferkey, R., Johnson, R.K., Rosenberg, M., and Livi, G.P. (1994). Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue. J. Biol. Chem. *269*, 32027–32030.

Weisman, R., and Choder, M. (2001). The fission yeast TOR homolog, tor1+, is required for the response to starvation and other stresses via a conserved serine. J. Biol. Chem. *276*, 7027–7032.

Wilkinson, M.G., Pino, T.S., Tournier, S., Buck, V., Martin, H., Christiansen, J., Wilkinson, D.G., and Millar, J.B. (1999). Sin1: an evolutionarily conserved component of the eukaryotic SAPK pathway. EMBO J. *18*, 4210–4221.

Yaffe, M.B., Leparc, G.G., Lai, J., Obata, T., Volinia, S., and Cantley, L.C. (2001). A motif-based profile scanning approach for genome-wide prediction of signaling pathways. Nat. Biotechnol. *19*, 348–353.

Zheng, X.F., Florentino, D., Chen, J., Crabtree, G.R., and Schreiber, S.L. (1995). TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. Cell *82*, 121–130.

2. 2. Additional results

AVO1 is not required for the rapamycin-sensitive TOR-sensitive function

Previously, we showed that AVO1 is required for the rapamycin-insensitive TOR2-unique signaling branch, the organization of the actin cytoskeleton. To test whether AVO1 is only involved in the TOR2-unique function, the effect of AVO1 depletion on the rapamycin-sensitive TOR signaling branch was examined. TOR controls the phosphorylation status and thereby the activity of NPR1 (Schmidt et al., 1998). Under favourable nutrient conditions TOR maintains NPR1 in a highly phosphorylated and inactive state. Upon TOR inactivation by rapamycin treatment NPR1 is dephosphorylated by SIT4 and activated. To check the effect of AVO1 on NPR1 phosphorylation *AVO1* was put under the control of a galactose-inducible and glucose-repressible promoter and the phosphorylation status of NPR1 was detected by gel mobility shift. NPR1 phosphorylation was similar in wild-type cells or cells overexpressing AVO1 (Fig. 2.1; compare lane 1, 2 to lane 5, 6). NPR1 phosphorylation was similarly unaltered by depletion of AVO1 (Fig. 2.1.; compare lane 3, 4 to lane 7, 8). As in wild-type cells, NPR1 was phosphorylated under favourable growth conditions and dephosphorylated upon treatment with rapamycin. Thus, AVO1 is not required for the regulation of NPR1 suggesting that AVO1 does not play a role in the rapamycin-sensitive TOR signaling branch.

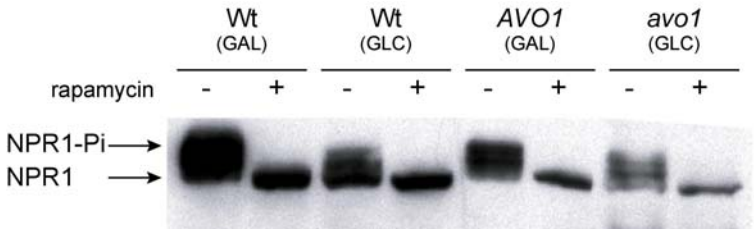


Figure 2.1. AVO1 does not affect the phosphorylation status of NPR1

Wild-type (TB50**a**) or *GAL1* promoter-*AVO1* (RL23-1c) strains carrying HA-NPR1 (pEJ23, YEplac181::HA-NPR1) were grown for 15 hr in either SGal/Gly-leu or SD-leu medium to log phase at 30°C and then either treated for 10 min with 100 ng/ml rapamycin or drug vehicle (90% ethanol, 10% Tween-20). Cells were harvested and lysed. Total cellular extracts (30 μg total protein) were fractionated on 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with anti-HA antibody.

Is AVO1 a conserved protein?

The components of the rapamycin-sensitive TORC1, KOG1 and LST8 are highly conserved from yeast to flies to humans. Similar to TOR1 and TOR2 in yeast, mTOR forms a complex with raptor (KOG1 in yeast) and mLST8 (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). Raptor and mLST8 seem to be important for the rapamycin-sensitive functions of mTOR, such as phosphorylation of 4E-BP1 and p70S6K. The components of the rapamycin-insensitive TORC2 do not have strong homologues in higher organisms. AVO1 displays a similarity to hSIN1, a human protein that was identified as a *RAS* suppressor in yeast (Colicelli et al., 1991). The overall identity of the two proteins is 19%. The two proteins are most similar in the C-terminus of AVO1, whereas the N-terminus of AVO1 is not conserved. hSIN1 exhibits a much smaller molecular weight (58kDa) compared to AVO1 (130kDa). Tissue blot analysis revealed that the expression level of *hSIN*1 was similar to that of *mTOR* (Loewith et al., 2002). Both are highly expressed in muscle, heart, liver and kidney. To test a possible interaction between hSIN1 and mTOR, recombinant, tagged hSIN1 and mTOR were transfected into HEK-239 cells to perform coimmunoprecipitations. Myc- tagged hSIN1 did not precipitate with HA- tagged mTOR in HEK-239 cells (data not shown). In collaboration with the group of Markus Rüegg (Biozentrum, Basel) a polyclonal antibody against hSIN1 was raised to check whether endogenous, untagged hSIN1 could interact with mTOR. However, endogenous hSIN1 was not able to interact with mTOR by coimmunoprecipitation in HEK-239 leading to the conclusion that hSIN1 does not interact with mTOR under the conditions we tested.

2. 3. Discussion

The additional data underscore the existence of two structurally and functionally distinct TOR complexes in yeast. AVO1, a member of TORC2 appears to be only required for the TOR2-unique signaling branch and did not affect the rapamycin-sensitive TOR signaling branch such as the phosphorylation status of NPR1 nor the expression of GLN3 and RTG1/3 target genes (Loewith et al., 2002).

Whether AVO1 is a conserved protein is unclear. We failed to detect an interaction between the putative AVO1 homologue hSIN1 and mTOR by coimmunoprecipitation.

However, there is evidence that some protein-protein interactions of TORC in mammalian cells are rather unstable and very sensitive to detergents (Hara et al., 2002; Kim et al., 2002). It is possible that our conditions are not suited to detect an association between hSIN1 and mTOR. It is also possible that the interaction between hSIN1 and mTOR is dependent on the cell type. Different TOR complexes could be formed depending on the function of mTOR in various tissues.

Nevertheless, it has been recently found, that there is a homologue of AVO3 in mammalian cells indicating the existence of different and possibly rapamycin-insensitive TOR complexes in mammalian cells which might also be important for the organization of the actin cytoskeleton (R. Loewith, E. Jacinto unpublished data).

3. Characterization of AVO1 and LST8

3. 1. Introduction

In yeast TOR exists in two complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) as shown in the first part of this thesis. These two complexes, which contain both common and distinct proteins, account for the specificity of TOR signaling. TORC1 mediates the rapamycin-sensitive signaling branch, which regulates transcription, translation and ribosome biogenesis. TORC2 signaling is rapamycin-insensitive and is required for the organization of the actin cytoskeleton. How TORC2 controls the organization of the actin cytoskeleton is not completely known. As introduced in the general introduction (Part 1), TOR2 is thought to regulate actin via the control of the RHO1 GTPase switch (Helliwell et al., 1998b; Schmidt et al., 1997). Upon activation of RHO1 by its exchange factor ROM2, activated RHO1 interacts with, and activates PKC1. Activated PKC1 subsequently signals to actin by activation the MAP kinase cascade consisting of BCK1, MKK1/2 and MPK1.

The yeast protein kinases YPK1 and YPK2, an essential pair of homologous kinases have been linked to the PKC1-controlled MAP kinase cascade (Schmelzle et al., 2002). YPKs are phosphorylated and thereby activated by the kinase pair PKH1 and PKH2 (Casamayor et al., 1999). Apart from regulating the YPKs, the PKHs also activate PKC1 through direct phosphorylation (Inagaki et al., 1999). The PKH - YPK signaling pathway is suggested to act downstream of a sphingolipid-derived signal (Sun et al., 2000).

The PKHs are the yeast orthologues of the mammalian phosphoinositide-dependent kinase (PDK1). Mitogenically activated PDK1 has been reported to phosphorylate a conserved residue in the activation loop of several members of the AGC kinases (cAMP-dependent protein kinase (PKA), protein kinase G (PKG), protein kinase C (PKC)) (Vanhaesebroeck and Alessi, 2000). Among other AGC kinases PDK1 activates p70S6K and serum- and glucocorticoid-induced kinase (SGK), the mammalian homologue of YPK1 and YPK2 indicating that the PKH-YPK pathway is conserved (Alessi et al., 1998; Park et al., 1999).

Here we study the role two TORC2 proteins AVO1 and LST8, mainly focusing on AVO1. Analyzing *avo1* and *lst8* mutants we confirm their positive function with TOR2 in the regulation of the actin cytoskeleton.

3. 2. Results

Growth and actin defects of *avo1* and *lst8* mutants are suppressed by activation of the RHO1-GTPase switch or the PKC1 MAP kinase cascade

Previously, we have reported that activation of the RHO1 GTPase switch by overexpression of a RHO1 GEF ROM2, the RHO1-related GTPase RHO2, or activated alleles of *PKC1*, *BCK1* or *MKK1* restored the viability of an *avo1* mutant strain (Loewith et al., 2002). We also found that deletion of *SAC7*, encoding a GAP for RHO1, suppressed the growth defect of *avo1* (data not shown). We did not observe any suppression of *avo1* mutants by overexpression of the TOR2-suppressors *SUR1* or *PLC1* nor by overexpression of other RHO1 effectors *FKS1*, encoding glucan synthase, *BNI1*, encoding the yeast formin or SKN7 (data not shown). Interestingly, overexpression of *YPK2*, encoding a kinase that has been previously implicated in the cell integrity pathway restored the growth defect of *avo1* cells (data not shown). Since YPK2 is phosphorylated and activated by the PKH1 and PKH2 protein kinases, which have also been linked to PKC1, we tested whether overexpression of the PKHs could rescue an *avo1* mutant. Overexpression of *PHKs* did not suppress *avo1*, possibly because the kinases are not in their activated state (data not shown).

To study the essential role of LST8 a conditional *LST8* mutant strain was created replacing the original *LST8* promoter with a glucose-repressible and galactose-inducible *GAL1* promoter. Because *tor2* and *avo1* mutant cells are suppressed by activation of the RHO1 GTPase switch or by hyperactivation of the PKC1 effector MAP kinase pathway, we tested whether overexpression of components in these pathways were also able to suppress an *lst8* mutant. Activation of the RHO1 GTPase switch by overexpression of *ROM2* or *RHO2* did not restore the growth defect of *lst8* mutant cells, whereas mutation of *SAC7* weakly suppressed the lethality of an *lst8* mutant (Fig. 3.1. and data not shown). Additionally, overexpression of *MSS4* that encodes a phosphatidylinositol kinase, and is a strong suppressor of *tor2* and *avo1* did not rescue the *lst8* mutant. As shown in Fig. 3.1. activation of the PKC1-MAP kinase cell integrity

pathway by overexpression of *PKC1, BCK1, MKK1* and constitutively activated alleles thereof lead to a partial suppression of the growth defect of *lst8* on glucose-containing media. Interestingly, a weak suppression of *lst8* mutant cells was observed in cells overexpressing *TOR2*, a result not observed in *avo1* cells. Surprisingly, the most potent multicopy suppressor of *lst8* was *YPK2*, which restored the growth of *lst8* cells to almost wild-type level. Thus, analysis of *avo1* and *lst8* multicopy suppressors underscores the positive role of AVO1 and LST8 in TORC2. The fact that LST8 is present in both complexes, in the rapamycin-sensitive TORC1 and in TORC2 might explain why the lethality of *lst8* can only be partially suppressed by known TORC2 suppressors.

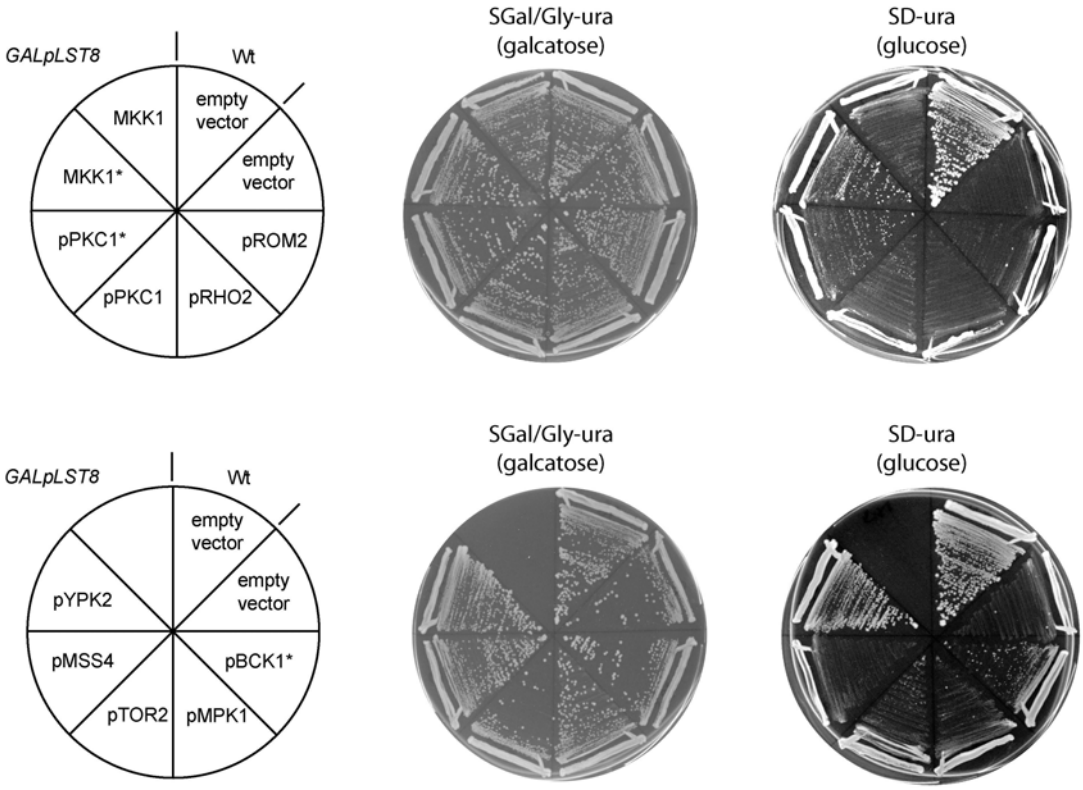


Figure 3.1. Growth defect of *lst8* mutant cells is suppressed by activation of the PKC1 MAP kinase cascade and *YPK2*

Wild-type (wt) (TB50**a**) and *GAL1* promoter-*LST8* cells transformed with empty vector, pRHO2, pPKC1, pPKC1*, pMKK1*, pMKK1, pBCK1*, pMPK1, pTOR2, pMSS4, or pYPK2 were streaked onto SGal/Gly-ura (galactose) and SD-ura (glucose) plates and incubated at 30°C.

Since AVO1 is required for organization of the actin cytoskeleton we tested if the identified growth suppressors could rescue the actin defect of *avo1* mutant cells. The distribution of the actin cytoskeleton of wild-type cells was compared to *avo1* mutant cells containing either empty control plasmid or a multicopy suppressor plasmid. Cells were grown under glucose conditions leading to the depletion of AVO1, and the distribution of the actin cytoskeleton was visualized by phalloidin staining. Wild-type cells exhibited a normal distribution of the cell cycle-dependent organization of the actin; in small-budded cells, the actin patches are concentrated at the bud and actin cables are oriented in the mother cell towards to bud (Fig. 3.2.). *avo1* mutant cells containing an empty control plasmid failed to properly localize actin as indicated by a random distribution of actin patches in the mother cell of small-budded cells (Fig. 3.2.). In *avo1* cells, overexpressing a constitutively active allele of *PKC1* partially restored the defect in the actin organization. These cells concentrated actin patches in the growth site, however, we did not observe actin cables in the mother cell of small-budded cells (Fig. 3.2.). We also looked at the actin cytoskeleton in *lst8* mutant cell overexpressing *YPK2*. However, as reported earlier the actin defect of *lst8* mutant cells was not as pronounced as in *avo1* mutant cells (Loewith et al., 2002) and we failed to detect a significant difference between *lst8* mutant cells transformed with empty plasmid or with pYPK2 (data not shown). The finding that activated PKC1 partially suppressed the actin defect of *avo1* mutant cells support an involvement of AVO1 in the PKC1-MAP kinase pathway.

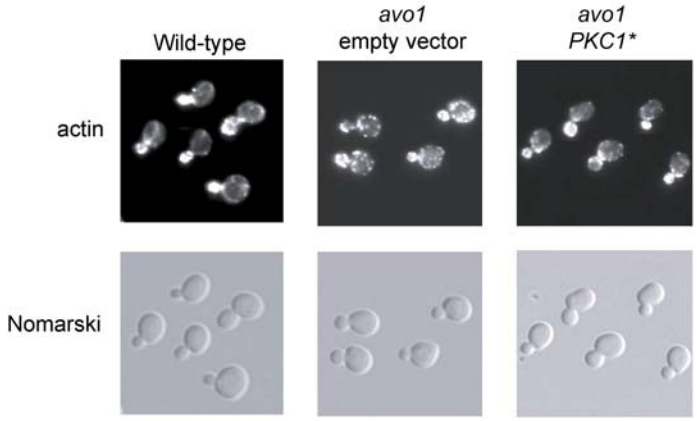


Figure 3.2. Actin defect of *avo1* is suppressed by activation of the PKC1 MAP kinase cascade.

Wild-type (TB50**a**) cells and *GAL1* promoter-*AVO1* (RL23-1c) transformed with empty plasmid or pPKC1* were pregrown in SGal/Gly (galactose) medium and shifted to YPD (glucose) medium for 15 hr, fixed, stained for actin with TRITC-phalloidin, and observed by fluorescence (actin, top panel) and Nomarski (bottom panel) microscopy.

AVO1 is required for full activation of MPK1 in response to heat shock

To further confirm the role of AVO1 in the cell integrity pathway we examined the activation of the PKC1-controlled MAP kinase MPK1 in AVO1-deficient cells. MPK1 is activated by various stresses, such as heat shock treatment (Heinisch et al., 1999). We monitored the kinetics of heat stress-induced MPK1 activation in AVO1-depleted cells. Activation of MPK1 was detected using an antibody that specifically recognizes the activated, dually phosphorylated form of MPK1. In wild-type cells, heat-induction leads to a transient activation of MPK1. Maximal activation was reached 30 to 60 min after shift from 24°C to 39°C, while MPK1 phosphorylation levels returned to basal levels within 120 min after the shift. This transient MPK1 activation correlates with the previously reported transient actin depolarization upon heat shock (Delley and Hall, 1999). The kinetics of MPK1 activation was similar in *avo1* mutant cells, but the level of activation was decreased compared to wild-type cells (Fig 3.3.). Total MPK1 levels, determined by immunoblotting were similar in wild-type and *avo1* cells at all time points. The residual activity of MPK1 observed in *avo1* could be due to incomplete depletion of *AVO1*. The finding that MPK1 activation in response to heat shock was reduced in *AVO1*-depleted cells suggests that AVO1 plays a positive role in the cell integrity pathway.

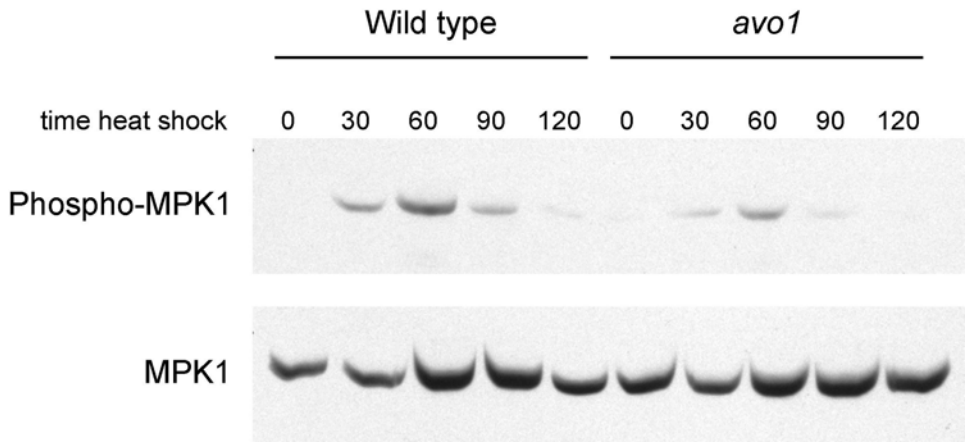


Figure 3.3. AVO1 is required for full activation of MPK1

Wild-type (wt) (TS99-5c) and *GAL1* promoter-*AVO1* (SW35-5c) cells expressing HA-tagged MPK1 were grown for 16 hr in glucose medium at 24°C (time 0). Cells were shifted to 39°C for the indicated times (30, 60, 90, 120 min) and activated MPK1 was detected by immunoblotting using a specific antibody that recognizes dually phosphorylated MPK1 (phospho-MPK1, top panel). Expression of HA-tagged MPK1 was analyzed by immunoblotting using anti-HA antibody (bottom panel).

AVO1 interactors

TOR2 controls the actin cytoskeleton by activation of the RHO-GTPase switch via the GEF ROM2 (Schmidt et al., 1997). The mechanism by which TOR2 regulates the ROM2 exchange activity is unknown. Therefore, we tested whether TOR2 might signal through its partner proteins, such as AVO1, to the GEF ROM2 and thereby regulate the activity of the GTPases RHO1 and RHO2. To check this possibility coimmunoprecipitation experiments with epitope-tagged versions of AVO1 and ROM2 were performed: we did not detect an interaction between AVO1 and ROM2 (data not shown). Using a yeast two-hybrid assay we examined if AVO1 interacts directly with the GTPase RHO1, which could possibly lead to an activation of RHO1. However, we failed to detect an interaction between AVO1 and the wild-type RHO1, nucleotide-free RHO1 (RHO1-G22A), nor activated, GTP-trapped RHO1 (RHO1-Q68H) (data not shown). Therefore, using these approaches we could not detect any interaction between AVO1 and already known components that are possibly regulated by TORC2 to organize the actin cytoskeleton.

In order to identify novel interactors of AVO1, which could link AVO1 to the organization of the actin cytoskeleton a large-scale purification of AVO1, was performed. The purification procedure relied on a TAP (tandem affinity purification)-tagged version of AVO1 expressed at endogenous levels. The TAP tag combines two different tags, an IgG binding domain of *Staphylococcus aureus* protein A and a Calmodulin binding peptide, separated by a cleavage site that is recognized by the TEV protease (Puig et al., 2001). Purified proteins were resolved by SDS-PAGE and identified by mass spectrometry. A mock purification using untagged AVO1 was also performed to identify proteins that specifically interact with AVO1. AVO1 copurified only with the previously identified components of TORC2, namely TOR2, AVO2 and AVO3. LST8 which is also a component of TORC2 could not be detected in the purification because the LST8 comigrates with a contaminating protein also found in the mock purification (data not shown).

In an additional attempt to identify AVO1 interacting proteins a yeast two-hybrid screen using full-length AVO1 as bait was performed. We reasoned that a yeast two-hybrid screen would allow the identification of unstable or transient interactors. Such weak protein-protein interactions are lost in a biochemical purification procedure since it involves several washing steps with quite high salt concentrations. Yeast cells

expressing the GAL4 DNA binding domain fused to the N-terminus of full length AVO1 was transformed with a plasmid library containing the prey fused to the GAL4 transcriptional activation domain. After selection and isolation of positive library plasmids, 13 candidates were retransformed into the yeast strain containing the *AVO1* bait plasmid to test the specificity of the positive plasmids. Two candidates were confirmed and sequenced. While the first clone contained only an intergenic region, the second clone contained sequence from *AVO3* (1.6 kb starting from nucleotide 456). To confirm the interaction *AVO3* (nt *456-2052*) and *AVO3* (nt *456-1266*) was cloned in the prey vector of the yeast two-hybrid system. We observed an interaction between full-length AVO1 and AVO3 (152-684) but not AVO3 (152-422) confirming the interaction of AVO1 with the N-terminal part of AVO3 by yeast two-hybrid (data not shown). The part of AVO1 responsible for the interaction with AVO3 is unknown as different AVO1 versions (AVO1(1-420), AVO1(380-790), AVO1(690-1176)) failed to interact with AVO3 by yeast two-hybrid assay suggesting that many binding regions are required for AVO1 to associate with AVO3 (data not shown). Thus, AVO1 binds to a region in the N-terminal part of AVO3 by yeast two-hybrid.

AVO1 contains a conserved domain essential for its function

AVO1 is a protein consisting of 1176 amino acids. Sequence analysis by SMART analysis (smart.embl-heidelberg.de) revealed that AVO1 does not contain any known domains or motifs. The weak RAS-interacting domain in the C-terminal part of AVO1 identified in an earlier analysis could not be confirmed. Alignment with putative homologues indicated that the most conserved region lies in the C-terminal part of AVO1, in particular in a region between amino acid 735 and 764 (Fig. 3.4.D). Data base analyses revealed that this region is unique for AVO1 and its putative homologues as it is not found in other proteins. The N-terminal part of AVO1 does not share any similarities with its putative homologues. To identify regions that are required for the function of AVO1, deletion mutants of AVO1 were constructed and transformed into an *avo*1 strain containing *AVO1* on an *URA3*-based plasmid. The growth phenotype of different *AVO1* mutants was assayed after counterselection on 5-fluorootic acid (5FOA) plates resulting in the loss of the *URA3*-based *AVO1*. Western blot analysis was performed to test whether all the mutant proteins are expressed (Fig. 3.4.B). Deletion of the 420 amino acids at the N-terminus did not affect the functionality of AVO1, while deleting the 486 amino acids at the C-terminus resulted in a slight

growth phenotype (Fig. 3.4.A). However, cells expressing AVO1 (1-790) did not show a defect in the organization of the actin cytoskeleton (data not shown). Mutants that lack the most conserved region located between amino acid 735 and 764 were not able to restore viability of *avo1* cells indicating that this region is important for the function of AVO1 (Fig. 3.4.C,D). Thus, C-terminal and N-terminal parts of AVO1 are not required for its function whereas a conserved domain between amino acid 735 and 764 is essential for the function of AVO1.

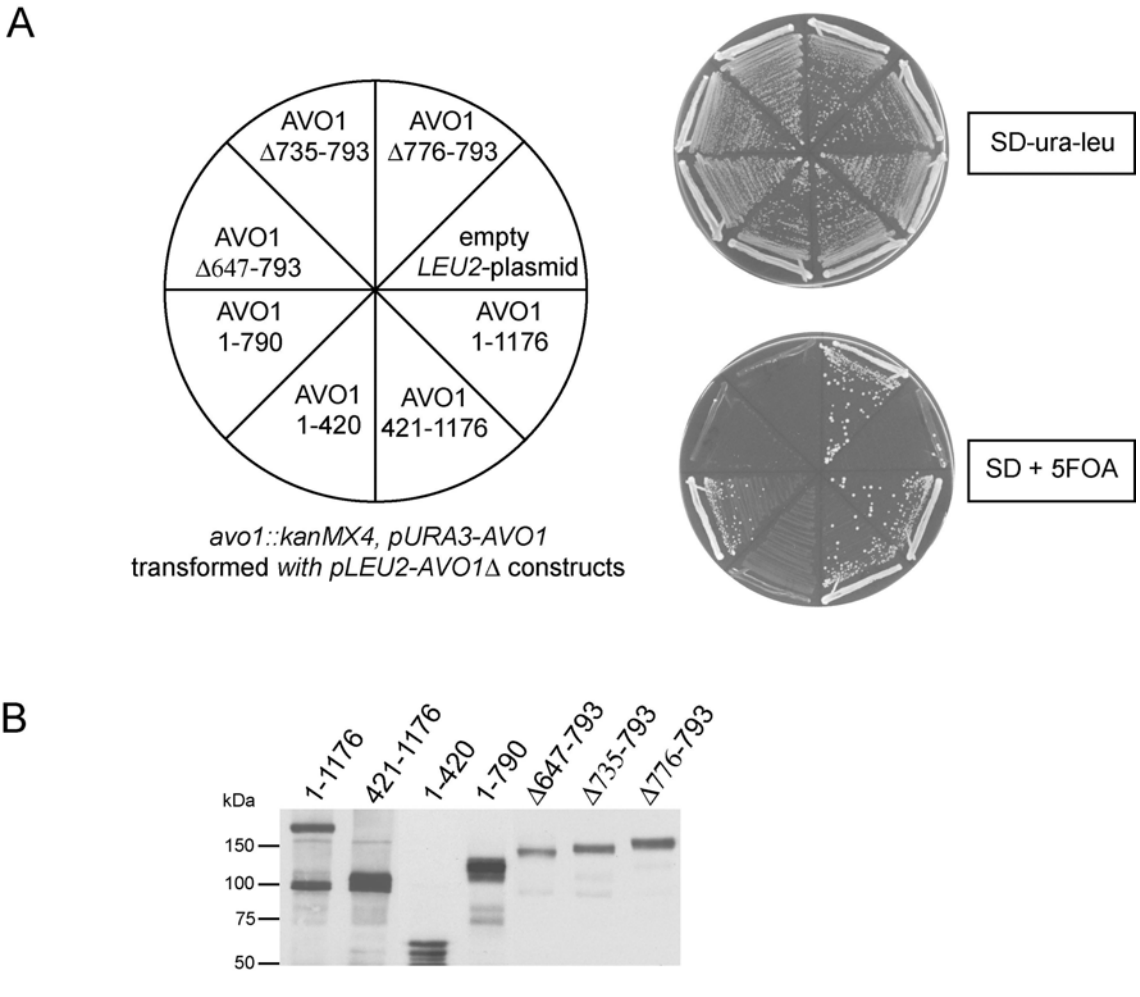
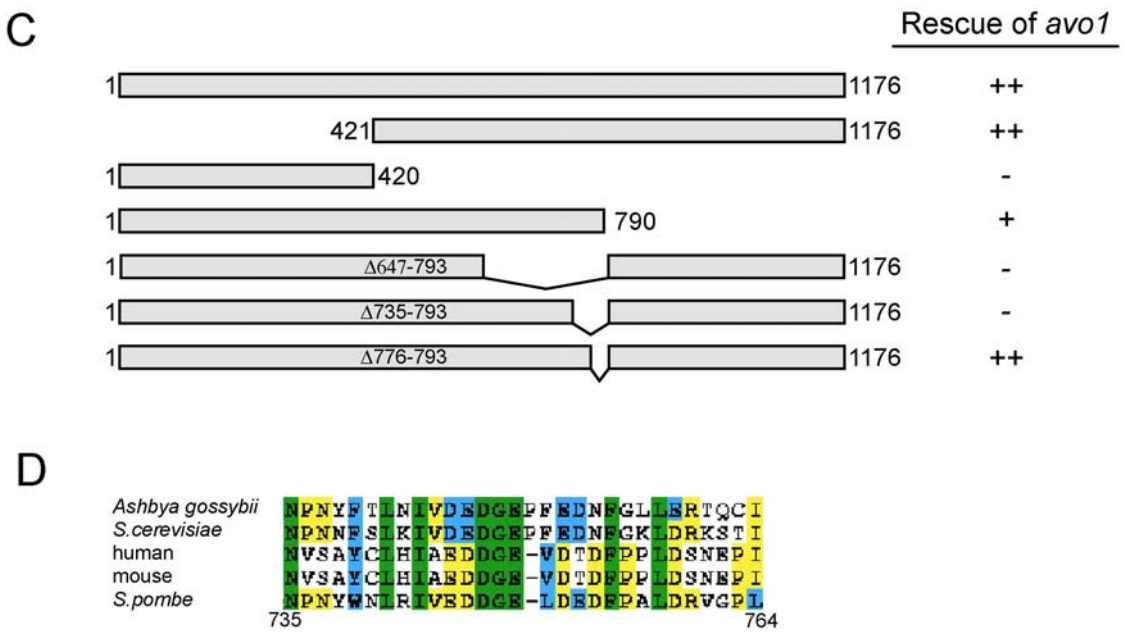


Figure 3.4. AVO1 contains a conserved domain essential for its function

A) *avo1* mutants expressing AVO1 from *URA3*-based plasmid (RL66-2d) were transformed with either empty plasmid, pAVO1(1-1176), pAVO1(421-1176), pAVO1(1-420), pAVO1(1-790), pAVO1 (Δ647-793), pAVO1 (Δ735-793), pAVO1 (Δ776-793) and streaked onto selective plates lacking uracil and leucine (SD-leu-ura) and onto plates containing 5-fluorootic acid (5FOA) to select against the *URA3*-based AVO1 plasmid.

B) Expression of the myc-, or HA-tagged constructs of AVO1 was checked by immunoblotting



C) Summary of the AVO1-constructs used in this study

D) Sequence alignment of a conserved region in AVO1 (from amino acid 735 to 764). Sequences of the putative AVO1 homologues in *Ashbya gossybii, Schizosaccharomyces pombe* (*S. pombe*), *mus musculus* (mouse) and *homo sapiens* (human) are aligned.

3. 3. Discussion

TORC2 consisting of the proteins TOR2, AVO1, AVO2, AVO3 and LST8 is required for the spatial control of cell growth, which is the organization of the actin cytoskeleton. TORC2 controls the cell cycle dependent organization of the actin cytoskeleton by activating the RHO1 GTPase via the exchange factor ROM2. Activated RHO1 signals to the actin cytoskeleton by binding to PKC1 leading to the activation of the cell integrity MAP kinase cascade. Here we analyzed the phenotypes of *avo1* and *lst8* mutant cells and characterized the AVO1 protein. We showed that upregulation of the RHO1 GTPase switch or the PKC1-MAP kinase cell integrity pathway suppressed the growth defect and partially rescued the actin defect of cells depleted for AVO1 or LST8 confirming a role of these two proteins in the signaling branch controlled by TORC2. Previously identified suppressors of *tor2* suppress an *lst8* mutant very poorly, likely because LST8 has multiple cellular functions, as it is not only a member of TORC2 but also part of the rapamycin-sensitive TORC1. The phenotype of LST8-defecient cells might therefore be a complex overlap of different functions. Surprisingly, multicopy *YPK2* restores growth of *lst8* cells very efficiently suggesting that overexpression of *YPK2* suppresses all essential functions of LST8. There is evidence for a relationship between the TORC2 signaling pathway and the PKH-YPK pathway because both pathways appear to impinge on the same readouts. First, PKHs are stimulated by sphingoid bases and *TOR2* as well as *AVO3* were identified as mutations that suppress *csg2*, a gene involved in sphingolipid biosynthesis (Dunn et al., 1998; Friant et al., 2001). Additionally, overexpression of *SUR1*, whose gene product also functions in sphingolipid biosynthesis, suppresses a *tor2* mutant (Helliwell et al., 1998a). Second, similar to mutants of TORC2, *ypk* mutant cells display an actin defect and reduced activation of the MAP kinase MPK1. The lethality of *ypk* cells is also suppressed by activation of the RHO1 GTPase switch as well as by the activation of the PKC1-MAP kinase signaling cascade (Schmelzle et al., 2002). Third, in *S. pombe* overexpression of *GAD8*, the orthologue of YPKs, suppresses the sterility of *tor1* mutant (Matsuo et al., 2003). The phosphorylation of GAD8 is dependent on TOR1 and KSG1, the orthologue of PKHs. TOR1 appears to be responsible for the phosphorylation of the turn motif and the hydrophobic motif while KSG1 directly phosphorylates the Thr in the activation loop. A similar situation is found in higher eukaryotes where p70S6K is phosphorylated by PDK1, the PKH orthologue, at the activation loop and by mTOR at the hydrophobic motif (Alessi et al., 1998; Burnett et al., 1998). Similarly, bacterial expressed YPKs

appear to be phosphorylated in a TORC2-dependent manner *in vitro* supporting a model in which YPKs receives signals from the sphingolipid-stimulated PKHs and the nutrient-controlled TOR kinase (R. Loewith, W. Oppliger unpublished). Thus, TORC2 might impinge on YPKs to control organization of the actin cytoskeleton. YPKs also perform different functions as *ypk1* mutant cells exhibit a defect in translation (Gelperin et al., 2002). Given the fact that YPKs are involved in both translation and actin cytoskeleton organization, processes controlled by TORC1 and TORC2, respectively, could explain the efficient suppression of *lst8* by multicopy YPK2.

Although the suppression of *lst8* by overexpression of *TOR2* is very weak, the result might indicate that LST8 is an upstream factor for the TOR signaling pathway. Since LST8 is present in both TOR complexes it may act as an upstream regulator of TOR by receiving signals from nutrients. Further experiments are needed to prove this hypothesis (see also Part 4 of this thesis).

We did not identify novel interacting proteins of TORC2 performing biochemical purifications and a yeast two-hybrid screen. Because TORC2 seems to be associated with membranes interaction of TORC2 to its substrates might be mediated through membranes (Kunz et al., 2000; Wedaman et al., 2003). Such interactions are lost in our biochemical purification procedures and likely cannot be detected by yeast two-hybrid. However, AVO1 appears to interact with the N-terminal part of AVO3 by yeast two-hybrid, but whether AVO1 interacts directly with AVO3 or through another protein is unknown. AVO3 contains six conserved regions that are essential for its function (R. Shioda, unpublished). The first conserved region at the N-terminus (350-480) of AVO3 overlaps with the AVO1 interaction domain suggesting a role of this conserved region in mediating interaction with AVO1.

Additionally, we identified a conserved region in AVO1 that is important for the function of AVO1. This region possibly meditates binding of AVO1 to TOR2 or other proteins in TORC2 or is required for proper folding of AVO1. We cannot exclude that there are other regions in AVO1 that are important for the functionality of AVO1. Further experiments need to be performed to test whether the AVO1 deletion mutants are still part of TORC2. Identification of AVO1 mutants associating with the complex but exhibiting a growth phenotype could be interesting for identifying downstream effectors of TORC2.

3. 4. Material and methods

Strains and media

The *S. cerevisiae* strains and plasmids used are listed in Table 3.1. and 3.2., respectively. All strains are isogenic derivatives of JK9-3d**a** or TB50**a**. Rich media, YPD or YPGal/Gly, and synthetic complete media, SD or SGal/Gly, were as described previously (Beck et al., 1999; Sherman, 1991). For TOR2-, and AVO1-depletion experiments, cells from logarithmically growing SGal/Gly or YPGal/Gly cultures were inoculated into SD or YPD medium, respectively. Glucose cultures were shaken for 15 hr before cells were harvested for analysis.

Genetic techniques

Restriction enzyme digests and ligations were performed according to standard methods. All enzymes and buffers were obtained commercially (Roche Diagnostics). *Escherichia coli* strains MH1 and DH5α were used for propagation and isolation of plasmids. Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983). PCR cassettes were used to generate gene deletions and modifications, as described (Longtine et al., 1998; Puig et al., 2001)

Actin staining

Logarithmically growing cells were fixed in formaldehyde (3.7%) and potassium phosphate buffer (100 mM; pH 6.5) and stained with tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin (Sigma) to visualize actin, as described previously (Benedetti et al., 1994).

Two-hybrid assays

Yeast two-hybrid assays were performed as described in (James et al., 1996). PJ69-4**a** cells were sequentially with a bait plasmid (pGBD-based) expressing a GAL4-BD (DNA binding domain) fusion protein and a prey plasmid or a library (pGAD-based) expressing a GAL4 AD (activation domain) fusion protein, and selected for the *HIS3* reporter on SD-ade-leu-trp plates.

MAP kinase assay

Yeast strains expressing HA-tagged MPK1 and *AVO1* under the control of the *GAL1* promoter were grown in YPGal/Gly. Per time point, 50 ml of YPD was inoculated,

grown at 24°C for 16 hr (to an OD_{600} of 0.4) and subsequently shifted to 39°C for the indicated time. Samples were chilled with crushed ice, harvested by centrifugation, washed with ice-cold water and stored at -80°C. The cells were resuspended in 0.5 ml lysis buffer (50 mM Tris, pH 7.5, 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerol phosphate, 5 mM sodium pyrophosphate, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 1 x Roche complete protease inhibitor cocktail) and lysed by vortexing 5 x 30 s with glass beads using a FastPrep machine (Savant Instruments). A total of 30 μg protein (for anti-activated MAPK detection) or 15 μg protein (for anti-HA detection) was loaded per lane for electrophoresis (10% acrylamide). After SDS-PAGE the proteins were eletroblotted to nitrocellulose. To detect the proteins the membrane was first incubated in blocking buffer (1 x TBS, 0.1% Tween-20, 5% milk) and then over night at 4°C with mouse anti-HA antibody (1:10000 diluted in blocking buffer) or rabbit anti-MAP kinase activated (New England Biolabs) (1:2000 diluted in blocking buffer). Primary antibodies were detected using the corresponding horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) with ECL reagents (Amersham Pharmacia Biotech).

TAP purification

TAP purification was performed as described in (Puig et al., 2001). Extracts were prepared from a 4 l culture expressing TAP-tagged AVO1 grown in YPD at 30°C to an OD_{600} of 0.8. Cells were chilled on ice for 30 min, collected by centrifugation, washed once in ice-cold water and lysed with a Bead Beater (Biospec products) in ~40 ml lysis buffer (1 x TBS, 10% glycerol, 0.5% Tween-20 plus inhibitors: 10 mM NaF, 10 mM NaN_3, 10 mM p-nitrophenylphosphate, 10 mM sodium pyrophosphate, 10 mM β-glycerophophate, 1 mM phenylmethylsulfonyl fluoride, 1 x Roche complete inhibitor cocktail). Extracts were precleared by 500 μl Sepharose CL 4B (Sigma), and processed according to (Puig et al., 2001) except that 0.5% Tween-20 was used as detergent and the proteins were not eluted from the calmodulin-beads but directly resuspended and boiled in 2 x SDS-PAGE sample buffer.

Immunoprecipitation

Protein extracts were prepared as described above. To immunoprecipitate epitope tagged protein 1 μl of either concentrated 12CA5 (anti-HA) or 9E10 (anti-myc) tissue culture supernatant was added and the tubes were rotated for 1 hr at 4°C. Afterwards 20 μl protein G-sepharose slurry (Sigma) was added and the tubes were rotated for

additional 2 hr at 4°C. Beads were collected by centrifugation, washed four times with 1 ml lysis buffer, and resuspended in 2 x SDS-PAGE sample buffer. After SDS-PAGE the proteins were electroblotted onto nitrocellulose, blocked in 5% milk in 1 x PBS 0.1% Tween-20, and incubated with primary antibody (12CA5 or 9E10 1:10000 in blocking solution) over night at 4°C. The membranes were washed and incubated for 1 hr at room temperature with anti-mouse horseradish peroxidase-conjugated secondary antibody. The tagged proteins were detected using ECL reagents (Amersham Pharmacia Biotech).

Table 3.1. Strains

Strain	Genotype
JK9-3d**a**	*MAT**a** leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
JK9-3dα	*MATα leu2-3,112 ura3-52 trp1 his4 rme1 HMLα*
TB50**a**	JK9-3d**a** *HIS4 his3*
TB50α	JK9-3dα *HIS4 his3*
PJ69-4a	*MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*
RL23-1c	TB50**a** *[kanMX4]-GAL1p-AVO1*
RL57-2d	TB50**a** *[kanMX4]-GAL1p-LST8*
TS99-5c	TB50**a** *MPK1-3HA-kanMX4*
SW35-5c	TB50**a** *[kanMX4]-GAL1p-AVO1 MPK1-3HA-kanMX4*
SW45-1b	TB50**a** *AVO1-TAP-KlTRP1*
SW71-3a	TB50**a** *[kanMX4]-GAL1p-LST8 sac7::kanMX4*
SW48-1b	TB50**a** *[kanMX4]-GAL1p-AVO1 sac7::kanMX4*
RL66-2d	TB50**a** *avo1::kanMX4* / pRL8-2-1: YEplac195::*myc-AVO1*
SW57-1d	TB50**a** *rom2::kanMX4 AVO1-TAP-KlTRP1* / pAS115: YCplac33::*3HA-ROM2*

Table 3.2. Plasmids

Plasmids	Description
pROM2	pAS30; *ROM2* in YEplac195 *(2μm, URA3)*, (Schmidt et al., 1997)
pRHO2	pC-186; *RHO2 (2μm, URA3)* (Madaule et al., 1987)
pPKC1	pSH24; *PKC1* in pSEY18 *(2μm, URA3)*, (Helliwell et al., 1998b)
pPKC1*	$PKC1^{R398P}$ in YCp50 *(CEN, URA3)*, (Nonaka et al., 1995)
pBCK1*	*BCK1-20* in pRS316 *(CEN, URA3)*, (Lee and Levin, 1992)
pMKK1	*MKK1* in YEp352 *(2μm, URA3)*
pMKK1*	$MKK1^{S386P}$ in YCplac33 *(CEN, URA3)*, (Watanabe et al., 1995)
pMPK1	*MPK1* in YEp352 *(2μm, URA3)*, (Kamada et al., 1995)
pTOR2	pJK3-3; *TOR2* in pSEY18 *(2μm, URA3)*, (Kunz et al., 1993)
pMSS4	pSH22; *MSS4* in pSEY18 *(2μm, URA3)*, (Helliwell et al., 1998a)
pYPK2	pTS96; YPK2 in YEplac195 *(2μm, URA3)*
pAVO1 (1-1176)	pSW59; expresses myc-tagged AVO1 (1-1176) from *AVO1* promoter. Cloned as 3.9-kb *Sal*I fragment into YEplac181::*AVO1*promoter*ATG-myc (2μm, LEU2)*
pAVO1 (421-1176)	pSW50; expresses myc-tagged AVO1(421-1176) from *AVO1* promoter. Cloned as 2.6-kb *Sal*I fragment into YEplac181::*AVO1*promoter*ATG-myc (2μm, LEU2)*
pAVO1 (1-420)	pSW11; expresses HA-tagged AVO1 (1-420) from *AVO1* promoter. Cloned as 1.5-kb *Xba*I-*Pst*I fragment into pHAC181 *(2μm, LEU2)*
pAVO1 (1-790)	pSW12; expresses HA-tagged AVO1 (1-790) from *AVO1* promoter. Cloned as 2.7-kb *Xba*I-*Pst*I fragment into pHAC181 *(2μm, LEU2)*

pAVO1 (Δ647-793)	pSW33; expresses HA-tagged AVO1 (Δ647-793) from *AVO1* promoter. Cloned as 2.2-kb *Xba*I-*Sal*I and 1.5-kb *Sal*I fragment into pHAC181 *(2μm, LEU2)*
pAVO1 (Δ735-793)	pSW32; expresses HA-tagged AVO1 (Δ735-793) from *AVO1* promoter. Cloned as 2.4-kb *Xba*I-*Sal*I and 1.5-kb *Sal*I fragment into pHAC181 *(2μm, LEU2)*
pAVO1 (Δ776-793)	pSW29; expresses HA-tagged AVO1 (Δ776-793) from *AVO1* promoter. Cloned as 2.5-kb *Xba*I-*Sal*I and 1.5-kb *Sal*I fragment into pHAC181 *(2μm, LEU2)*
pGBD::AVO1	pSW7; *AVO1* cloned as 3.5-kb *Sma*I-*Sal*I fragment into pGBD-C1 (*ADH1* promoter; *2μm, TRP1*)
pGBD::AVO1(1-420)	pSW8; *AVO1 (nt 1-1260)* cloned as 1.3-kb *Sma*I-*Pst*I fragment into pGBD-C1 (*ADH1* promoter; *2μm, TRP1*)
pGBD::AVO1(380-790)	pSW9; *AVO1 (nt 1140-2370)* cloned as 1.2-kb *Bam*HI-*Pst*I fragment into pGBD-C1 (*ADH1* promoter; *2μm, TRP1*)
pGBD::AVO1(690-1176)	pSW10; *AVO1 (nt 2070-3528)* cloned as 1.5-kb *Sal*I-*Sal*I fragment into pGBD-C1 (*ADH1* promoter; *2μm, TRP1*)
pGAD::RHO1	pSW19; $RHO1^{C206S}$ cloned as 0.6-kb *Bam*HI-*Bgl*II fragment into pGAD-C1 (*ADH1* promoter; *2μm, LEU2*) from pETE25 (Schmidt et al., 1997)
pGAD::RHO1(Q86H)	pSW20; $RHO1^{Q86H\ C206S}$ cloned as 0.6-kb *BamH*I-*Bgl*II fragment into pGAD-C1 (*ADH1* promoter; *2μm, LEU2*) from pETE27 (Schmidt et al., 1997)
pGAD::RHO1(G22A)	pSW21; $RHO1^{G22A\ C206S}$ cloned as 0.6-kb *BamH*I-*Bgl*II fragment into pGAD-C1 (*ADH1* promoter; *2μm, LEU2*) from pAS92 (Schmelzle et al., 2002)
pGAD::AVO3 (152-684)	pSW30; *AVO3 (nt 456-2052)* cloned as 1.6-kb *Cla*I-*Pst*I fragment into pGAD-C1 (*ADH1* promoter; *2μm, LEU2*)
pGAD::AVO3 (152-422)	pSW31; *AVO3 (nt 456-1266)* cloned as 0.8-kb *Cla*I-*Pst*I fragment into pGAD-C1 (*ADH1* promoter; *2μm, LEU2*)

4. Functional analysis of TORC2

4. 1. Introduction

TOR signaling complexes

Previously, we identified two TOR complexes (TORC1 and TORC2) in yeast, and at least one of these (TORC1) is conserved in man. While TORC1 mediates the rapamycin-sensitive signaling branch, TORC2 signaling is rapamycin-insensitive and responsible for the control of the actin cytoskeleton. TORC1 consists of four proteins, TOR1 or TOR2, KOG1, TCO89 and LST8, and with the exception of TCO89, all of these proteins are evolutionarily conserved. TORC2 comprises seven proteins, TOR2, AVO1, AVO2, AVO3, LST8 and the recently identified BIT61 and BIT2 (Loewith et al., 2002; Reinke et al., 2004).

Raptor is the highly conserved mammalian orthologue of KOG1. It has a molecular mass of 150 kDa and was identified independently by three different groups (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). At its N-terminus raptor and its orthologues contain a domain named RNC (raptor N-terminal conserved) consisting of three blocks with at least 67-79% sequence similarity. The RNC domain appears to be unique to raptor and its orthologues, as it is not found in other protein sequences in public databases. The RNC domain is followed by three HEAT repeats and the C-terminal third of raptor contains seven WD40 repeats.

mLST8 is a protein of 36 kDa and consists almost entirely of seven WD40 repeats (Kim et al., 2003; Loewith et al., 2002). The WD40 repeat is characterized by a set of conserved residues within a repeat length of around 40 amino acids. mLST8 shows sequence similarity to the β subunits of heterotrimeric G proteins. In analogy to the G protein β1 subunit that contains six WD40 repeats folding into a cylindrical β-propeller structure, mLST8 is thought to have a β-propeller structure.

Raptor interacts with the N-terminal, HEAT repeats containing part of mTOR underscoring the function of HEAT repeats as mediators of protein-protein interactions (Kim et al., 2002). The association between raptor and mTOR involves multiple sites in raptor suggesting that mTOR and raptor make extensive contacts with each other. However, the mTOR-raptor interaction is very sensitive to detergents. A stable

association of both proteins can only be detected in buffer containing no detergents or 0.3% CHAPS; lysis buffers with Triton X-100 or NP-40 as detergent eliminates the interaction (Hara et al., 2002; Kim et al., 2002).

Raptor, like its orthologue in yeast KOG1, appears to be a positive regulator of TOR since raptor is required for the optimal phosphorylation of 4E-BP1 and p70S6K in nutrient-stimulated cells. The mechanism by which raptor modulates these downstream effectors is controversial. The first model, presented by the group of Sabatini, proposes a mechanism by which raptor and mTOR form a nutrient-sensitive complex and raptor both positively and negatively regulates mTOR function (Kim et al., 2002). Nutrients seem to affect the stability of mTOR-raptor complex. Deprivation of leucine or other nutrients such as glucose but not removal of growth factors leads to a stabilization of mTOR-raptor interaction and to a decrease in p70S6K phosphorylation, while readdition of nutrients reverses the effect. This inverse correlation between the stability of mTOR-raptor association and the phosphorylation of p70S6K lead to the suggestion that a strong interaction between raptor and mTOR results in an inhibition of the mTOR kinase activity. Apart from this negative regulation, raptor positively modulates mTOR as raptor downregulation by siRNA reduces p70S6K phosphorylation leading to a model in which raptor interacts with mTOR under all nutrient conditions but with different affinities. Under good nutrients conditions raptor forms a rather unstable complex with mTOR that is required for mTOR function. On the other hand, under nutrient-poor conditions mTOR-raptor complex is stabilized resulting in the inhibition of mTOR kinase activity.

In contrast to this model the group of Yonezawa fails to detect a nutrient-sensitive interaction between raptor and mTOR (Hara et al., 2002). In addition, experiments in yeast indicated that TOR1 or TOR2 forms a stable complex with KOG1 independently of the nutritional state of the cell (Loewith et al., 2002). Raptor might rather function as a scaffold protein bringing mTOR kinase in proximity to its substrates. Consistent with this model raptor is able to bind to 4E-BP1 and p70S6K1. The binding of raptor to 4E-BP1 increases if the rapamycin-sensitive phosphorylation sites in 4E-BP1 are changed to alanine and decreases if the same amino acids are mutated to glutamic acid indicating a preferential binding of raptor to the nonphosphorylated form of 4E-BP1. Reducing the amount of raptor by RNA interference results in a decrease in 4E-BP1 phosphorylation underscoring the requirement of raptor for the phosphorylation of 4E-

BP1 (Hara et al., 2002). The RAIP motif (in the N-terminus) and the TOS motif (in the C-terminus) are both required for the efficient phosphorylation of 4E-BP1 (Beugnet et al., 2003; Schalm and Blenis, 2002). Mutation of the TOS motif in 4E-BP1 abolishes the binding of 4E-BP1 to raptor indicating the requirement of a functional TOS motif in the assembly of mTOR-raptor-4E-BP1 complex (Schalm et al., 2003). Thus, the TOS motif seems to mediate the association between 4E-BP1 to raptor supporting the model in which raptor acts as a scaffold protein that presents the mTOR kinase to its downstream substrates.

A second interactor of mTOR, mLST8 exerts a positive role on mTOR function (Kim et al., 2003). Unlike raptor, mLST8 binds to the mTOR kinase domain in a constitutive, detergent-, and nutrient-insensitive manner. Depletion of mLST8 using siRNA blocks the activation of p70S6K in response to growth factors and nutrients. On the other hand, overexpression of mLST8 stimulates mTOR activity towards p70S6K and enhances the association of raptor to mTOR, which is inconsistent with the model that strong binding of raptor would inhibit mTOR activity. While raptor does not affect the binding of mLST8 to mTOR, depletion of mLST8 destabilizes the interaction between raptor and mTOR implying a role of mLST8 in the formation of the mTOR complex. The function of raptor and mLST8 in the mTOR complex, especially in respect to the formation of a nutrient-sensitive complex remains controversial.

TORC2 in yeast

During my thesis I was interested in the function of the components that form TORC2. While the phenotypical analysis of TORC2 partners indicated that they perform a positive role in the rapamycin-insensitive TOR2-unique signaling branch, the molecular function and mechanism by which these proteins control the TOR2-unique branch remained to be elucidated.

TORC2 contains at least five proteins, TOR2, AVO1, AVO2, AVO3 and LST8, and forms a stable complex. So far we were not able to identify other components of TORC2 or more transient interactors of the complex, which could serve as signaling molecules. Nevertheless, the group of Ted Powers identified an additional component of TORC2, named BIT61(Reinke et al., 2004). BIT61 is a nonessential protein and

bit61 mutant cells do not show any obvious growth defect under different growth conditions. Database analysis revealed that BIT61 has a homologue in the yeast genome, YBR270C (named BIT2), which could be redundant to BIT61. Although BIT2 interacts with TOR2, a double mutant of *bit61bit2* behaves like a wild- type when grown under various growth conditions (Ryo Shioda, unpublished). The role of these two proteins in TORC2 is unknown.

Recently, novel components of TORC2 signaling pathway have been identified. In a global yeast two-hybrid screen two uncharacterized proteins, YIL105C and YNL047C were identified as interactors of AVO2 (Uetz et al., 2000). Additionally, *YIL105C* was pulled out as a genetic interactor of *MSS4* in a synthetic lethal screen (Audhya et al., 2004). Sequence analysis revealed that YNL047C is 53% identical to YIL105C. Whereas the single deletion mutants are viable the double *yil105c ynl047c* mutant is lethal indicating a redundancy of the two proteins (Audhya et al., 2004). YIL105C and YNL047C were named SLM1 and SLM2, respectively. The SLM proteins contain a lipid binding PH domain and preferentially bind to $PI4,5P_2$. They localize to the cell periphery and the localization is at least partly dependent on the lipid kinase MSS4 as the SLMs show an enhanced cytoplasmic localization in *mss4* mutant cells. Thus, MSS4-generated $PI4,5P_2$ is important for the recruitment of SLMs to the plasma membrane. Similar to *mss4* mutant cells a temperature sensitive *slm1ts slm2* exhibits a defect in the organization of the actin cytoskeleton at restrictive temperature. This actin defect is suppressed by overexpression of multicopy *PKC1* but not by *BCK1* or *MKK1*. Additionally, deletion of *SAC7*, which encodes a GAP for RHO1, could rescue the lethality of a *slm1 slm2* double mutant. *PKC1* could only restore viability of the temperature-sensitive *slm1* strain when grown at 26°C but not under heat shock conditions implying that other RHO1 effectors may also be required. SLM1 and SLM2 are able to interact with each other and are phosphoproteins. As predicted by the yeast two-hybrid analysis SLM1 coimmunoprecipitates with AVO2, although the interaction is very unstable leading to the suggestion that the SLM proteins might be substrates for TORC2 rather than stable components of TORC2. Indeed, TORC2 phosphorylates recombinant SLM1 and SLM2 *in vitro* (Audhya et al., 2004). This suggests that the SLMs are substrates of TORC2 through which TORC2 signals to the actin cytoskeleton. Thus, the SLM proteins seem to require two different signals to perform their function. One signal is generated by the lipid kinase MSS4 and leads to the proper localization of the two proteins. The second signal comes from TORC2 that

phosphorylates both proteins. Further work has to be performed to clarify the significance of the TORC2-mediated phosphorylation of the SLMs as well as their function in the organization of the actin cytoskeleton.

TORC2 consisting of the proteins TOR2, AVO1, AVO2, AVO3 and LST8 is a stable complex (Loewith et al., 2002). The interaction of the different proteins is constitutive and is not sensitive to nutrient conditions. Phenotypic analyses revealed that TORC2 is important for the organization of the actin cytoskeleton. The function of the different proteins in the complex as well as the architecture of the TORC2 is unknown. Here we analyze the architecture of TORC2 as well as the function of components of TORC2 in yeast. We provide evidence that AVO1 and possibly AVO3 act as scaffold proteins important for the integrity of TORC2 and are required for efficient phosphorylation of downstream target proteins. LST8 appears to be involved in modulating the kinase activity of TOR2.

4. 2. Results

TORC2 exists in an oligomeric form

Gel filtration experiments indicated that TOR2 forms a stable complex of 2 M Da (Loewith et al., 2002). Summing the molecular weights of TOR2 and its partners results in a mass of 0.78 M Da (including BIT61 and BIT2) suggesting that TORC2 might exist in a multimeric state. To check whether the components of TORC2 can interact with themselves coimmunoprecipitation experiments were performed. As shown in Fig. 4.1.A) every component of TORC2 tested is able to oligomerize suggesting that TORC2 exists in an oligomeric state. Most likely TORC2 dimerizes which would be consistent with the molecular weight observed by gel filtration.

Under nitrogen starvation conditions TOR signaling is inhibited. The mechanism by which TOR is inhibited is unknown. Since nitrogen starvation does not affect the association of TOR2 with its partners, the activity of TORC2 could be regulated through its dimerization. To test this possibility TOR2 dimerization was analyzed in cells that were starved of nitrogen for 60 min. The interaction of TOR2 with itself was unaffected upon nitrogen starvation (Fig. 4.1.B). Thus, TORC2 exists in a dimeric or higher order state which is unaffected by nitrogen starvation.

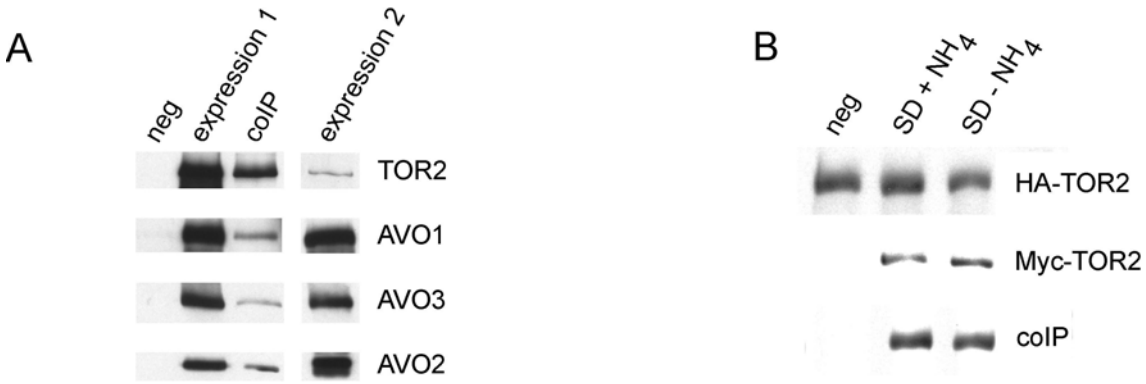


Figure 4.1. TORC2 exists in an oligomeric form

A) Lysates from cells expressing HA-TOR2 and myc-TOR2 (SW70 transformed with pAN54), AVO1-HA and AVO1-TAP (SW110), AVO3-HA and AVO3-myc (SW112) or AVO2-HA and AVO2-myc (SW111) were subjected to immunoprecipitation with anti-myc or pulldown with IgG-sepharose and probed with anti-myc or anti-ProteinA to check expression of myc- or TAP-tagged proteins (lane 4, expression 2) and with anti-HA (lane 3, coIP) to test for coimmunoprecipitation. Expression of HA-tagged proteins was detected by immunoprecipitation with anti-HA and probed with anti-HA (lane 2, expression 1). As negative control lysates from cells expressing HA-TOR2 (SW70), AVO1-HA (RL69-1c), AVO3-HA (RL42-1c) or AVO2-HA (RL39-1a) were subjected to immunoprecipitation with anti-myc and probed with anti-HA (lane 1).

B) Lysates from cells expressing HA-TOR2 and mycTOR2 (SW70 transformed with pAN54) grown in medium containing ammonium as nitrogen source (lane 2) or nitrogen starved for 1 hr (lane 3) were subjected to immunoprecipitation with anti-myc. Immunoprecipitates were probed with anti-HA to detect coimmunoprecipitated TOR2 (bottom panel, coIP). As negative control lysate from cells expressing HA-TOR2 (SW70) transformed with empty plasmid were subjected to immunoprecipitation with anti-myc and probed with anti-HA (lane 1, bottom panel, coIP). The expression of HA or myc-tagged TOR2 detected by immunoprecipitation is shown in the top panel and middle panel, respectively.

AVO1 and AVO3 are important for the structural integrity of TORC2

To study the contribution of TORC2 components to TORC2 architecture we shut off expression of TORC2 components and assayed complex integrity by looking at coimmunoprecipitation of the remaining components. Since all the proteins of TORC2 except AVO2 are essential we depleted cells of an essential partner by replacing its own promoter with the *GAL1* promoter and turning off its expression in glucose-containing media. Interaction of TORC2 components was examined by comparing cells grown in galactose-media to cells depleted of a TORC2 component grown in glucose-media. Cells depleted for a protein will also be referred as mutant cells.

To investigate the role of AVO1 in the architecture of TORC2 possible interactions between the different TORC2 proteins were analyzed in *avo1* mutants by coimmunoprecipitations. The expression levels of the analyzed proteins did not vary by changing the media from galactose to glucose. As shown in Fig. 4.2. depletion of AVO1 affected the integrity of TORC2. AVO2 as well as AVO3 no longer interact with TOR2 (Fig. 4.2.A,B) and were also not able to associate with each other (Fig. 4.2.C) when AVO1 was depleted. On the other hand, the association of TOR2 and LST8 was not dependent on the presence of AVO1 (Fig. 4.2.D). Because TOR2 and LST8 are both components of TORC1 coimmunoprecipitation of this two proteins could be misleading. To ensure that the interaction between TOR2 and LST8 in *avo1* was not due to TORC1, cell extracts were depleted for TORC1 by precipitating KOG1, a component of TORC1. TAP-tagged KOG1 was precipitated by incubating the cell extract with IgG-sepharose prior to the coimmunoprecipitation experiment. TOR2 still associated with LST8 in absence of AVO1 and TORC1 indicating that the major part of TOR2 is in TORC2 (data not shown). Thus, AVO1 appears to be important for the association of AVO2 and AVO3 to TORC2 and does not affect the interaction between TOR2 and LST8. Consistent with this result AVO2 and AVO3 interactions with LST8 were lost in *avo1* mutant cells (Fig. 4.2.E,F).

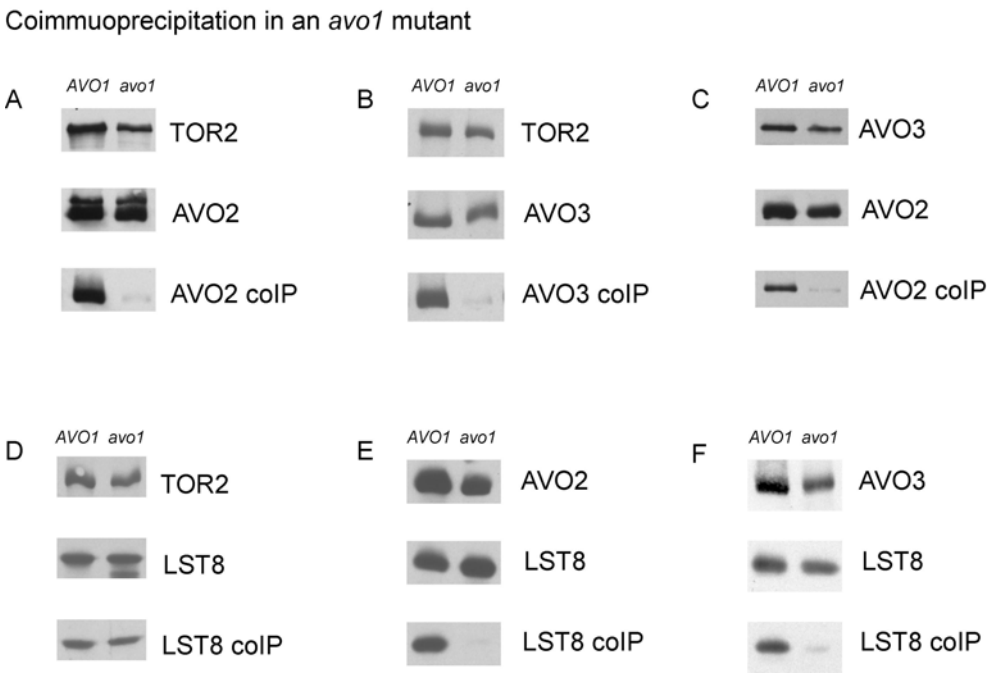


Figure 4.2. AVO1 is required for AVO2 and AVO3, but not for LST8 to associate with TORC2

Lysates from cells expressing *GAL1* promoter-*AVO1* and HA- or myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-myc. Cells expressing AVO1 grown in galactose medium (lane 1) were compared to AVO1-depleted cells grown for 15 h in glucose medium (lane 2). Immunoprecipitates were probed with anti-myc or anti-H, respectively to detect the coimmunoprecipitated partner protein (bottom panel). The expression level of the HA or myc-tagged TORC2 component is shown in the top panel and middle panel, respectively.

A) *GAL1pAVO1 HA-TOR2 AVO2-myc* (SW76-5b), B) *GAL1pAVO1 HA-TOR2 AVO3-myc* (SW68-3b), C) *GAL1pAVO1 AVO3-HA AVO2-myc* (SW73-4b), D) *GAL1pAVO1 HA-TOR2 LST8-myc* (SW75-10c), E) *GAL1pAVO1 LST8-HA AVO2-myc* (SW69-4d), F) *GAL1pAVO1 LST8-HA AVO3-myc* (SW74-11b)

In *avo3* mutant cells the association of TOR2 and AVO2 was impaired (Fig. 4.3.A), while the interaction of TOR2 with LST8 was unaffected (Fig. 4.3.B). Likewise AVO3 was required for AVO1 and AVO2 to interact (Fig. 4.3.C) and the interaction between AVO1 or AVO2 and LST8 was abolished in *avo3* mutant cells (Fig. 4.3.D,E). Therefore the binding of AVO1 and AVO2 to TORC2 is dependent on the presence of AVO3 suggesting that AVO2 binds through both AVO1 and AVO3 to TORC2.

Figure 4.3. AVO3 is required for AVO1 and AVO2, but not LST8 to associate to TORC2

Lysates from cells expressing *GAL1* promoter-*AVO3* and HA- or myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-myc. Cells expressing AVO3 grown in galactose medium (lane 1) were compared to AVO3-depleted cells grown for 15 hr in glucose medium (lane 2). Immunoprecipitates were probed with anti-myc or anti-HA, respectively to detect the coimmunoprecipitated partner protein (bottom panel). The expression level of the HA or myc-tagged TORC2 component is shown in the top panel and middle panel, respectively. A) *GAL1pAVO3 HA-TOR2 AVO2-myc* (SW72-5a), B) *GAL1pAVO3 HA-TOR2 LST8-myc* (SW77-5d), C) *GAL1pAVO3 AVO1-HA AVO2-myc* (SW65-11c), D) *GAL1pAVO3 AVO1-HA LST8-myc* (SW67-3b), E) *GAL1pAVO3 LST8-HA AVO2-myc* (SW78-3a)

Consistent with this model AVO3 interacted with TOR2 in an *avo2* mutant (Fig. 4.4.A) and deletion of *AVO2* did not disturb the association between TOR2 and LST8 (Fig 4.4.B). On the other hand, we observed that removal of LST8 destabilized but did not completely abolish the TOR2-AVO3 or the TOR2-AVO2 interaction (Fig. 4.4.C,D).

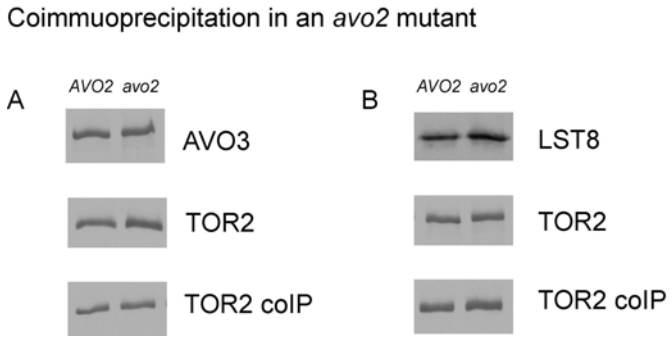


Figure 4.4. Effect of *avo2, lst8 and tor2* on the integrity of TORC2

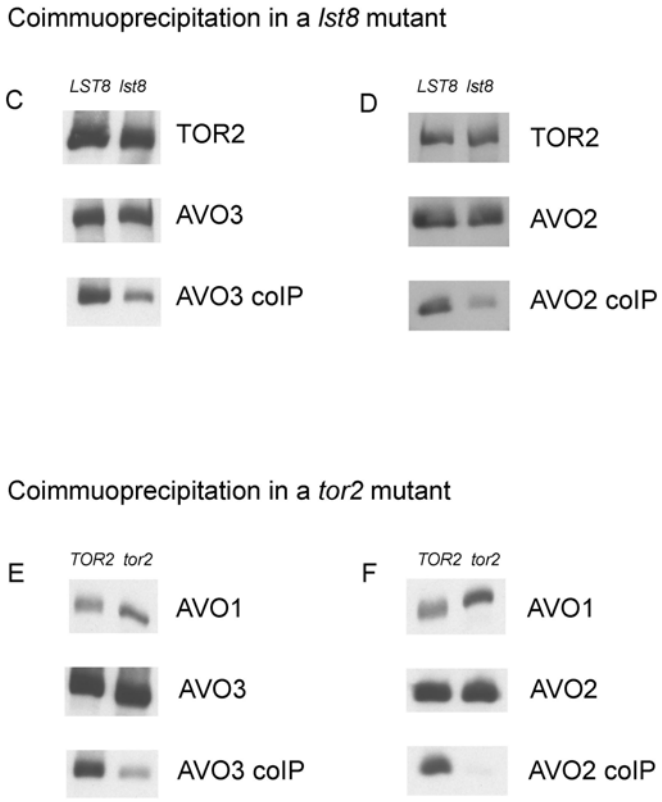
A, B) AVO2 is not required for AVO1 and LST8 to interact with TOR2

Lysates from wild-type (lane1) or *avo2* mutant cells (lane 2) expressing HA- or myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-myc. Immunoprecipitates were probed with anti-HA to detect the coimmunoprecipitated partner protein (bottom panel). The expression level of the HA or myc-tagged TORC2 component is shown in the top panel and middle panel, respectively.

A) *HA-TOR2 AVO3-myc* (SW62-7a), *avo2 HA-TOR2 AVO3-myc* (SW102-8b), B) *HA-TOR2 LST8-myc* (SW63-1d), *avo2 HA-TOR2 LST8-myc* (SW103-2d)

To examine whether AVO1 and AVO3 can interact independently of TOR2 forming a subcomplex, the association between AVO1, AVO2 and AVO3 was studied in TOR2-depleted cells. As shown in Fig. 4.4.E,F the interaction between AVO1 and AVO3 was destabilized and AVO1 no longer bound to AVO2 in absence of TOR2 leading to the conclusion that TOR2 is required for the interaction of AVO1 to AVO2 and AVO3.

Thus, AVO1, AVO3 and TOR2 appear to function as important structural elements of TORC2 and are required for AVO2 to bind to the complex. LST8 binds directly to TOR2 (see below) independently of AVO1, AVO2 and AVO3 function.



C, D) Depletion of LST8 destabilizes the interaction between AVO3 and AVO2 with TOR2

Lysates from cells expressing *GAL1* promoter-*LST8* and HA- or myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-myc. Cells expressing LST8 grown in galactose medium (lane 1) were compared to LST8-depleted cells grown for 15 hr in glucose medium (lane 2). Immunoprecipitates were probed with anti-myc to detect the coimmunoprecipitated partner protein (bottom panel). The expression level of the HA or myc-tagged TORC2 component is shown in the top panel and middle panel, respectively.

C) *GAL1pLST8 HA-TOR2 AVO3-myc* (SW104-3c), D) *GAL1pLST8 HA-TOR2 AVO2-myc* (SW104-3c)

E, F) AVO1 does not interact with AVO3 or AVO2 in cells depleted for TOR2

Lysates from cells expressing *GAL1* promoter-*TOR2* and HA- or myc-tagged versions of two TORC2 components were subjected to immunoprecipitations with anti-HA or anti-myc. Cells expressing TOR2 grown in galactose medium (lane 1) were compared to TOR2-depleted cells grown for 15 hr in glucose medium (lane 2). Immunoprecipitates were probed with anti-myc to detect the coimmunoprecipitated partner protein (bottom panel). The expression level of the HA or myc-tagged TORC2 component is shown in the top panel and middle panel, respectively.

E) *GAL1pTOR2 AVO1-HA AVO3-myc* (SW105-3d), F) *GAL1pTOR2 AVO1-HA AVO2-myc* (SW106-5a)

LST8 binds to the C-terminal part of TOR2 while AVO3 associates with the N-terminal part of TOR2

To determine which domains in TOR2 mediate the interaction between the different partner proteins coimmunoprecipitation experiments were performed. N-terminally tagged versions of TOR2 were expressed from high copy plasmids in strains containing epitope tagged TOR2 partner proteins. LST8 was able to interact with the C-terminal half of TOR2 containing the FAT, FRB and the kinase domain, but not with the N-terminal half of TOR2 (Fig. 4.5.A). The binding of AVO1 and AVO3 to TOR2 appeared to be more complex because in cells expressing endogenous TOR2 we failed to detect an interaction of these two proteins with either the N-terminal or the C-terminal half of TOR2. Therefore, endogenous TOR2 was depleted using a strain that contains endogenous TOR2 under the control of the *GAL1* promoter. In cells grown in glucose-media we observed a weak interaction between AVO3 and the N-terminal half of TOR2 consisting of the HEAT repeats but not with the C-terminal half of TOR2 (Fig. 4.5.B). However, using this approach we did not detect an interaction between AVO1 and the two TOR2 constructs suggesting that AVO1 might bind to TOR2 via multiple contacts with various domains of TOR2. Thus, AVO3 seems to associate with the HEAT repeats of TOR2, while LST8 avidly binds to the C-terminal half of TOR2.

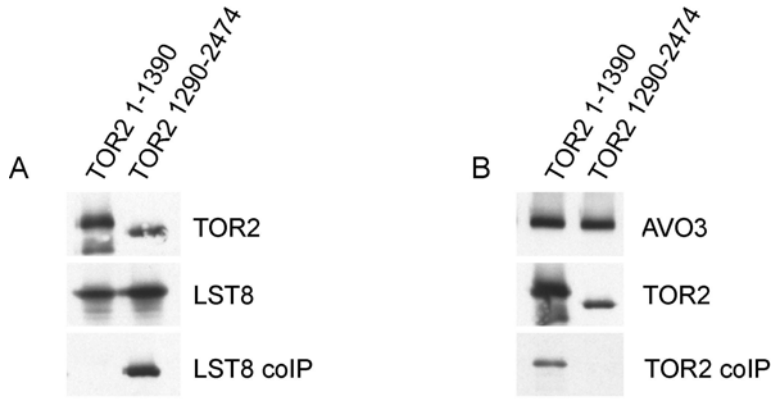


Figure 4.5. Interaction domains of TOR2 with LST8 and AVO3

A) LST8 binds to the C-terminal part of TOR2.

Cells containing myc-tagged LST8 (RL59-2d) were transformed with pSW61 expressing the N-terminal half of HA-tagged TOR2 (amino acids 1-1390) and pSW62 expressing the C-terminal half of HA-tagged TOR2 (amino acids 1290-2474). Lysates were incubated with anti-HA and subsequently probed with anti-myc to detect coimmunoprecipitated LST8 (bottom panel).

B) AVO3 associates with the N-terminal part of TOR2

Cells containing *GAL1* promoter-*TOR2* and myc-tagged AVO3 (SW94-1a) were transformed with pSW61 expressing the N-terminal half of HA-tagged TOR2 (amino acids 1-1390) and pSW62 expressing the C-terminal half of HA-tagged TOR2 (amino acids 1290-2474). Lysates were incubated with anti-myc and subsequently probed with anti-HA to detect coimmunoprecipitated TOR2 (bottom panel).

LST8 controls TOR2 kinase activity and AVO1 functions as scaffold protein

The coimmunoprecipitation experiments indicated that AVO1 as well as AVO3 are important structural proteins to maintain the integrity of the TORC2 without affecting the binding of LST8 to TOR2. To study the contribution of TORC2 components to kinase activity the activity of immunopurified TORC2 depleted of various partner proteins was determined in an *in vitro* kinase assay using as a substrate 4E-BP1 (a substrate of mammalian TOR). To control for specificity, activity from a mock purification was compared to the activity of immunopurified TOR2. We did not detect phosphorylation of 4E-BP1 in the mock assay and obtained proteins of TORC2 but not TORC1 using immunopurified TORC2 demonstrating the feasibility of the assay (see also Fig. 5.2. in

Part 5 and data not shown). As a further control the phosphorylation status of 4E-BP1 phosphorylation was analyzed using a kinase dead version of TOR2. Compared to wild-type TOR2, kinase dead TOR2 drastically reduced phosphorylation of 4E-BP1 indicating that 4E-BP1 is phosphorylated in a TOR2-dependent manner (Audhya et al., 2004).

In vitro 4E-BP1 is unstructured and phosphorylated by a number of unrelated kinases. Therefore we consider 4E-BP1 to be a nonphysiological substrate for TOR2 in yeast, which allows studying the catalytic activity of TOR2 independently of the integrity of TORC2. To test linearity of the *in vitro* kinase assay time course experiments were performed. In wild-type as well as in cells depleted for AVO1 or LST8 the rate of 4E-BP1 phosphorylation was linear up to 30 min incubation time (data not shown). The activity of TOR2 towards 4E-BP1 was determined by performing kinase reactions in the presence of different concentrations of 4E-BP1 and subsequent quantification of 4E-BP1 phosphorylation. The phosphorylation was normalized to the amount of TOR2 present in the kinase reaction determined by immunoblotting. The kinetic analysis is shown as a Lineweaver-Burk double reciprocal plot that allows the determination of the K_M (Michealis-Menten constant; -1/X intercept) and the relative V_{max} (maximal velocity; 1/Y intercept). The K_M and relative V_{max} of 4E-BP1 in *avo1* mutants was not significantly changed compared to wild-type cells (Fig. 4.6.C). In *lst8* mutant cells we observed a slight increase in the K_M and a 2-fold decrease in relative V_{max} compared to wild-type cells indicating that in the absence of LST8 the catalytic kinase activity of TOR2 is perturbed (Fig. 4.6.C). Because AVO1 depletion, which results in the disruption of TORC2 integrity, did not change the TOR2-mediated phosphorylation of 4E-BP1 we conclude that AVO1 is not required for the regulation of TORC2 catalytic activity. Depletion of LST8 in contrast, suggests that LST8 modulates the kinase activity of TOR2.

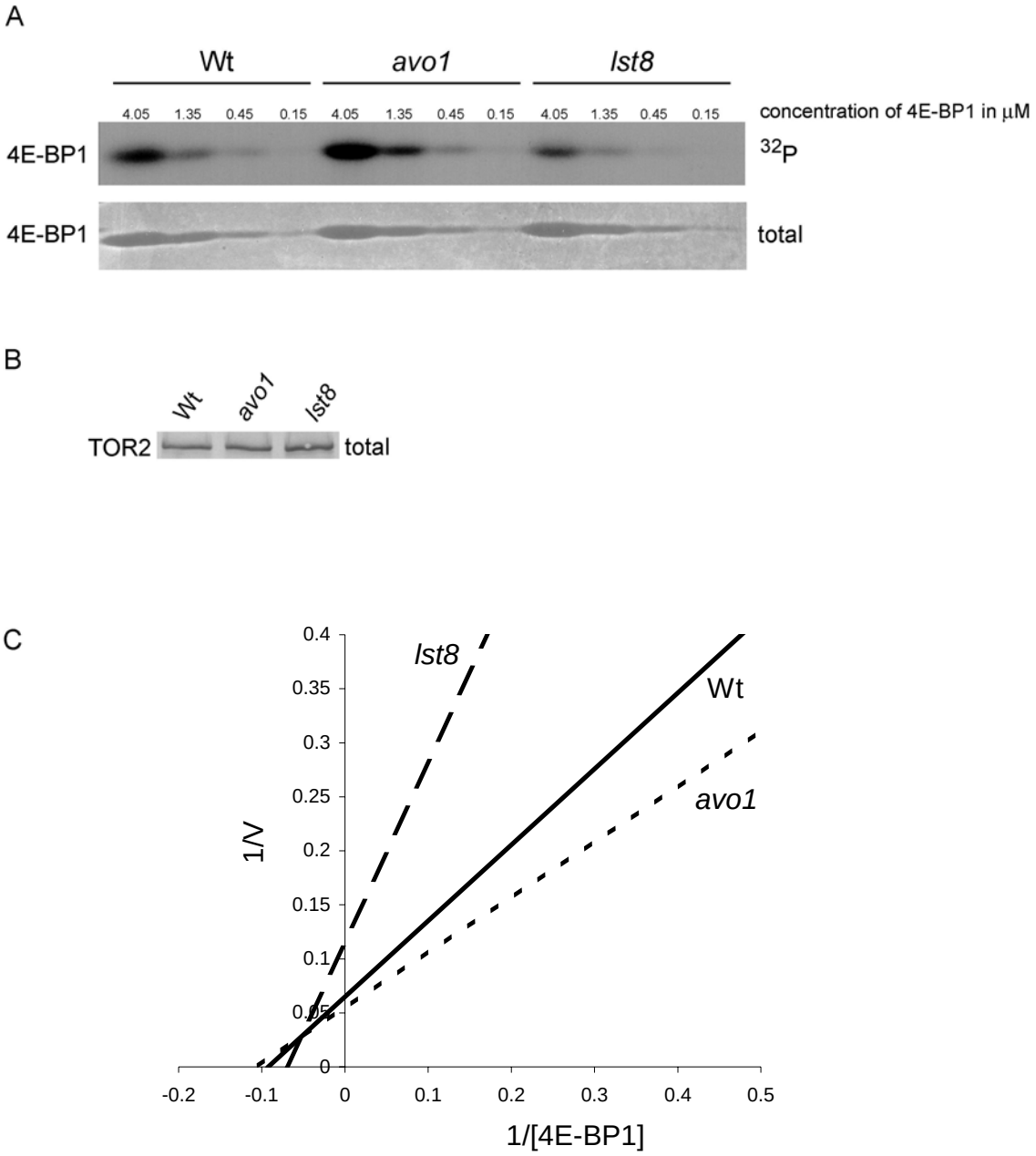


Figure 4.6. LST8 controls TOR2 kinase activity

In vitro kinase assays were performed using HA-TOR2 immunopurified from wild-type (SW70), *GAL1* promoter-*AVO1* (SW84-1d) or -*LST8* (SW100-1a) cells grown in glucose medium for 15 hr to deplete AVO1 or LST8. Purified HA-TOR2 was incubated with different concentrations of 4E-BP1 (from 0.15 - 0.45 μM) in the presence of ^{32}P-ATP.

A) Phosphorylation (top panel) and amount of 4E-BP1 (bottom panel)

B) Expression of HA-TOR2 purified from wt, *avo1* and *lst8* cells detected by immunoblotting

C) Analysis of the normalized data shown as Lineweaver-Burk plot. X-axis represents the reciprocal value of 4E-BP1 concentrations in μM and Y-axis shows the reciprocal value of 4E-BP1 phosphorylation in arbitrary units. X intercept defines $-1/K_M$, and Y intercept $1/V_{max}$.

To investigate if the integrity of TORC2 affects the accessibility of *in vivo* substrates, kinase assays using recombinant SLM1 were performed. SLM1 was recently found to be a substrate of TORC2 (Audhya et al., 2004). To estimate the K_M of the kinase reaction, various concentrations of bacterial expressed SLM1 were incubated with TORC2 purified from wild-type, *avo2* or *avo1* cells in the presence of ^{32}P-ATP. Quantification of the phosphorylation and data analysis in the Lineweaver-Burk plot exhibited a 4-fold increase of the K_M in *avo1* but only a slight increase in *avo2* cells when compared to the K_M in wild-type cells (Fig. 4.7.C). The increased K_M for SLM1 without changing relative V_{max} of the reaction indicates that the depletion of AVO1 inhibits binding of SLM1 to the TOR2 kinase. Thus, AVO1 appears to act as scaffold and adaptor protein facilitating an efficient binding of substrates to TORC2.

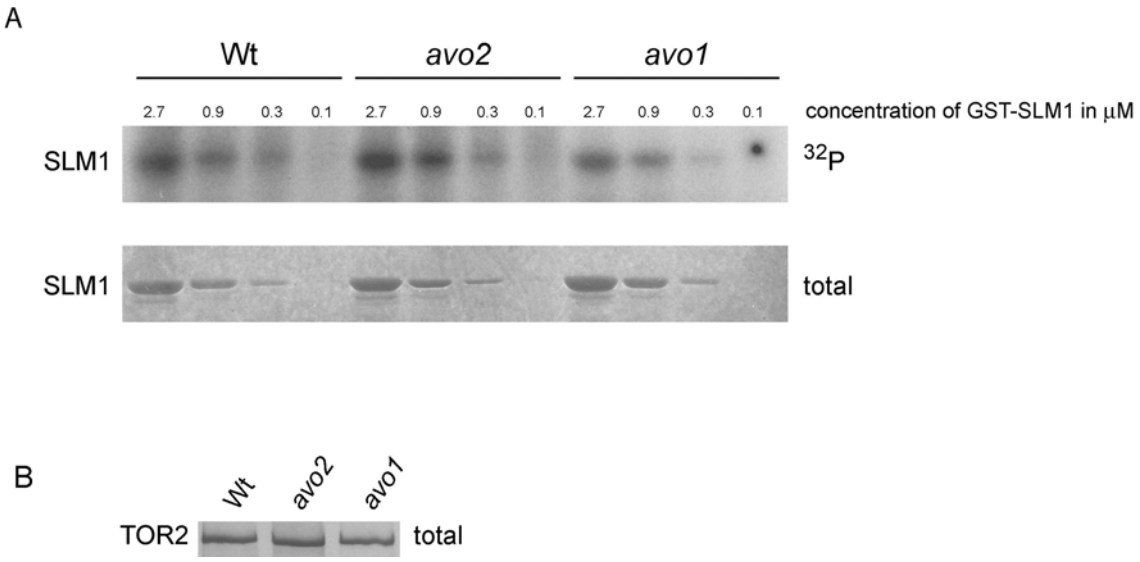


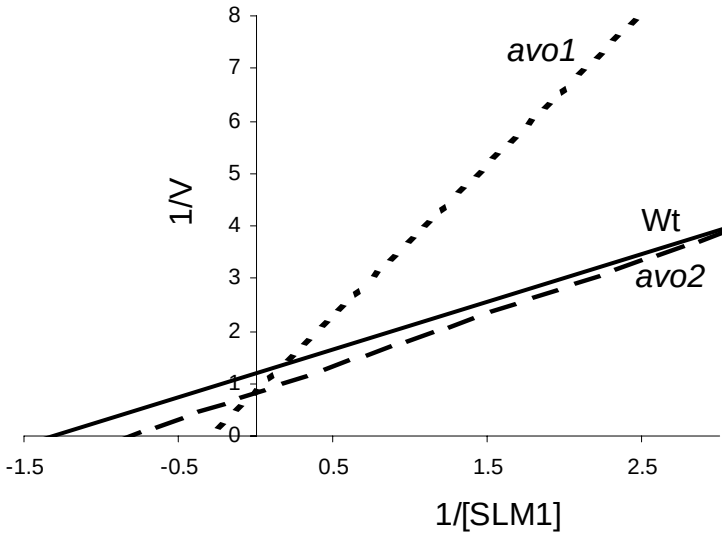
Figure 4.7. AVO1 functions as scaffold and adaptor protein

In vitro kinase assays were performed using immunopurified TOR2 from wild type (SW70), *avo2* (SW92-5a) or *GAL1* promoter-*AVO1* (SW84-1d) containing cells grown in glucose medium for 15 hr to deplete AVO1. Immunopurified TORC2 was incubated with different concentrations of recombinant GST-SLM1 (from 0.1 - 2.7 μM) in the presence of ^{32}P-ATP.

A) Phosphorylation (top panel) and amount of SLM1 (bottom panel)

B) Expression of HA-TOR2 purified from wt, *avo1* and *avo2* cells detected by immunoblotting

C



C) Analysis of the normalized data shown as Lineweaver-Burk plot. X-axis represents the reciprocal value of GST-SLM1 concentrations in μM and Y-axis shows the reciprocal value of SLM1 phosphorylation in arbitrary units. X intercept defines $-1/K_M$, and Y intercept $1/V_{max}$.

4. 3. Discussion

Here we analyzed the architecture and the function of different components of TORC2. We showed that TORC2 exists in an oligomeric form and that AVO1 and AVO3 are scaffold and likely also adaptor proteins, whereas LST8 controls the catalytic activity of TOR2 protein kinase.

The dimerization of TORC2 could be important for regulation of its activity. It has been recently reported that the activity of ATM, a kinase, that belongs like TOR to the phosphatidylinositide 3-kinase (PI3K)-related superfamily, is regulated by its dimerization (Bakkenist and Kastan, 2003). In its inactive state ATM exists as a dimer. Upon irradiation leading to autophosphorylation and activation of ATM, the ATM dimer is dissociated and active ATM monomers are able to phosphorylate their substrates. Although the regulation of TORC2 is different in the sense that TORC2 is dimerized in its active form dimerization could be an important regulatory mechanism. However, we did not see a change in the dimerization state of TOR2 under conditions where TOR signaling is inhibited. It remains to be elucidated which role the different partner proteins play in the dimerization of TORC2.

Based on coimmunoprecipitation of TORC2 partner proteins in the absence of an individual partner we propose a model in which AVO1 and AVO3 bind at least partly to the HEAT repeats of TOR2 cooperatively mediating the binding of AVO2 and possibly BIT61, BIT2 (preliminary data) to the complex, whereas LST8 binds to the C-terminal half of TOR2 independently of AVO1, AVO2 or AVO3 (model Fig. 4.8.). AVO2 might serve as an adaptor protein for substrates, such as SLM1, as SLM1 copurifies with AVO2. Given the fact that *AVO2* is a nonessential gene and does not exhibit any growth phenotypes under various conditions efficient phosphorylation of substrates must also occur in *avo2* mutants. Likewise we did not observe a drastic change in the K_M for SLM1 to TOR2 in an *avo2* mutant. The protein-protein interactions occurring in TORC2 appear to be rather complex and the association of a given protein to TORC2 may involve binding to different partner proteins through various domains, as the interaction between AVO1, AVO2 and AVO3 is impaired in the absence of TOR2.

In *in vitro* kinase assays using 4E-BP1 and SLM1 as substrates for the TOR2 protein kinase we could discriminate different functions for AVO1 and LST8. We showed that

the TORC2-mediated phosphorylation of 4E-BP1, a nonphysiological substrate for TORC2, is independent of AVO1, but requires the presence of LST8 as indicated by the decrease in the relative V_{max} for in the absence of LST8. Thus, LST8 seems to regulate the catalytic activity of TOR2, likely by direct binding to the TOR2 kinase domain. Importantly, the disruption of TORC2 observed upon AVO1 or AVO3 depletion did not disturb the interaction between TOR2 and LST8 suggesting that the integrity of TORC2 is not needed for TOR2 to be active. In this respect the destabilization of TORC2 in LST8-depleted cells did not account for the inhibition of the TOR2 kinase activity towards 4E-BP1 and might rather be a consequence of the inhibitory effect upon LST8 depletion. The finding that depletion of AVO1 resulted in a decreased affinity of TOR2 for SLM1 leads to the conclusion that AVO1 might act as an adaptor protein. Consistent with the coimmunoprecipitation experiments AVO1 and possibly AVO3 are important structural elements forming a stable complex with TOR2 and thereby provide the structural basis for binding and efficient phosphorylation of downstream factors.

What is the function of LST8? *LST8* was first identified in a synthetic lethal screen with *sec13* and was shown to be required for transport of amino acid permeases, such as the general amino acid GAP1 from Golgi to plasma membrane (Roberg et al., 1997). However, the effect of LST8 on GAP1 sorting appeared to be indirect and a consequence of LST8 acting in the TOR signaling pathway (Chen and Kaiser, 2003). LST8 is a constituent of both TORC1 and TORC2 acting positively with TOR in either complex (Chen and Kaiser, 2003; Loewith et al., 2002). Therefore, LST8 might perform similar functions in TORC1 and TORC2. It has been reported that LST8 and TOR1 are localized at endosomal and Golgi membranes and LST8 could be important for the proper localization of TORCs. However, in *lst8* mutants TOR1 still associated with internal membranes indicating that LST8 does not mediate localization of TOR1 to membranes (Chen and Kaiser, 2003). Our data suggest that LST8 bound to the TOR2 kinase domain is required for TOR2 kinase activity which is consistent with the function of mLST8 in mammals (Kim et al., 2003). The mechanism by which LST8 regulates TOR2 kinase activity is not known but LST8 could be important for proper folding of the kinase domain or could compete with inhibitory factors for binding to the TOR2 kinase domain.

What is upstream of TORC2? In yeast TOR responds to nutrients, in particular to the quantity and quality of the nitrogen source. Through which mechanism the nutrient availability is sensed by TOR is unknown. Although TORC1 and TORC2 control different signaling processes within a cell, cell growth has to occur in a coordinated manner. Therefore both TOR complexes might respond to the same signal. Since LST8 is the only TOR-interacting protein present in both complexes it could be an important factor for sensing upstream signals. Data presented in Part 3 of this thesis indicate a function for LST8 upstream of TOR2 as overexpression of *TOR2* weakly suppresses the growth phenotype of *lst8* mutants. LST8 might therefore receive upstream signals and accordingly modulate the kinase activity of both TORC1 and TORC2.

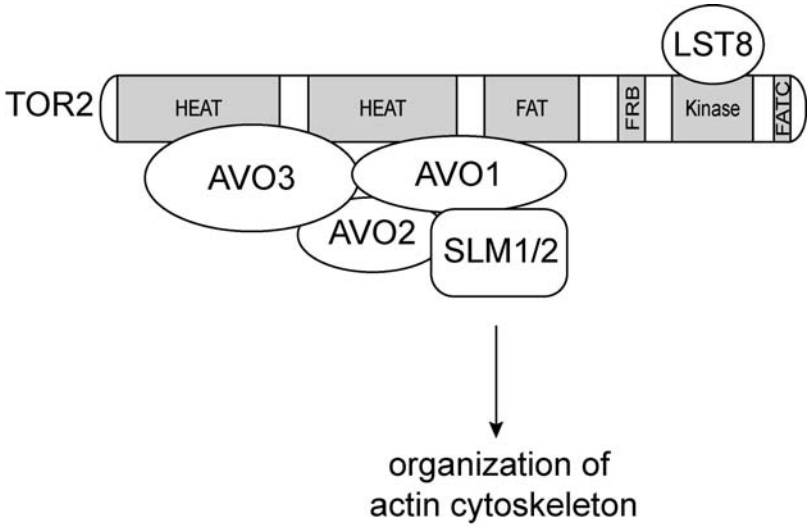


Figure 4.8. Model

AVO1 and AVO3 act as scaffold and adaptor proteins maintaining the integrity of TORC2 and the association with downstream factors, like SLM1 and SLM2 to (TORC2). LST8 through binding to the C-terminal part of TOR2 regulates kinase activity of TOR2. Oligomerization of TORC2 is not shown in this model.

4. 4. Materials and methods

Strains, plasmids, and media

The *S. cerevisiae* strains and plasmids used are listed in Table 4.1. and 4.2., respectively. All strains are isogenic derivatives of JK9-3d**a** or TB50**a**. Rich media, YPD or YPGal/Gly, and synthetic complete media, SD or SGal/Gly, were as described previously (Beck et al., 1999; Sherman, 1991). Nitrogen starvation experiments were performed with synthetic media as previously described (Schmidt et al., 1998). For TOR2-, AVO1-, AVO3-, LST8-depletion experiments, cells from logarithmically growing SGal/Gly or YPGal/Gly cultures were inoculated into SD or YPD medium, respectively. Glucose cultures were shaken for 15 hr before cells were harvested for analysis.

Genetic techniques

Restriction enzyme digests and ligations were performed according to standard methods. All enzymes and buffers were obtained commercially (Roche Diagnostics). *Escherichia coli* strains MH1 and DH5α were used for propagation and isolation of plasmids. Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983). PCR cassettes were used to generate gene deletions and modifications, as described (Longtine et al., 1998; Puig et al., 2001).

Immunoprecipitations and TAP pull-downs

A 200 ml culture was grown in YPGal/Gly or YPD at 30°C for 15 hr to OD_{600} of 0.8, harvested by centrifugation and washed with cold water. The pellet was resuspended in 2 ml lysis buffer (1 x PBS, 10% glycerol, 0.5% Tween-20 plus inhibitors: 10 mM NaF, 10 mM NaN_3, 10 mM p-nitrophenylphosphate, 10 mM sodium pyrophosphate, 10 mM β-glycerophophate, 1 mM phenylmethylsulfonyl fluoride, 1 x Roche complete inhibitor cocktail). Cells were lysed by vortexing 5 x 30 s with glass beads using a FastPrep machine (Savant Instruments). Extracts were cleared with a 5 min, 2900 x g spin. An aliquot of extract containing 3 mg protein was adjusted to 1 ml with lysis buffer plus inhibitors. To immunoprecipitate the epitope tagged protein 1 μl of either concentrated 12CA5 (anti-HA) or 9E10 (anti-myc) tissue culture supernatant was added and the tubes were rotated for 1 hr at 4°C. Afterwards 20 μl protein G-sepharose slurry (Sigma) was added and the tubes were rotated for additional 2 hr at 4°C. For TAP pull-downs 20 μl IgG-sepharose 6 fast flow (Amersham Pharmacia Biotech) was added, and tubes were rotated for 3 hr at 4°C. Beads were collected by centrifugation, washed four times

with 1 ml lysis buffer, and resuspended in 2 x SDS-PAGE sample buffer. For coimmunoprecipitations the sample was split in half, one aliquot was used for expression control, the other half to detect the coimmunoprecipitated protein and subjected to SDS-PAGE. After electrophoresis the proteins were electroblotted onto nitrocellulose, blocked in 5% milk in 1 x PBS 0.1% Tween-20, and incubated with primary antibody (12CA5 or 9E10 1:10000 in blocking solution, or anti-ProteinA 1:5000 in blocking buffer) over night at 4°C. The membranes were washed and incubated for 1 hr at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody. The tagged proteins were detected using ECL reagents (Amersham Pharmacia Biotech).

Purification of GST-tagged proteins

XL-1 blue cells transformed with pGEX-4T::SLM1 were grown in 1 l LB/Amp at 37°C to an OD_{600} of 0.4 and expression of GST-SLM1 was induced by incubation with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3.5 hr at 37°C. Cells were lysed in lysis buffer (1 x PBS, 10% glycerol, 0.5% Tween-20, 1 mM phenylmethylsulfonyl fluoride, 1 x Roche complete inhibitor cocktail) by sonication. Cleared extracts were incubated with 600 µl glutathione sepharose beads (Pharmacia) and rotated at 4°C for 3 hr. After washing with lysis buffer the protein was eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0) and dialysed against 1 x PBS, 20% glycerol, 0.5% Tween-20.

Kinase assays

Cells expressing HA-TOR2 in different mutant backgrounds maintained on YPD and YPGal/Gly plates, respectively were inoculated into YPGal/Gly liquid media and cultured over night. Per strain (4 assays) 4 l of YPD were inoculated and grown at 30°C for 15 h to an OD_{600} of 0.8 and the chilled on ice for 30 min. Cells were collected by centrifugation, washed once in ice-cold water and lysed with a Bead Beater (Biospec products) in ~20 ml lysis buffer (1 x PBS, 10% glycerol, 0.5% Tween-20 plus inhibitors: 10 mM NaF, 10 mM NaN_3, 10 mM p-nitrophenylphosphate, 10 mM sodium pyrophosphate, 10 mM β-glycerophophate, 1 mM phenylmethylsulfonyl fluoride, 1 x Roche complete inhibitor cocktail). The lysate was cleared with a 5 min, 2900 x g spin. Lysates were normalized to ~20 ml and ~300 mg protein per strain (4 kinase reactions) and passed over 200 µl Sepharose CL-4B (Sigma) : ProteinA-sepharose (Amersham) (3:1), which has been previously equilibrated in lysis buffer. To the flow through was

added 150 μl anti-HA crosslinked to Protein A-sepharose beads. This mixture was rotated for 3 hr at 4°C after which time the beads were collected in a column and washed with 30 ml lysis buffer. Antibody-beads were equally split amongst 5 tubes. To one aliquot 30 μl 2 x SDS-PAGE sample buffer was added and was used for western blotting. To the remaining aliquots either GST-LIT2 ranging from 0.1 - 2.7 μM or 4E-BP1 (Stratagene) ranging from 0.15 - 4.05 μM in 50 μl kinase buffer (lysis buffer with 20% glycerol). For time course experiments 30 μg GST-LIT2 or 1 μg 4E-BP1 was added. 6 μl of 10 x goodies (40 mM $MnCl_2$, 100 mM dithiothreitol, 10 x Roche complete inhibitor cocktail - EDTA, 100 mM NaN_3, 100 mM NaF, 100 mM p-nitrophenylphosphate, 100 mM β-glycerophosphate) were added before the reaction. The kinase reaction was started with the addition of 4 μl ATP mix (1.2 mM ATP, 2.5 μCi/ μl γ^{32}P-ATP [3000 Ci/mmol] in kinase buffer. Tubes were mixed (1200 rpm) at 30°C (incubation time for reactions with GST-LIT2: 20 min, incubation time for reactions with 4E-BP1: 10 min). The reactions were terminated with the addition of 15 μl 5 x SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE (5-20%) and radioactivity was quantified using GeneSnap software (SynGene).

Table 4.1. Strains

Strain	Genotype
JK9-3d**a**	*MAT**a** leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
JK9-3dα	*MATα leu2-3,112 ura3-52 trp1 his4 rme1 HMLα*
TB50**a**	JK9-3d**a** *HIS4 his3*
TB50α	JK9-3dα *HIS4 his3*
RL39-1a	TB50**a** *AVO2-3HA-kanMX4*
RL42-1c	TB50**a** *AVO3-3HA-kanMX4*
RL59-2d	TB50**a** *LST8-13myc-kanMX4*
RL69-1c	TB50**a** *AVO1-3HA-kanMX4*
SW62-7a	TB50**a** *HA-TOR2 AVO3-13myc-kanMX4*
SW63-1d	TB50α *HA-TOR2 LST8-13myc-kanMX4*
SW65-11c	TB50**a** *[HIS3MX6]-GAL1p-AVO3 AVO1-3HA-kanMX4 AVO2-13myc-kanMX4*

SW67-3b	TB50α *[HIS3MX6]-GAL1p-AVO3 AVO1-3HA-kanMX4 LST8-13myc-kanMX4*
SW68-3b	TB50**a** *[HIS3MX6]-GAL1p-AVO1 3HA-TOR2 AVO3-13myc-kanMX4*
SW69-4d	TB50**a** *[HIS3MX6]-GAL1p-AVO1 LST8-3HA-kanMX4 AVO2-13myc-kanMX4*
SW70	TB50**a** *3HA-TOR2*
SW72-5a	TB50**a** *[HIS3MX6]-GAL1p-AVO3 3HA-TOR2 AVO2-13myc-kanMX4*
SW73-4b	TB50**a** *[HIS3MX6]-GAL1p-AVO1 AVO3-3HA-kanMX4 AVO2-13myc-kanMX4*
SW74-11b	TB50**a** *[HIS3MX6]-GAL1p-AVO1 LST8-3HA-kanMX4 AVO3-13myc-kanMX4*
SW75-10c	TB50**a** *[HIS3MX6]-GAL1p-AVO1 3HA-TOR2 LST8myc-kanMX4*
SW76-5b	TB50**a** *[HIS3MX6]-GAL1p-AVO1 3HA-TOR2 AVO2-13myc-kanMX4*
SW77-5d	TB50**a** *[HIS3MX6]-GAL1p-AVO3 3HA-TOR2 LST8-13myc-kanMX4*
SW78-3a	TB50**a** *[HIS3MX6]-GAL1p-AVO3 LST8-3HA-kanMX4 AVO2-13myc-kanMX4*
SW84-1d	TB50**a** *[HIS3MX6]-GAL1p-AVO1 3HA-TOR2*
SW92-5a	TB50**a** *3HA-TOR2 avo2::kanMX4*
SW94-1a	TB50**a** *[HIS3MX6]-GAL1p-TOR2 AVO3-13myc-kanMX4*
SW100-1a	TB50**a** *[HIS3MX6]-GAL1p-LST8 3HA-TOR2*
SW102-8b	TB50**a** *avo2::kanMX4 3HA-TOR2 AVO3-13myc-kanMX4*
SW103-2d	TB50α *avo2::kanMX4 3HA-TOR2 LST8-13myc-kanMX4*
SW104-3c	TB50**a** *[kanMX4]-GAL1p-LST8 3HA-TOR2 AVO3-13myc-kanMX4*
SW105-3d	TB50α *[HIS3MX6]-GAL1p-TOR2 AVO1-3HA-kanMX4 AVO3-13myc-kanMX4*
SW106-5a	TB50**a** *[HIS3MX6]-GAL1p-TOR2 AVO1-3HA-kanMX4 AVO2-13myc-kanMX4*
SW108-4a	TB50α *[kanMX4]-GAL1p-LST8 3HA-TOR2 AVO2-13myc-kanMX4*
SW109-3d	TB50**a** *[HIS3MX6]-GAL1p-AVO1 3HA-TOR2 LST8-13myc-kanMX4 KOG1-TAP-kanMX4*
SW110	TB50**a/**α *AVO1-3HA-kanMX4 AVO1-TAP-KITRP1*
SW111	TB50**a/**α *AVO2-3HA-kanMX4 AVO2-13myc-kanMX4*
SW112	TB50**a/**α *AVO3-3HA-kanMX4 AVO3-13myc-kanMX4*

Table 4.2. Plasmids

Plasmids	Description
pAN54	expresses myc-tagged TOR2 from *TOR2* promoter, YCplac111::*myc-TOR2* (*CEN, LEU2*)
pRL42-1	expresses GST-tagged SLM1 (YIL105c), pGEX-4T::*SLM1*
pSW61	expresses HA-tagged TOR2 (1-1390) from *TOR2* promoter. Cloned as 4.7-kb *Bam*HI-*Pst*I fragment into YEplac195::*CYC1* terminator (*2μ, URA3)*
pSW62	expresses HA-tagged TOR2 (1290-2474) from *TOR2* promoter. Cloned as 4.1-kb *Sac*I-*Pst*I fragment into YEplac195::*TOR2* promoter *ATG-3HA* (*2μ, URA3)*

5. TOR and its role as protein kinase

5. 1. Introduction

TOR as protein kinase

TOR is the founding member of phosphatidylinositol kinase-related kinases (PIKK) family of protein kinases (Keith and Schreiber, 1995). PIKKs share similarity to the kinase domain of phosphatidylinositol kinases (PIK), but none of them are believed to act as a lipid kinase. Rather, PIKKs are thought to act as Ser/Thr protein kinases. TOR too is a protein kinase as immunoprecipitated mTOR autophosphorylates (Brunn et al., 1996; Chen et al., 1995) and is able to phosphorylate 4E-BP1 and p70S6K *in vitro* (Brunn et al., 1997; Burnett et al., 1998). The phosphorylation occurs in an mTOR-dependent manner since mTOR containing a point mutation changing Asp2338 to Ala shows no kinase activity. This Asp residue is conserved in both protein and lipid kinases, where it is required for kinase activity (Hunter, 1995). Mutation of Asp2338 to Ala also inhibits mTOR autophosphorylation on Ser2481 (Peterson et al., 2000).

In vitro mTOR has been reported to phosphorylate five sites in p70S6K, Ser371, Thr389, Thr421, Ser424, and possibly Ser411, with Thr389 being the preferred site (Burnett et al., 1998; Isotani et al., 1999; Saitoh et al., 2002). 4E-BP1 is phosphorylated at two sites by mTOR *in vitro*, Thr37 and Thr46 (Brunn et al., 1997; Fadden et al., 1997; Gingras et al., 1999). The phosphorylation sites fall into two different groups. The hydrophobic motif, Thr389-Tyr, and the Ser/Thr-Pro motif found in all the other phosphorylation sites of p70S6K and 4E-BP1. Since there is no common recognition motif in the amino acid sequence of mTOR substrates, other structural determinates or accessibility of substrates to mTOR mediated by protein-protein interactions might also be important for an efficient phosphorylation of substrates. Nevertheless, an effect of so far unknown kinases or phosphatases which associate to mTOR or are regulated by mTOR can also not be ruled out.

In yeast the function of TOR1 and TOR2 as protein kinases is unclear. Although TOR1 and TOR2 are able to phosphorylate 4E-BP1 (Alarcon et al., 1999), direct substrates for the TOR kinases in yeast have not been reported until very recently (Audhya et al., 2004). It is well established that TORC1 controls at least some of the rapamycin-sensitive readouts via inhibition of phosphatases (Schmelzle and Hall, 2000). Under

good nutrient conditions TORC1 inhibits the phosphatase SIT4 by promoting its association with TAP42. Upon inhibition of TORC1, either by rapamycin or nitrogen starvation, catalytically active SIT4 is released from TAP42 promoting the dephosphorylation of its target proteins such as the protein kinase NPR1, the transcription factor GLN3 and the TAP42 interacting protein TIP41. TOR1 has been reported to control the association of SIT4 to TAP42 via direct phosphorylation of TAP42 (Jiang and Broach, 1999), however this result remains controversial. The protein TIP41 was identified in a yeast two-hybrid screen as an interactor of TAP42 (Jacinto et al., 2001). TIP41 is a negative regulator of the TOR signaling pathway and mediates inhibition of the TOR signaling pathway by binding and inhibiting TAP42. Upon rapamycin treatment TIP41 is dephosphorylated in a SIT4-dependent manner leading to an enhanced binding of TIP41 to TAP42 and thereby inactivating TAP42 and amplifying SIT4 phosphatase activity. Since the phosphorylation status of TIP41 and NPR1 is regulated in a TOR-dependent manner it has been suggested that TOR might directly, or via a TOR-regulated kinase, control the phosphorylation of these proteins. Recently, the phosphorylation sites of NPR1 were identified (D. Bonenfant unpublished). NPR1 contains 22 rapamycin-sensitive phosphorylation sites and the motifs of these sites are similar to the rapamycin-sensitive sites in p70S6K and 4E-BP1. However, *in vitro* kinase assays indicated that TOR1 does not directly phosphorylate NPR1 suggesting that another kinase phosphorylates NPR1 and TOR may control the phosphorylation status of NPR1 exclusively via the phosphatase SIT4.

Analysis of phosphorylation sites

Identification of phosphorylation sites is still challenging despite recent advances in methodology. The most commonly used technique to analyze a phosphoprotein involves *in vivo* or *in vitro* labelling with ^{32}P-phosphate. The radiolabelled protein is then digested with a protease and the peptides are separated either by high performance liquid chromatography or by two-dimensional phosphopeptide mapping. The phosphorylation site is then determined by Edman sequencing. Alternatively, phosphorylation sites can also be traced by using mass spectrometric methods. The analysis of proteins by mass spectrometry involves proteolytic digestion of the protein of interest, which is followed by measuring the mass of the generated peptides. The different peptides are then identified by comparing the observed masses obtained by

mass spectrometry to the predicted masses based on the specificity of the protease that was used to digest the protein of interest. Singly phosphorylated peptides exhibit a mass that is 80 Da greater than the predicted mass from the peptide sequence since they contain an HPO_4 group. The phosphorylation site in a phosphopeptide can be determined by fragmentation of the peptide in the collision cell of the mass spectrometer (Bonenfant et al., 2003a). A major problem of this method is the complexity of the data that increases with the molecular weight of the protein. In addition, electrospray ionization produces multiple signals for each peptide due to multiple charging. To reduce the complexity procedures have been developed to detect phosphopeptides in a selective manner. One of these methods involves the enrichment of phosphopeptides in a complex mixture by immobilized metal-affinity chromatography (IMAC) (Ficarro et al., 2002). IMAC beads selectively bind phosphopeptides which can be eluted from the beads with Tris-buffer (pH 8.0) or alkaline phosphatase treatment. The eluted peptide is then analyzed by mass spectrometry.

Identification of substrates for a specific kinase

There are different procedures to identify a substrate for a kinase of interest. One approach, called KESTREL (kinase substrate tracking and elucidation) is based on a kinase reaction using cell extracts and the kinase of interest in the presence of ^{32}P-ATP (Knebel et al., 2001). Another assay makes use of ATP derivates that can only be used as substrates by mutated forms of the protein kinase of interest (Bishop et al., 2001).

The major problem performing a kinase reaction with whole cell extract and the kinase of interest is the high background due to endogenous kinases that are present in the cell extract. In KESTREL the conditions of the kinase assay have been optimized resulting in a reduction of the background (Knebel et al., 2001). To maximize the sensitivity of the kinase reaction ATP-depleted cell extracts are incubated with a high concentration of the kinase of interest using ^{32}P-ATP of high specific radioactivity. The incubation time is kept short and the use of MnATP instead of MgATP is preferred at least for kinases that can use both forms of ATP with similar efficiency. In addition, performing the kinase reaction with cell extract that is purified prior to the kinase reaction by a single step purification, such as ion-exchange chromatography minimized the signal to noise ratio. The substrates are detected by autoradiography and are further purified in order to be identified by mass spectrometry.

Another method was developed by the group of Kevan Shokat (Bishop et al., 2001). This approach allows the specific labelling of the substrates of a kinase of interest in the cell extract. The method is based on the mutation of a conserved bulky residue in the ATP-binding pocket of a given kinase to a glycine or alanine. Only this mutated kinase is then able to bind bulky ATP analogues that fit into the mutant-binding pocket but cannot bind to wild-type kinases. A kinase reaction can be performed using cell extract that contains a single analogue-sensitive kinase and the radiolabelled ATP-analogue resulting in the specific labelling of the direct substrate of the kinase of interest, as only this modified kinase is able to use the ATP-analogue to phosphorylate its substrate. However, computer-based modelling of the active site of the kinase as well as elaborate chemical synthesis of the analogues is required for the design of the ATP-analogues.

Here we focus on TOR and its role as a protein kinase in yeast. We tried to map TOR-regulated phosphorylation sites in TIP41 as well as AVO1 and AVO3 by mass spectrometric methods. To gain further insight into TOR signaling we started to develop an assay to identify substrates for the TOR kinases. This work was done in collaboration with Paul Jenoe, Division of Biochemistry, Biozentrum, Basel.

5. 2. Results

Phosphorylation of TIP41

To map phosphorylation sites TIP41 was expressed as a GST-tagged fusion protein from a *GAL1* promoter-containing plasmid in yeast cells. After 2 hours induction with galactose GST-TIP41 was purified via glutathione-affinity chromatography, and the protein was digested with either trypsin or endoprotease LysC. The generated peptides were analyzed by electrospray mass spectrometry (LC/MS) and compared to the predicted masses based on the specificity of the protease. Combining trypsin and LysC digestion 90% of the peptides were observed in the LC/MS analysis missing only one Ser or Thr containing peptide. Examining the spectra for the presence of peptides whose masses were increased by 80 Da (or multiples thereof), two candidates were found: peptide T39 and T40 (data not shown). Alternatively, the tryptic fragments of GST-TIP41 were subjected to IMAC (immobilized metal-affinity chromatography) selection. IMAC-beads charged with Fe^{3+} selectively bind phosphopeptides. The bound peptides were eluted from the IMAC-beads with Tris-buffer (pH 8.0) and analyzed by MALDI-TOF mass spectrometry. Three candidate phosphopeptides were identified: T39, T38-39, T40 (T38-39 represents an incompletely digested peptide) confirming the results obtained by LC/MS analysis (Fig. 5.1.). When elution from the IMAC-beads was performed in the presence of alkaline phosphatase, the mass of the three peptides decreased by 80 Da indicating that the peptides selected by IMAC are indeed phosphorylated (Fig. 5.1.).

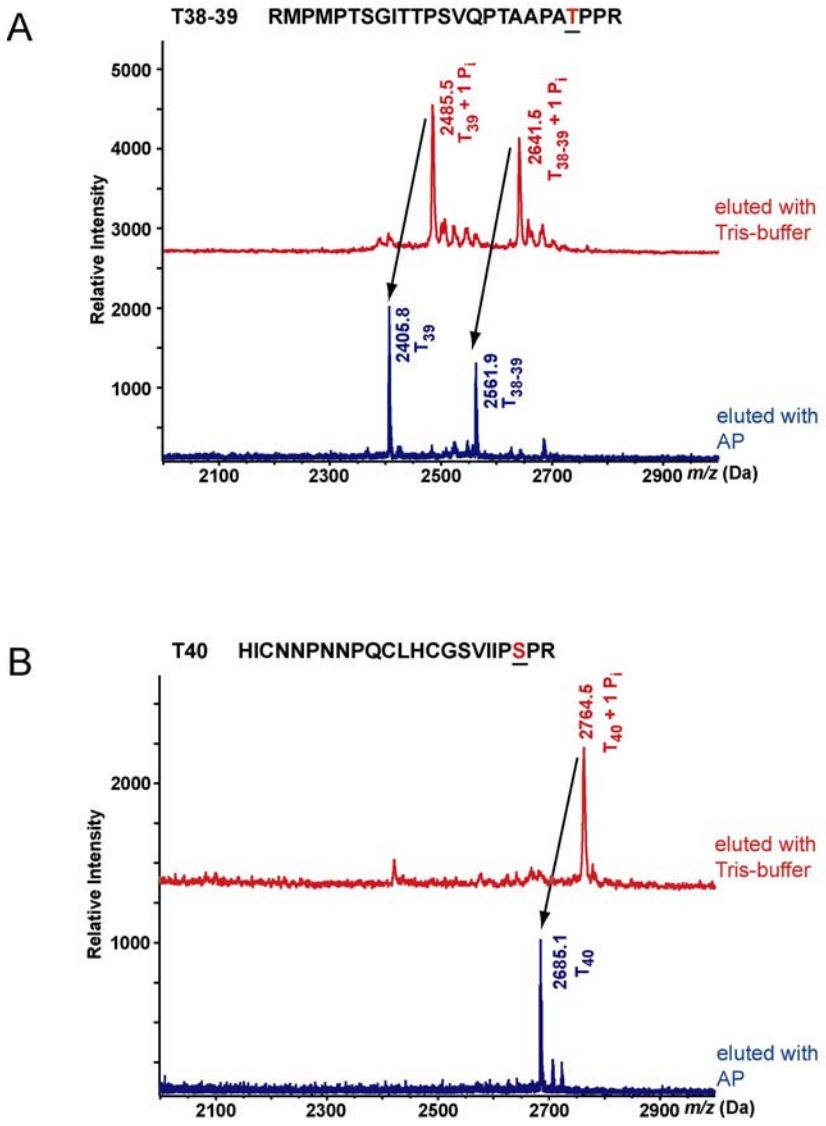


Figure 5.1. IMAC phosphopeptide selection from tryptic digest of GST-TIP41.

A) MALDI-TOF spectrum of T39 and T38-39 phosphopeptides eluted from IMAC beads in presence of Tris-buffer (pH 8.0) (upper spectrum) or in presence of alkaline phosphatase (AP) (lower spectrum).

B) MALDI-TOF spectrum of T40 phosphopeptide eluted from IMAC beads in presence of Tris-buffer (pH 8.0) (upper spectrum) or in the presence of alkaline phosphatase (AP) (lower spectrum)

To determine the precise phosphorylation sites the phosphorylated peptides T39 and T40 were analyzed by electrospray ionisation tandem mass spectrometry (LC-MS/MS). Ions of the specific peptide (T39 or T40) were selected in the first quadrupole of the mass spectrometer and subsequently subjected to fragmentation in the collision cell leading to the generation of ions from which the sequence of the peptide can be deduced. Phosphorylated fragment ions can be identified by a shift of 80 Da (or divided by the corresponding charge state of the fragment ions). Comparing the MS/MS spectra of the phosphorylated peptide with its unphosphorylated counterpart allows one to pinpoint the residue from which the mass shift occurred. We identified Thr55 and Ser79 as phosphorylation sites of TIP41 (data not shown).

The function and the rapamycin-sensitivity of the two phosphorylation sites remain to be elucidated. However, the two phosphorylation sites identified possess a Ser/Thr-Pro motif that is similar to rapamycin-sensitive sites in p70S6K, 4E-BP1 and NPR1. Since mTOR is thought to directly phosphorylate p70S6K and 4E-BP1 we suggested that TIP41 is phosphorylated by the TOR kinases. To investigate this possibility *in vitro* kinase assays with TOR complex 1 (TORC1) and bacterial expressed GST fusion proteins of wild-type TIP41 and TIP41(81-355) lacking the N-terminal phosphorylation sites were carried out. No phosphorylation of wild-type TIP41 or TIP41(81-355) was observed leading to the conclusion that TIP41 is likely phosphorylated by a kinase other than TOR1/2 (data not shown).

AVO1 and AVO3 are phosphoproteins

AVO1 and AVO3 appear to be phosphorylated by TORC2 *in vitro* (R. Loewith, W. Oppliger unpublished). The other proteins of TORC2, BIT61 and AVO2 were phosphorylated to a minor extent, whereas LST8 was not phosphorylated (Fig. 5.2.A). To test whether phosphorylation of AVO1 and AVO3 is dependent on TOR2, kinase assays with a kinase dead version of TOR2 (TOR2-KD) were performed. Phosphorylation of AVO1 and AVO3 was decreased in a TOR2-KD strain indicating that TOR2 is responsible for *in vitro* phosphorylation of AVO1 and AVO3 (Fig. 5.2.B).

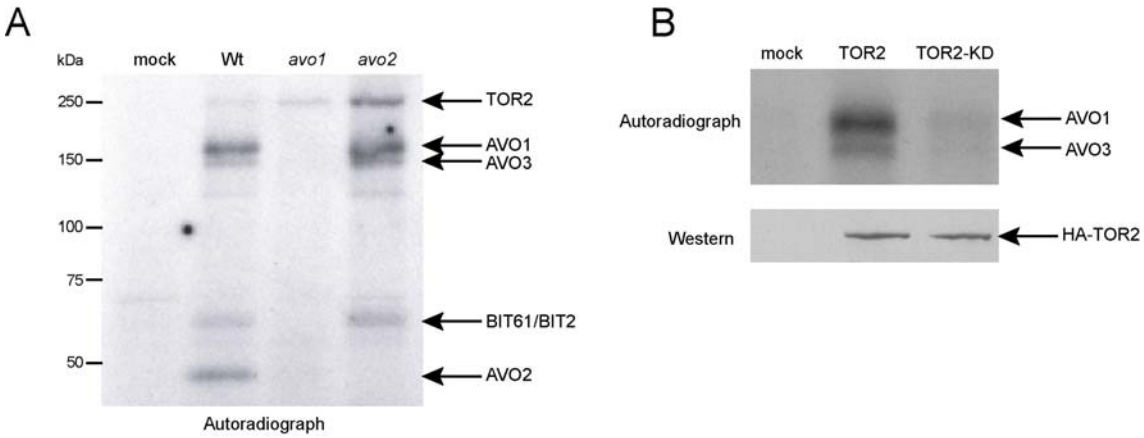


Figure 5.2. AVO1 and AVO3 are phosphorylated in a TOR2-dependent manner

A) *In vitro* kinase assays were performed using immunopurified TORC2 from wild-type (SW70), *avo2* (SW92-5a) or *GAL1* promoter-*AVO1* (SW84-1d) containing cells grown in glucose medium for 15 hr to deplete AVO1. As a control mock purification of untagged strain (TB50**a**) was performed.

B) *In vitro* kinase assays were performed using TORC2 purified from TAP-tagged AVO2 *GAL1* promoter-*TOR2* (SW80-1d) transformed with either empty plasmid (lane 1, mock), pHA-TOR2 (lane 2) or pHA-TOR2-KD (lane 3). Cells were grown for 16 hr in glucose medium to deplete endogenous TOR2. Upper panel: phosphorylation of AVO1 and AVO3, lower panel: expression of HA-TOR2 (lane 2) or HA-TOR2-KD (lane 3) detected by immunoblotting.

To identify the TORC2-mediated phosphorylation sites of AVO1 and AVO3 we developed an assay in which the phosphorylation sites of AVO1 and AVO3 were labelled with ^{32}P, subsequently isolated and analyzed by mass spectrometry. To label the phosphorylation sites of AVO1 and AVO3 TORC2 was purified with a TAP-tagged version of AVO2 and subjected to kinase reaction in the presence of ^{32}P-ATP. After gel electrophoresis the radioactive AVO1 and AVO3 bands were cut and proteolytic cleaved by trypsin. The peptides were separated on a reverse-phase column and the amount of radioactivity was determined. The fractions containing high amount of radioactivity representing phosphorylated peptides were analyzed by MALDI-TOF mass spectrometry. We were not able to obtain reproducible results using this approach. The major problems were first the low abundance of AVO1 and AVO3 and second the low stoichiometry of the phosphorylation making it very difficult to identify phosphorylated peptides.

(This part was done in collaboration with P. Jenoe, R. Loewith, W. Oppliger)

Approach to identify substrates of TOR2

To identify substrates of TORC2 an approach similar to KESTREL was developed. To eliminate the background due to endogenous protein kinases we chemically digested yeast protein extract with cyanogenbromide (CNBr) to fragment and inactivate endogenous protein kinases. Yeast extracts were prepared in the absence of phosphatase inhibitors to ensure that potential substrates are present in an unphosphorylated from. SDS was included to solubilize membrane proteins. To remove SDS the proteins were precipitated by deoxycholate-TCA precipitation and subsequently digested with CNBr. Since methionine is a rare amino acid we expected to obtain relatively large peptides ensuring that the structural determinants for substrate recognition would be preserved. The CNBr digest was fractionated by reverse-phase chromatography. Separate fractions were incubated with purified TORC2 in the presence of ^{32}P-ATP and subjected to electrophoresis on Tris-tricine gels. In a control experiment a mock kinase assay was performed to validate the specificity of the approach (Fig. 5.3.A). Radiolabelled bands specifically phosphorylated by TORC2 demonstrate the feasibility of the approach (Fig. 5.3.B; band 1 to 5). Preliminary analysis of the radioactive band 5 by MALDI-TOF revealed that the band contained a mixture of several proteins consisting of heat shock proteins SSA1 and SSB1, the two ribosomal proteins RS9A and RS7B, pyruvate kinase PYK1, and the inorganic diphosphatase IPP1. However, further purification of the bands using chromatographic methods is required to clarify the identity of the labelled protein.

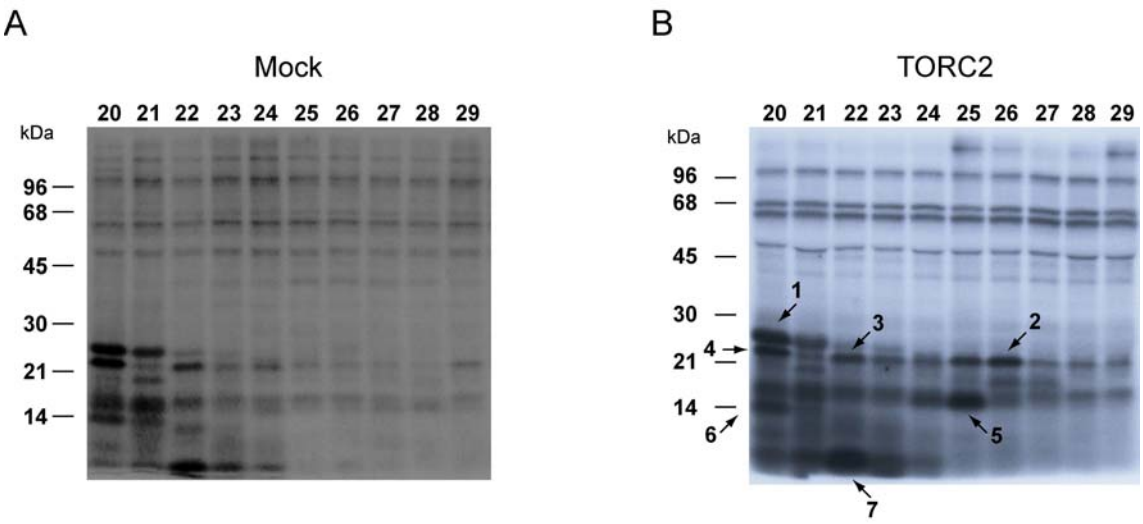


Figure 5.3. TORC2 *in vitro* kinase assays with a CNBr peptide library generated from wild-type strain (TB50a).

TORC2 was purified from strain RL126-5d expressing TAP-tagged AVO2 and incubated with different fractions of a CNBr digest of yeast protein extract that was chromatographed on a reverse-phase column.
A) Autoradiograph of kinase assays from mock purification (untagged strain)
B) Autoradiograph of kinase assays from TORC2 purification.

5. 3. Discussion

To study the role of TOR as a protein kinase we analyzed the TOR-dependent phosphorylation sites of TIP41 by mass spectrometric methods. We identified two phosphorylation sites at the N-terminus of TIP41, Thr55 and Ser79. However, the stoichiometry of the phosphorylation appeared to be quite low and we had problems to confirm the phosphorylation sites when we analyzed TIP41 expressed from its own promoter instead of using TIP41 overexpressed from a galactose inducible plasmid. Whether or not the identified phosphorylation sites are rapamycin-sensitive, and also the *in vivo* role of these sites remain to be elucidated. TIP41 is a conserved protein, but the function of its homologue in higher eukaryotes has not been studied so far. The N-terminal part of TIP41 that contains the identified phosphorylation sites is not conserved indicating that the phosphorylation sites are also not conserved. Which

kinase phosphorylates TIP41 remains unknown, as TORC1 did not directly phosphorylate TIP41 *in vitro*. Thus, TORC1 may only control phosphorylation of TIP41 via SIT4 phosphatase whereas another kinase phosphorylates TIP41. A similar situation is observed in 4E-BP1 where rapamycin sensitivity of phosphorylation sites not always correlates with the ability of TOR to phosphorylate these sites *in vitro* (Gingras et al., 2001). It is therefore likely, that rapamycin-sensitive sites are not only directly phosphorylated by TOR but also controlled by TOR-regulated kinases or phosphatases.

We encountered similar problems analyzing the phosphorylation of AVO1 and AVO3. The stoichiometry of the phosphorylation as well as the expression level of these proteins appeared to be too low to obtain reproducible results. Since AVO1 and AVO3 were phosphorylated in a TOR2-dependent manner *in vitro* AVO1 and AVO3 might be direct targets of the TOR2 protein kinase. Phosphorylation of these two proteins might be important for downstream signaling events such as attachment of other substrates to TORC2. The significance of AVO1 and AVO3 phosphorylation and the *in vivo* role of these phosphorylations remain to be elucidated.

To identify novel substrates for the TOR kinases we performed *in vitro* kinase assays using a yeast peptide library. We identified 5 bands that were phosphorylated in TORC2-dependent manner. One band was analyzed and as expected it contained a mixture of proteins which are highly expressed. It should be noted that no attempts were made to specifically isolate the radioactively labelled peptides that would reveal the identity of the labelled protein(s), but could be carried out by reverse-phase chromatography. However, identifying the inorganic diphosphatase IPP1 in the radiolabelled band is interesting, since it has been recently reported that polyphosphates may play a regulatory role in the activation of mTOR (Wang et al., 2003). The developed assay appears to be feasible and can also be applied to identify substrates of other protein kinases.

5. 4. Material and Methods

Strains, plasmids, and media

The *S. cerevisiae* strains and plasmids used are listed in Table 5.1. and 5.2. respectively. All strains are isogenic derivatives of JK9-3d**a** or TB50**a**. Rich media, YPD or YPGal/Gly, and synthetic complete media, SD or SGal/Gly, were as described previously (Beck et al., 1999; Sherman, 1991). For TOR2-, and AVO1-depletion experiments, cells from logarithmically growing SGal/Gly, SRaf/Gly or YPGal/Gly cultures were inoculated into SD or YPD medium, respectively. Glucose cultures were shaken for 15 hr before cells were harvested for analysis.

Genetic techniques

Restriction enzyme digests and ligations were performed according to standard methods. All enzymes and buffers were obtained commercially (Roche Diagnostics). *Escherichia coli* strains MH1 and DH5α were used for propagation and isolation of plasmids. Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983). PCR cassettes were used to generate gene deletions and modifications, as described (Longtine et al., 1998; Puig et al., 2001)

Protein purification

TB50**a** cells transformed with pGAL-GST-TIP41 (pSW63) were grown in SRaf/Gly-leu to an OD_{600} of 0.4. Expression of GST-TIP41 was induced by adding galactose (2% end concentration) and incubated for 2 hr at 30°C. Extracts prepared as described below were passed over a column packed with glutathione Sepharose 4B (Amersham Biosciences) which had been equilibrated with lysis buffer. After washing the column thoroughly with 100 mM Tris, pH 8.0, 150 mM NaCl, GST-TIP41 was eluted from the column with 100 mM Tris, pH 8.0, 150 mM NaCl containing 10 mM reduced glutathione (Sigma). The purity of the protein was checked by SDS polyacrylamide gel electrophoresis. The protein pool was divided into equal aliquots and stored at -20°C. Purification of bacterial expressed proteins was performed as described in part III.

Peptide Nomenclature

Peptides generated by trypsin cleavage are labelled with T. The peptides are numbered sequentially according to their position based on the N-terminal methionine of GST-TIP41.

Mass spectrometry

Enzymatic digestions and LC-MS and LC-MS/MS was performed as described (Schneider et al., 1999). Selection of phosphopeptides and MALDI-TOF was performed as described (Bonenfant et al., 2003b).

Generation of yeast peptide library

Wild-type cells (TB50**a**) were grown in 50 ml YPD to OD_{600} of 0.6, collected by centrifugation and resuspended in 0.5 ml lysis buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 x Roche complete inhibitor cocktail). Cells were lysed by vortexing 5 x 30 s with glass beads using a FastPrep machine (Savant Instruments). Extracts were cleared with a 5 min, 2900 x g spin and dialysed with a Float-A-Lyzer (10000 Da MWCO, Spectrum Labs) against 100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS. Per 100 μl homogenate 2.2 μl Na-deoxycholate (10 mg/ml) was added and incubated on ice for 30 min. Proteins were precipitated by adding 42 μl 20% TCA per 100 μl homogenate, 30 min on ice After centrifugation at 12000 rpm for 5 min, SDS was extracted from the pellet with three 1 ml washes of acetone-acetic acid-triethylamine 90:5:5. Afterwards the pellet was dried in a speed vac (Konigsberg and Henderson, 1983). The protein pellet was solubilized in formic acid. Final concentration of formic acid was brought to 70% with water. To cleave the proteins an excess of white crystalline cyanogen bromide to between 2- and 100-fold molar excess over methionyl residues was added and incubated at room temperature for 24 h. The reaction was terminated by injecting it onto an RP-HPLC for desalting. RP-HPCL was performed using a Vydac 214TP52 column with a 60 min gradient consisting of 0.4 – 76% acetonitrile in 0.1% TFA at flow rate of 100 μl/min, collecting fractions every minute.

Kinase assays

SW80-1d cells transformed with either empty plasmid, pHA-TOR2 or pHA-TOR2-KD were inoculated into SRaf/Gly-trp liquid media and cultured over night. 5 l of SD-trp were subsequently inoculated to an initial OD_{600} of 0.008. Cultures were grown at 30°C for 16 hr (to an OD_{600} of ~1.0) and then chilled on ice for 30 minutes. Cells were collected by centrifugation, washed once in ice-cold water and lysed (7 x 30 sec) with a Bead Beater (Biospec products) in ~40 ml lysis buffer (1 x PBS, 10% glycerol, 0.5% Tween-20 plus inhibitors: 10 mM NaF, 10 mM NaN_3, 10 mM p-nitrophenylphosphate, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM

phenylmethylsulfonyl fluoride, 1 x Roche complete inhibitor cocktail). Glass beads (0.5 mm) were washed with an additional 10 ml of lysis buffer with inhibitors and pooled lysates were cleared with a 5 min., 2900 x g spin. Lysates were normalized to ~50 ml and ~375 mg protein and passed over 125 μl of Sepharose CL-4B (Sigma) which had been previously equilibrated in lysis buffer. To the flow through was added 150 μl IgG-Sepharose (Amersham Bioscience; prewashed with lysis buffer). This mixture was rotated for 2 hr at 4°C after which time the beads were collected in a column and washed with 45 ml lysis buffer. IgG beads from each of the three strains were then equally split to two 1.5 ml eppendorf tubes and all liquid was removed with a Hamilton syringe. To one aliquot from each of the three strains was added 30 μl 1 x SDS-PAGE sample buffer. These samples were used for western blotting. To the remaining beads were added 50 μl kinase assay buffer (lysis buffer with 20% glycerol) and 6 μl of 10 x goodies (40 mM $MnCl_2$, 100 mM dithiothreitol, 10 x Roche Protease inhibitor cocktail - EDTA, 100 mM NaN_3, 100 mM NaF, 100 mM p-nitrophenylphosphate, 100 mM β-glycerophosphate). The reaction was started with the addition of 4 μl ATP mix (1.2 mM ATP, 2.5 μCi/μl γ^{32}P-ATP [3000 Ci/mmol] in kinase buffer). Tubes were mixed (1200 rpm) at 30°C and the reactions were terminated after 10 min with the addition of 15 μl 5 x SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE (5-20%) and radioactivity was quantified using GeneSnap software (SynGene). Kinase assays using TORC2 purified from TAP-tagged AVO2 strain (RL126-5d) were performed accordingly. Purification of HA-TOR2 by immuno-affinitiy was performed as described in Part 4.

Table 5.1. Strains

Strain	Genotype
JK9-3d**a**	*MAT**a** leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
TB50**a**	JK9-3d**a** *HIS4 his3*
SW80-1d	TB50**a** *[HIS3MX6]-GAL1p-TOR2 AVO2-TAP-kanMX4*
RL126-5d	TB50**a** *AVO2-TAP-KlTRP1*
SW70	TB50**a** *3HA-TOR2*
SW84-1d	TB50**a** *[HIS3MX6]-GAL1p-AVO1 3HA-TOR2*
SW92-5a	TB50**a** *3HA-TOR2 avo2::kanMX4*

Table 5.2. Plasmids

Plasmids	Description
pHA-TOR2	expresses HA-tagged TOR2 from *TOR2* promoter; pRS314::*3HA-TOR2* (*CEN, TRP1*) (Jiang and Broach, 1999)
pHA-TOR2-KD	expresses HA-tagged TOR2-kinase dead from *TOR2* promoter; pRS314::*3HA-TOR2*^{D2998E} (*CEN, TRP1*) (Jiang and Broach, 1999)
pGAL-GST-TIP41	pSW63; expresses GST-TIP41 under the control of the *GAL1* promoter. Cloned as 1.1-kb *Bam*HI-*Sal*I fragment into YCplac111::*GAL1* promoter-*GST* (*CEN, LEU2*).
pGST-TIP41	pEJ122; pGEX-4T::*TIP41*
pGST-TIP41(81-355)	pSW36; *TIP41* (nt *243-1065*) cloned as 0.8-kb *Bam*HI-*Sal*I fragment into pGEX-4T

6. Perspectives

TOR is a major controller of cell growth in *S. cerevisiae*. TOR signaling is rather complex and includes regulation of many different pathways. The identification of two distinct TOR complexes, TORC1 and TORC2 begins to explain how specificity and diversity is achieved in TOR signaling. Given the fact that components of the TOR complexes stably interact with each other suggests that a major role of these proteins is to maintain structural integrity of the complex. Indeed, our data on TORC2 indicate that AVO1 and AVO3 are required for the stability of the complex and also function as adaptor proteins required for recruitment of substrates for TORC2.

What are the substrates of the TOR kinases in yeast? The knowledge about downstream processes controlled by TOR has rapidly increased over the past few years. However, direct substrates of the TOR protein kinases have not been found until very recently in yeast (Audhya et al., 2004). We identified several candidate substrates of the TOR2 kinase. One possible substrate is YPK2 and it would be interesting to study the role of YPKs in TOR signaling. In analogy to the situation in higher eukaryotes and *S. pombe*, YPKs have already been implicated to act as potential substrates for TOR, however, how YPKs signal to downstream effector pathways such as the PKC1-MAP kinase cascade is not understood. Other putative substrates of TOR2 include proteins within TORC2, such as AVO1 and AVO3, which are both phosphorylated in a TOR2-dependent manner *in vitro.* The *in vivo* role of AVO1 and AVO3 phosphorylation is unknown. In this respect it would be interesting to map the phosphorylation sites of these two proteins. Mutation of the phosphorylation sites into phosphomimetic and non-phosphorylatable sites would clarify the significance of these phosphorylation events *in vivo*. Phosphorylation of AVO1 and AVO3 could create a docking site for substrates of TORC2. Applying a proteomic approach using yeast peptide library as described in Part 5 could lead to the identification of potential substrates for TORC1 and TORC2.

How is TOR signaling regulated in response to nutrients? Our work suggests that LST8 modulates TOR2 kinase activity, possibly through binding directly to the kinase domain of TOR2. Since LST8 is present in both complexes it is likely that LST8 also regulates kinase activity of TORC1. TORC1 and TORC2 might therefore respond to upstream signals through LST8, whereas specificity of downstream signaling events is determined by the different partner proteins of both complexes.

7. Bibliography

Abraham, R.T. 2001. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev*. 15:2177-96.

Alarcon, C.M., J. Heitman, and M.E. Cardenas. 1999. Protein kinase activity and identification of a toxic effector domain of the target of rapamycin TOR proteins in yeast. *Mol Biol Cell*. 10:2531-46.

Alberts, A.S., N. Bouquin, L.H. Johnston, and R. Treisman. 1998. Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein beta subunits and the yeast response regulator protein Skn7. *J Biol Chem*. 273:8616-22.

Alessi, D.R. 2001. Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. *Biochem Soc Trans*. 29:1-14.

Alessi, D.R., M.T. Kozlowski, Q.P. Weng, N. Morrice, and J. Avruch. 1998. 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. *Curr Biol*. 8:69-81.

Andrade, M.A., and P. Bork. 1995. HEAT repeats in the Huntington's disease protein. *Nat Genet*. 11:115-6.

Audhya, A., and S.D. Emr. 2002. Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev Cell*. 2:593-605.

Audhya, A., R. Loewith, A.B. Parsons, L. Gao, M. Tabuchi, H. Zhou, C. Boone, M.N. Hall, and S.D. Emr. 2004. Genome-wide synthetic lethal analysis identifies new PI4,5P_2 effectors that function in regulation of actin organization. *submitted to EMBO J.*

Avruch, J., C. Belham, Q. Weng, K. Hara, and K. Yonezawa. 2001. The p70 S6 kinase integrates nutrient and growth signals to control translational capacity. *Prog Mol Subcell Biol*. 26:115-54.

Bakkenist, C.J., and M.B. Kastan. 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*. 421:499-506.

Barbet, N.C., U. Schneider, S.B. Helliwell, I. Stansfield, M.F. Tuite, and M.N. Hall. 1996. TOR controls translation initiation and early G1 progression in yeast. *Mol Biol Cell*. 7:25-42.

Beck, T., P.A. Delley, and M.N. Hall. 2001. Control of the actin cytoskeleton by extracellular signals. *Results Probl Cell Differ*. 32:231-62.

Beck, T., and M.N. Hall. 1999. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature*. 402:689-92.

Beck, T., A. Schmidt, and M.N. Hall. 1999. Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. *J Cell Biol*. 146:1227-38.

Benedetti, H., S. Raths, F. Crausaz, and H. Riezman. 1994. The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol Biol Cell*. 5:1023-37.

Beugnet, A., X. Wang, and C.G. Proud. 2003. Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1. *J Biol Chem*. 278:40717-22.

Bierer, B.E., P.S. Mattila, R.F. Standaert, L.A. Herzenberg, S.J. Burakoff, G. Crabtree, and S.L. Schreiber. 1990. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci U S A*. 87:9231-5.

Biondi, R.M., A. Kieloch, R.A. Currie, M. Deak, and D.R. Alessi. 2001. The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *Embo J*. 20:4380-90.

Bishop, A.C., O. Buzko, and K.M. Shokat. 2001. Magic bullets for protein kinases. *Trends Cell Biol*. 11:167-72.

Bonenfant, D., T. Mini, and P. Jenoe. 2003a. Mass spectrometric analysis of protein phosphorylation. *The Protein Protocols Handbook, edited by John M. Walker*.

Bonenfant, D., T. Schmelzle, E. Jacinto, J.L. Crespo, T. Mini, M.N. Hall, and P. Jenoe. 2003b. Quantitation of changes in protein phosphorylation: a simple method based on stable isotope labeling and mass spectrometry. *Proc Natl Acad Sci U S A*. 100:880-5.

Bosotti, R., A. Isacchi, and E.L. Sonnhammer. 2000. FAT: a novel domain in PIK-related kinases. *Trends Biochem Sci*. 25:225-7.

Brunn, G.J., C.C. Hudson, A. Sekulic, J.M. Williams, H. Hosoi, P.J. Houghton, J.C. Lawrence, Jr., and R.T. Abraham. 1997. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science*. 277:99-101.

Brunn, G.J., J. Williams, C. Sabers, G. Wiederrecht, J.C. Lawrence, Jr., and R.T. Abraham. 1996. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *Embo J*. 15:5256-67.

Burnett, P.E., R.K. Barrow, N.A. Cohen, S.H. Snyder, and D.M. Sabatini. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A*. 95:1432-7.

Cafferkey, R., M.M. McLaughlin, P.R. Young, R.K. Johnson, and G.P. Livi. 1994. Yeast TOR (DRR) proteins: amino-acid sequence alignment and identification of structural motifs. *Gene*. 141:133-6.

Cardenas, M.E., N.S. Cutler, M.C. Lorenz, C.J. Di Como, and J. Heitman. 1999. The TOR signaling cascade regulates gene expression in response to nutrients. *Genes Dev*. 13:3271-9.

Casamayor, A., P.D. Torrance, T. Kobayashi, J. Thorner, and D.R. Alessi. 1999. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr Biol*. 9:186-97.

Chant, J. 1999. Cell polarity in yeast. *Annu Rev Cell Dev Biol*. 15:365-91.

Chen, E.J., and C.A. Kaiser. 2003. LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway. *J Cell Biol*. 161:333-47.

Chen, J., X.F. Zheng, E.J. Brown, and S.L. Schreiber. 1995. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proc Natl Acad Sci U S A*. 92:4947-51.

Cherkasova, V.A., and A.G. Hinnebusch. 2003. Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev*. 17:859-72.

Choi, J., J. Chen, S.L. Schreiber, and J. Clardy. 1996. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science*. 273:239-42.

Choi, K.M., L.P. McMahon, and J.C. Lawrence, Jr. 2003. Two motifs in the translational repressor PHAS-I required for efficient phosphorylation by mammalian target of rapamycin and for recognition by raptor. *J Biol Chem*. 278:19667-73.

Chook, Y.M., and G. Blobel. 1999. Structure of the nuclear transport complex karyopherin-beta2-Ran x GppNHp. *Nature*. 399:230-7.

Colicelli, J., C. Nicolette, C. Birchmeier, L. Rodgers, M. Riggs, and M. Wigler. 1991. Expression of three mammalian cDNAs that interfere with RAS function in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 88:2913-7.

Colombani, J., S. Raisin, S. Pantalacci, T. Radimerski, J. Montagne, and P. Leopold. 2003. A nutrient sensor mechanism controls Drosophila growth. *Cell*. 114:739-49.

Cosentino, G.P., T. Schmelzle, A. Haghighat, S.B. Helliwell, M.N. Hall, and N. Sonenberg. 2000. Eap1p, a novel eukaryotic translation initiation factor 4E-associated protein in Saccharomyces cerevisiae. *Mol Cell Biol*. 20:4604-13.

Crespo, J.L., K. Daicho, T. Ushimaru, and M.N. Hall. 2001. The GATA transcription factors GLN3 and GAT1 link TOR to salt stress in Saccharomyces cerevisiae. *J Biol Chem*. 276:34441-4.

Crespo, J.L., and M.N. Hall. 2002. Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae. *Microbiol Mol Biol Rev*. 66:579-91, table of contents.

Crespo, J.L., T. Powers, B. Fowler, and M.N. Hall. 2002. The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. *Proc Natl Acad Sci U S A*. 99:6784-9.

Danaie, P., M. Altmann, M.N. Hall, H. Trachsel, and S.B. Helliwell. 1999. CLN3 expression is sufficient to restore G1-to-S-phase progression in Saccharomyces cerevisiae mutants defective in translation initiation factor eIF4E. *Biochem J*. 340 (Pt 1):135-41.

De Craene, J.O., O. Soetens, and B. Andre. 2001. The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. *J Biol Chem*. 276:43939-48.

Delley, P.A., and M.N. Hall. 1999. Cell wall stress depolarizes cell growth via hyperactivation of RHO1. *J Cell Biol*. 147:163-74.

Dennis, P.B., A. Jaeschke, M. Saitoh, B. Fowler, S.C. Kozma, and G. Thomas. 2001. Mammalian TOR: a homeostatic ATP sensor. *Science*. 294:1102-5.

Dennis, P.B., N. Pullen, S.C. Kozma, and G. Thomas. 1996. The principal rapamycin-sensitive p70(s6k) phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases. *Mol Cell Biol*. 16:6242-51.

Di Como, C.J., and K.T. Arndt. 1996. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev*. 10:1904-16.

Dilova, I., C.Y. Chen, and T. Powers. 2002. Mks1 in concert with TOR signaling negatively regulates RTG target gene expression in S. cerevisiae. *Curr Biol*. 12:389-95.

Dunn, T.M., D. Haak, E. Monaghan, and T.J. Beeler. 1998. Synthesis of monohydroxylated inositolphosphorylceramide (IPC-C) in Saccharomyces cerevisiae requires Scs7p, a protein with both a cytochrome b5-like domain and a hydroxylase/desaturase domain. *Yeast*. 14:311-21.

Edinger, A.L., C.M. Linardic, G.G. Chiang, C.B. Thompson, and R.T. Abraham. 2003. Differential effects of rapamycin on mammalian target of rapamycin signaling functions in mammalian cells. *Cancer Res*. 63:8451-60.

Etienne-Manneville, S., and A. Hall. 2002. Rho GTPases in cell biology. *Nature*. 420:629-35.

Fadden, P., T.A. Haystead, and J.C. Lawrence, Jr. 1997. Identification of phosphorylation sites in the translational regulator, PHAS-I, that are controlled by insulin and rapamycin in rat adipocytes. *J Biol Chem*. 272:10240-7.

Fang, Y., M. Vilella-Bach, R. Bachmann, A. Flanigan, and J. Chen. 2001. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science*. 294:1942-5.

Ficarro, S.B., M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, and F.M. White. 2002. Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. *Nat Biotechnol*. 20:301-5.

Fingar, D.C., and J. Blenis. 2004. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene*. 23:3151-71.

Friant, S., R. Lombardi, T. Schmelzle, M.N. Hall, and H. Riezman. 2001. Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. *Embo J*. 20:6783-92.

Gao, X., Y. Zhang, P. Arrazola, O. Hino, T. Kobayashi, R.S. Yeung, B. Ru, and D. Pan. 2002. Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat Cell Biol*. 4:699-704.

Garami, A., F.J. Zwartkruis, T. Nobukuni, M. Joaquin, M. Roccio, H. Stocker, S.C. Kozma, E. Hafen, J.L. Bos, and G. Thomas. 2003. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell*. 11:1457-66.

Gelperin, D., L. Horton, A. DeChant, J. Hensold, and S.K. Lemmon. 2002. Loss of ypk1 function causes rapamycin sensitivity, inhibition of translation initiation and synthetic lethality in 14-3-3-deficient yeast. *Genetics*. 161:1453-64.

Gingras, A.C., S.P. Gygi, B. Raught, R.D. Polakiewicz, R.T. Abraham, M.F. Hoekstra, R. Aebersold, and N. Sonenberg. 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev*. 13:1422-37.

Gingras, A.C., B. Raught, and N. Sonenberg. 2001. Regulation of translation initiation by FRAP/mTOR. *Genes Dev*. 15:807-26.

Goldberg, Y. 1999. Protein phosphatase 2A: who shall regulate the regulator? *Biochem Pharmacol*. 57:321-8.

Gorner, W., E. Durchschlag, M.T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis, and C. Schuller. 1998. Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev*. 12:586-97.

Gorner, W., E. Durchschlag, J. Wolf, E.L. Brown, G. Ammerer, H. Ruis, and C. Schuller. 2002. Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *Embo J*. 21:135-44.

Groves, M.R., and D. Barford. 1999. Topological characteristics of helical repeat proteins. *Curr Opin Struct Biol*. 9:383-9.

Guo, W., F. Tamanoi, and P. Novick. 2001. Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat Cell Biol*. 3:353-60.

Han, J.W., R.B. Pearson, P.B. Dennis, and G. Thomas. 1995. Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70s6k by inducing dephosphorylation of the same subset of sites. *J Biol Chem*. 270:21396-403.

Hara, K., Y. Maruki, X. Long, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, and K. Yonezawa. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell*. 110:177-89.

Hara, K., K. Yonezawa, Q.P. Weng, M.T. Kozlowski, C. Belham, and J. Avruch. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem*. 273:14484-94.

Hardie, D.G., and S.A. Hawley. 2001. AMP-activated protein kinase: the energy charge hypothesis revisited. *Bioessays*. 23:1112-9.

Hardwick, J.S., F.G. Kuruvilla, J.K. Tong, A.F. Shamji, and S.L. Schreiber. 1999. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc Natl Acad Sci U S A*. 96:14866-70.

Harrison, J.C., E.S. Bardes, Y. Ohya, and D.J. Lew. 2001. A role for the Pkc1p/Mpk1p kinase cascade in the morphogenesis checkpoint. *Nat Cell Biol*. 3:417-20.

Heinisch, J.J., A. Lorberg, H.P. Schmitz, and J.J. Jacoby. 1999. The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in Saccharomyces cerevisiae. *Mol Microbiol*. 32:671-80.

Heitman, J., N.R. Movva, and M.N. Hall. 1991a. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*. 253:905-9.

Heitman, J., N.R. Movva, P.C. Hiestand, and M.N. Hall. 1991b. FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 88:1948-52.

Helliwell, S.B., I. Howald, N. Barbet, and M.N. Hall. 1998a. TOR2 is part of two related signaling pathways coordinating cell growth in Saccharomyces cerevisiae. *Genetics*. 148:99-112.

Helliwell, S.B., A. Schmidt, Y. Ohya, and M.N. Hall. 1998b. The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. *Curr Biol*. 8:1211-4.

Helliwell, S.B., P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M.N. Hall. 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol Biol Cell*. 5:105-18.

Hidalgo, M., and E.K. Rowinsky. 2000. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene*. 19:6680-6.

Hinnebusch, A.G., and K. Natarajan. 2002. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryot Cell*. 1:22-32.

Hunter, T. 1995. When is a lipid kinase not a lipid kinase? When it is a protein kinase. *Cell*. 83:1-4.

Inagaki, M., T. Schmelzle, K. Yamaguchi, K. Irie, M.N. Hall, and K. Matsumoto. 1999. PDK1 homologs activate the Pkc1-mitogen-activated protein kinase pathway in yeast. *Mol Cell Biol*. 19:8344-52.

Inoki, K., Y. Li, T. Xu, and K.L. Guan. 2003a. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev*. 17:1829-34.

Inoki, K., Y. Li, T. Zhu, J. Wu, and K.L. Guan. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol*. 4:648-57.

Inoki, K., T. Zhu, and K.L. Guan. 2003b. TSC2 mediates cellular energy response to control cell growth and survival. *Cell*. 115:577-90.

Isotani, S., K. Hara, C. Tokunaga, H. Inoue, J. Avruch, and K. Yonezawa. 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. *J Biol Chem*. 274:34493-8.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol*. 153:163-8.

Jacinto, E., B. Guo, K.T. Arndt, T. Schmelzle, and M.N. Hall. 2001. TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. *Mol Cell*. 8:1017-26.

Jacinto, E., and M.N. Hall. 2003. Tor signalling in bugs, brain and brawn. *Nat Rev Mol Cell Biol*. 4:117-26.

James, P., J. Halladay, and E.A. Craig. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*. 144:1425-36.

Jefferies, H.B., S. Fumagalli, P.B. Dennis, C. Reinhard, R.B. Pearson, and G. Thomas. 1997. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *Embo J*. 16:3693-704.

Jiang, Y., and J.R. Broach. 1999. Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. *Embo J*. 18:2782-92.

Kamada, Y., T. Funakoshi, T. Shintani, K. Nagano, M. Ohsumi, and Y. Ohsumi. 2000. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol*. 150:1507-13.

Kamada, Y., U.S. Jung, J. Piotrowski, and D.E. Levin. 1995. The protein kinase C-activated MAP kinase pathway of Saccharomyces cerevisiae mediates a novel aspect of the heat shock response. *Genes Dev*. 9:1559-71.

Keith, C.T., and S.L. Schreiber. 1995. PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science*. 270:50-1.

Kim, D.H., D.D. Sarbassov, S.M. Ali, J.E. King, R.R. Latek, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*. 110:163-75.

Kim, D.H., D. Sarbassov dos, S.M. Ali, R.R. Latek, K.V. Guntur, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini. 2003. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell*. 11:895-904.

Kimura, N., C. Tokunaga, S. Dalal, C. Richardson, K. Yoshino, K. Hara, B.E. Kemp, L.A. Witters, O. Mimura, and K. Yonezawa. 2003. A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes Cells*. 8:65-79.

Klionsky, D.J., and Y. Ohsumi. 1999. Vacuolar import of proteins and organelles from the cytoplasm. *Annu Rev Cell Dev Biol*. 15:1-32.

Knebel, A., N. Morrice, and P. Cohen. 2001. A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38delta. *Embo J*. 20:4360-9.

Komeili, A., K.P. Wedaman, E.K. O'Shea, and T. Powers. 2000. Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. *J Cell Biol*. 151:863-78.

Konigsberg, W.H., and L. Henderson. 1983. Removal of sodium dodecyl sulfate from proteins by ion-pair extraction. *Methods Enzymol*. 91:254-9.

Krause, S.A., and J.V. Gray. 2002. The protein kinase C pathway is required for viability in quiescence in Saccharomyces cerevisiae. *Curr Biol*. 12:588-93.

Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N.R. Movva, and M.N. Hall. 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell*. 73:585-96.

Kunz, J., U. Schneider, I. Howald, A. Schmidt, and M.N. Hall. 2000. HEAT repeats mediate plasma membrane localization of Tor2p in yeast. *J Biol Chem*. 275:37011-20.

Kuroda, A., K. Nomura, R. Ohtomo, J. Kato, T. Ikeda, N. Takiguchi, H. Ohtake, and A. Kornberg. 2001. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in E. coli. *Science*. 293:705-8.

Kuruvilla, F.G., A.F. Shamji, and S.L. Schreiber. 2001. Carbon- and nitrogen-quality signaling to translation are mediated by distinct GATA-type transcription factors. *Proc Natl Acad Sci U S A*. 98:7283-8.

Lee, K.S., and D.E. Levin. 1992. Dominant mutations in a gene encoding a putative protein kinase (BCK1) bypass the requirement for a Saccharomyces cerevisiae protein kinase C homolog. *Mol Cell Biol*. 12:172-82.

Liu, Z., T. Sekito, M. Spirek, J. Thornton, and R.A. Butow. 2003. Retrograde signaling is regulated by the dynamic interaction between Rtg2p and Mks1p. *Mol Cell*. 12:401-11.

Loewith, R., and N.M. Hall. 2004. TOR signaling in yeast: temporal and spatial control of cell growth. *Cell growth: control of cell size, Cold Spring Harbor Laboratory Press*.

Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J.L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, and M.N. Hall. 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell*. 10:457-68.

Longtine, M.S., A. McKenzie, 3rd, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast*. 14:953-61.

Lorenz, M.C., and J. Heitman. 1995. TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. *J Biol Chem*. 270:27531-7.

Luke, M.M., F. Della Seta, C.J. Di Como, H. Sugimoto, R. Kobayashi, and K.T. Arndt. 1996. The SAP, a new family of proteins, associate and function positively with the SIT4 phosphatase. *Mol Cell Biol*. 16:2744-55.

Madaule, P., R. Axel, and A.M. Myers. 1987. Characterization of two members of the rho gene family from the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 84:779-83.

Magasanik, B., and C.A. Kaiser. 2002. Nitrogen regulation in Saccharomyces cerevisiae. *Gene*. 290:1-18.

Manning, B.D., A.R. Tee, M.N. Logsdon, J. Blenis, and L.C. Cantley. 2002. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell*. 10:151-62.

Martin, K.A., and J. Blenis. 2002. Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. *Adv Cancer Res*. 86:1-39.

Matsuo, T., Y. Kubo, Y. Watanabe, and M. Yamamoto. 2003. Schizosaccharomyces pombe AGC family kinase Gad8p forms a conserved signaling module with TOR and PDK1-like kinases. *Embo J*. 22:3073-83.

Meyuhas, O. 2000. Synthesis of the translational apparatus is regulated at the translational level. *Eur J Biochem*. 267:6321-30.

Mothe-Satney, I., D. Yang, P. Fadden, T.A. Haystead, and J.C. Lawrence, Jr. 2000. Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression. *Mol Cell Biol*. 20:3558-67.

Nave, B.T., M. Ouwens, D.J. Withers, D.R. Alessi, and P.R. Shepherd. 1999. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J*. 344 Pt 2:427-31.

Noda, T., and Y. Ohsumi. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem*. 273:3963-6.

Nojima, H., C. Tokunaga, S. Eguchi, N. Oshiro, S. Hidayat, K. Yoshino, K. Hara, N. Tanaka, J. Avruch, and K. Yonezawa. 2003. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J Biol Chem*. 278:15461-4.

Nonaka, H., K. Tanaka, H. Hirano, T. Fujiwara, H. Kohno, M. Umikawa, A. Mino, and Y. Takai. 1995. A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevisiae. *Embo J*. 14:5931-8.

Oldham, S., J. Montagne, T. Radimerski, G. Thomas, and E. Hafen. 2000. Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. *Genes Dev*. 14:2689-94.

Park, J., M.L. Leong, P. Buse, A.C. Maiyar, G.L. Firestone, and B.A. Hemmings. 1999. Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *Embo J*. 18:3024-33.

Pearson, R.B., P.B. Dennis, J.W. Han, N.A. Williamson, S.C. Kozma, R.E. Wettenhall, and G. Thomas. 1995. The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *Embo J*. 14:5279-87.

Perry, J., and N. Kleckner. 2003. The ATRs, ATMs, and TORs are giant HEAT repeat proteins. *Cell*. 112:151-5.

Peterson, R.T., P.A. Beal, M.J. Comb, and S.L. Schreiber. 2000. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J Biol Chem*. 275:7416-23.

Potter, C.J., L.G. Pedraza, H. Huang, and T. Xu. 2003. The tuberous sclerosis complex (TSC) pathway and mechanism of size control. *Biochem Soc Trans*. 31:584-6.

Powers, T., and P. Walter. 1999. Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. *Mol Biol Cell*. 10:987-1000.

Proud, C.G. 2002. Regulation of mammalian translation factors by nutrients. *Eur J Biochem*. 269:5338-49.

Proud, C.G. 2004. mTOR-mediated regulation of translation factors by amino acids. *Biochem Biophys Res Commun*. 313:429-36.

Pruyne, D., and A. Bretscher. 2000a. Polarization of cell growth in yeast. *J Cell Sci*. 113 (Pt 4):571-85.

Pruyne, D., and A. Bretscher. 2000b. Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states. *J Cell Sci*. 113 (Pt 3):365-75.

Puig, O., F. Caspary, G. Rigaut, B. Rutz, E. Bouveret, E. Bragado-Nilsson, M. Wilm, and B. Seraphin. 2001. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods*. 24:218-29.

Raught, B., A.C. Gingras, and N. Sonenberg. 2001. The target of rapamycin (TOR) proteins. *Proc Natl Acad Sci U S A*. 98:7037-44.

Reinke, A., S. Anderson, J.M. McCaffery, J. Yates, 3rd, S. Aronova, S. Chu, S. Fairclough, C. Iverson, K.P. Wedaman, and T. Powers. 2004. TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in Saccharomyces cerevisiae. *J Biol Chem*. 279:14752-62.

Roberg, K.J., S. Bickel, N. Rowley, and C.A. Kaiser. 1997. Control of amino acid permease sorting in the late secretory pathway of Saccharomyces cerevisiae by SEC13, LST4, LST7 and LST8. *Genetics*. 147:1569-84.

Saitoh, M., N. Pullen, P. Brennan, D. Cantrell, P.B. Dennis, and G. Thomas. 2002. Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site. *J Biol Chem*. 277:20104-12.

Saucedo, L.J., and B.A. Edgar. 2002. Why size matters: altering cell size. *Curr Opin Genet Dev*. 12:565-71.

Saucedo, L.J., X. Gao, D.A. Chiarelli, L. Li, D. Pan, and B.A. Edgar. 2003. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol*. 5:566-71.

Schalm, S.S., and J. Blenis. 2002. Identification of a conserved motif required for mTOR signaling. *Curr Biol*. 12:632-9.

Schalm, S.S., D.C. Fingar, D.M. Sabatini, and J. Blenis. 2003. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr Biol*. 13:797-806.

Schmelzle, T., T. Beck, D.E. Martin, and M.N. Hall. 2004. Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Mol Cell Biol*. 24:338-51.

Schmelzle, T., and M.N. Hall. 2000. TOR, a central controller of cell growth. *Cell*. 103:253-62.

Schmelzle, T., S.B. Helliwell, and M.N. Hall. 2002. Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast. *Mol Cell Biol*. 22:1329-39.

Schmidt, A., T. Beck, A. Koller, J. Kunz, and M.N. Hall. 1998. The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. *Embo J*. 17:6924-31.

Schmidt, A., M. Bickle, T. Beck, and M.N. Hall. 1997. The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell*. 88:531-42.

Schmidt, A., and M.N. Hall. 1998. Signaling to the actin cytoskeleton. *Annu Rev Cell Dev Biol*. 14:305-38.

Schmidt, A., J. Kunz, and M.N. Hall. 1996. TOR2 is required for organization of the actin cytoskeleton in yeast. *Proc Natl Acad Sci U S A*. 93:13780-5.

Schneider, U., T. Mini, P. Jeno, P.A. Fisher, and N. Stuurman. 1999. Phosphorylation of the major Drosophila lamin in vivo: site identification during both M-phase (meiosis) and interphase by electrospray ionization tandem mass spectrometry. *Biochemistry*. 38:4620-32.

Schreiber, S.L. 1991. Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science*. 251:283-7.

Sekulic, A., C.C. Hudson, J.L. Homme, P. Yin, D.M. Otterness, L.M. Karnitz, and R.T. Abraham. 2000. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res*. 60:3504-13.

Shamji, A.F., F.G. Kuruvilla, and S.L. Schreiber. 2000. Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins. *Curr Biol*. 10:1574-81.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol*. 194:3-21.

Smith, A., M.P. Ward, and S. Garrett. 1998. Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. *Embo J*. 17:3556-64.

Sophianopoulou, V., and G. Diallinas. 1995. Amino acid transporters of lower eukaryotes: regulation, structure and topogenesis. *FEMS Microbiol Rev*. 16:53-75.

Stan, R., M.M. McLaughlin, R. Cafferkey, R.K. Johnson, M. Rosenberg, and G.P. Livi. 1994. Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue. *J Biol Chem*. 269:32027-30.

Stocker, H., and E. Hafen. 2000. Genetic control of cell size. *Curr Opin Genet Dev*. 10:529-35.

Sun, Y., R. Taniguchi, D. Tanoue, T. Yamaji, H. Takematsu, K. Mori, T. Fujita, T. Kawasaki, and Y. Kozutsumi. 2000. Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. *Mol Cell Biol*. 20:4411-9.

Tee, A.R., D.C. Fingar, B.D. Manning, D.J. Kwiatkowski, L.C. Cantley, and J. Blenis. 2002. Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc Natl Acad Sci U S A*. 99:13571-6.

Tee, A.R., B.D. Manning, P.P. Roux, L.C. Cantley, and J. Blenis. 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr Biol*. 13:1259-68.

Tee, A.R., and C.G. Proud. 2002. Caspase cleavage of initiation factor 4E-binding protein 1 yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. *Mol Cell Biol*. 22:1674-83.

ter Schure, E.G., N.A. van Riel, and C.T. Verrips. 2000. The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae. *FEMS Microbiol Rev*. 24:67-83.

Thevelein, J.M., and J.H. de Winde. 1999. Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae. *Mol Microbiol*. 33:904-18.

Torres, J., C.J. Di Como, E. Herrero, and M.A. De La Torre-Ruiz. 2002. Regulation of the cell integrity pathway by rapamycin-sensitive TOR function in budding yeast. *J Biol Chem*. 277:43495-504.

Uetz, P., L. Giot, G. Cagney, T.A. Mansfield, R.S. Judson, J.R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, and J.M. Rothberg. 2000. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. *Nature*. 403:623-7.

van Slegtenhorst, M., M. Nellist, B. Nagelkerken, J. Cheadle, R. Snell, A. van den Ouweland, A. Reuser, J. Sampson, D. Halley, and P. van der Sluijs. 1998. Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. *Hum Mol Genet*. 7:1053-7.

Vandenbol, M., J.C. Jauniaux, and M. Grenson. 1990. The Saccharomyces cerevisiae NPR1 gene required for the activity of ammonia-sensitive amino acid permeases encodes a protein kinase homologue. *Mol Gen Genet*. 222:393-9.

Vanhaesebroeck, B., and D.R. Alessi. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J*. 346 Pt 3:561-76.

Wang, L., C.D. Fraley, J. Faridi, A. Kornberg, and R.A. Roth. 2003. Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammary cancer cells. *Proc Natl Acad Sci U S A*. 100:11249-54.

Wang, X., L.E. Campbell, C.M. Miller, and C.G. Proud. 1998. Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem J*. 334 (Pt 1):261-7.

Warner, J.R. 1999. The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci*. 24:437-40.

Watanabe, Y., K. Irie, and K. Matsumoto. 1995. Yeast RLM1 encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway. *Mol Cell Biol*. 15:5740-9.

Wedaman, K.P., A. Reinke, S. Anderson, J. Yates, 3rd, J.M. McCaffery, and T. Powers. 2003. Tor kinases are in distinct membrane-associated protein complexes in Saccharomyces cerevisiae. *Mol Biol Cell*. 14:1204-20.

Xu, G., G. Kwon, C.A. Marshall, T.A. Lin, J.C. Lawrence, Jr., and M.L. McDaniel. 1998. Branched-chain amino acids are essential in the regulation of PHAS-I and p70 S6 kinase by pancreatic beta-cells. A possible role in protein translation and mitogenic signaling. *J Biol Chem*. 273:28178-84.

Zabrocki, P., C. Van Hoof, J. Goris, J.M. Thevelein, J. Winderickx, and S. Wera. 2002. Protein phosphatase 2A on track for nutrient-induced signalling in yeast. *Mol Microbiol*. 43:835-42.

Zhang, H., J.P. Stallock, J.C. Ng, C. Reinhard, and T.P. Neufeld. 2000. Regulation of cellular growth by the Drosophila target of rapamycin dTOR. *Genes Dev*. 14:2712-24.

Zhang, Y., X. Gao, L.J. Saucedo, B. Ru, B.A. Edgar, and D. Pan. 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol*. 5:578-81.

Zheng, X.F., D. Florentino, J. Chen, G.R. Crabtree, and S.L. Schreiber. 1995. TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. *Cell*. 82:121-30.

Curriculum vitae

Name	Stephan Wullschleger
Date of Birth	December 29, 1974
Place of Origin	Zofingen (AG), Switzerland
June 1995	Matura, Kantonsschule Zofingen (AG), Switzerland
October 1995 - March 2000	Diploma in Biology II (Biochemistry) Biozentrum, University of Basel, Switzerland
September 1998 - July 1999	Diploma thesis with PD Dr. Heinz Müller Departments of Research and Gynecology University Hospital of Basel, Switzerland 'Estrogen receptor regulation by heregulins in the breast cancer cell line MCF7'
July 2000 - June 2004	Ph.D. thesis with Prof. Michael N. Hall Biozentrum, Division of Biochemistry University of Basel, Switzerland 'Characterization of TOR complex 2 (TORC2) in *Saccharomyces cerevisiae*'

Publications

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M. N. (2002)
Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Molecular Cell. 10 (3): 457-468*

Wullschleger, S., Loewith, R., Oppliger, W., and Hall, M. N.
Characterization of AVO1, a component of TOR complex 2 (TORC2)
Manuscript in preparation

Erklärung

Ich erkläre hiermit, dass ich diese Dissertation ‘Characterization of TOR complex 2 (TORC2) in *Saccharomyces cerevisiae*’ nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Fakultät eingereicht habe.

Stephan Wullschleger