Functional analysis of TOR complex 2 and its control of sphingolipid biosynthesis in *Saccharomyces cerevisiae*

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Summary

The target of rapamycin (TOR) kinase is a central controller of cell growth. In yeast, the TOR proteins function as key components of two highly conserved multiprotein complexes, TORC1 and TORC2. TORC1 is sensitive to the immunosuppressive and anti-cancer drug rapamycin, and controls temporal aspects of cell growth through regulation of transcription and translation. It contains TOR1 or TOR2, and in addition KOG1, LST8, and TCO89. TORC2 is a rapamycin insensitive complex that mediates spatial control of cell growth via actin cytoskeleton organization. It contains TOR2, AVO1, AVO2, AVO3, BIT61 and LST8.

This thesis has two parts. In the first part, we describe a molecular analysis of the TORC2 subunit AVO3. We find that AVO3 is localized at the cell periphery, consistent with the localization of TOR2. AVO3 has six distinct regions that are highly conserved from yeast to human. AVO3 and, in particular, five of its conserved regions are required for the TORC2 function of signaling via the cell integrity pathway and polarization of the actin cytoskeleton. AVO3 also interacts with the novel TORC2 component BIT2, and is required for full activation of the TORC2 downstream target MPK1. This part of the thesis therefore describes a positive role for AVO3 in TORC2, to support growth and actin organization.

In the second part of this thesis, we focus on a new readout of TORC2, crosstalk with the sphingolipid biosynthesis pathway. Sphingolipids and their metabolites are known as building components of cellular membranes, and also as signaling molecules mediating cell growth, endocytosis, actin regulation, and stress response. Here we show that TORC2 temperature sensitive mutants, *tor2*^{ts} and *avo3-1*, are synthetically lethal at the permissive temperature, with partial inhibition of sphingolipid biosynthesis by the antibiotic myriocin. At non-permissive temperature, the TORC2 mutants show reduced *de novo* synthesis of sphingolipids. Consistent with this, a general reduction of sphingolipid contents is also observed in steady state. In contrast, rapamycin treatment and loss of TORC1 function do not induce a reduction in sphingolipid

biosynthesis. Our findings in this part of the thesis indicate that TORC2, but not TORC1, mediate sphingolipid biosynthesis.

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Contents

Preface

This thesis focuses mainly on functional analysis of TOR complex 2 (TORC2) in yeast *Saccharomyces cerevisiae*. As a result, many exciting findings concerning TOR complex 1 (TORC1) are overlooked in this thesis, even though the importance of TORC1 on cell growth is recognized.

As the mammalian TORC1 field has been tugged by findings in yeast TORC1, so could the findings in yeast TORC2 contribute to understanding of the structure and function of mammalian TORC2.

This thesis is divided into three parts. The first part, the introduction, provides an overview of the two major players of this thesis, yeast TORC2 signaling pathway and sphingolipid biosynthetic pathway.

The second part, the results, represents two major findings that were made during this study. The first key finding was that AVO3, a component of TORC2, is essential for TORC2 function. The second was that TORC2 signaling pathway controls sphingolipid biosynthesis. This study was carried out in close collaboration with Karsten Meyer and the help of Xue Li Guan, and Markus R. Wenk at Singapore.

In the last part, based on our findings and results as well as those of other labs, the functions of TORC2 are discussed in a broader context. Although this part is hypothetical, it gives some perspective to understanding the "TORC2 network".

PART I

Introduction

Cell growth is a fundamental property of cells

Cell growth, defined as accumulation of cell mass, is a fundamental property of cells, together with cell division and cell death. Cell growth and cell division are considered to be distinct processes. During formation of egg and neuronal spiral code, for example, cells accumulate cell mass without dividing. On the other hand, during egg cleavage, cells divide without any accumulation of cell mass. These examples clearly show that the two fundamental processes, cell growth and cell division, are distinct.

Since cell growth governs cell size, it must be tightly regulated. Imagine a cell that its size decreases its size 0.98 times during the cell cycle. The cell would become smaller than a single atom after several thousand cell cycles. Imagine a cell that increases its size 1.02 times during cell cycle. The cell would be larger than the earth after several thousand cell cycles. Thus, similar to cell division, cells have specific mechanisms that regulate accumulation of mass.

Cell growth implicates mass accumulation and surface expansion

Mass accumulation during cell growth is accompanied by expansion of cell surface, i.e. plasma membrane. Mathematically, when the cubic volume of an ideal sphere is doubled, the surface area is increased 1.69 times; therefore cells need to synthesize a sufficient quantity of lipids during each cell growth period. In addition to the plasma membrane, cells contain other membrane systems, such as mitochondria, Golgi, endoplasmic reticulum, and other vesicles which are also required for duplication. In other words, cells have to coordinate synthesis of those membranes, and particularly synthesis of lipids, with cell growth. Thus, cells most likely have a system(s) that regulates lipid biogenesis.

TOR as a central controller of cell growth

The target of rapamycin

The target of rapamycin protein, TOR, plays a key role in the regulation of cell growth. TOR was originally identified in *Saccharomyces cerevisiae* as a mutant that is resistant to rapamycin, a drug known as immunosuppressant, anti-proliferative, and anti-cancer agent (Heitman, Movva et al. 1991). TOR is a structurally and functionally conserved in eukaryotes. It contains a kinase domain which has strong similarity to the catalytic domains of phosphoinositide-3 kinase (PI3K) and phosphatidylinositol-4 kinase (PI4K) (Fruman, Meyers et al. 1998; Crespo and Hall 2002). Two other conserved domains, HEAT repeats and FAT domain, are involved in mediating protein interaction. Functionally, TOR regulates cell growth in response to nutrients.

TOR complex 1 as a temporal controller of cell growth

The TOR proteins in *Saccharomyces cerevisiae* are part of two distinct multiprotein complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Loewith, Jacinto et al. 2002; Wedaman, Reinke et al. 2003; Reinke, Anderson et al. 2004). The activity of both TOR proteins depends on formation of these complexes (Wullschleger, Loewith et al. 2005).

In response to nutrients, TORC1, which contains TOR1 or TOR2 (and is therefore termed "the shared pathway"), and associated partners KOG1, TCO89, and LST8, plays an essential role in temporal control of cell growth. TORC1 positively controls starvation-specific transcription and translation via a phosphatase switch composed of the type 2A-related phosphatase SIT4, TAP42, and TIP41 (Di Como and Arndt 1996; Jiang and Broach 1999; Jacinto, Guo et al. 2001; Cherkasova and Hinnebusch 2003). Under good nitrogen conditions, TOR inactivates the type 2A-related phosphatase SIT4 by binding SIT4 to TAP42 (Di Como and Arndt 1996; Jiang and Broach 1999; Jacinto, Guo et al. 2001). In the absence of nutrients, TORC1 is inactive and mediate release of SIT4 from TAP42. Released, and therefore active SIT4, dephosphorylates and activates target proteins such as the transcription factor GLN3 and the kinase NPR1, which are involved in synthesizing alternative nutrient sources (Beck and Hall 1999; Jacinto, Guo et al. 2001). TOR also mediates ribosomal protein gene transcription, by

regulating the subcellular localization of a corepressor CRF1 via protein kinase A (PKA) and the PKA-regulated kinase YAK1 (Martin, Soulard et al. 2004). In addition, TOR regulates autophagy, by controlling ATG1 kinase-dependent organization of elements on the pre-autophagosomal membrane (Mizushima, Yamamoto et al. 2001; Suzuki, Kirisako et al. 2001; Kim, Huang et al. 2002).

Mammalian TOR complex 1 (mTORC1) controls several pathways that collectively determine the cell mass. mTORC1 acts as an assembler of growth signals, such as growth hormones, amino acids availability, and energy, and regulates several cellular processes including translation, transcription, ribosome biosynthesis, and autophagy (Hay and Sonenberg 2004; Wullschleger, Loewith et al. 2006). mTORC1 is sensitive to rapamycin, and consists of mTOR, raptor, and mLST8, which are the mammalian homologs of TOR1/TOR2, KOG1 and LST8, respectively (Hara, Maruki et al. 2002; Kim, Sarbassov et al. 2002).

TOR complex 2 as a spatial controller of cell growth

While TORC1 is responsible for temporal control of cell growth, TORC2 signaling pathway mediates a spatial control of cell growth, including actin cytoskeleton organization and regulation of endocytosis in a cell-cycle dependent manner. TORC2 is rapamycin insensitive complex. Intensive work on TORC2 and its downstream targets have revealed parts of this complex signaling network, however there is still much unknown. The TORC2 and its downstream cascades are drawn in Figure I-1.

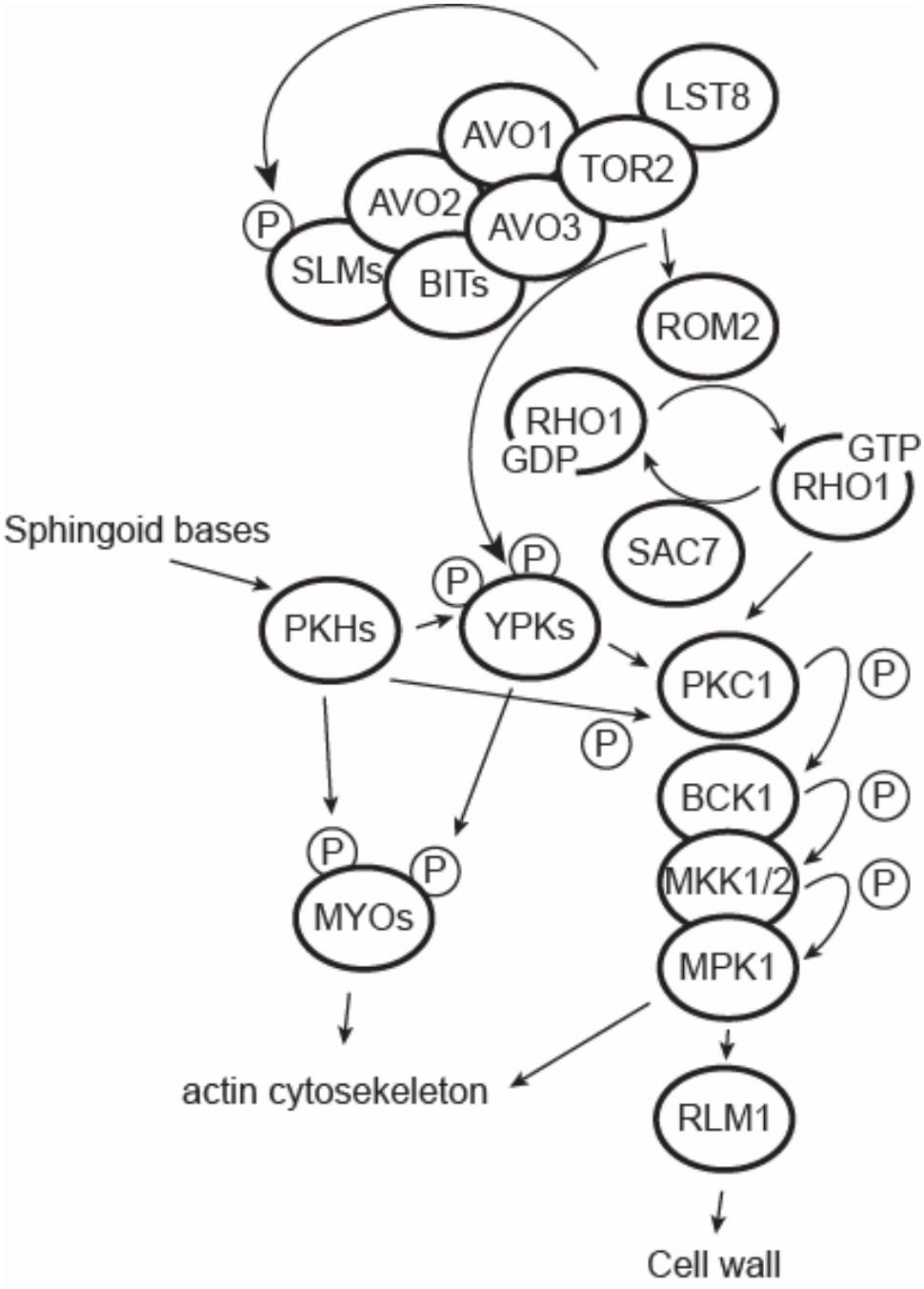


Figure I-1. The TOR complex 2 signaling network.

"P" indicates phosphorylation. See text for detail.

TORC2 in *S. cerevisiae*

Yeast TOR2, as well as TOR1, are ~280 KDa proteins that contain several domains. An N-terminal domain comprised of up to 20 tandem-repeated HEAT motifs, that mediate protein-protein interactions in multiprotein complexes (Groves and Barford 1999). The FAT domain, occupying the region between the HEAT repeats and a C-terminal catalytic domain, also mediates protein-protein interactions, and perhaps also acts as a scaffold. In addition, based on gel filtration elution profile, TOR2 appears as two species, one of 1.5–2 MDa and a second of 0.7–0.8 MDa (Loewith, Jacinto et al. 2002; Wullschleger, Loewith et al. 2005). Consistent with the findings above, TOR2 is indeed found in a multiprotein complex (Loewith, Jacinto et al. 2002; Wedaman, Reinke et al. 2003; Reinke, Anderson et al. 2004). TORC2 is composed of TOR2, but not TOR1 (and is therefore termed "the unique pathway"), AVO1, AVO2, AVO3, LST8, BIT61, BIT2, SLM1, and SLM2 (Loewith, Jacinto et al. 2002; Wedaman, Reinke et al. 2003; Audhya, Loewith et al. 2004; Fadri, Daquinag et al. 2005). The complex mediates actin cytoskeleton organization, and therefore is essential for spatial control of cell growth (Loewith, Jacinto et al. 2002).

TOR2, *LST8*, *AVO1*, and *AVO3* are essential genes. SLM1 and SLM2 are redundant and perform an essential function, since a double deletion mutant is lethal (Audhya, Loewith et al. 2004). Consistent with the phenotypes of TOR2-depleted cell, LST8-, AVO1-, and AVO3-depleted cells exhibit a depolarized actin cytoskeleton and show cell cycle arrest at G_2/M transition (Loewith, Jacinto et al. 2002; Ho, Shiau et al. 2005). *AVO2*, *BIT61*, and *BIT2* are non-essential genes, and deletion mutants do not show any phenotype under good nutrient conditions. Function of the partner proteins is not well-characterized except contribution to TORC2 kinase activity (Wullschleger, Loewith et al. 2005).

Downstream targets of TORC2 in yeast

ROM2, RHO, PKC1, and MPK1-MAP kinase cascade

The TORC2 signaling pathway begins with activation of the guanine nucleotide exchange factor ROM2 by an unknown mechanism (Schmidt, Bickle et al. 1997). Activated ROM2 in turn converts small GTPase RHO1 and RHO2 to their active, GTP

bound state. GTP-bound RHO can bind and phosphorylate PKC1 (Kamada, Qadota et al. 1996). PKC1 regulates organization of the actin cytoskeleton via the MPK1-MAP kinase cascade (Schmidt, Bickle et al. 1997; Helliwell, Howald et al. 1998; Loewith, Jacinto et al. 2002). The MPK1-MAP kinase cascade composes of MAPKKK, BCK1, two redundant threonine/tyrosine MAPKKs, MKK1 and MKK2, and the MAPK MPK1/SLT2 (Costigan, Gehrung et al. 1992; Nonaka, Tanaka et al. 1995; Kamada, Qadota et al. 1996; Delley and Hall 1999).

It is known that the MPK1-MAP kinase cascade mainly maintains cell integrity by regulating cell wall biosynthesis in response to stresses (Levin 2005). MPK1 is activated by dual phosphorylation of its two conserved theronine (Thr190) and tyrosine (Tyr192) residues (Lee, Irie et al. 1993). The activated-MPK1 induces transcription of genes involved in cell wall biosynthesis such as *CHS3*, encoding a chitin synthase, and *FKS1*, a component of β-1,3-glucan synthase (Jung and Levin 1999). RLM1 and SWI4 are identified as the transcription factors of MPK1 (Watanabe, Takaesu et al. 1997; Garcia, Bermejo et al. 2004; Levin 2005). RLM1 up-regulates expression of cell wall-related genes. RLM1 interacts with MPK1 for activation by phosphorylation (Watanabe, Irie et al. 1995; Jung, Sobering et al. 2002). However, loss of MPK1 function leads to more severe phenotypic defects than a *rlm1* deletion mutant, suggesting that MPK1 has additional effectors (Jung and Levin 1999). SWI4 is the transcription factor of the SWI4-SWI6 complex that regulates late G_1-specific transcription targets. SWI6 is a transcription cofactor of SWI4, and phosphorylated in vivo in an MPK1-dependent manner in response to cell wall stress (Madden, Sheu et al. 1997). Interestingly, the cell cycle regulated phosphorylation site of SWI6, Ser160, is within a consensus MAP kinase phosphorylation site, and a *swi6* mutant that lacks this site is not phosphorylated by MPK1 (Baetz, Moffat et al. 2001). This suggests that MPK1 may regulate the SWI4-SWI6 complex through the cell cycle. Consistent with this notion is the fact that MPK1 activity fluctuates through the cell cycle, peaking in G_1 (Zarzov, Mazzoni et al. 1996). It is also unknown how MPK1 mediates actin cytoskeleton organization.

MAPK phosphatases

As the phosphorylation cascades of MAP kinases positively regulate their activity,

dephosphorylation of MAP kinases plays an important role in their regulation. MAPK phosphatases remove a phosphate from phospho-tyrosine and/or phospho-threonine residues of MAP kinases, and thereby downregulate the activity of MAP kinases. MSG5 is a dual-specificity (Tyr and Ser/Thr) protein phosphatase involved in regulation of the MPK1 activity. MSG5 was originally isolated as a suppressor of G_1 cell cycle arrest induced by pheromone-stimulation and disruption of *GPA1*, α subunit of G protein coupled to mating factor receptors (Doi, Gartner et al. 1994). Overexpression of MSG5 suppresses the growth-inhibitory effect of overexpression of $MKK1^{P386}$, an activated-form of MKK1 MAPK kinase (Watanabe, Irie et al. 1995). In contrast, disruption of *MSG5* increases the phosphorylation state of MPK1 upon heat shock (Martin, Rodriguez-Pachon et al. 2000; Flandez, Cosano et al. 2004). MSG5 binds and dephosphorylates MPK1. Reciprocally, MPK1 phosphorylates MSG5 as a consequence of the activation of MPK1. Under activating conditions, a decrease in the affinity between MSG5 and MPK1 is observed, suggesting that the mechanism by which MPK1 controls MSG5 is via the modulation of protein-protein interactions. The other MAPK phosphatases, such as PTP2, PTP3, and SDP1, are also known to act on MPK1, and are required in a passive way to provide basal signal to the MAP kinase (Theodosiou and Ashworth 2002).

YPK1 and YPK2

YPK1 and *YPK2* were originally isolated as genes which share 44-46% identity with mammalian PKC isozymes throughout their putative catalytic domains (Chen, Lee et al. 1993). Double deletion mutant *ypk1 ypk2* is lethal. Expression of rat serum- and glucocorticoid-inducible kinase (SGK) can rescue the *ypk1 ypk2* mutant, but other AGC kinases, such as mouse protein kinase B (PKB) or rat p70 S6 kinase, can not suppress the lethality, suggesting that yeast YPK function similarly to mammalian SGK (Casamayor, Torrance et al. 1999).

YPK kinases are phosphorylated by TOR2 and PKH kinases (Roelants, Torrance et al. 2002; Kamada, Fujioka et al. 2005; Liu, Zhang et al. 2005). TOR2 directly phosphorylates the turn motif and the hydrophobic motif of YPK2, and regulates YPK2 kinase activity, which is required for regulation of actin organization(Kamada, Fujioka et al. 2005). PKH kinase, PKH1 and PKH2, are yeast orthologues of the mammalian

PDK1 protein kinase. PKH kinases phosphorylate the conserved residue Thr501 in YPK2 (Casamayor, Torrance et al. 1999), which is also required for endocytosis and actin organization (Friant, Lombardi et al. 2001; deHart, Schnell et al. 2002). PKH kinases are activated by sphingoid bases, and phosphorylate YPK kinases and PKC1 (Friant, Lombardi et al. 2001; Roelants, Torrance et al. 2002; Liu, Zhang et al. 2005).

Loss of YPK1 alone is sufficient to cause slow growth and a defect in the depolarization of the actin cytoskeleton (Chen, Zheng et al. 1995; Roelants, Torrance et al. 2002; Schmelzle, Helliwell et al. 2002). These defects are suppressed by overexpression of the Rho GEF TUS1, or expression of activated-PKC1 (Roelants, Torrance et al. 2002; Schmelzle, Helliwell et al. 2002). In addition, YPK deficient cells are defective in MAP kinase activation in response to heat shock, indicating that YPK proteins mediate actin cytoskeleton organization via the MPK1-MAP kinase cascade (Schmelzle, Helliwell et al. 2002). Another downstream target of YPK proteins is a type I myosin MYO5 (Grosshans, Grotsch et al. 2006). Deletion of *MYO5* has little effect on growth, but together with a deletion of *MYO3*, encoding the other type I myosin, severe defect in growth, actin cytoskeleton organization, and endocytosis are observed (Geli and Riezman 1996; Anderson, Boldogh et al. 1998; Jonsdottir and Li 2004). MYO5 interacts with YPK2, and its myosin motor domain is phosphorylated by YPK2 *in vitro*. In addition, a deletion of *MYO5* causes a synthetic α-factor uptake defect in a *YPK2* deletion mutant (Grosshans, Grotsch et al. 2006). MYO5 also interacts with PKH1 and PKH2, and a *MYO5* deletion mutant shows synthetic growth defect in a PKH deficient cells (Grosshans, Grotsch et al. 2006). These results suggest that YPK kinases and probably PKH kinases directly control MYO5 activity that modulates actin cytoskeleton and endocytosis.

In summary, YPK kinases act as an effecter of TORC2 and PKH kinases, and mediate actin cytoskeleton organization and endocytosis together with MPK1-MAP kinase cascade and the type I myosins.

SLM1 and SLM2

SLM1 and SLM2 were originally identified as interacting proteins of AVO2 by a global two-hybrid screening (Uetz, Giot et al. 2000). The screening has also provided an

interesting connection; SLM1 and SLM2 can also interact with AVO3 via BIT2. SLM1 and SLM2 are 53%indentical and functionally redundant proteins, but expression levels of SLM1 is about 10 times higher that those of SLM2 (Audhya, Loewith et al. 2004). Both proteins contain Pleckstrin homology (PH) domains, which are known for their ability to bind phosphoinositides and to drive membrane recruitment of their host proteins.

Disruption mutant of *SLM1* is synthetic lethal with *mss4*^{ts}, a temperature sensitive mutant of a single phosphoinositide kinase in yeast (Audhya, Loewith et al. 2004). This genetic interaction is interesting because overexpression of *MSS4* is a suppressor of *tor2*^{ts} (Helliwell, Howald et al. 1998). Double deletion mutant *slm1 slm2* is lethal. Lethality of the double deletion mutant can be suppressed by additional deletion of *SAC7*, a RHO1 guanine nucleotide activating protein, both at permissive and non-permissive temperature, suggesting that elevated RHO1-GTP levels are sufficient to bypass the requirement for SLM1 and SLM2 for cell viability (Audhya, Loewith et al. 2004). SLM temperature sensitive strains, *slm1*^{ts} *slm2*Δ, show actin depolarization and the depolarization of actin is suppressed by overexpression of PKC1 (Audhya, Loewith et al. 2004; Fadri, Daquinag et al. 2005). SLM1 and SLM2 are phosphorylated by TORC2 protein kinase activity *in vitro*, and phosphorylation of SLM proteins requires TORC2 protein kinase activity *in vivo*. In *tor2*^{ts} strain, SLM1 does not localize the cell periphery where SLM1 in the wild type localizes. Thus these data suggest that SLM1 and SLM2 act on downstream of TORC2 in signaling to the actin cytoskeleton, and represent the first substrates of TORC2 protein kinase activity (Audhya, Loewith et al. 2004).

Recently, it was proposed that SLM1 and SLM2 are substrates of the calcineurin phosphatase required for heat stress-induced endocytosis of the yeast uracil permease FUR1 (Bultynck, Heath et al. 2006). SLM1 and SLM2 lacking the calcineurin recognition site is unable to be dephosphorylated, and the phosphorylated SLM proteins accumulate in time dependent manner upon heat stress. This phosphorylation is also observed in *tor2*^{ts} mutant, suggesting that phosphorylation of SLM proteins upon heat stress are carried out by other kinases. During heat shock, YPK and PKH kinases are activated by sphingolipid intermediates phytosphingosine (Friant, Lombardi et al. 2001).

Inhibition of whole sphingolipid biosynthesis by antibiotic myriocin demonstrates less phosphorylation of SLM proteins, indicating that certain kinases activated phytosphingosine are required for phosphorylation of SLM proteins (Bultynck, Heath et al. 2006).

Taken together, SLM proteins could play a role in an effecter of TORC2 signaling, phosphoinositide signaling, and stress response cascade, and mediates actin cytoskeleton organization.

TORC2 readouts

Actin cytoskeleton organization

The actin cytoskeleton organization is a well-established readout of TORC2. In *Saccharomyces cerevisiae*, there are three distinct structures of actin, actin cables, an actinmyosin contractile ring, and cortical patches (Adams and Pringle 1984; Pruyne and Bretscher 2000). Actin cables, composed of actin (ACT1), fimbrin (SAC6), and tropomyosin (TPM1, TPM2), is organized in parallel towards the incipient and growing bud (Pruyne and Bretscher 2000). The cables serve as tracks for myosin V–based transport system that delivers vesicles, organelles and mRNA to the bud. The actin-myosin contractile ring transiently forms at the mother-daughter neck and is important for cytokinesis. Actin patches are associated with invaginations of the plasma membrane and are composed of filamentous actin and many other proteins (Mulholland, Preuss et al. 1994; Pruyne and Bretscher 2000). These structures appear at the cortex with a short lifetime (5 to 20 sec) (Smith, Swamy et al. 2001; Kaksonen, Sun et al. 2003) and are highly motile (Doyle and Botstein 1996; Waddle, Karpova et al. 1996; Carlsson, Shah et al. 2002; Kaksonen, Sun et al. 2003). The actin cytoskeleton carries out reconstruction and provides mechanical strength in response to changes in the external environment, and is also implicated in various other cellular processes (Pruyne and Bretscher 2000; Pruyne and Bretscher 2000). In addition, both cables and patches change their arrangement in a cell cycle-dependent manner. The cortical patches, for instance, localize to the cell periphery during the S phase, and to the small and growing bud during the G_2/M phase (Casamayor and Snyder 2002). The functions of actin patches are not clearly defined, but they are likely to act on part of endocytic

events.

Endocytosis

Several reports have argued that TOR is involved in endocytosis (deHart, Schnell et al. 2003; Schmelzle, Beck et al. 2004). Endocytosis plays a key role not only in uptake of nutrients and pathogens from extracellular environment, but also in control of the protein and lipid composition of the plasma membrane and regulation of signaling pathways. Endocytosis requires remodeling of the cell cortex during its internalization, i.e. invagination of the plasma membrane induced by endocytic coat proteins. In addition, actin cytoskeleton organization, especially actin cortical patches, plays a critical role in endocytic internalization (Engqvist-Goldstein and Drubin 2003). It can be suspected that TOR has a linkage to endocytosis via actin cytoskeleton control.

Indeed, Hicke and colleagues isolated a TOR2 mutant *tor2*^{G2128R} which has defect in endocytosis. α-factor internalization in *tor2*^{G2128R} cells was significantly delayed compared with TOR2 wild type cells (deHart, Schnell et al. 2002), and Lucifer yellow uptake to the vacuole in the mutant was not be observed (deHart, Schnell et al. 2003). In the mutant cells, actin was frequently depolarized, with actin patches seen throughout the mother cell, instead of polarized towards the bud.

Consisted with *tor2*^{G2128R} results, disruption of *rom2*, a downstream target of TOR2, also decreased efficiency of α-factor internalization (deHart, Schnell et al. 2003). A mutant of *ypk1*, another down stream target of TOR2 (Kamada, Fujioka et al. 2005), was also identified as defective in α-factor internalization through ROM2-RHO1 (deHart, Schnell et al. 2002; deHart, Schnell et al. 2003), suggesting that YPK1 is required for receptor internalization. All of the findings described above indicate that TOR2 could be involved in endocytosis in yeast.

TORC2 in other species

Currently, mammalian TORC2 (mTORC2) and *Dictyostelium* TORC2 are only identified. However, *Drosophila*, *Candida Sp*., A*shbya gossypii, Schizosaccharomyces pombe* have a putative homolog of *AVO3*. There is possibility that these species posses a functionally and structurally equivalent complex to TORC2. AVO3 homolog is not found yet in plants and *C. elegance*.

Mammalian

Mammalian TOR complex 2 (mTORC2) is proposed to mediate actin cytoskeleton organization like the yeast TORC2. mTORC2 contains mTOR, rictor (also known as mAVO3), and mLST8, orthologs of the yeast TOR2, AVO3, and LST8, respectably (Jacinto, Loewith et al. 2004; Sarbassov, Ali et al. 2004). mTORC2 associates and phosphorylates Akt/PKB on Ser473 within the carboxyl-terminal hydrophobic motif (Hresko and Mueckler 2005; Sarbassov, Guertin et al. 2005). Function and regulation mechanism of mTORC2 are not clearly defined yet.

Dictyostelium discoideum

Dictyostelium discoideum, a soil-living amoeba, has unique advantages for studying the molecular mechanisms underlying cytokinasis and chemotaxis. Pianissimo, the *D. discoideum* homolog of AVO3, encodes a cytosolic regulator of receptor- and G protein-mediated activation of adenylate cyclase ACA (Chen, Long et al. 1997). The AVO1 homolog RIP3 (Ras-interacting protein) was also isolated as a component of the Ras regulatory network, that is required for signal relay and proper chemotaxis (Lee, Parent et al. 1999). Unlike the counterparts in yeast, both genes in *D. discoideum* are not essential for growth. However, disruption of each gene eliminates ACA activity in response to an exogenous cAMP signal, and impairs the ability for chemotaxis (Chen, Long et al. 1997; Lee, Parent et al. 1999). Recently, mass spec analysis and co-immunoprecipitations revealed that Pianissimo and RIP3 interact with the *Dictiosterium* homolog of TOR and LST8, indicating that TORC2 is conserved in *D. discoideum* (Lee, Comer et al. 2005; Sasaki and Firtel 2006). Dd-LST8 disruption mutant is defective in ACA activation and fails in chemotaxis, similar to either *pia* and *rip3* mutants (Lee, Comer et al. 2005; Sasaki and Firtel 2006). Taken together, these data indicates that TORC2 in *D. discoideum* positively regulates ACA activity in response to exogenous cAMP signals, and mediates cytokinasis and chemotaxis. Although the finding that TORC2 might be involved in RAS-cAMP signaling pathway is not confirmed yet in the other organisms, remodeling actin cytoskeleton and maintaining polarity in *Dictyostelium* represent a common function of TORC2 which is found in other organisms.

Sphingolipids as a membrane component and signaling molecule

Sphingolipids are essential components of cellular membranes in eukaryotic cells. First described by Johann L.W. Thudichum in 1884 as an enigmatic compound found in brain tissue (Thudichum 1884), sphingolipids are now recognized as a building block of the cellular membrane, a signal molecule of intracellular signal transduction, and a substance of several disorders.

Sphingolipid structure

All sphingolipids contain a ceramide which consists of a long-chain sphingoid base (in general, sphingosine, sphinganine, or phytosphingosine, see below) and a fatty acid molecule attached by an amide bond to carbon 2 in the sphingoid base.

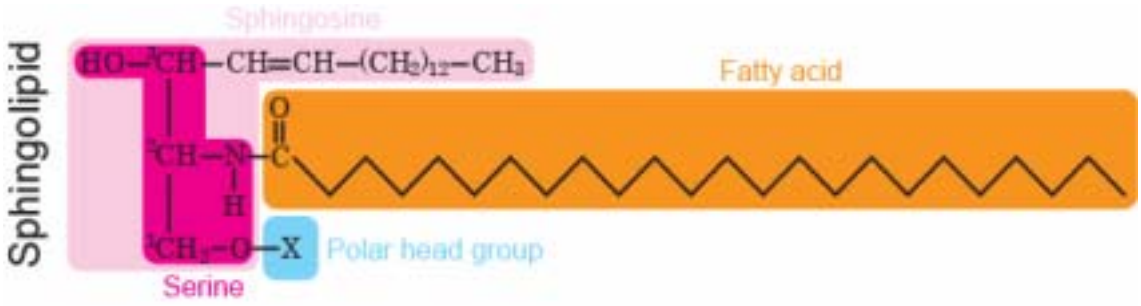


Figure I-2. Molecular structure of sphingolipid

The structure of sphingolipids contains sphingoid base (shown as sphingosine in this figure) that is linked to a fatty acid molecule through an amide bond, thereby forming the ceramide unit. Addition at the polar head group provides property of the sphingolipids. In yeast, inostolphosphate, mannosyl inositolphsphate, or mannosyl diinositolphophatephosphocholine to ceramide leads to inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), and mannosyldiinositol phosphorylceramide ($M(IP)_2C$). In mammal, phosphocholine or carbohydrates to ceramide, for example, leads to sphingomyelin or glycosphingolipids, respectively

The sphingoid base normally consists of 18- or 20- carbon alkyl chain, in which two atoms from L-serine and the others from fatty acyl CoA, however the yeast serine palmitoyltransferase, the first enzyme of sphingolipid biosynthesis, can use C12, and C14 fatty acyl CoA as a substrate as well (Pinto, Wells et al. 1992). The sphingoid base often has a modification with either a hydroxyl group at position 4 or a *trans*-double bond at carbon 4 and 5 (note that sphingoid base with the *trans*-double bond is called sphingosine, whereas sphingoid base without the *trans*-double bond is called sphinganine). The length of the fatty acid is from 14 to 30, probably providing some physical characterizations that enable sphingolipids to form 'lipid rafts'.

Sphingolipids have a polar head group which provides unique properties to the lipids. Distinguished by the polar head group, yeast have three major complex sphingolipids (Dickson and Lester 2002): inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), and mannosyldiinositol phosphorylceramide ($M(IP)_2C$).

In mammals sphingolipids can also be categorized by the polar head group into three classes; ceramide, sphingomyelin, and glycosylceramide, although there are huge structural variations amongst head groups (Futerman and Hannun 2004).

Sphingolipid biosynthesis in yeast

The sphingolipid biosynthesis in yeast consists of several catalytic steps in the ER and Golgi. The first reaction of the synthesis, condensation of L-serine and palmitoyl-CoA, is known as a rate-limiting step of the reaction chain, and the final product is mannosyldiinositol phosphorylceramide ($M(IP)_2C$).

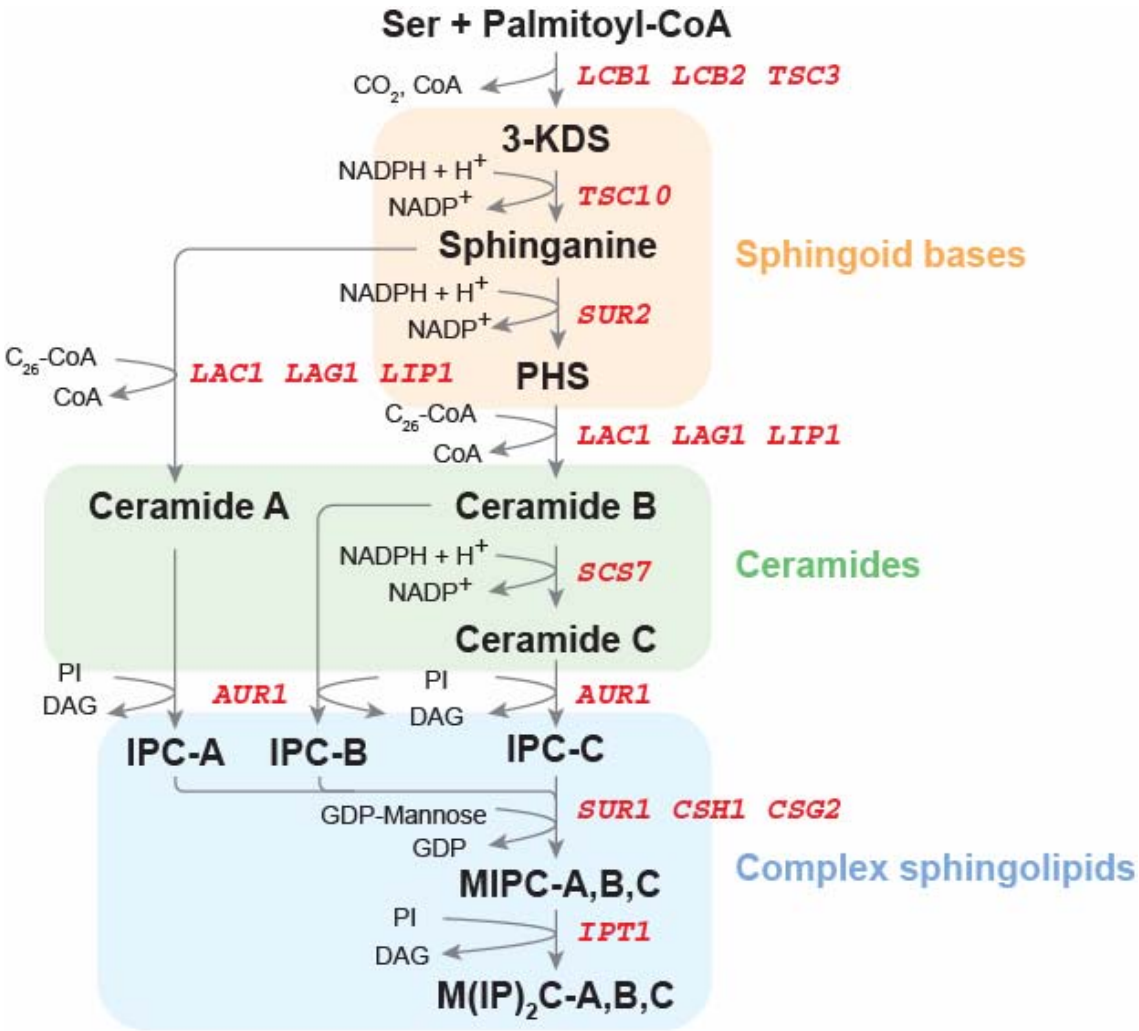


Figure I-3. Schematic overview of the yeast sphingolipid biosynthetic pathway

To avoid confusion, complex sphingolipids B'and D species are not illustrated here. The B'species are hydoxylation form of ceramide A catalyzed by SCS7. The D species are the C species with hydroxylated long chain fatty acid.

3-KDS 3-ketodihydrosphingosine, PHS phytosphingosine, IPC inositol phosphorylceramide, MIPC mannose inositol phosphorylceramide, M(IP)2C mannose di-inositol phosphorylceramide PI phosphatidylinositol, DAG diacylglycerol

Serine palmitoyltransferase

The first step of sphingolipid biosynthesis in *S. cerevisiae* as well as mammals involves condensation of L-serine and palmitoyl-CoA by the serine palmitoyltransferase, a

rate-limiting enzyme of the *de novo* sphingolipid biosynthesis. The product of this reaction is 3-ketodihydroshingosine (3-KDS) (Pinto, Wells et al. 1992; Nagiec, Baltisberger et al. 1994). The enzymatic activity of the serine palmitoyltransferase depends *in vitro* on supply of its substrates, serine and palmitoyl-CoA, but not on its products either 3-KDS or sphinganines, suggesting that either feedback inhibition or repression of enzyme synthesis could be very mild (Pinto, Wells et al. 1992). The serine palmitoyltransferase is supposed to be a heterotrimer composed of the two homologous units LCB1 and LCB2, and a small protein TSC3 (Gable, Slife et al. 2000). LCB1 and LCB2 are required for SPT activity in all eukaryotes (Hanada 2003). Sequence analysis reveals that both proteins have at least one potentail transmembrane domain at their N-terminus and an aminotransferase motif around 320 aa. Disruption of either of these genes cause lethality, which can be suppressed by exogenous sphingoid bases (Pinto, Wells et al. 1992). TSC3, an 80 amino acid protein, was identified as a component of the serine palmitoyltransferase complex that optimizes the serine palmitoyltransferase activity upon heat stress (Gable, Slife et al. 2000). So far, TSC3 orthologues are found only in *S. cerevisiae*. Disruption of *TSC3* induces a heat sensitive phenotype, indicating that sphingolipids are required for heat stress response. TSC3 may bind and deliver palmitoyl-CoA to the SPT, thus increasing its activity (Gable, Slife et al. 2000; Monaghan, Gable et al. 2002).

The serine palmitoyltransferase activity is inhibited by myriocin/ISP-1, initially isolated as an antibiotic and immunosuppressant from *Myriococcum albomyces, Isaria sinclairi,* and *Mycelia sterilia* (Miyake, Kozutsumi et al. 1995). The inhibitory effect of myriocin on the serine palmitoyltransferase might be caused by the structure of the molecule which is an analogue of its product sphingosine. Consisted with this, myriocin-containing affinity chromatography carried out a screening of myriocin binding protein and isolated LCB1 and LCB2 (Chen, Lane et al. 1999). The serine palmitoyltransferase localizes at the endoplasmic reticulum confirmed by GFP fusions with LCB1 and LCB2 (Habeler, Natter et al. 2002; Jockusch, Voigt et al. 2003; Han, Gable et al. 2004), therefore the reaction of the serine palmitoyltransferase would occur in the ER

3-keto reductase

After the initial condensation of L-serine and palmitoyl-CoA into 3-KDS, 3-KDS is immediately reduced using NADPH by the 3-keto reductase TSC10 into sphinganine, also called dihydrosphingosine (DHS) (Pinto, Srinivasan et al. 1992; Beeler, Bacikova et al. 1998). This reaction also occurs in the ER. Deletion of *TSC10* makes yeast auxotroph to sphinganine or its hydroxylation form sphingosine (Beeler, Bacikova et al. 1998).

Enzymes that modify sphingoid bases

Sphinganine can be modified in three ways. The first way is hydroxylation at the C-4 position by SUR2, producing phytosphingosine (PHS, also called 4-hydroxyshinganine) (Grilley, Stock et al. 1998). The second way is phosphorylation at C-1 position of the sphingosine backbone by sphingoid base kinase LCB4 and LCB5 providing sphinganine-1-phosphate (DHS-1P) (Nagiec, Skrzypek et al. 1998). The third way is condensation with a very long chain fatty acid by ceramide synthase converting into sphinganine-derived ceramide (Guillas, Kirchman et al. 2001; Schorling, Vallee et al. 2001; Vallee and Riezman 2005).

Phytosphingosine is the primary sphingoid base in yeast (Grilley, Stock et al. 1998). This intermediate of sphingolipid has unique property that 20 μM of exogenous phytosphingosine inhibits cell growth, while structurally similar or metabolically related molecules including 3-KDS, sphinganine, C2-phytoceramide, and stearylamine did not affect cell growth (Chung, Mao et al. 2001). Also, phytosphingosine inhibit uptake of uracil, tryptophan, leucine, and histidine (Chung, Mao et al. 2001).

Phytosphingosine is a product of hydroxylation of sphinganine catalyzed by SUR2, with NADPH as electron donor (Grilley, Stock et al. 1998). SUR2 is an integral membrane protein localizing in the ER (Habeler, Natter et al. 2002; Jockusch, Voigt et al. 2003). SUR2 is also able to hydroxylate sphinganine-derived ceramide into phytosphingosine-derived ceramide (Grilley and Takemoto 2000). As expected, disruption of *SUR2* induces accumulation of IPC-A and IPC-B' (Haak, Gable et al. 1997; Grilley, Stock et al. 1998), which are sphinganine derivate sphingolipids without hydroxylation at C4 position, and also did influence cellular phospholipid levels

(Cliften, Wang et al. 1996). However, disruption of *SUR2* does not affect cell viability. Together with the fact that sphinganine-derived ceramide and its derivatives are rarely found in the wild type strain W303 (Grilley, Stock et al. 1998), even though phytosphingosine is the primary sphingoid base, there is redundancy between phytosphingosine and sphinganine, and even between phospholipids (Cliften, Wang et al. 1996).

Sphinganine-1-phosphate and phytosphingosine-1-phosphate (S1P) are bioactive lipid metabolites that have been implicated in many biological processes (Chalfant and Spiegel 2005). One example of function of S1P is cell growth and cell death. Abnormal accumulation of S1P induces cell death. Double disruption of *dpl1 ysr2*, both encode proteins in degradation of S1P, were inviable, but additional disruption of *lcb4*, responsible for about 97% of total phosphorylation activity of S1P (Nagiec, Skrzypek et al. 1998), can suppress the phenotype (Kim, Fyrst et al. 2000; Zhang, Skrzypek et al. 2001). Furthermore, overexpression of either LCB4 or LCB5 in the triple mutant *dpl1 ysr2 lcb4* induced growth inhibition, suggesting that S1P accumulation can inhibit cell growth. LCB4 was localized to the ER whereas LCB5 was found to co-fractionate with Golgi membranes (Funato, Lombardi et al. 2003).

In mammals, function of S1P is much investigated than yeast. S1P controls numerous aspects of cell physiology, including cell survival and mammalian inflammatory responses (Spiegel and Milstien 2003; Chalfant and Spiegel 2005). Mammalian can sense extracellular S1P as a signal through its interaction with a family of five specific G-protein-coupled receptors (GPCRs), S1P1 to S1P5 (Spiegel and Milstien 2003; Goetzl and Rosen 2004). Furthermore, similarly to other potent lipid mediator, S1P also has intracellular function independent of these receptors (Spiegel and Milstien 2003).

Ceramide synthase

As mentioned above, hydroxylation of sphinganine produces phytosphingosine. In the ER, phytosphingosine is further modified to ceramide by the ceramide synthase. Two types of ceramide synthases are known. One is a minor acyl-CoA-independent ceramide synthase encoded by *YPC1* and *YDC1* that are able to catalyze both synthesis

and breakdown of ceramide (Mao, Xu et al. 2000; Mao, Xu et al. 2000). The other is the acyl-CoA-dependent ceramide synthase, that is composed of LAG1, LAC1, and LIP1 (Guillas, Kirchman et al. 2001; Schorling, Vallee et al. 2001; Vallee and Riezman 2005). LAG1 and LAC1 that share 87.3% similarity at the amino acid level are functionally equivalent. A double deletion mutant of *lag1 lac1* has reduced sphingolipid levels because of less activity of the acyl-CoA-dependent ceramide synthase (Guillas, Kirchman et al. 2001; Schorling, Vallee et al. 2001). Double mutation is lethal or results in very slow growth, probably due to accumulation of phytosphingosine and S1P (Schorling, Vallee et al. 2001).

IPC synthase

IPC is synthesized by a ceramide phosphoinositol transferase, also called IPC synthase. This synthase are encoded on *AUR1* named after aureobasidin A resistance 1. *AUR1* was identified as a gene whose mutation provides resistance for antifungal drug aureobasidin A (LY295337) (Heidler and Radding 1995). It consists of 401 amino acids and contains a PAP2 domain, which is found in the enzyme type 2 phosphatidic acid phosphatases. Subsequent studies revealed that disruption mutants of *AUR1* fail to synthesize complex sphingolipid species and accumulates ceramide (Nagiec, Nagiec et al. 1997), indicating that *AUR1* encodes an IPC synthase. AUR1 localizes primarily in the Golgi and not in the ER (Levine, Wiggins et al. 2000). Taken together with the fact that IPC localizes at the Golgi fraction and that ceramide exists in the ER fraction, AUR1 converts ceramide to IPC probably in the Golgi (Funato and Riezman 2001). Deletion of *AUR1* induces microtubule disassembly and chitin delocalization, a similar phenotype to aureobasidin A treatment (Hashida-Okado, Ogawa et al. 1996). The mechanism that causes this phenotype is unclear, but the findings suggest a connection between sphingolipids biogenesis and cell morphology.

MIPC synthase

MIPC is a mannosylated form of IPC. *SUR1*, encoding MIPC synthase subunit, has been originally identified as the suppressor mutant of *rvs161*, whose phenotypes are reduced viability upon starvation and sensitivity to several drugs with non-related structure (Desfarges, Durrens et al. 1993). Later on, the gene was independently isolated as *CSG1*, by screening for growth phenotypes in the presence of 100 mM

calcium (Fu, Beeler et al. 1994). Disruption mutant of *sur1/csg1* fails to accumulate glycogen upon stationary phase entry, and has dumbbell-like morphology in stationary phase (Desfarges, Durrens et al. 1993). It was therefore speculated that *SUR1* might play a role in maintenance of the cell membrane. Indeed, *SUR1* mutant cells show an overall decrease of the phospholipid amounts and modifications in the relative contents of some phospholipid classes (Desfarges, Durrens et al. 1993). Consequential studies revealed that *sur1* mutants have reduced MIPC and $M(IP)_2C$ amounts, and accumulate IPC species, suggesting that *SUR1* is required for IPC monnosylation (Beeler, Fu et al. 1997). Amino acids 56 – 148 in SUR1, located between transmembrane domain I and II, are homologous to the catalytic domain of the OCH1 and HOC1-encoded α-1, 6-mannosyltransferase, supporting the idea that SUR1 is a mannosyltransferase of IPC (Beeler, Fu et al. 1997). Recently, CSH1 has been identified as a mannosyltransferase component which has redundant functions of SUR1 (Uemura, Kihara et al. 2003; Lisman, Pomorski et al. 2004). CSH1 is highly homologous to SUR1 (71% similarity and 65% identity) and CSH1 protein sequence displays similarity to the sequence in the mannosyltransferases as well as SUR1. Single deletion mutants of either *CSH1* or *SUR1* can synthesize MIPC and $M(IP)_2C$, however a double deletion of *CSH1* and *SUR1* completely blocks MIPC and $M(IP)_2C$ synthesis (Uemura, Kihara et al. 2003; Lisman, Pomorski et al. 2004), indicating that these proteins have redundant functions as mannosyltransferases of IPC. A further study of SUR1 and CSH1 has demonstrated that these two proteins have different substrate specificity (Uemura, Kihara et al. 2003).

The other subunit of MIPC synthase is CSG2. *CSG2* was isolated as a calcium sensitive mutant but not strontium sensitive, indicating that it does not have defects in the vacuolar transport system, which would cause both calcium and strontium sensitivity (Beeler, Gable et al. 1994). *CSG2* encodes a putative membrane protein with nine transmembrane domains. Amino acid 95-106 within the first cytoplasmic loop of CSG are homologous to a consensus Ca^{2+}-binding loop of the EF-hand. Although the growth rate of *csg2* null mutants is similar to wild type in the absence of calcium, concentrations higher than 10 mM calcium decreases the growth rate of *csg2* at 26°C. In the presence of calcium, *csg2* mutant accumulate Ca^{2+}, probably in a nonvacuolar organelle (Beeler, Gable et al. 1994). Disruption mutants of *csg2* fail to

synthesize MIPC, but since CSG2 has no homologous sequence to mannosyltransferase reactive site, function of CSG2 is speculated to be regulatory subunit of MIPC synthase (Zhao, Beeler et al. 1994; Beeler, Fu et al. 1997; Uemura, Kihara et al. 2003; Lisman, Pomorski et al. 2004). Physical interaction between CSG2 and both CSH1 or SUR1 can support this hypothesis (Uemura, Kihara et al. 2003).

VRG4 that encodes a GDP-mannose transporter is also required for monnosylation of IPC, since VRG4 delivers GDP-mannose from the cytosol to lumen of the Golgi (Dean, Zhang et al. 1997).

$M(IP)_2C$ synthase

A further modification of MIPC, addition of an inositol phosphate to the head group of MIPC, takes place at the Golgi. This modification that produces mannose di-inositolphosphotidyl ceramide [$M(IP)_2C$] is performed by insitolphosphotransferase 1 (IPT1). *IPT1* was identified by sequence comparison as the only putative homolog of the *AUR1* in the *S. cerevisiae* genome sequence data base (Dickson, Nagiec et al. 1997). Mutants baring disruption of IPT1 fails to produce $M(IP)_2C$, and accumulates its precursor MIPC, indicating that IPT1 is indeed the $M(IP)_2C$ synthase. Although $M(IP)_2C$ is the most abundant sphingolipid found in the plasma membrane, an IPT1 disruptant did not show any growth defect either in rich media or several stress conditions including heat shock (52°C), high salt stress (0.75 M NaCl), and low pH (pH4.1), indicating that $M(IP)_2C$ is not really necessary for responding to these stresses (Dickson, Nagiec et al. 1997). Recent studies provided some interesting findings toward elucidation of $M(IP)_2C$ function. Disruption of *IPT1* confers tolerance to exogenous zymocin, a trimeric protein toxin complex which inhibits cell growth (Zink, Mehlgarten et al. 2005). In contrast, overexpression of IPT1 enhances zymocin sensitivity. Since exogenous zymocin has to be taken up to perform its toxicity, it is speculated that $M(IP)_2C$ participates in endocytosis (Zink, Mehlgarten et al. 2005).

Sphingolipid catabolism in yeast

As many other degradation mechanisms, study of sphingolipid catabolism in yeast is less focused than its anabolism. On other hand, sphingomyelinases in mammals, which hydrolyze the phosphodiester linkage of sphingomyelin to produce ceramide and

phosphorylcholine, are well-studied because the enzyme acts as key regulators of the intracellular levels of ceramide (Smith and Merrill 2002) and apoptosis and cell differentiation and cell proliferation (Claus, Russwurm et al. 2000; Gulbins and Li 2006). In *S. cerevisiae*, at least four classes of sphingolipid degradation enzymes have been identified which depredate sphingolipid and its derivatives.

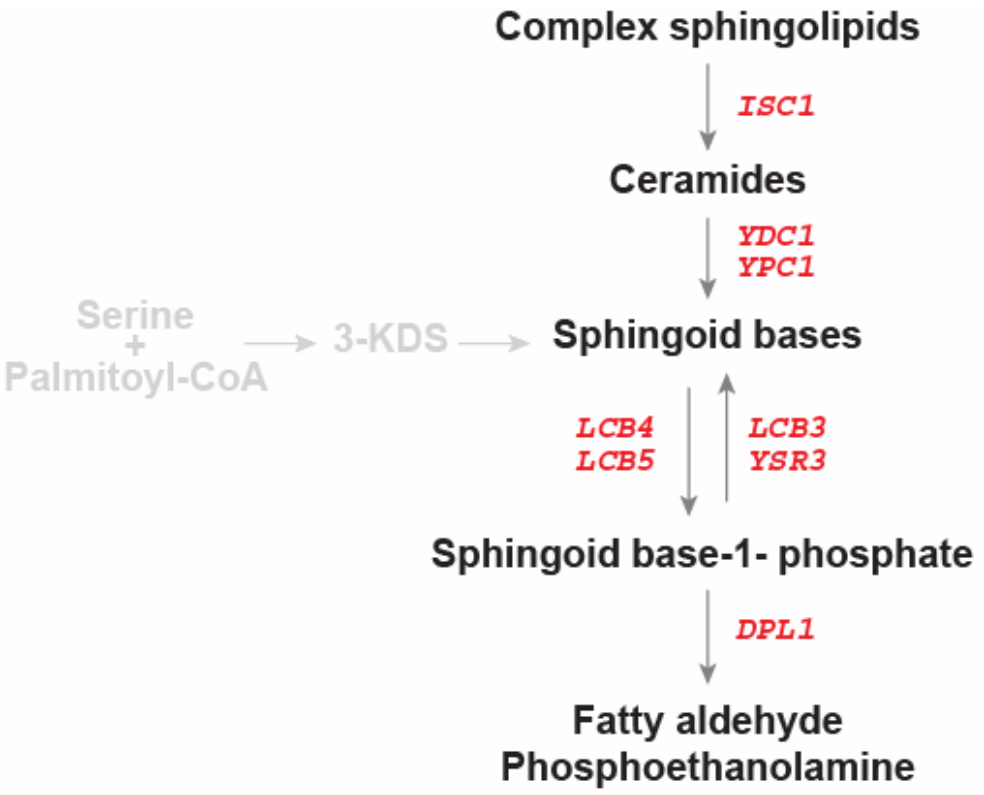


Figure I-4. the yeast sphingolipid catabolism

See text for detail.

Inositol phosphosphingolipid phospholipase C

Yeast Inositol phosphosphingolipid phospholipase ISC1 has 30% identity to neutral

sphingomyelinase of mammals, and shares several common features including hydrolytic activity, the requirement of Mg^{2+} for optimal activity, optimal neutral pH, and the conserved P-loop-like domain found in the entire family of sphingomyelinases (Sawai, Okamoto et al. 2000). ISC1 can hydrolyze phosphoceramides, i.e. all complex sphingolipids, to generate ceramide. Deletion strain of *ISC1* is loss of sphogolipase activity, suggesting that ISC1 is the only shingolipase in yeast. Regulation of ISC1 activity *in vivo* is supposed to depend on mitochondrial lipid phosphatidylglycerol and cardiolipin (Vaena de Avalos, Okamoto et al. 2004; Vaena de Avalos, Su et al. 2005).

Ceramidase

Two ceramidase are identified in *S. cerevisiae*. YPC1 preferentially hydrolyzes phytoceramide (Mao, Xu et al. 2000), whereas YDC1 hydrolyzes dihydroceramide preferentially and phytoceramide only slightly (Mao, Xu et al. 2000). Deletion of *YPC* and *YDP1* or both did not apparently affect growth, suggesting neither gene is essential (Mao, Xu et al. 2000). However, the *ydc1* deletion mutant, but not the *ypc1* deletion mutant, is sensitive to heat stress, supporting the idea that the two enzymes have distinct physiological functions (Mao, Xu et al. 2000).

Sphingoid base phosphate phosphatase

Sphingoid base phosphate phosphatase LCB3/LBP1/YSR2 was separately identified as a mutant that confers resistance to sphingosine (Mandala, Thornton et al. 1998), and that accumulates phosphorylated sphingoid bases (Mao, Wadleigh et al. 1997). The deletion of *LCB3* results in the accumulation of phosphorylated sphingoid bases, reduced ceramide levels, and fails to incorporate exogenous dihydrosphingosine into sphingolipids (Mao, Wadleigh et al. 1997; Mandala, Thornton et al. 1998). The homolog of *LCB3*, *YSR3/LBP2* also encodes sphingoid base phosphate phosphatase that specifically dephosphorylates sphinganine 1-phosphate (Mao, Wadleigh et al. 1997).

Sphingoid base phosphate lyase

In addition, yeast express sphingoid base phosphate lyase encoded by *DPL1*, which cleaves the substrates to yield ethanolamine-1-phosphate and hexadecanal (Saba, Nara et al. 1997). Disruption mutants of *DPL1* accumulate sphingoid base phosphates (Kim, Fyrst et al. 2000) at slightly elevated levels (Skrzypek, Nagiec et al. 1999; Kim, Fyrst et

al. 2000). This effect is further enhanced in *dpl1 lcb3* double mutants with 500 times greater levels of sphingoid base phosphate relative to wild type (Skrzypek, Nagiec et al. 1999; Kim, Fyrst et al. 2000). Although the functions of sphingoid base phosphate in yeast are largely unknown, recent evidence suggests a role for these lipids in resistance to heat stress, in the shift from fermentative to respiratory growth, and in messengers of calcium signaling (Skrzypek, Nagiec et al. 1999; Kim, Fyrst et al. 2000; Birchwood, Saba et al. 2001).

Sphingolipid function in yeast

As a building blocks of membranes

Sphingolipids are components of cellular membranes. Cellular membranes are composed of a mixture of lipids and proteins, as suggested by the fluid mosaic model proposed by Singer and Nicolson back in 1972 (Singer and Nicolson 1972). Studies on membranes conducted in the past 24 years draw a much more detailed picture of the membrane structures (Engelman 2005), revealing that membranes are typically very crowded with proteins, and that their bilayers vary considerably in thickness. Together with phospholipids, sphingolipids form the lipid bilayers which distinguish the intracellular environment from the extracellular environment. The lipid composition of a typical plasma membrane in *S. cerevisiae* contains 30% sphingolipid species and 70% phospholipid species in weight (Patton and Lester 1991). As both lipid species have similar molecular weight and mass, sphingolipids are speculated to occupy 30% of the lipid surface area of the cell membrane.

It is known that sphingolipids can aggregate with themselves in cellular membranes and, with sterol-backbone lipids, form separate domains which are less fluid than the bulk phospholipids. These sphingolipid-based microdomains, or "lipid rafts", offer active spots where protein-protein interactions are enhanced, and where signal transduction, lipid and protein trafficking, and enzymatic reactions are accelerated (Simons and Ikonen 1997; Edidin 2003; Munro 2003; Simons and Vaz 2004).

As signaling molecules

Sphingolipids are known to function as signaling molecules that regulate many cellular processes, including cell growth, endocytosis, and stress response.

Cell growth

Sphingolipids are essential for cell growth and survival, since yeast strains defective in sphingolipid biosynthesis are non-viable, unless exogenous sphingoid bases are supplied. Disruption of a subunit of serine palmitoyltransferase, either *lcb1* or *lcb2*, is lethal (Dickson, Wells et al. 1990). TSC10, the 3-ketosphinganine reductase responsible to the next reaction of serine palmitoyltransferase, is also essential for growth (Beeler, Bacikova et al. 1998). Double disruption of *lac1* and *lag1*, components of ceramide synthase, shows very slow growth (Barz and Walter 1999; Schorling, Vallee et al. 2001), and single deletion of the other component of ceramide synthase *lip1* shows very slow growth as well as the double disruption *lac1 lag1* (Vallee and Riezman 2005). Consistent with the deletion mutants, inhibition of the synthase activity by the specific inhibitor of ceramide synthase Fumonisin A leads to a severe growth defect (Wu, McDonough et al. 1995), indicating ceramide are required for effective vegetative growth. IPC synthase encoded by *AUR1* is also an essential for growth, and an inhibitor of AUR1, aureobasidin A, blocks cell growth (Heidler and Radding 1995; Hashida-Okado, Ogawa et al. 1996; Nagiec, Nagiec et al. 1997; Hashida-Okado, Yasumoto et al. 1998). While the sphingolipids mentioned above are required for cell growth, MIPC and M(IP)2C, the end products of the sphingolipids biosynthesis pathway are not essential for growth. Inactivation of either MIPC synthase by *csg2* deletion (Beeler, Gable et al. 1994) or M(IP)2C synthase by *ipt1* deletion did not significantly slow cell growth under good growth conditions (Dickson, Nagiec et al. 1997). These findings suggest that sphingolipids are important molecules for cell growth, especially sphingoid bases, ceramide, and IPC, which are critical.

How do sphingolipids contribute cell growth? The complete explanation is still unknown, but the findings described above support the existence of other functions for sphingolipids except as building blocks of cellular membranes. Another function of sphingolipids is as signaling molecules, which response to environmental changes.

One crucial study with the serine palmitoyltransferase inhibitor myriocin/ISP-1 has given a clue. Myriocin inhibits serine palmitoyltransferase activity, thereby blocking cell growth. A multi-copy suppressor screening with the drug identified *YPK1*, a gene encoding a serine/threonine kinase (Sun, Taniguchi et al. 2000). A functional homolog

of YPK1 is a mammalian protein kinase SGK, which is phosphorylated by PDK1, a downstream kinase of phosphatidylinositol 3-kinase (PI3K), suggesting that sphingolipids participate in a signal transduction pathway.

In mammalian cells, the PDK1-PKB signaling cascade downstream of PI3K mediates the physiological effects of insulin and other growth factors. PDK1 can phosphorylate a conserved residue within the activation loop of the AGC subfamily of protein kinases, containing PKB, PKC isozymes, p70 S6 kinase, and SGK (Vanhaesebroeck and Alessi 2000; Biondi 2004; Long, Muller et al. 2004; Mora, Komander et al. 2004). Together with PKB, SGK mediates the readouts of PI3K signaling, such as cell survival and cell cycle progression (Vanhaesebroeck and Alessi 2000; Mora, Komander et al. 2004).

Kinase dead mutants of YPK1 fail to suppress the growth defect upon myriocin treatment, suggesting that the kinase activity of the YPK1 is essential for the suppression (Sun, Taniguchi et al. 2000). YPK1 is a phospho-protein and the phosphorylation of YPK1 is decreased upon myriocin-induced sphingolipid depletion. Addition of exogenous sphinganine to myriocin-treated cells suppresses the reduction in the phosphorylation of YPK1, indicating that intercellular sphingolipids mediates the phosphorylation of YPK1 (Sun, Taniguchi et al. 2000).

Which protein(s) can phosphorylate YPK1? It has been shown previously that PKH1 can phosphorylate Thr504 in YPK1 (Casamayor, Torrance et al. 1999). *PKH1* and its homolog *PKH2* encode protein kinases with catalytic domains closely related to those of human and *Drosophila* PDK1 (Casamayor, Torrance et al. 1999). Similar to YPK1, overexpression of PKH1 suppresses the myriocin-induced cell growth defect (Sun, Taniguchi et al. 2000), suggesting that sphingolipids can act as a signaling molecules and upregulate YPK1 kinase activity via PKH. Indeed, nanomolar concentrations of sphingoid base increase PKH1 and PKH2 kinase activity *in vitro* (Friant, Lombardi et al. 2001), indicating that PKH could be a link between the lipid molecules and a signaling cascade consisted by kinases. PKH1 and PKH2 also phosphorylate PKC1 *in vitro* (Friant, Lombardi et al. 2001). PKC1 in yeast, the only yeast homolog of the mammalian protein kinase C (Levin, Fields et al. 1990), regulates MPK1-MAP kinase cascade and mediates the cell wall synthesis and actin cytoskeleton organization. In

summary, sphingoid base, one of the intermediate of sphingolipids metabolites, plays a key role as a signaling molecule which activate PKH kinases and its substrates YPKs and PKC1 (Casamayor, Torrance et al. 1999; Friant, Lombardi et al. 2001; Schmelzle, Helliwell et al. 2002).

Endocytosis

The initial observation that sphingolipids mediate endocytosis came up with a genetic screening for endocytosis mutants (Munn and Riezman 1994); one of those mutants was allelic to the *LCB1* (Sutterlin, Doering et al. 1997). The endocytic defect of the *lcb1* mutant, named *lcb1-100*, could be overcome by exogenous sphingoid base (Zanolari, Friant et al. 2000). Even when endogenous sphingolipid synthesis was inhibited, and exogenous sphingolipid could not be given a further modification, sphingoid bases were able to suppress the endocytic defect (Zanolari, Friant et al. 2000). These results suggest that sphingoid base is required for the internalization step of endocytosis. Consistent with the study that showed that sphingoid base can activate PKH1 and PKH2, overexpression of either PKH1 or PKH2 can suppress the *lcb1-100* endocytic defect (both fluid phase and receptor mediated internalization) and temperature sensitivity (Friant, Lombardi et al. 2001; deHart, Schnell et al. 2002). In addition, a temperature sensitive mutant *pkh-ts*, harboring a chromosomal deletion of *PKH2* (*pkh2::LEU2*) and a temperature-sensitive *pkh1-ts* mutant allele, $pkh1^{D398G}$ (Inagaki, Schmelzle et al. 1999), failed in fluid phase and receptor mediated internalization at non-permissive temperature 37°C (Friant, Lombardi et al. 2001). Not only PKH kinases, but also YPK1 and YPK2 were found to be required for endocytosis (deHart, Schnell et al. 2002). These findings suggest that sphingoid bases mediate endocytosis via the protein kinases PKH1 and PKH2.

Since endocytosis is tightly coupled with actin assembly (Engqvist-Goldstein and Drubin 2003), it is conceivable that sphingolipids mediate actin cytoskeleton organization. Indeed, the serine palmitoyltransferase temperature sensitive mutant *lcb1-100* showed actin depolarization at non-permissive temperature. This actin defect was suppressed by additional sphingoid bases (Zanolari, Friant et al. 2000) and by overexpression of either PKH1, PKH2, YCK2 or PKC1 (Friant, Zanolari et al. 2000; Friant, Lombardi et al. 2001). Thus, sphingoid bases and PKH-PKC signaling cascade

seems to be required for proper actin organization, but it is not clear yet how the cascade regulates actin organization. Notably, PKC1 and its downstream MPK-MAPK signaling pathway are involved in actin regulation (Delley and Hall 1999; Levin 2005), so it is suspected that the signaling cascade at least partially participates in the regulation mechanism of actin cytoskeleton by sphingolipids.

Stress response

Sphingolipids are also required for stress response. Studies of serine palmitoyltransferase mutant strains (*lcb1-100* and 7R4), which lack newly synthesized sphingolipids at non-permissive temperature, have demonstrated that these lipids play a role in resisting heat, osmotic, and low pH stresses (Patton and Lester 1991) since the mutants are hypersensitive to these stresses. Following studies on a wild type strain showed, upon heat stress, a transient (up to 20 min) increase in the concentration of phytosphingosine and sphinganinel, and a more stable increase in ceramide This accumulation was not observed in the mutants (Dickson, Nagiec et al. 1997). Treatment of sphinganine at the permissive temperature 25°C induced expression of STRE (stress response element) regulated genes, which are also induced by heat stress, suggesting that sphingolipids mediate heat stress response (Dickson, Nagiec et al. 1997; Cowart, Okamoto et al. 2003). Increase of ceramide upon heat stress was inhibited by either australigungin or Fumonisin B1, both are ceramide synthase inhibitors, indicating that accumulation of sphingolipid intermediates (sphinganine, phytosphingosine, and ceramide) by heat stress probably results in *de novo* synthesis (Jenkins, Richards et al. 1997; Wells, Dickson et al. 1998). The transient change in these intermediates is consistent with the idea that these intermediates act as signaling molecules. Interestingly, mutants lacking increased *de novo* sphingolipids upon heat stress were found to lack the transient heat stress induced G_0/G_1 arrest (Shin, Matsumoto et al. 1987; Jenkins and Hannun 2001), suggesting that newly synthesized sphingolipid metabolites also mediate the cell cycle arrest.

The mechanism by which sphingolipids contribute to heat stress response is gradually beginning to be understood. Upon heat stress, in the *lcb1-100* strain, there was no induction of the major heat shock proteins (Friant, Meier et al. 2003), although transcription and nuclear export of mRNA of heat shock protein was not affected.

Translation initiation was strongly down-regulated, as demonstrated by pulse-chaise experiment and polysome profiling (Meier, Deloche et al. 2005). The defects in translation initiation in the mutant were not observed in a ceramide synthase mutant (*lac1 lag1*^{ts}) or a sphingoid bases kinase mutant (*lcb4 lcb5*), suggesting that sphingoid bases have a role in heat stress response (Meier, Deloche et al. 2005). Disruption of the yeast eIF4E-binding protein EAP1 restored translation initiation and synthesis of heat shock proteins, suggesting that sphingoid bases during heat stress regulate translation initiation at a cap dependent step. In addition, the PKH-YPK signaling cascade, a downstream target of sphingoid bases, is partially required for the stability of eIF4G (Meier, Deloche et al. 2005). Taken together, the results of the studies presnetd above suggest that sphingoid bases are required for heat stress response, probably because the lipids promote translation of heat shock proteins via PKH-YPK signaling cascade and a cap dependent translation initiation step. It would be interesting to sturdy that, for example, how PKH signaling reaches EAP1, or the contribution of the other signaling pathway on regulation of the translation initiation upon heat stress response.

Not only *de novo* biogenesis of sphingolipids, ceramide and sphingoid bases can be generated by degradation of complex sphingolipid (hydrolysis pathway). So far, contribution of the hydrolysis pathway to the heat shock response seems less impact than that of *de novo* pathway. ISC1 hydrolyzes complex sphingolipids to ceramide. At 39°C for 1 h, disruption mutant of *isc1* showed cell cycle arrest like wild type suggesting that ceramide produced by ISC1 is not likely to contribute cell cycle regulation in response to heat stress (Cowart, Okamoto et al. 2006). However, the mutant showed decrease of the C_{24}-, $C_{24:1}$- and C_{26}-dihydroceramide species (Cowart, Okamoto et al. 2006), suggesting that possibly sphingolipid hydrolysis pathway participates in heat shock response at different level of that of *de novo* sphingolipid biogenesis.

As substances of several disorders

One obvious reason that sphingolipids draw attention is that the lipids are involved in several human disorders, including acid sphingomyelinase deficiency types A and B, and Krabbe diseases.

The acid sphingomyelinase deficienies types A and B, also called Niemann-Pick disease, are an autosomal recessive disorders due to mutation of the sphingomyelinase gene (Vanier 2002).

Krabbe disease (or globoid cell leukodystrophy) is an autosomal recessive disorder resulting from the deficiency of galactocerebrosidase (Wenger, Victoria et al. 1999). The galactocerebrosidase is a lysosomal β-galactosidase responsible for the hydrolysis of several galactolipids, including galactosylceramide and psychosine (galactosylsphingosine). Deficiency of the enzyme induces accumulation of its substrates, and accumulation of psychosine may be the cause of the pathology and symptoms observed in this disease (Kobayashi, Shinoda et al. 1987; Wenger, Victoria et al. 1999).

Not only the two disorders written above, but also Tay-Sachs disease, Farber Disease, and Gaucher disease are known as autosomal recessive disorders caused by deficiency of enzymes in sphingolipid metabolism. These genetic disorders confirm the importance of sphingolipids and the existence of regulation mechanisms of sphingolipid metabolism.

Aim of This Thesis

The TOR signaling pathway, conserved from yeast to mammals, is essential for cell growth.

The aim of this thesis is to find and describe additional functions of TOR in the model organism *Saccharomyces cerevisiae*, and to gain further understanding of how TOR contributes to cell growth. We specifically focus on the TOR complex 2 signaling branch, and study a novel readout, sphingolipid biosynthesis.

PART II

Results

II-1. Analysis of AVO3 function in TOR complex 2

SUMMARY

The atypical and highly conserved protein kinase TOR is a central controller of growth. In yeast, TOR2 is found in two functionally and structurally distinct complexes, TORC1 and TORC2. TORC2 contains TOR2, AVO1, AVO2, AVO3, BIT61 and LST8. Here we describe a molecular analysis of the TORC2 subunit AVO3. AVO3 has six distinct regions that are highly conserved from yeast to human. AVO3 and, in particular, five of its conserved regions are required for the TORC2 function of signaling via the cell integrity pathway and polarization of the actin cytoskeleton. AVO3 also interacts with the novel TORC2 component BIT2. Our results suggest that AVO3 performs a positive role in TORC2 to support growth and actin organization.

INTRODUCTION

TOR, the target of rapamycin, plays a crucial role in the regulation of cell growth by coupling the accumulation of mass to nutrient and other environmental cues (Barbet, Schneider et al. 1996; Schmelzle and Hall 2000; Rohde, Heitman et al. 2001; Jacinto and Hall 2003; Hay and Sonenberg 2004). TOR was originally defined in *Saccharomyces cerevisiae* with the identification of mutations that confer resistance to the anti-fungal, immunosuppressant and anti-cancer drug rapamycin (Heitman, Movva et al. 1991). TOR proteins are functionally and structurally conserved in all eukaryotes and are the founding members of the phosphatidylinositol kinase-related protein kinase (PIKK) family. These proteins contain several known or putative protein-protein interaction domains, and most function as components of multiprotein complexes (Bakkenist and Kastan 2004).

In budding yeast, there are two TOR genes, *TOR1* and *TOR2*, and the TOR proteins function in two distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Loewith, Jacinto et al. 2002; Chen and Kaiser 2003; Wedaman, Reinke et al. 2003; Reinke, Anderson et al. 2004). TORC1 is composed of KOG1, TCO89, LST8 and either TOR1 or TOR2. When growth conditions are favorable, TORC1 promotes mass accumulation by stimulating ribosome biogenesis, translation initiation and nutrient uptake, and by inhibiting autophagy (Noda and Ohsumi 1998; Crespo, Powers et al. 2002). TORC2 contains AVO1, AVO2, AVO3/TSC11, BIT61, LST8 and TOR2, but not TOR1. Although *AVO3* was originally named *TSC11* (temperature sensitive suppressor of *csg2*) (Beeler, Bacikova et al. 1998), we prefer to use the name *AVO3* to avoid confusion with mammalian TSC (tuberous sclerosis complex) proteins that negatively regulate mTOR activity (Jacinto and Hall 2003). While TORC1 mediates temporal control of cell growth, TORC2 regulates the cell-cycle dependent polarization of actin cytoskeleton and thus controls spatial aspects of cell growth.

Loss of TORC2 function leads to a depolarization of the actin cytoskeleton – actin patches are evenly distributed in the mother and daughter cell (Schmidt, Kunz et al. 1996). This defect in cell cycle-dependent actin organization is suppressed by hyperactivation of the TORC2-regulated cell integrity pathway (Helliwell, Schmidt et al. 1998; Loewith, Jacinto et al. 2002). TORC2 regulates this pathway via activation

of the guanine nucleotide exchange factor ROM2 (Schmidt, Bickle et al. 1997). ROM2 converts the small GTPases RHO1 and RHO2 to an active, GTP-bound state. GTP-bound RHO binds and activates PKC1 (Nonaka, Tanaka et al. 1995). PKC1, in turn, signals to the actin cytoskeleton via a MAP kinase cascade composed of the MAPKKK BCK1, the MAPKKs MKK1 and MKK2, and the MAPK MPK1/SLT2 (Lee and Levin 1992; Irie, Takase et al. 1993; Lee, Irie et al. 1993; Helliwell, Schmidt et al. 1998).

Here we investigate the function of AVO3, an essential component of TORC2. Disruption of AVO3 mimics a TOR2 deficiency, and AVO3 defects are suppressed by overexpression of signaling proteins downstream of TORC2. Thus, AVO3 functions positively with TOR2 to regulate the cell integrity pathway and the cell cycle-dependent polarization of the actin cytoskeleton. AVO3 is conserved throughout eukaryotic evolution and we show that several domains that are particularly well conserved are essential for the function of AVO3. Lastly, we show that the non-essential TORC2 components AVO2, BIT61 and its homolog BIT2 perform positive roles in TORC2 signaling.

EXPERIMENTAL PROCEDURES

Yeast strains, plasmids and media

The complete genotypes of yeast strains used in this study are listed in Table I. Plasmids used in this study are listed in Table II. Standard techniques and media were used (Sherman 1991; Barbet, Schneider et al. 1996). All cultures were incubated at 30°C unless otherwise indicated.

Actin staining

Cells growing logarithmically in YPD 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, Raths et al. 1994). Actin cytoskeleton organization was assessed in several hundred small-budded cells, as described previously (Delley and Hall 1999).

Spot assay

Logarithmically growing cells were harvested and resuspended in 10 mM TRIS pH 7.4. The resuspended cells were diluted in a fivefold dilution series. 4 µl of each dilution (10x, 50x, 250x, 1250x and 6750x diluted) were spotted on an YPD plate. Growth was scored after two days at 30°C.

MPK1 phosphorylation assay

YPD cultures of logarithmically growing cells were heat-shocked at 39°C and harvested. Cell extracts were prepared as described previously (Martin, Rodriguez-Pachon et al. 2000). Protein concentrations of extracts were determined by using Bradford Assay (Bio-RAD). Samples were denatured by addition of 5X SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer and heating at 96°C for 5 min. A total of 25 µg of protein (for MPK1 protein detection) or 40 µg of protein (for phosphorylated MAPK detection) was loaded for standard SDS-PAGE (10% acrylamide) and Western blot analyses. For immunodetection, a mouse anti-HA antibody (12CAS) and rabbit phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1000; Cell Signaling) were used. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse (anti-HA) or anti-rabbit (anti-phospho MAPK) antibody and detection by ECL reagents (Amersham Pharmacia Biotech).

Immunoprecipitation and Western blotting

YPD cultures of logarithmically growing cells were harvested, and cell extracts and co-immunoprecipitation were prepared as described previously (Loewith, Jacinto et al. 2002). Western blotting and immunodetection were performed as described above for the MAP kinase assay.

RESULTS

AVO3 is essential and required for actin cytoskeleton organization

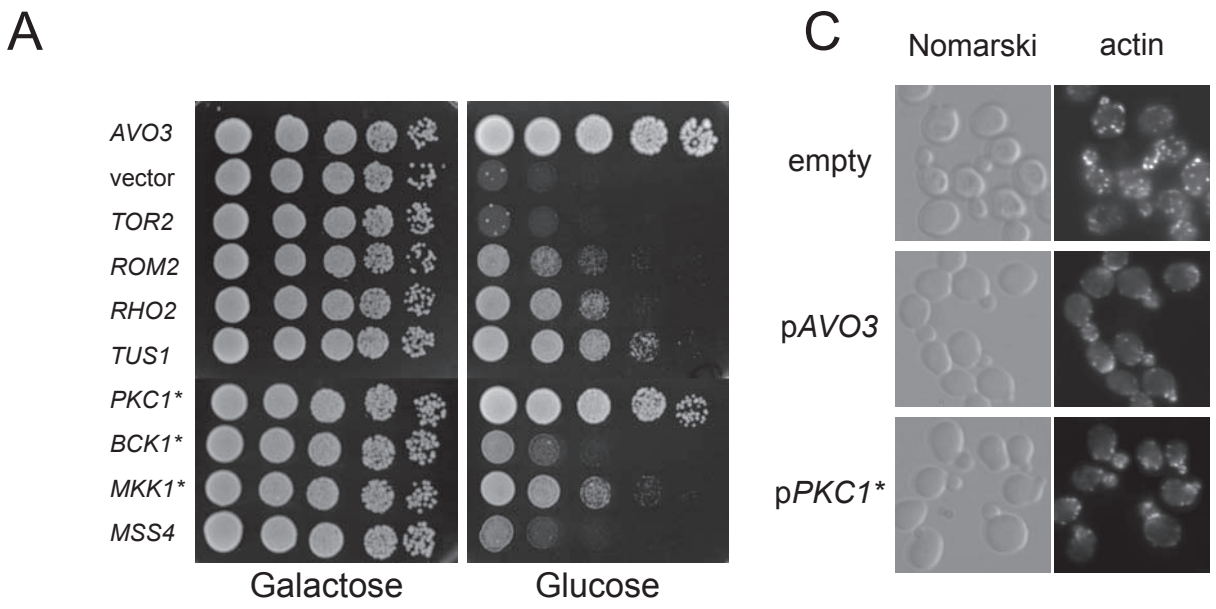
TORC2 regulates polarization of the actin cytoskeleton, through RHO small GTPases, protein kinase C (PKC1) and the MPK1 MAP kinase cascade (Helliwell et al., 1998b; Loewith et al., 2002; Wedaman et al., 2003). Since AVO3 is an essential protein (Winzeler et al., 1999) and a subunit of TORC2 (Loewith et al., 2002; Wedaman et al., 2003), it likely plays a role in TORC2 signaling. To investigate this possibility, we

examined if an *avo3* mutant phenocopies a *tor2* mutant. We constructed a strain that expresses HA-tagged AVO3 under the control of the galactose-inducible, glucose repressible *GAL1* promoter and assayed this strain for the ability to form colonies. As expected, this strain showed a severe growth defect when cells were grown in glucose-containing media (data not shown). Previous work (Helliwell et al., 1998b) demonstrated that the growth and actin defects of a *tor2* mutant are suppressed by hyperactivation of the RHO-PKC1-MAP kinase cascade. To examine whether the growth of AVO3-depleted cells is restored by overexpression of *TOR2* or known *tor2* suppressors, we transformed plasmids carrying these genes into the *GAL1p-AVO3* strain and spotted the cells on glucose containing, solid medium (Figure II-1-1A). Although overexpression of *TOR2* did not suppress the *avo3* growth defect, the growth defect was suppressed by multicopy *ROM2, RHO2, TUS1,* activated-*PKC1*, activated-*BCK1,* activated-*MKK1 and MSS4*, suggesting that, like TOR2 and AVO1 (Loewith et al., 2002), AVO3 positively regulates signaling through the RHO-PKC1-MPK1 pathway.

We next investigated whether AVO3 is required for polarization of the actin cytoskeleton. Since actin polarization is a rapid process relative to colony formation, we first determined the time course of AVO3 depletion in *GAL1p-AVO3* cells shifted from galactose- to glucose-containing media. The HA-tagged AVO3 protein decreased in a time dependent manner, and was undetectable by immunoblot within 12 to 16 hours after the shift to glucose medium (Figure II-1-1B). Within 12-16 hours of incubation in glucose medium, the AVO3-depleted cells lost actin polarization (Figure II-1-1C). This actin defect was suppressed by *AVO3* (Figure II-1-1C, middle panels), by overexpression of activated-PKC1 (Figure II-1-1C, bottom panels) or by the above multicopy suppressors of the *avo3* growth defect (*RHO2, TUS1,* activated-*BCK1,* activated-*MKK1*) (data not shown). Thus, an *avo3* mutation mimics *tor2* and *avo1* mutations, suggesting that AVO3 functions positively with TOR2 and AVO1 to support growth and polarization of the actin cytoskeleton.

AVO3 is required for full MPK1 activation

To confirm that AVO3 signals through the MPK1 MAPK cascade, we examined heat-induced activation of MPK1 in cells depleted for AVO3. To assay MPK1 activation, we monitored the phosphorylation state of MPK1 using a phospho-specific



- **Figure II-1-1**

AVO3 is essential and required for actin cytoskeleton organization. (A) Multicopy of *ROM2, RHO2, TUS1,* activated-*PKC1*, activated-*BCK1*, activated-*MKK1* and *MSS4* restore growth in AVO3-depleted cells. *GAL1p-AVO3* cell (RS61-4B) containing empty vector (YEplac195) or *AVO3* (pRS1), *TOR2* (pJK3-3), *ROM2* (pAS30), *RHO2* (pRHO2), *TUS1* (pTS38), activated-*PKC1* (YCp50::*PKC1*(R398P)), activated-*BCK1* (pRS316::BCK1-20), activated-*MKK1* (YCplac33::*MKK1*(S386P)) and *MSS4* (pSH22) were incubated on either galactose- or glucose-containing plate. Asterisk indicates activated-proteins. (B) HA-AVO3 abundance in *GAL1p-AVO3* cells decreased in a time dependent manner upon shift from galactose- to glucose-containing media. *GAL1pHA-AVO3* cells (RS61-5B) were incubated in glucose-containing media for the indicated times. HA-AVO3 was immunoprecipitated and detected by anti-HA antibody. Equal amounts of protein extracts were used for immunoprecipitation. (C) Actin depolarization of AVO3-depleted cells was suppressed by activated PKC1. *GAL1p-AVO3* cells with empty vector, p*AVO3*, or p*PKC1** were incubated in YPD for 16 h. After fixing with formaldehyde, the cells were treated with rhodamin-phalloidin to stain actin. Actin was visualized by fluorescence microscopy using TRITC filters. Cells were visualized by Nomarski optics.

antibody that specifically recognizes the dually phosphorylated and thereby activated form of MPK1. Wild type and *GAL1p-AVO3* cells were incubated at 24°C for 16 hours in glucose-containing medium (YPglucose) to deplete AVO3 protein (in the *GAL1p-AVO3* strain) and then shifted to 39°C to induce phosphorylation and activation of MPK1 (Martin et al., 2000) (Figure II-1-2). MPK1 phosphorylation showed similar induction kinetics in both strains however the magnitude of MPK1 phosphorylation was significantly reduced in AVO3-depleted cells. Thus, AVO3 is required for heat-shock induced phosphorylation of MPK1, confirming that AVO3 signals positively through the MPK1 MAPK pathway.

AVO3 localizes at the cell periphery

To gain a better understanding of AVO3 and TORC2 function, we observed the subcellular localization of AVO3. Previous studies have revealed that TOR2 is mainly enriched in the plasma membrane fraction on sucrose density gradient (Kunz et al., 2000), and localized at the cell periphery (Kunz et al., 2000; Wedaman et al., 2003). Since AVO3 physically interacts with TOR2 (Loewith et al., 2002; Wedaman et al., 2003), we hypothesized that AVO3 localize at the cell periphery as well. To address this, indirect immunofluorescence was performed to visualize AVO3. We constructed a C-terminus conjugated 13Myc-tagged AVO3 (AVO3-Myc) using PCR-recombination method (Longtine et al., 1998). AVO3-Myc was expressed under control of endogenous promoter. AVO3-Myc was functional, since the tagged-protein suppressed lethality of depleted-AVO3 (data not shown). Indirect immunofluorescence against the Myc epitope revealed that AVO3 localized at the cell periphery, and formed patch structures (Figure II-1-3). This peripheral localization of AVO3 is consistent with the previous studies that demonstrate TOR2 localization (Kunz et al., 2000; Wedaman et al., 2003), and the patch structure of AVO3 might correspond to the clusters of gold particle which visualized endogenous TOR2 under electron microscopy (Wedaman et al., 2003). Even in small budded cells, the AVO3 patch structures are distributed both in the mother cell and the bud tip, suggesting that AVO3 localization is independent of cell cycle (Figure II-1-3). The AVO3 peripheral localization was stable upon stress conditions; starvation, high temperature (37°C), osmostress, and 0.005% SDS. Rapamycin and myriocin (inhibitor of serine

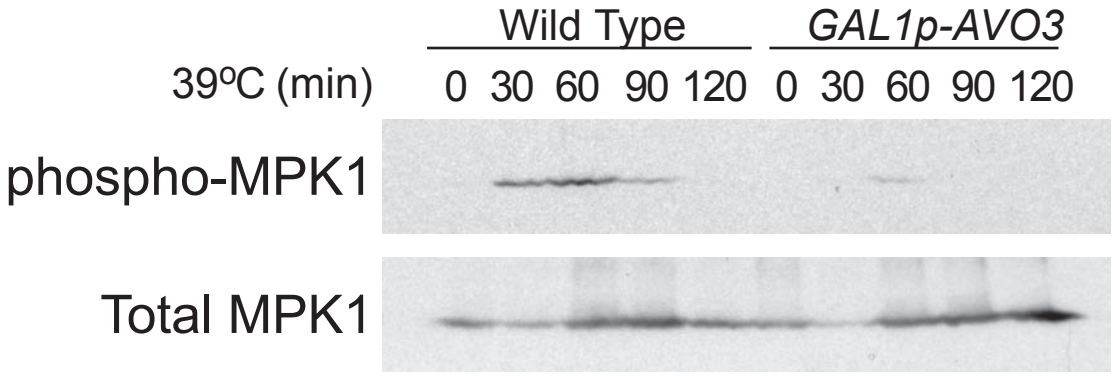


Figure II-1-2

AVO3 is required for full MPK1 activation. After 16 h growth in glucose medum (YPD) at 24°C, wild type (TS99-5C) and GAL1p-AVO3 (RS97-5A) were incubated at 39°C for indicated times (0, 30, 60, 90 and 120 min). Phospho-MPK1 was detected with antibody against phospho-p44/42 MAP kinase (see Experimental procedures). For total MPK1, anti-HA antibody was used because MPK1 in both strains is HA-tagged.

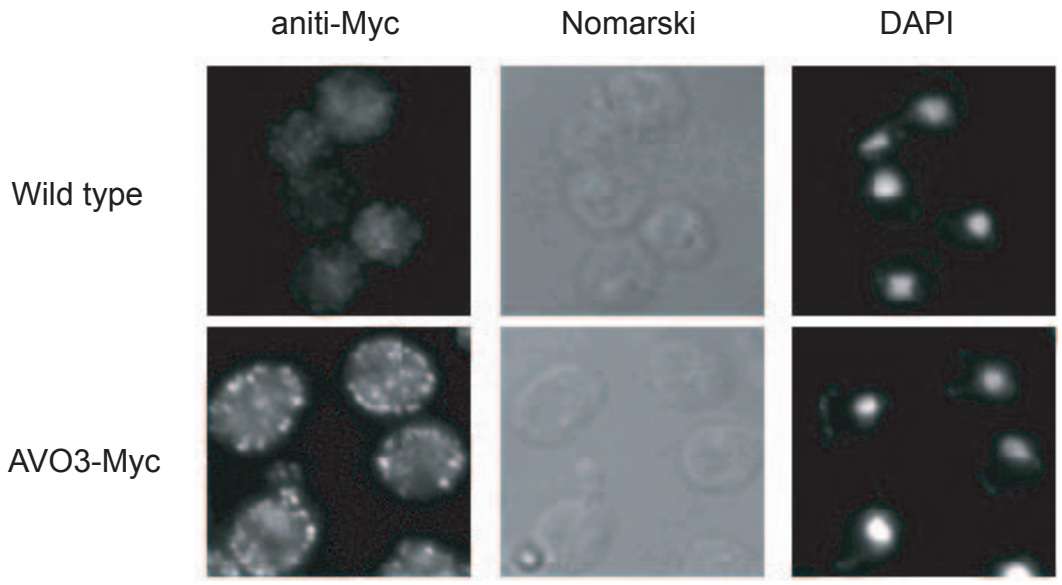


Figure II-1-3

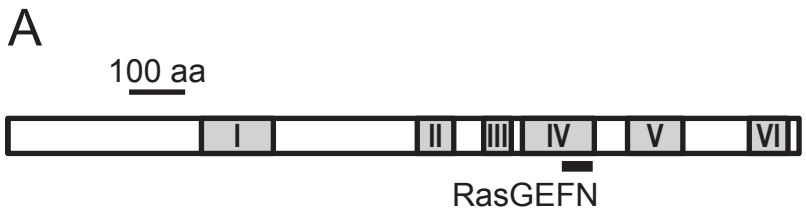
Cells were grown in YPD to midlog phase and fixed with 3% formaldehyde. AVO3 localizaiton was examined by indirect immunofluorescence on whole fixed cell. Wild type: TB50, AVO3-Myc: RL40-1c

palmitoyltransferase) also did not affect on the localization of AVO3, suggesting that AVO3 constantly might localizes at the cell periphery.

AVO3 has six highly conserved regions

To obtain further insight into AVO3 function, we compared the amino acid sequences of AVO3 orthologs to identify conserved regions that might be particularly important for AVO3 function. A global alignment program called T-coffee (http://www.ch.embnet.org/software/TCoffee.html) (Notredame et al., 2000) was used to compare putative AVO3 orthologs from six genera (*Candida* sp*., Schizosaccaromyces pombe, Neurospore crassa, Dictyostelium discoideum, Drosophila melanogaster, Homo sapiens*). The T-coffee analysis revealed that AVO3 orthologs contain at least 6 conserved regions (AVO3 Conserved Regions, ACR I to VI), corresponding to amino acids 351-480, 740-806, 870-905, 930-1050, 1145-1220 and 1340-1406 in *S. cerevisiae* AVO3 (Figure II-1-4A). ACRII and V (Figure II-1-4B) are more highly conserved than ACRI, III, IV and VI. ACRI, II, III, IV, V and VI are 51%, 66%, 47%, 41%, 66% and 41% similar between *S. cerevisiae* and human, respectively. The alignment also demonstrated that yeast AVO3, compared to fly and human sequences, contains a ~150 amino acid extension at the N-terminus and lacks a ~500 amino acid insertion between ACRV and VI (Jacinto et al., 2004). Individual BLAST searches with each conserved region failed to reveal any significantly related peptide sequence in other proteins. The alignment data indicate that AVO3 is evolutionally conserved and suggest that it might have a unique function.

To determine if AVO3 has defined domains, the yeast AVO3 amino acid sequence was also analyzed with the Conserved Domain Database (CDD) program (http://www.ncbi.nlm.nih.gov:80/Structure/cdd/cdd/.shtml). This analysis revealed that AVO3 has two domains at the C-terminus, so-called RasGEFN and KOG3694 domains. The RasGEFN domain is commonly found N-terminal to the catalytic site of Ras guanine nucleotide exchange factors (RasGEF). RasGEFN is thought to play a structural role in maintaining the RasGEF catalytic site in a precise position (Boriack-Sjodin et al., 1998). The putative RasGEFN domain in *S. cerevisiae* AVO3, amino acids 990 to 1047, falls within the C-terminal half of ACRIV and is of questionable significance as it was not detected with the BLAST searches described



B

ACRII

```
S.cer: 745  DSKVLQTKDFTRWNWNIINELLEGPLLNKKQLEELVKSTKFIRRLLVFYRPLRLRFSNVN 804
            DS+VLQ K+   WNWN+I  +L+ P +N +  ++  +  +F+RRLL FY+P    ++N++
H.sap: 509  DSQVLQHKENLEWNWNLIGTILKWPNVNLRNYKD-EQLHRFVRRLLYFYKPSSKLYANLD 567
```

ACRV

```
S.cer: 1151 AALWCVGFIGSTELGIGLLDNYSLVEDIIEVAYNASVTSVRFTAFYVLGLISMTREGCEI 1210
            A+LW +G IGS+  G+ LL   +++ DI+++A    V S+R T  YVLGLI+ T++GC+I
H.sap: 900  ASLWALGNIGSSNWGLNLLQEENVIPDILKLAKQCEVLSIRGTCVYVLGLIAKTKQGCDI 959
```

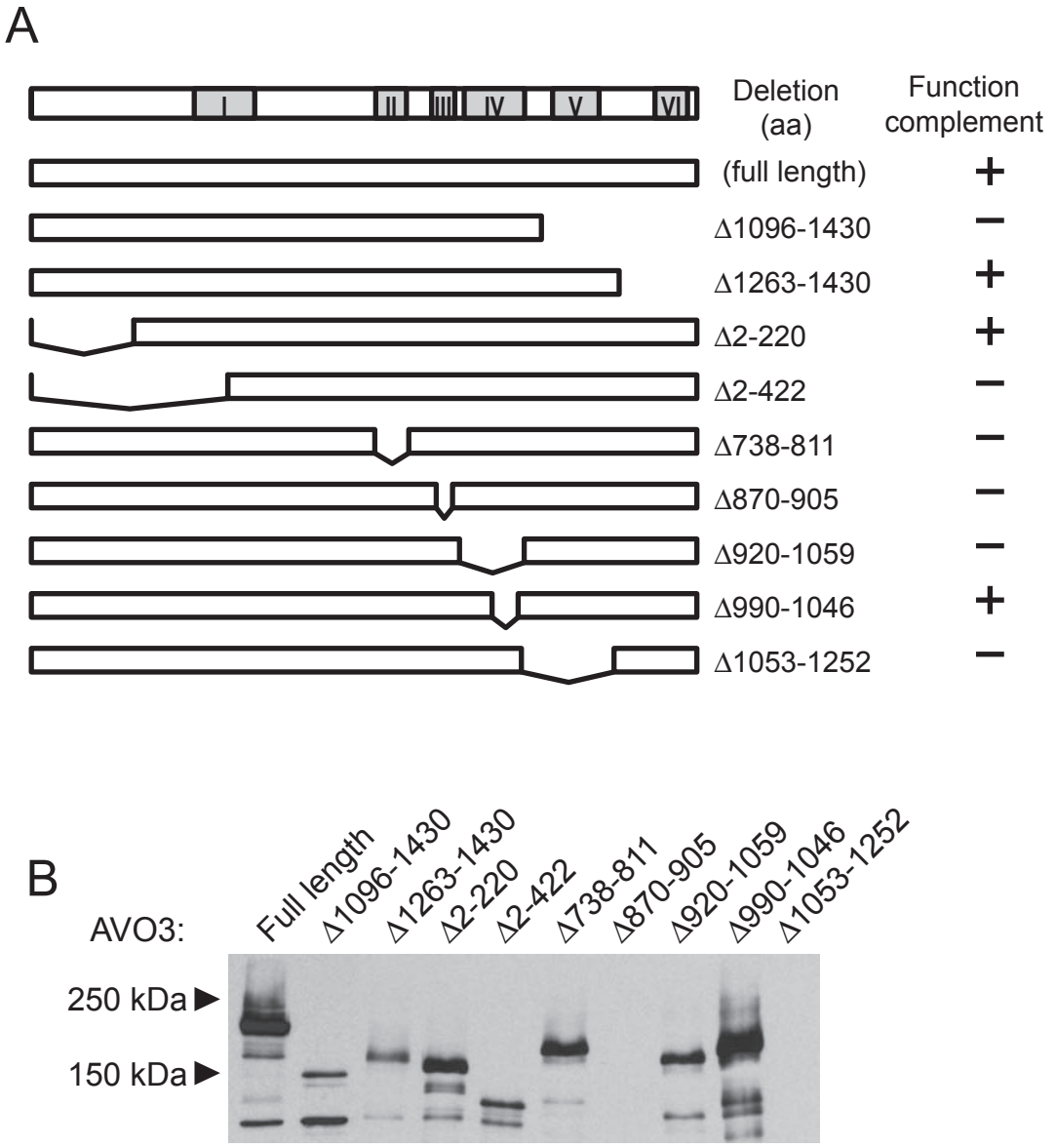
Figure II-1-4

AVO3 has six highly conserved regions. (A) Schematic diagram of AVO3 conserved regions (ACR) I to VI are shown in grey boxes, and corresponding amino acids of ACRI-VI are 350-480, 740-806, 870-905, 930-1050, 1145-1220 and 1340-1406, respectably. (B) An amino acid sequence alignment of ACRII and ACRV in *S. cerevisiae* (*S.cer*) and *H. sapiens* (*H.sap*). In the middle line of each alignments, identical residues are shown in single letter code and similar residues are shown in "+". The numbers correspond to the amino acid position of each protein.

above nor is it found in AVO3 orthologs outside of *S. cerevisiae*. KOG3694 (eukaryotic ortholog groups 3694) has been defined solely by computational analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, KOG v1.00, (Tatusov et al., 2003)) and is of unknown function. KOG3694 corresponds to ACRV and is found in all AVO3 orthologs but not in other proteins. KOG3694 is unrelated to the TORC1 subunit KOG1.

To investigate the function of each conserved region of AVO3, we performed a deletion analysis on *S. cerevisiae AVO3*. *AVO3-myc* plasmids individually containing in-frame deletions of each ACR, the N-terminal extension, or the RasGEFN domain (Figure II-1-5A) were constructed. Sequence analyses confirmed that each construct contained the desired deletion. Immunoblot analyses with anti-Myc confirmed that all constructs except those lacking ACRIII and ACRV expressed stable, deletion variants of AVO3 (Figure II-1- 5B). To determine which regions are essential for AVO3 function, the deletion alleles were introduced into the *GAL1p-AVO3* strain, and the transformants were assayed for growth on YPglucose agar (Figure II-1- 5A, right column). This assay revealed that ACRII and ACRIV are essential for AVO3 function and a region spanning amino acids 221-422 containing the N-terminal part of ACRI is also essential. However, although ACRIV is essential, deletion of the overlapping RasGEFN domain did not compromise AVO3 function, indicating that the RasGEFN domain is not the essential part of ACRIV. ACRVI and the N-terminus of AVO3 are not essential for AVO3 function. AVO3 deletion variants lacking ACRIII and ACRV were undetectable suggesting that ACRIII and ACRV are required, directly or indirectly, for AVO3 stability. Thus, amino acids 221-422 (containing a part of ACRI), ACRII, III, IV and V, but not ACRVI and the N-terminal extension of *S. cerevisiae* AVO3 are essential for AVO3 function. The RasGEFN domain is not required for AVO3 function.

Examination of the actin cytoskeleton in the above mutants revealed a direct correlation between inability to grow at the nonpermissive condition (glucose medium) and a defect in polarization of the actin cytoskeleton (data not shown). Thus, amino acids 221-422, ACRII, III, IV and V are essential for the ability of AVO3 to support both cell growth and actin regulation.



FigureII-1-5

(A) AVO3 deletion allele. Deleted amino acids are indicated next to each allele. Functional complement indicates whether the deletion allele can complement the growth and actin polarization defects of *GAL1p-AVO3* cells (RS61-5B). (B) Expression of AVO3 deletion variants. Protein expression was detected by immunoblot after co-immunoprecipitations of the myc-epitope fused to each AVO3 deletion variants.

AVO3 interacts with BIT2 in TORC2

Powers and colleagues recently identified BIT61 (YJL058c) as a component of TORC2 (Reinke et al., 2004). Disruption of BIT61 confers hypersensitivity to rapamycin but otherwise fails to confer a phenotype under a variety of growth conditions (Reinke et al., 2004). The uncharacterized S. cerevisiae protein BIT2 (Binding protein of TOR2, YBR270c) shares 44% identity with BIT61 (Figure II-1- 6A). The putative protein BIT2 derives it name from BIT61 and has yet to be shown to bind TOR2. BIT2 or BIT61 homologs are found in other fungi but not in higher eukaryotes, suggesting that BIT2 and BIT61 perform function(s) unique to fungi. Global yeast two-hybrid analyses (Ito et al., 2001; Uetz et al., 2000) indicated that both BIT2 and BIT61 interact with AVO3. These two-hybrid analyses also indicated that BIT2 binds to the TORC2 substrates SLM1 and SLM2. These observations suggest that BIT2 is a novel component of TORC2. To confirm that BIT2 is a component of TORC2, we examined whether BIT2 associates with TOR2 and AVO3 in vivo. Immunoprecipitated myc-tagged BIT2 co-precipitated both HA-tagged TOR2 and HA-tagged AVO3 (Figure II-1- 6B). Furthermore, BIT2 binds to the other TORC2 partners AVO1 and AVO2, also as determined by co-immunoprecipitation experiments (data not shown). Thus, BIT2 is indeed a component of TORC2.

AVO3 functions positively with BIT2, BIT61 and AVO2 in TORC2

Like *AVO2* and *BIT61*, *BIT2* encodes a nonessential protein. To further examine the role of these proteins in TOR signaling, we investigated the phenotype of mutants containing all possible combinations of *BIT2, BIT61* and *AVO2* wild type and null alleles. Single and double mutants were crossed to generate all possible combinations of the double and triple disruptions to assay synthetic effects on cell growth. We did not observe any significant growth defects when the single, double or triple disruptants were incubated at low temperature (15°C), high temperature (37°C or 39°C), high osmolarity (1 M Sorbitol), or in the presence of a salt stress (1.2 M NaCl, 0.3 M LiCl), or a cell wall stress (0.005% SDS) (data not shown). However, all the above null mutants, with the exception of the *bit61* and *bit2* single mutants, exhibited a growth defect when combined with an *AVO3* deficiency. *bit2 GAL1p-AVO3* cells and *bit61 GAL1p-AVO3* cells grew like *GAL1p-AVO3* cells, but the *bit2 bit61* double mutation or

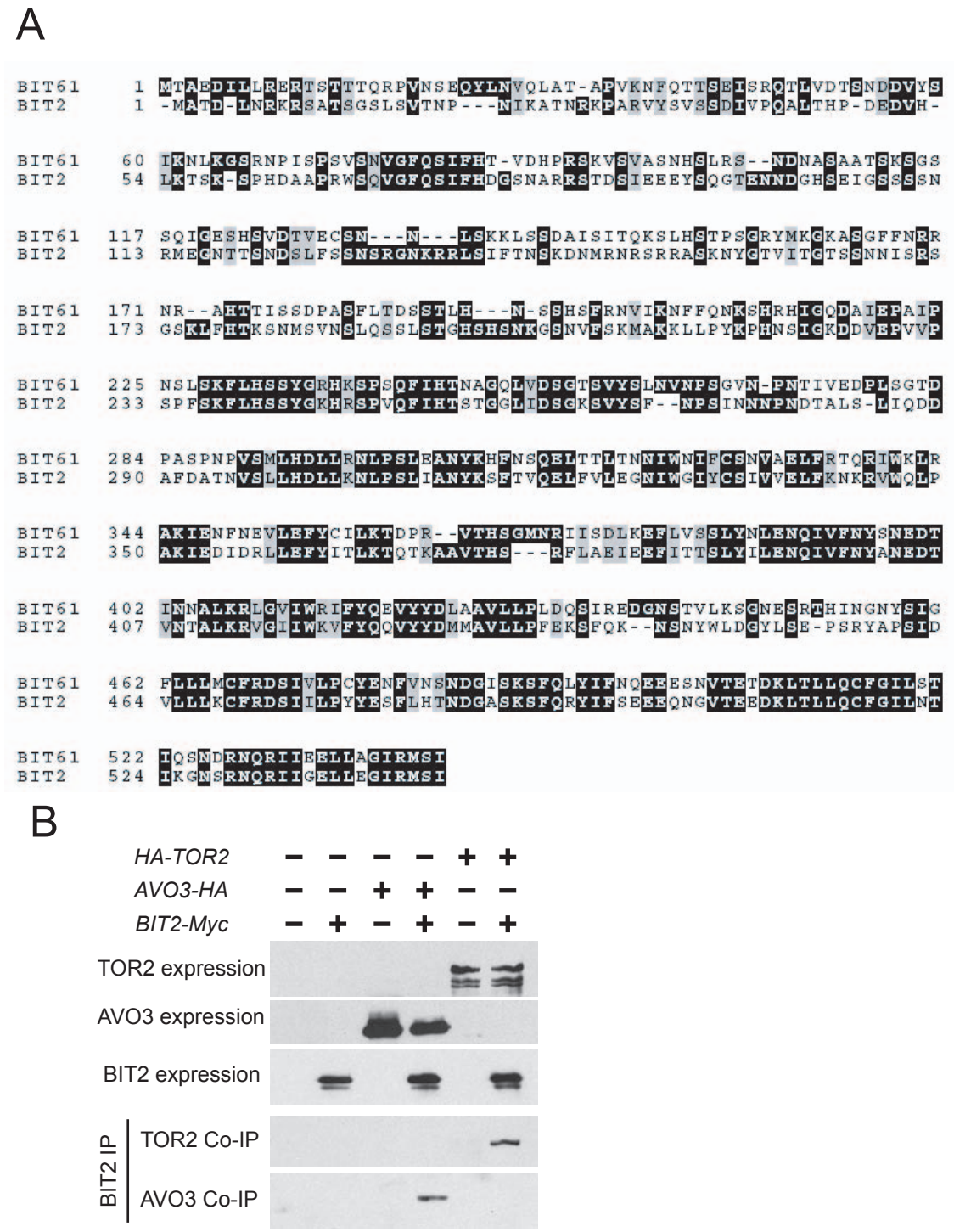


Figure II-1-6

BIT2 is a component of TORC2. (A) Alignment of the amino acid sequences of BIT61 and BIT2. The alignment was generated using the ClustalW and BOXSHADE. Black boxes indicate identical residues and grey boxes show amino acid similarities. (B) BIT2 associates with AVO3 and TOR2. Lysates from wild type (TB50a, lane 1), cells expressing myc-tagged BIT2 (RL155-1D, lane 2), cells expressing HA-tagged AVO3 (RL42-1C, lane 3), cells co-expressing HA-tagged AVO3 and myc-tagged BIT2 (RL163-8A, lane 4), HA-tagged TOR2 (SF43-1C, lane 5) or co-expressing HA-tagged TOR2 and myc-tagged BIT2 (RL163-8A, lane 6) were immunoprecipitated with anti-HA antibody (top two panels), or with anti-myc antibody (bottom three panels). Immunoblots were performed with anti-HA antibody (top two panels and bottom two panels), or with anti-myc antibody (middle panel).

the *avo2* single mutation exacerbated the growth defect of *GAL1p-AVO3* cells grown on media containing raffinose as the sole carbon source where the *GAP1* promoter is only weakly active (Figure II-1- 7A). Furthermore *bit2 bit61 avo2 GAL1p-AVO3* cells exhibited an even more severe growth defect compared to either *bit2 bit61 GAL1p-AVO3* or *avo2 GAL1p-AVO3* cells grown on raffinose. Introduction of an *avo2* deletion into an *avo3* temperature sensitive strain further reduced the temperature tolerance of the strain (data now shown). Thus, combined with the results of the above binding studies, BIT2, BIT61 and AVO2 function positively with AVO3 in TORC2. Furthermore, as suggested by the synthetic phenotype of *bit2* and *bit61* mutations and the homology of the BIT proteins, BIT2 and BIT61 may have redundant functions. Finally, our observations also suggest that BIT2 or BIT61 and AVO2 perform distinct functions in TORC2.

To further investigate the function of these non-essential TORC2 proteins, we examined the actin cytoskeleton in the above mutant cells containing all possible combinations of *BIT2*, *BIT61* and *AVO2* wild type and null alleles and *GAL1p-AVO3*. Cells grown in galactose medium were shifted to YPglucose and harvested after 4, 8, or 10 hours. Actin defects appeared earlier in the *bit2 bit61 GAL1p-AVO3*, *avo2 GAL1p-AVO3* and *bit2 bit61 avo2 GAL1p-AVO3* cells compared to *GAL1p-AVO3* cells (Figure II-1- 7B). Thus, although BIT2, BIT61 and AVO2 likely perform separate functions, both of these functions, like that of AVO3, seem to be required for polarization of the actin cytoskeleton.

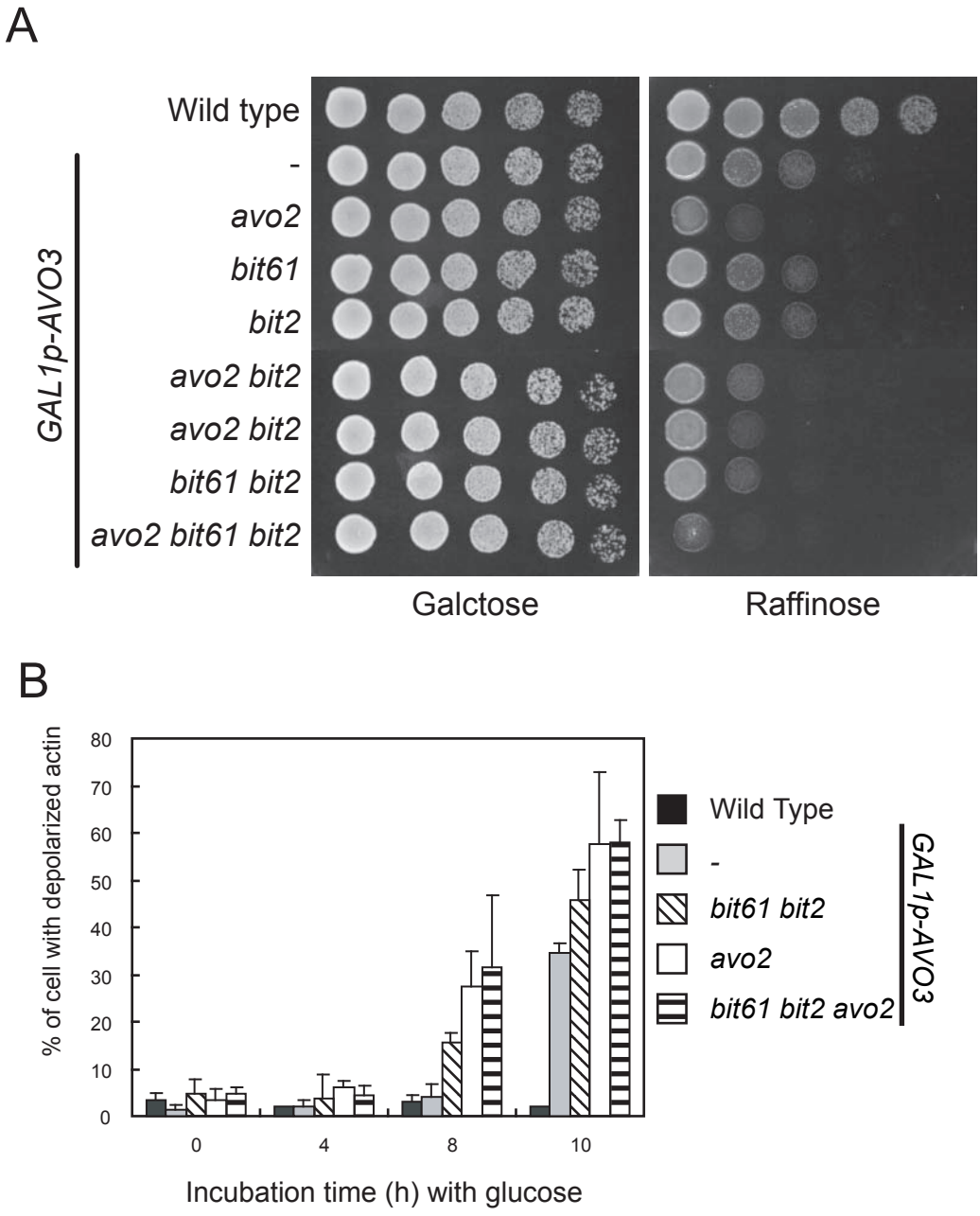


Figure II-1-7

(A) AVO3, BIT2, BIT61 and AVO2 function positively in TORC2. (A) Wild type (TB50a), *GAL1p-AVO3* (RS61-4B), *avo2 GAL1p-AVO3* (RS89-25A), *bit61 GAL1p-AVO3* (RS94-23A), *bit2 GAL1p-AVO3* (RS93-7D), *avo2 bit61 GAL1p-AVO3* (RS91-1A), *avo2 bit2 GAL1p-AVO3* (RS92-4D), *bit61 bit2 GAL1p-AVO3* (RS95-21C) and *avo2 bit61 bit2 GAL1p-AVO3* (RS90-1A) were spotted onto galactose (left panel) or raffinose (right panel) containing plates and incubated at 30° C for 2 days (for galactose) or 4 days (for raffinose). (B) Disruptants of *bit61 bit2 avo2 GAL1p-AVO3* represents actin depolarization in earlier time point. After several hour pre-incubation with galactose, cells were incubated with YPglucose for the indicated hours. Fixing, staining and observing cells were performed as Figure 1B. Percentage of small-budded cells in which actin was depolarized was counted as described in the experimental procedure.

Discussion

We have demonstrated that AVO3, an essential component of TORC2, acts similarly to TOR2 and AVO1 to positively regulate cell growth and actin cytoskeleton organization. TORC2 regulates actin organization via the RHO-PKC1-MAP kinase cascade (the cell integrity pathway), and indeed disruption of AVO3 results in reduced signaling through this pathway. However, the mechanism by which TORC2 regulates this pathway remains unclear. Direct substrates of this kinase complex are SLM1 and SLM2 (Audhya, Loewith et al. 2004), but how SLM function relates to RHO function is unknown.

What is the molecular function of AVO3? AVO3 lacks a defined functional or catalytic domain but does have six regions that have been highly conserved among AVO3 orthologs. Five of these six regions are essential for AVO3 function but the roles of these domains remain to be elucidated. At least some of the conserved domains may be required for interaction with other TORC2 components as TORC2 is also conserved(Jacinto, Loewith et al. 2004; Sarbassov, Ali et al. 2004). Conserved regions ACRIII and ACRV are required for stability of AVO3, but the instability of AVO3 deletion variants lacking these two regions may be due to an inability of the two mutant proteins to assemble into TORC2. If AVO3 performs a scaffolding role, as suggested by multiple conserved domains, or performs a catalytic role remains to be determined.

Is AVO3 a link between TORC2 signaling and sphingolipid signaling? AVO3 was originally identified as tsc11, a temperature sensitive mutation that suppresses the calcium sensitive growth phenotype of a csg2 mutant (Beeler, Bacikova et al. 1998). Interestingly, this suppressor screen also identified a temperature sensitive mutation in TOR2/TSC14 as a suppressor of csg2 (Beeler, Bacikova et al. 1998). CSG2 encodes a subunit of the inositol phosphorylceramide (IPC) mannosyltransferase (Zhao, Beeler et al. 1994; Uemura, Kihara et al. 2003; Lisman, Pomorski et al. 2004). csg2 mutants accumulate the sphingolipid IPC-C and die in the presence of >20 mM Ca2+ (Beeler, Gable et al. 1994; Zhao, Beeler et al. 1994; Haak, Gable et al. 1997; Kohlwein, Eder et al. 2001). Most csg2 suppressors act by reducing IPC-C levels. However, this does not appear to be the mechanism by which avo3/tsc11 or tor2/tsc14 suppresses csg2

(unpublished data). The sphingolipid composition of an avo3 temperature sensitive mutant at permissive and nonpermissive temperatures was not significantly different from that of a parental wild type strain (unpublished data). This suggests that TORC2 does not act upstream of IPC-C synthesis. It is possible that TORC2 signals in parallel to or downstream of IPC-C and that hyperactivation of one or both of these pathways is toxic. The yeast PDK1 homologs PKH1 and PKH2 are activated in vitro by sphingolipid precursors and activate PKC1 and the PKC1-effector MAPK pathway, which are also a downstream effectors of TORC2 (Helliwell, Schmidt et al. 1998; Inagaki, Schmelzle et al. 1999; Friant, Lombardi et al. 2001), supporting the notion of parallel signaling. The relationship between TORC2 and sphingolipid metabolism remains to be defined. It is also unclear how downregulation of TORC2 signaling suppresses the calcium sensitivity of a csg2 mutant.

We have also provided evidence that BIT2 is a component of TORC2. BIT2, its homolog BIT61, and AVO2 are all non-essential components of TORC2, suggesting that they perform peripheral roles in TORC2. Although, simultaneous loss of BIT2, BIT61 and AVO2 did not affect cell growth in either good nutrient conditions or under stress conditions, loss of BIT2 and BIT61 or AVO2 exacerbated the growth defect of an avo3 mutant. This suggests that BIT2, BIT61 and AVO2, have positive functions in TORC2. Notably, global two-hybrid analyses suggest that BIT2 and AVO2 interact with the homologous proteins SLM1 and SLM2 (Uetz, Giot et al. 2000; Ito, Chiba et al. 2001). SLM1 and SLM2 are substrates of TORC2 (Audhya, Loewith et al. 2004). Thus it seems plausible that BIT2, BIT61 and AVO2 might act as adaptors to facilitate the association of TOR2 with some of its substrates.

Acknowledgements

We acknowledge support from the Swiss National Science Foundation and the Canton of Basel to M.N.H.

Table I. Strains used in this study

Strain	Genotype
TB50**a**	MAT**a** leu2-3,112 ura3-52 trp1 his3 rme1 HML**a** leu
RL37-4a-Nat	TB50**a** *avo2::NatMX*
RL42-1C	TB50**a** AVO3-3HA::KanMX4
RL140-2D	TB50**a** BIT2-13Myc::KanMX4
RL155-1D	TB50**α** BIT2-13Myc::KanMX4 tor2::KanMX4 / pRS3143HA-TOR2
RL163-8A	TB50**α** BIT2-13Myc::KanMX4 AVO3-3HA::KanMX
RS4-2D	TB50**a** [*KanMX4*]-*GAL1p-AVO3*
RS61-5B	TB50**a** [HIS3MX]-GAL1p-3HA-AVO3
RS89-25A	RS61**α** *avo2::NAT*
RS90-8A	RS61**a** avo2::NAT bit2::KanMX bit61::KanMX
RS91-1A	RS61**α** avo2::NAT bit61::KanMX
RS92-4D	RS61**a** avo2::NAT bit2::KanMX
RS93-7D	RS61**a** *bit2::KanMX*
RS94-23A	RS61**α** *bit61::KanMX*
RS95-21C	RS61**a** bit2::KanMX bit61::KanMX
RS97-5A	TB50**a** [KanMX4]-GAL1p-AVO3 MPK1-3HA::KanMX
SF43-1C	TB50**a** *tor2::kanMX4* / pRS314*3HA-TOR2*
SW70	TB50**a** *3HA-TOR2*
TS99-5C	TB50**a** MPK1-3HA::KanMX4

Table II. Vectors used in this study

Plasmid	Description (reference)
YEplac195	(2µm URA3) (Gietz and Sugino 1988)
YCplac33	(CEN URA3) (Gietz and Sugino 1988)
pRS1	express AVO3 from the AVO3 promoter (CEN URA3) - a 5.0 kb PCR fragment containing the AVO3 locus (-445 to +4379) was cloned into the SmaI and the SacI sites of YCplac33.
pJK3-3	expresses TOR2 (2µm URA3) (Kunz, Henriquez et al. 1993)
pAS30	expresses ROM2 (2µm URA3) (Helliwell, Schmidt et al. 1998)
pRHO2	expresses RHO2 (2µm URA3) (Madaule, Axel et al. 1987)
pTS38	expresses TUS1 (2µm URA3) (Schmelzle, Helliwell et al. 2002)
YCp50::*PKC1*(R398P)	expresses an activated allele of PKC1 (CEN URA3) (Nonaka, Tanaka et al. 1995)
pRS316::BCK1-20	expresses an activated allele of BCK1 (CEN URA3) (Lee and Levin 1992)
YCplac33::*MKK1*(S386P)	expresses a hyperactive mutation of MKK1 (CEN URA3) (Watanabe, Irie et al. 1995)
pSH22	expresses MSS4 (2µm URA3) (Helliwell, Schmidt et al. 1998)
pRS28	expresses Myc-tagged AVO3
pRS42	expresses myc-tagged AVO3(Δ1096-1430)
pRS43	expresses myc-tagged AVO3(Δ1263-1430)
pRS44	expresses myc-tagged AVO3(Δ2-220)
pRS45	expresses myc-tagged AVO3(Δ2-422)
pRS47	expresses myc-tagged AVO3(Δ738-811)
pRS48	expresses myc-tagged AVO3(Δ870-905)
pRS49	expresses myc-tagged AVO3(Δ920-1059)
pRS50	xpresses myc-tagged AVO3(Δ990-1046)
pRS52	expresses myc-tagged AVO3(Δ1053-1252)

II-2. TOR complex 2 mediates sphingolipid biosynthesis in *Saccharomyces cerevisiae*

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Running title: TORC2 mediates sphingolipid biosynthesis

Summary

Sphingolipids and their metabolites are known as building components of cellular membranes, and also as signaling molecules mediating cell growth, endocytosis, actin regulation, and stress response. Here we report a crosstalk between sphingolipid biosynthesis and the TOR signaling pathway in *Saccharomyces cerevisiae*. The target of rapamycin (TOR) kinase is a central component of two highly conserved multiprotein complexes, TORC1 and TORC2. TORC1 is sensitive to the immunosuppressive and anti-cancer drug rapamycin, and controls temporal aspects of cell growth through regulation of transcription and translation. TORC2 is a rapamycin insensitive complex that mediates spatial control of cell growth via actin cytoskeleton organization. We show that TORC2 temperature sensitive mutants, *tor2*^{ts} and *avo3-1*, are synthetically lethal at the permissive temperature, with partial inhibition of sphingolipid biosynthesis by the antibiotic myriocin. At non-permissive temperature, the TORC2 mutants show reduced *de novo* synthesis of sphingolipids. Consistent with this, a general reduction of sphingolipid contents is also observed in steady state. In contrast, rapamycin treatment and loss of TORC1 function do not induce a reduction in sphingolipid biosynthesis. Our findings indicate that TORC2, but not TORC1, mediate sphingolipid biosynthesis.

Introduction

Cell growth, defined as accumulation of cell mass, is a fundamental property of cells, together with cell division and cell death. In response to nutrient, many processes of cell growth, including transcription, translation, ribosome biogenesis, autophagy, and actin cytoskeleton organization are regulated by the well-conserved protein kinase TOR (Target Of Rapamycin) (Barbet, Schneider et al. 1996; Schmelzle and Hall 2000; Rohde, Heitman et al. 2001; Jacinto and Hall 2003; Hay and Sonenberg 2004; Martin and Hall 2005; Wullschleger, Loewith et al. 2006). TOR constitutes two distinct protein complexes, TORC1 and TORC2 (Loewith, Jacinto et al. 2002), which are structurally and functionally conserved from yeast to mammals (Wullschleger, Loewith et al. 2005). In *S. cerevisiae,* TORC1, sensitive to the immunosuppressive and anti-cancer drug rapamycin, consists of either TOR1 or TOR2, KOG1, TCO89, and LST8 (Loewith, Jacinto et al. 2002; Reinke, Anderson et al. 2004). TORC1 controls translation and transcription via the type 2A-related phosphatase SIT4 and its regulatory subunit TAP42 (Di Como and Arndt 1996; Jiang and Broach 1999; Jacinto, Guo et al. 2001). In the absence of nutrients, TORC1 is inactive: under these conditions, SIT4 is released from TAP42, and thereby becomes active. Released SIT4 dephosphorylates and activates targets such as the transcription factor GLN3 and the kinase NPR1, which are involved in synthesizing alternative nutrient sources (Beck and Hall 1999; Jacinto, Guo et al. 2001). In the presence of nutrients, in addition to maintaining SIT4 in an inactive state, TORC1 mediates ribosome biogenesis, by switching the forkhead-like transcription factor FHL1 from binding its corepressor CRF1 to its coactivator IFH1. This regulation mechanism is mediated by protein kinase A (PKA) and the PKA-regulated kinase YAK1 (Martin, Soulard et al. 2004). In addition, TORC1 mediates autophagy by controlling the ATG1 kinase-dependent organization of the pre-autophagosomal membrane (Mizushima, Yamamoto et al. 2001; Suzuki, Kirisako et al. 2001; Kim, Huang et al. 2002). TORC2, the rapamycin insensitive complex, is composed of TOR2, AVO1, AVO2, AVO3, BIT61, and LST8 (Loewith, Jacinto et al. 2002; Wedaman, Reinke et al. 2003; Fadri, Daquinag et al. 2005). The complex mediates cell-cycle dependent actin cytoskeleton organization, via RHO1-PKC1 and the SLT2/MPK1-MAPK cascade (Schmidt, Bickle et al. 1997; Helliwell, Schmidt et al. 1998; deHart, Schnell et al. 2003). In addition, it has been suggested to mediate

endocytosis (deHart, Schnell et al. 2003). Recent studies have identified two substrates of TORC2, SLM1/2 and YPK2, both are involved in actin cytoskeleton regulation (Audhya, Loewith et al. 2004; Fadri, Daquinag et al. 2005; Kamada, Fujioka et al. 2005). SLM1 and SLM2 contains a PH domain, and act downstream of TORC2 and the phosphatidylinositol-4-phosphate 5-kinase MSS4 (Audhya, Loewith et al. 2004; Fadri, Daquinag et al. 2005). YPK2, the yeast homolog of SGK1 (serum- and glucocorticoid-activated kinase), is phosphorylated at the hydrophobic motif by TORC2 (Kamada, Fujioka et al. 2005). YPK2 is also phosphorylated at the T loop by PKH2 (the yeast homolog of PDK1), which acts downstream of sphingolipids in yeast (Casamayor, Torrance et al. 1999; Inagaki, Schmelzle et al. 1999; Friant, Lombardi et al. 2001). It is unclear whether SLM1/2 or YPK2 signal through RHO1 or a parallel pathway to the actin cytoskeleton.

Sphingolipids are serine-backbone lipids found in cellular membranes in all eukaryotes. Sphingolipids and their derivatives function as building components of cellular membranes, but also as signaling molecules that regulate many cellular processes, including cell growth, endocytosis, actin regulation, and stress response (Dickson and Lester 2002; Obeid, Okamoto et al. 2002; Jenkins 2003; Sims, Spassieva et al. 2004). In yeast, *LCB1* and *LCB2*, encoding the two subunits of a serine-palmitoyl transferase which catalyzes the first step of sphingolipid synthesis, are essential for growth (Pinto, Wells et al. 1992; Nagiec, Baltisberger et al. 1994). Other genes that encode enzymes involved in sphingolipid synthesis, such as the 3-ketosphinganine reductase *TSC10* (Beeler, Bacikova et al. 1998), a ceramide synthase subunits *LIP1* (Vallee and Riezman 2005), and the inositol-phosphoryl ceramide (IPC) synthase *AUR1* (Heidler and Radding 1995) are also essential, suggesting that sphingolipid biosynthesis is required for cell growth. Sphingolipid intermediates sphinganine (DHS) and phytosphingosine (PHS) are known to mediate endocytosis and actin cytoskeleton regulation (Dickson and Lester 2002; Sims, Spassieva et al. 2004), probably since both processes are tightly coupled (Engqvist-Goldstein and Drubin 2003). For example, an *lcb1* mutant that fails to uptake α-factor and Lucifer yellow (Munn and Riezman 1994), also shows actin depolarization (Zanolari, Friant et al. 2000; Schmelzle, Helliwell et al. 2002). The endocytic defect and actin disorganization of the *lcb1* mutant can be overcome by

supplementing the growth media with either DHS or PHS (Zanolari, Friant et al. 2000), or by overexpression of *PKH1*, *PKH2*, *YPK1*, and *YPK2*, which act downstream of DHS and PHS (Friant, Lombardi et al. 2001; deHart, Schnell et al. 2002). PHS and PKH kinases are also involved in translation initiation upon heat stress, and are required for the recovery from heat shock (Meier, Deloche et al. 2005).

Interestingly, many TOR functions overlap sphingolipids functions. As described above, cell growth, actin cytoskeleton regulation and endocytosis are mediated by both TOR signaling and sphingolipids biosynthesis. Furthermore, some genetic links between the two pathways have been described (Beeler, Bacikova et al. 1998; Helliwell, Howald et al. 1998). A *TORC2* temperature sensitive mutant (*tor2*^{ts}) can be suppressed by overexpression of *SUR1*, a gene encodeing a subunit of mannosyl inositolphosphoryl ceramide (MIPC) synthase (Helliwell, Howald et al. 1998). Reciprocally, *tor2*^{ts} and *AVO3* temperature sensitive mutant, *avo3-1*, can suppress the calcium sensitivity of a disruption mutant of *CSG2*, another subunit MIPC synthase. Although these suppression mechanisms are not clear, the findings suggest a crosstalk(s) between TORC2 signaling and the sphingolipid biosynthetic process.

Here, we describe that TORC2 mediates sphingolipid biogenesis in *S. cerevisiae*. TORC2 deficient cells showed synthetic lethality upon treatment with myriocin, a specific inhibitor of the serine-palmitoyl transferase. In addition, TORC2, but not TORC1 deficient cells showed less accumulation of complex sphingolipids and ceramide, indicating that sphingolipid biosynthesis is mediated specifically by TORC2. Our findings provide a novel target of TORC2 signaling pathway.

Results

TORC2 deficient cells are sensitive to myriocin

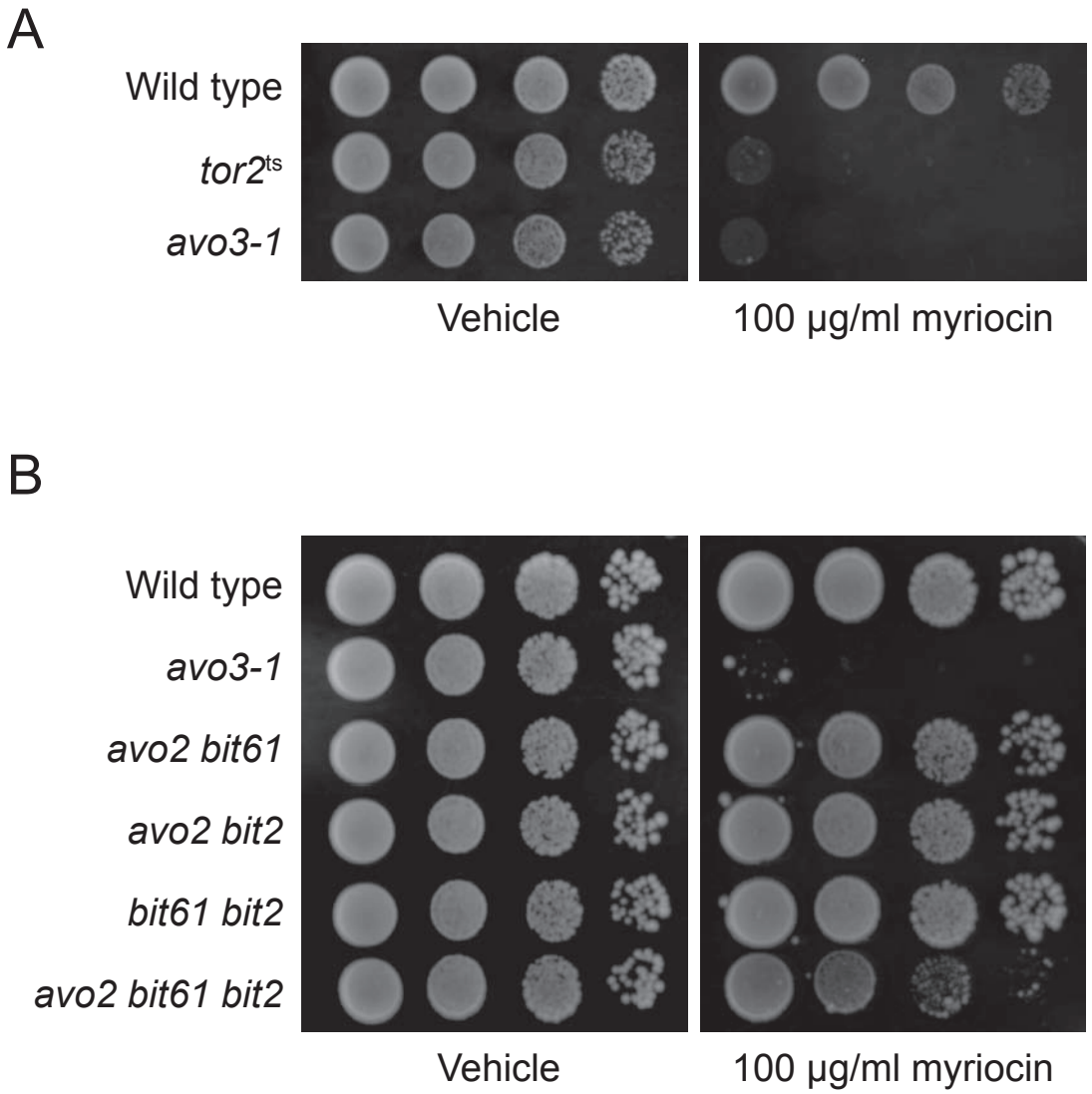
The initial observation that indicated a crosstalk(s) between TORC2 signaling module and sphingolipid biosynthesis was a genetic interaction: overexpression of *SUR1*, a subunit of MIPC synthase could suppress lethality of *tor2*^{ts} mutant (Helliwell et al., 1998a). Further genetic evidence came from the identification of mutants of TORC2 components, *tor2*^{ts} and *avo3-1*, as suppressors of calcium sensitivity, which was induced by disruption of *CSG2*, another subunit of MIPC synthase (Beeler et al., 1998). Taken

together with the functional overlaps between the two pathways (see introduction), these evidence support the existence of a crosstalk between TORC2 and sphingolipids. To confirm this hypothesis, we investigated the cell viability of TORC2 deficient cells upon myriocin treatment. Since myriocin is a specific inhibitor of serine palmitoyltransferase, which catalyses the first step of sphingolipid biosynthesis (Sun et al., 2000), then if both pathways are cooperating, myriocin treatment could give a synthetic effect on cell growth for TORC2 deficient cells. TORC2 deficient cells, both *tor2*^{ts} and *avo3-1*, did not grow upon 100 ng/ml myriocin treatment at permissive temperature 34°C, whereas the wild type strain formed colonies (Figure II-2- 1A). Colony formation of TORC2 deficient cells was defective both in a myriocin concentration-dependent and in a temperature-dependent manner (data not shown), suggesting that TORC2 and sphingolipid biogenesis are indeed cooperating to control growth.

TORC2 components, AVO2, BIT61, and BIT2 contribute to myriocin tolerance

The non-essential proteins AVO2, BIT61, and BIT2 (YBR270c) were isolated as interacting proteins of TOR2 in two-hybrid screens (Ito et al., 2001; Uetz et al., 2000), and in co-immunoprecipitations (Fadri et al., 2005; Loewith et al., 2002; Reinke et al., 2004). BIT61 and BIT2 are highly related proteins, sharing 45% amino acid identity and 61% similarity. Although no growth defects were observed under rich medium conditions neither for the single deletion mutant of *avo2*, *bit61*, or *bit2*, nor for the *avo2 bit61* double mutant (Reinke et al., 2004), the double mutant showed a modest growth defect when treated with 1.0 ng/ml rapamycin (Reinke et al., 2004), and promotion of cytosolic localization of SLM1 (Fadri et al., 2005). These findings suggest that the non-essential TORC2 components participate in TORC2 function under certain conditions, even though their contribution seems not to be indispensable.

To determine whether AVO2, BIT61, and BIT2 are required for the cooperation of TORC2 and sphingolipids, we tested the myriocin tolerance of deletion mutants in all possible combinations of these three genes. Although all single and double deletion mutants did not show significant growth defect at 34°C with 100 ng/ml myriocin (under which *avo3-1* showed a growth defect), the *avo2 bit61 bit2* triple mutant failed to grow (Figure II-2- 1B). The growth defect of the triple mutant was more severe with higher



Figures II-2-1

TORC2 deficient cells are sensitive to myriocin. (A) Saturated cultures of wild type, *tor2*ts, or *avo3-1* cells were serially diluted and spotted onto YPD plus methanol (vehicle), or YPD plus 100 µg/ml myriocin, and grown for two days at 34° C. (B) Saturated cultures of wild type, *avo3-1*, *avo2 bit61*, *avo2 bit2*, *bit61 bit2*, and *avo2 bit61 bit2* cells were serially diluted and spotted onto YPD plus methanol (vehicle), or YPD plus 100 µg/ml myriocin, and grown for three days at 30° C.

myriocin doses (data not shown). *avo2 bit61* and *avo2 bit2* double mutants, but not *bit61 bit2*, showed growth defects at 24°C when treated with 250 ng/ml myriocin (data not shown). The synthetic growth defect of *avo2 bit61 bit2* and myriocin suggests that these TORC2 components could be required for the cooperation between TORC2 and sphingolipids.

AVO3 domains are required for the cooperation with sphingolipid biogenesis

AVO3, an essential TORC2 component (Loewith et al., 2002; Wedaman et al., 2003; Wullschleger et al., 2005), has several conserved domains identified by sequence alignments of AVO3 orthologs from *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, *Drosophila melanogaster* and *Mus musculus* (Jacinto et al., 2004) and from six fungi (Ho et al., 2005). To determine whether these domains are required for the cooperation between TORC2 and sphingolipids, we examined the effect of myriocin treatment on domain-specific deletion mutants of AVO3. Following the Jacinto paper (Jacinto et al., 2004), the six conserved domains of yeast AVO3 were chosen for disruption (Figure II-2- 2A). AVO3 lacking domains I, II, III, IV or V failed to suppress the *avo3-1* temperature sensitivity at the non-permissive temperature (Figure II-2- 2B), suggesting that these domains are critical for the function of AVO3. The disruption mutant of domain VI, however, was able to rescue *avo3-1* growth at non-permissive temperature. Furthermore, disruption of domain VI, but not of the other domains, was able to suppress the *avo3-1* growth defect upon myriocin treatment, similar to full length AVO3. The domain VI deletion mutant was also able to suppress the actin depolarization phenotypes of *avo3-1* (data not shown). Domain VI locates very close to the stop codon (1340-1410 aa of 1430 aa), and the similarity of the domain between *S. cerevisiae* and *H. sapience* is 40%, but despite this high similarity, the domain is not likely to be essential for AVO3 function. Taken together, these findings suggest that the conserved domains I - V of AVO3 are required for its function, and in particular for the cooperation with sphingolipid biogenesis to control cell growth.

TORC2 deficient cells show less accumulation of sphingolipids

The synthetic growth defect described above has led us to further investigate the cooperation between TORC2 signaling and sphingolipid biogenesis. Since the genetic interaction between TORC2 and MIPC synthase indicates a modulation of synthesis of

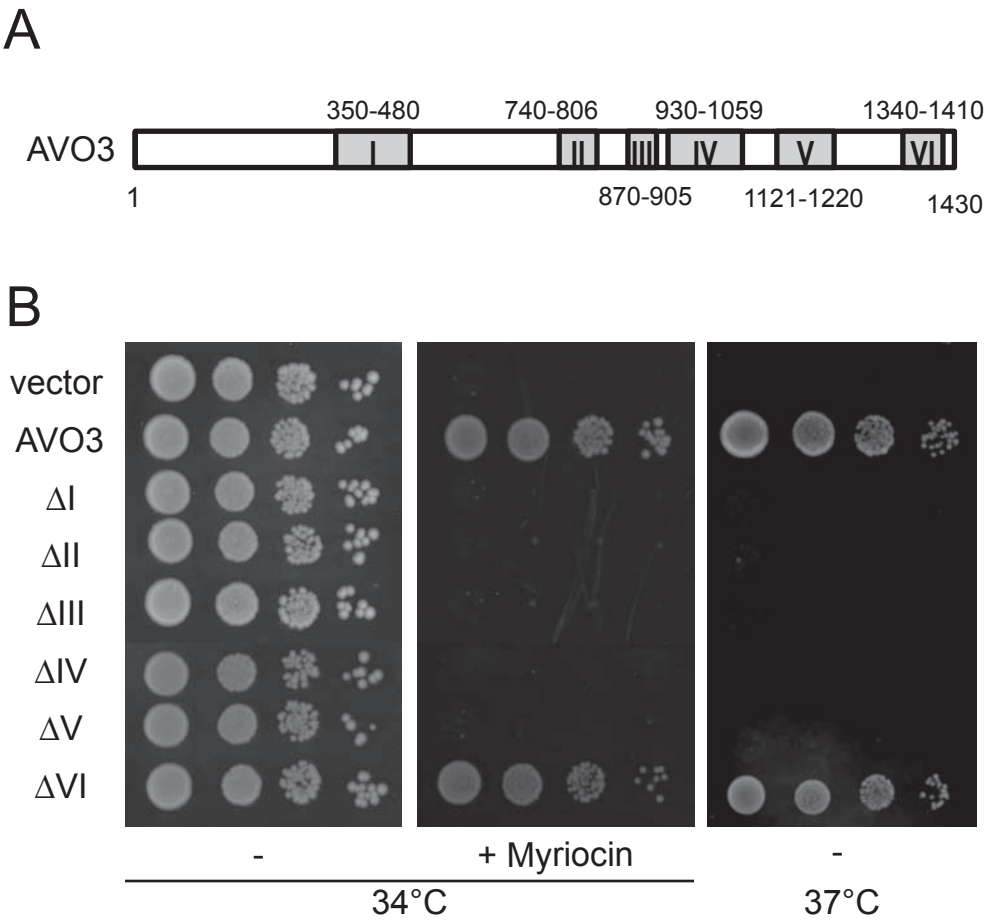


Figure II-2-2

Functional analysis of AVO3 conserved regions. (A) Schematic diagram of AVO3 conserved regions. The conserved regions I to VI are shown in grey boxes, the corresponding amino acids are 350-480, 740-806, 870-905, 930-1050, 1145-1220 and 1340-1406, respectively. (B) Saturated cultures of *avo3-1* cells carrying the indicated plasmid were serially diluted, spotted onto YPD plus methanol (vehicle) or 100 µg/ml myriocin, and grown at the conditions indicated. .

the complex sphingolipids inositol phosphorylceramide (IPC), MIPC, and mannosyl diinositolphosphoryl ceramide ($M(IP)_2C$) (Beeler et al., 1998; Helliwell et al., 1998a), we hypothesized that the level of complex sphingolipids would be influenced under TORC2 deficiency. To address this hypothesis, newly-synthesized complex sphingolipids in TORC2 deficient cells were measured using tritium-labeled inositol, since at least one inositol is contained in each of the complex sphingolipids. The wild type and TORC2 deficient cells *tor2*^{ts} and *avo3-1* were incubated at the non-permissive temperature (37°C) for six hours to decrease TORC2 activity (Ho et al., 2005; Schmidt et al., 1996). At this time point, both *tor2*^{ts} and *avo3-1* cells stopped growing and showed actin depolarization, but were still able to form colonies (data not shown). After two hours of labeling with [^{3}H]-inositol at the non-permissive temperature, lipids were extracted. Same amount of labeled material was loaded onto a thin layer chromatography (TLC), separated, and visualized on X-ray films. After the six hour incubation at the non-permissive temperature, IPC levels in *avo3-1* were reduced whereas the level in wild type was constant (Figure II-2- 3). Consistent with this reduction, the levels of MIPC and $M(IP)_2C$, the products of IPC modification, were also decreased in comparison with the wild type levels. Notably, phosphatidylinositol (PI) level did not significantly change during the incubation, suggesting that PI can be able to be synthesized even though TORC2 is inactive. Another TORC2 deficient mutant, *tor2*^{ts} also showed a similar reduction in complex sphingolipids under the same experimental conditions, although this reduction was lower than *avo3-1* (data not shown), perhaps due to the different mutant alleles. For further confirmation, the lipid profile of the TORC2 deficient cells in steady-state was analyzed using mass spectrometry-based lipid profiling (Figure II-2- 4). Consistent with the reduction in newly-synthesized complex sphingolipids on the TLC plate, IPC levels in steady-state in the TORC2 deficient cells were decreased (Figure II-2- 4). In contrast, MIPC were slightly elevated, though there was a species-dependent response. Another major difference in the lipid levels of TORC2 deficient cells besides the sphingolipid reduction was lyso-PI accumulation (Figure II-2- 3), and this accumulation was also observed in the steady state lipid profile (Figure II-2- 4). Interestingly, the lipid profiling demonstrated reduction of phosphatidylserine levels in the TORC2 deficient mutants. Taken together, our results indicate that TORC2 activity is required for

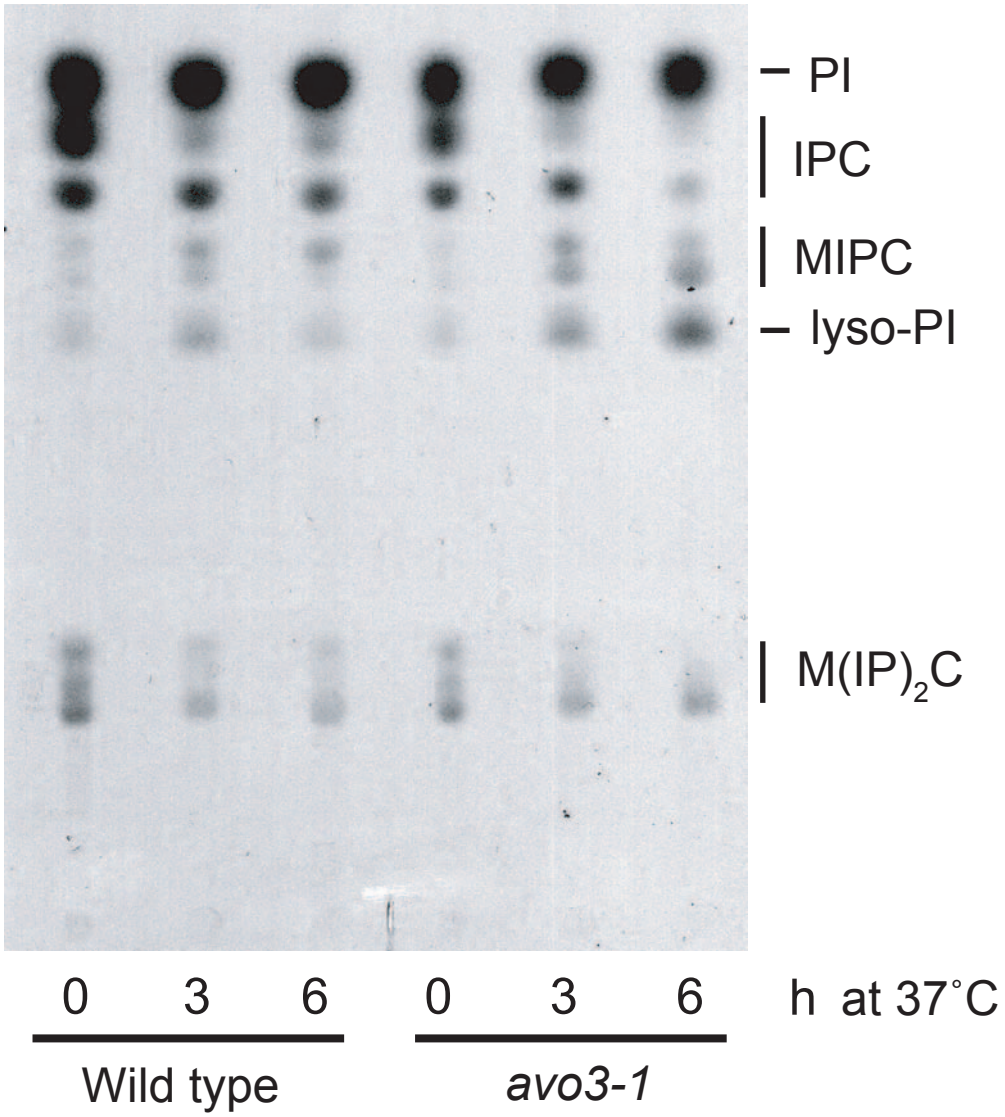


Figure II-2-3

Complex sphingolipid levels decrease in TORC2 deficient cells. Wild type and *avo3-1* cells were incubated for the indicated times at non-permissive temperature, and then labelled with [3H]-inositol for two hours, also at non-permissive temperature. Lipids were extracted and separated by TLC with chloroform, methanol, and 0.25% potassium chloride (55/45/10, v/v/v), and then visualized by autoradiography. PI: phosphatidylinositol, IPC: Inositol phosphorylceramide, MIPC: mannosyl inositolphosphoryl ceramide, M(IP)2C: mannosyl diinositolphosphoryl ceramide

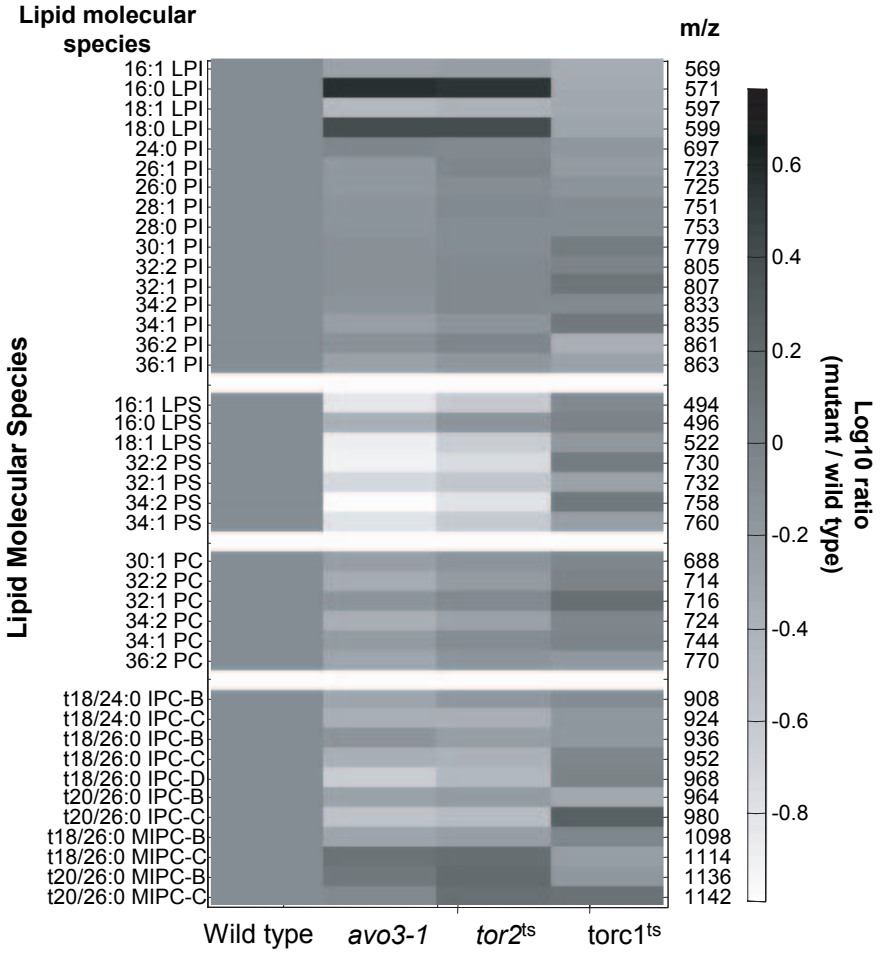


Figure II-2-4

Steady state levels of IPC and phosphatidylserine in TORC2 deficient cells are decreased. Analysis of phospholipids and complex sphingolipid levels in *avo3-1*, *tor2*ts and torc1ts relative to a wild type strain was performed using the mass spectrometry-based lipid profiling. Levels of lipid were quantified using relevant internal standards and calculated relative to the wild type strain for the individual molecular species. LPI: lyso-phosphatidylinositol, LPS; lyso-phosphatidylserine, PS: phosphatidylserine, PC: phosphatidylcholine, the other abbreviations are in *figure 3*.

sphingolipid synthesis.

TORC1 does not participate in the sphingolipid biogenesis

TORC1, a distinct protein complex from TORC2, regulates cell growth in response to nutrients in eukaryotes. Yeast TORC1 regulates starvation-specific transcription and translation via a phosphatase switch composed of the type 2A-related phosphatase SIT4, TAP42, and TIP41 (Cherkasova and Hinnebusch, 2003; Di Como and Arndt, 1996; Jacinto et al., 2001; Jiang and Broach, 1999), and ribosomal protein gene transcription by control of the subcellular localization of a corepressor CRF1 via protein kinase A (PKA) and the PKA-regulated kinase YAK1 (Martin et al., 2004). Does TORC1 participate in sphingolipid biosynthesis like TORC2? To answer this question, we investigated whether TORC1 inactivation would cause a decrease in sphingolipid biogenesis. TORC1 inactivation was performed in two ways; rapamycin treatment of wild type cells, and incubation at non-permissive temperature of a TORC1 temperature sensitive strain (torc1^{ts}).

Rapamycin, known as an anti-fungal, anti-tumor and immunosuppressive drug, is a specific inhibitor of TORC1 (Loewith et al., 2002). Wild type strain was incubated at 24°C for one hour with rapamycin, and then cells were labeled with [^{3}H]-inositol for two hours. Lipids were extracted and separated by TLC. One hour treatment of rapamycin inhibited TORC1 activity (Loewith et al., 2002; Martin et al., 2004), but did not affect complex sphingolipid biosynthesis (Figure II-2- 5), suggesting that TORC1 does not mediate complex sphingolipid biosynthesis.

The torc1^{ts} strain is defective in both TOR1 and TOR2 genes, but contains a plasmid encoding for a *tor2* mutant that can carry out the TORC2 unique function, but not the TORC1 shared function (Helliwell et al., 1998a). Therefore, at non-permissive temperature, the torc1^{ts} cells are inactivated only for TORC1 signaling but not TORC2. Under the conditions where TORC2 deficiency arises (six hours at 37°C), the torc1^{ts} cells were large and rounded, indicating the torc1^{ts} are indeed defective in TORC1 function (data not shown) (Helliwell et al., 1998a). Lipid profiling analysis of the torc1^{ts} cells, as measured by mass spectrometry, demonstrated that complex sphingolipids were not reduced at the non-permissive temperature (Figure II-2- 4),

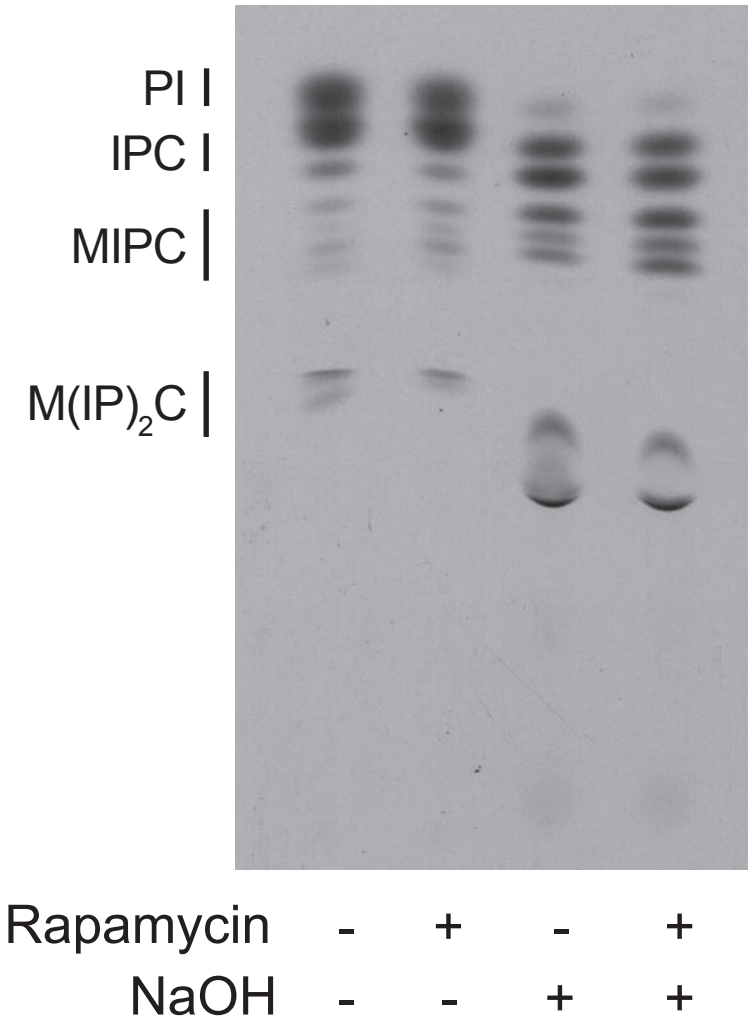


Figure ii-2-5

Rapamycin does not affect sphingolipid synthesis. Wild type strain at the mid-log phase was incubated with 200 ng/ml rapamycin for 30 min, and then labelled with [3H]-inositol for two hours in the presence of rapamycin. Lipids were extracted and subjected to alkaline hydrolysis with 0.6 M NaOH for 150 min at 30° C. Then, lipids were separated by TLC with chloroform, methanol, and 4.2 M ammonium hydroxide (9/7/2, v/v/v), and visualized by autoradiography. For the abbreviations of lipids, see *Figure 3*.

suggesting that inactivation of TORC1 does not influence sphingolipid biosynthesis. Importantly, this finding excludes a non specific effect of the long term heat stress on sphingolipid biosynthesis in temperature sensitive strains, since the long time heat stress on torc1^{ts} did not lead to a general reduction in sphingolipid biosynthesis. Thus, only TORC2, but not TORC1 or heat stress, mediates sphingolipid biosynthesis.

TORC2 deficient cells show a decrease in sphingoid bases and ceramide.

How does TORC2 mediate complex sphingolipid biosynthesis? One possibility is that TORC2 mediates synthesis of sphingolipid intermediates, thereby influencing complex sphingolipid biosynthesis (Figure II-2- 6A). Sphingolipid biosynthesis in yeast begins with condensation of L-serine and palmitoyl-CoA by serine palmitoyltransferase, which consists of three subunits in yeast, LCB1, LCB2, and TSC3 (Gable et al., 2000; Pinto et al., 1992b), and two subunits in mammals, LCB1 and LCB2 (Hanada, 2003). This reaction appears to be the rate-limiting step of the whole sphingolipid biosynthesis process, and a key factor of the reaction is intracellular concentration of its substrates serine and palmitoyl-CoA (Pinto et al., 1992b). This condensation generates a sphingoid base called 3-ketodihydrosphingosine (3-KDS). 3-KDS is reduced immediately by TSC10 into DHS, also called sphinganine (Pinto et al., 1992b). DHS is then mainly hydroxylated into PHS at the C-4 position by SUR2 (Grilley et al., 1998). DHS and PHS can be converted into ceramide at the ER by ceramide synthase (Guillas et al., 2001; Schorling et al., 2001; Vallee and Riezman, 2005). Then, ceramide is transported by vesicular and non-vesicular transport to the Golgi, where ceramide is transformed into the complex sphingolipids IPC, MIPC and M(IP)$_2$C (Dickson and Lester, 2002; Sims et al., 2004). To investigate whether TORC2 mediates synthesis of sphingolipid intermediates, sphingoid bases (3-KDS, DHS, and PHS) and ceramide in wild type, *tor2*^{ts}, and *avo3-1* cells were measured. Similar to the inositol labeling, wild type and mutant strains were incubated at 37°C for six hours to inactivate TORC2 and then labeled with [^{3}H]-palmitic acid or [^{3}H]-L-serine for two hours. During labeling, both radioactive molecules were taken up by wild type and TORC2 deficient strains without significant difference (data not shown). Again, the amount of [^{3}H] of the extracted lipids was counted by a scintillation counter, and same amounts of [^{3}H] were loaded onto a TLC. Palmitate labeling has demonstrated that both TORC2 deficient

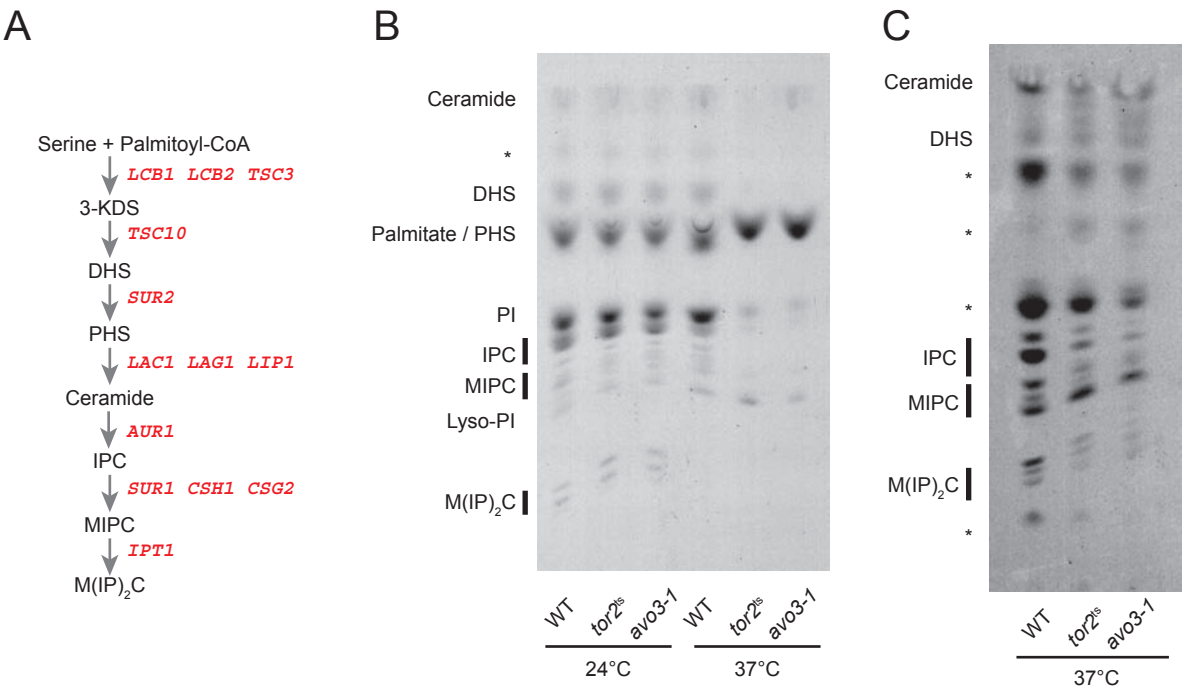


Figure II-2-6

TORC2 deficient cells show downregulation in *de novo* sphingolipid biosynthesis. (A) Schematic diagram of yeast sphingolipid biosynthetic pathway. 3-KDS: 3-ketodihydrosphingosine, DHS: dihydrosphingosine, PHS: phytosphingosine. (B) [3H]-palmitate labeling was performed on wild type, *tor2*ts, and *avo3-1* cells after a six hour incubation at non-permissive temperature. Lipid extraction and separation were the same as in *figure 4*. The asterisks indicate unidentified lipids. (C) [3H]-L-serine labeling was performed on wild type, *tor2*ts, and *avo3-1* cells. The experimental conditions were the same as in (B).

strains accumulated palmitate and showed almost complete loss of synthesis of other sphingolipid metabolites (Figure II-2- 6B), suggesting that TORC2 mutants are unable to utilize palmitate. Serine labeling showed a reduction in complex sphingolipids in TORC2 deficient cells (Figure II-2- 4C), consistent with the results of inositol labeling. In addition, ceramide in TORC2 deficient cells was slightly reduced, and sphingoid bases were undetectable (Figure II-2- 6C). Since the accumulation of a substrate is expected when an enzyme is defective (Beeler et al., 1998; Haak et al., 1997; Schorling et al., 2001), the findings suggest that the first step, mediated by serine palmitoyltransferase, failed to occur in TORC2 deficient cells.

TORC2 does not mediate the serine palmitoyltransferase activity at the translational, transcriptional, and post-transcriptional levels.

The findings described above led us to investigate serine palmitoyltransferase. The activity of serine palmitoyltransferase is the first and apparently rate-limiting step of sphingolipid metabolism (Obeid et al., 2002), therefore the reduction in sphingolipid biosynthesis in TORC2 deficient cells could be explained by defects in serine palmitoyltransferase. A microarray dataset of *tor2*^{ts} at 37°C for six hours revealed that TOR2 has 108 transcriptional readouts (that change more than two fold compared to wild type), but these readouts did not include the serine palmitoyltransferase components LCB1, LCB2, or TSC3 (data not shown). There was also no significant difference in the protein levels of serine palmitoyltransferase components between the wild type and TORC2 deficient cells after a six hour incubation at non-permissive temperature (data not shown). Physical interaction between LCB1 and LCB2 (Gable et al., 2000) was observed in the mutant strain as well as the wild type in the conditions where TORC2 deficient cells demonstrate reduction of sphingolipids (data not shown). In addition, no phosphorylation on LCB1 and LCB2 was observed by anti-phospho antibodies in both the wild type and the mutants, suggesting that TORC2 does not mediate the serine palmitoyltransferase activity at the translational, transcriptional, and post-transcriptional level.

Discussion

We have shown that deficiency of TORC2 results in reduction of sphingolipid metabolites. TORC2 mutants, *tor2*^{ts} and *avo3-1*, as well as *avo2 bit61 bit2* are synthetically lethal upon myriocin treatment at sub-lethal concentrations. In *tor2*^{ts} and *avo3-1* mutants, sphingolipid metabolites are reduced. The findings indicate that TORC2 is required for sphingolipid biosynthesis.

TORC2 inactivation was induced upon shift of yeast cells to 37°C, a non-permissive temperature, for six hours. Under these conditions actin depolarization is observed (Schmidt, Kunz et al. 1996; Loewith, Jacinto et al. 2002). Previous studies have shown that heat stress induces sphingolipid synthesis and *de novo* sphingolipid metabolites are required for heat shock response (Jenkins and Hannun 2001; Dickson and Lester 2002; Jenkins 2003). However, TORC2 mediated sphingolipid biosynthesis is not likely to be influenced by heat stress. First, as described above, reduction of sphingolipid biosynthesis only appeared in *tor2*^{ts} and *avo3-1*, but not in torc1^{ts} cells, suggesting that temperature sensitivity did not affect sphingolipid biosynthesis upon long term exposure to heat stress. Second, while the induction of DHS and PHS by heat stress is transient and occurs within 30 minutes of heat stress onset (Dickson, Nagiec et al. 1997), reduction of sphingolipids was only observed after six hour of incubation at the non-permissive temperature (Figure II-2- 3). Third, in the beginning of the temperature shift and for up to three hours, the growth curve and the ratio of actin depolarized cells in TORC2 deficient cells were not significantly different from the wild type (data not shown), suggesting that the initial adaptation to heat stress is similar to the wild type. Taken together, TORC2-mediated sphingolipid biosynthesis is appears not to be a secondary result of heat stress response; therefore TORC2 is indeed required for sphingolipid biosynthesis.

To display the phenotypes of TORC2 deficient cells takes at least six hours at the non-permissive temperature (Schmidt, Kunz et al. 1996; Loewith, Jacinto et al. 2002; Ho, Shiau et al. 2005). Why does inactivation of TORC2 take such a long time? There is no certain answer yet, but it is plausible that TORC2 is very stable; therefore it takes a long time to denature its components. Another possible explanation might be that the yeast takes up sphingolipids from the growth media during the first period of

incubation, and only when these are no longer available we can detect a reduction in intracellular sphingolipids. We cannot exclude the possibility that the sluggish response is allele specific of the *tor2*^{ts} and *avo3-1* mutants, as we previously reported several allele-specific differences between TORC2 mutants. For instance, four classes of *tor2*^{ts} mutants were initially isolated, and each showed different colony size after 2.5 days at 37°C (Helliwell, Howald et al. 1998). Another mutant, *tor2*^{G2128R}, demonstrateed an endocytic defect (deHart, Schnell et al. 2003), while *tor2*^{ts} and *avo3-1* do not show endocytic phenotype (data not shown), suggesting that different mutants might be defective in different TORC2 functions. It appears that TOR has multiple functions (Martin and Hall 2005; Wullschleger, Loewith et al. 2006), many of them still not well characterized; the different mutants might prove helpful in shedding light on these functions, as well as elucidating new readouts.

How does TORC2 mediate sphingolipid biosynthesis? So far, the regulation mechanism of sphingolipid biogenesis is not well understood. The only conditions known to change sphingolipid synthesis are heat shock and substrate availability, both of which appear not to play a role in the crosstalk between TORC2 and sphingolipids. Our findings indicate that TORC2 is not likely to control the biosynthesis at the mRNA and protein levels of serine palmitoyltransferase. Previous studies have shown that serine and palmitoyl-CoA are rate-limiting factors of the activity of serine palmitoyltransferase in yeast (Pinto, Wells et al. 1992) and in mammals (Hanada 2003). Therefore, a possible model is that TORC2 acts upstream of serine palmitoyltransferase, perhaps on its substrates. However, the concentration of serine in TORC2 deficient cells is similar to the wild type at non-permissive temperature (data not shown), suggesting that the decrease in sphingolipid biosynthesis in TORC2 deficient cells is not due to a change in serine concentration. Based on the finding that TORC2 deficient cells were not able to utilize palmitate (Figure 5B), it is speculated that TORC2 deficient cells are defective either in the affinity of the serine palmitoyltransferase to its substrate palmitate CoA, or in acyl-CoA transferases, which convert palmitate to palmitate-CoA. FAA1-3 are the yeast acyl-CoA transferases, and FAA1 prefers C12:0 to C16:0 fatty acids, including palmitate, as substrates *in vitro* (Johnson, Knoll et al. 1994). The regulation mechanism of FAA1 and the other FAAs is not known.

Recently it has been proposed that SLM1 and SLM2 may act downstream of sphingoid bases, and are required for endocytosis during heat stress (Bultynck, Heath et al. 2006). SLM proteins bind TORC2 and are also required for actin cytoskeleton organization (Audhya, Loewith et al. 2004; Fadri, Daquinag et al. 2005), suggesting that SLM proteins participate in TORC2 function. Several observations in these studies support our findings. First, deletion mutants of either *slm1* or *slm2* are sensitive to myriocin (Bultynck, Heath et al. 2006), consistent with our finding that TORC2 deficiency induces myriocin sensitivity. Second, heat stress-induced phosphorylation of the SLM proteins is independent of TORC2. Heat stress and phytosphingosine treatment can induce phosphorylation of SLM proteins. In addition, more than 90% of the heat-stress-induced phosphorylation of SLM proteins is inhibited by myriocin (Bultynck, Heath et al. 2006), suggesting that heat stress-induced phosphorylation of the SLM proteins is dependent on *de novo* sphingolipid biosynthesis, and thereby on sphingolipid-dependent protein kinases (Bultynck, Heath et al. 2006). However, the phosphorylation levels of the SLM proteins in *tor2*^{ts} are similar to the wild type after a four hour incubation at 38°C, indicating that the phosphorylation of the SLM proteins during heat stress is independent of TORC2 (Bultynck, Heath et al. 2006). Notably, this finding also indicates that the heat stress response of *tor2*^{ts} is similar to the wild type up to at least four hours.

TOR is a central controller of cell growth, and cell growth implicates mass accumulation and surface expansion. Since sphingolipids are mainly found in the plasma membrane (Patton and Lester 1991) and are required for cell growth (Dickson and Lester 2002), our finding that sphingolipid biosynthesis is mediated by TOR is therefore consistent with the previous findings, and presents further evidence for TOR function as a central controller of cell growth. The detailed mechanism by which TORC2 regulates sphingolipid biosynthesis remains to be studied.

Acknowledgements

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Materials and methods

Strains, plasmids and media

The complete genotypes of yeast strains used in this study are listed in Table I. Standard techniques and media were used (Sherman 1991). Rich media, YPD and SDYE, and synthetic complete media with 2% glucose (SD) has been described previously (Sherman 1991).

Spot assay

Cells in mid-log phase were diluted in a ten-fold dilution series (10x, 100x, 1000x, and 10000x). 4 μl of each dilution were spotted onto a YPD plate containing the indicated drags. Growth was scored after two or three days at 24°C or the indicated temperature.

Constructions of the domain-specific AVO3 deletion mutants

The domain-specific deletion mutants of *AVO3* were constructed by the two-step overlapping PCR method. For the first PCR, two sets of primers were designed. The N-terminus set was for amplification from -500 bp of the start codon of *AVO3* to a nucleotide just before the domain to be deleted. The reverse primer had a 40 bp extension at the 3' end which complements a sequence just after the deletion site. The C-terminal set was for amplification from just after the deletion site to +500 bp of the terminal codon. The forward primer of the C-terminus set contained a 40 bp extension at the 3' end which complements a sequence just before the deletion site. With a yeast genome as a template, the first PCR was performed, and the PCR products were subjected to the second PCR. The second PCR was done with the first PCR products as templates and primers. The second PCR products were purified and inserted into the *Saccharomyces/ E.coli* plasmid vector YCplac33.

Lipid labeling, extraction, and thin layer chromatography

[^{3}H]*myo*-inositol, [^{3}H]palmitate, and [^{3}H]serine labeling of yeast cells was performed for two hours at 24°C or 37°C. Lipids were extracted, and analyzed by thin layer chromatography as described (Zanolari, Friant et al. 2000). Briefly, 20 OD_{600} equivalent of cells grown in SD media with [^{3}H]*myo*-inositol or [^{3}H]palmitate were

harvested, and labeling was stopped by addition of NaN3/NaF mixture. The cells were broken by vortex with glass beads and chloroform/methanol/water (10/10/3) to extract lipids. The supernatant were treated with 0.6 M NaOH at 30 min for at least 90 min and neutralized by 0.6 M acetic acid. Lipids in the supernatants were purified by BuOH. Same amounts of [^{3}H], counted by a scintillation counter, were loaded onto a thin layer chromatography.

Mass spectrometry-based lipid profiling

The mass spectrometry-based approach to profile lipids was performed as described (Guan and Wenk 2006). Briefly, 25 OD_{600} equivalent of cells were resuspended in 2 ml of 95% ethanol/water/diethyl ether/pyridine/ammonium hydroxide (15:15:5:1:0.018) with internal standards. Cells were broken by glass beads vortexing, and lipids were extracted and desalted by butanol extraction. The sphingolipid-enriched fraction was subjected to mild alkaline hydrolysis to degrade ester bonds of glycerophospholipids. Lipids were then extracted with chloroform and dried under nitrogen. For characterization/identification as well as quantification of lipid molecular species, electrospray-ionization mass spectrometry and tandem mass spectrometry was performed. Obtained data sets were normalized, and warped against a reference set. Relative difference in the lipid compositions of the mixtures can be computed by calculating the logarithm (log10) of the ratio of ion intensities relative to control samples.

Table I. Strains used in this study

Strain	Genotype
TB50**a**	MAT**a** leu2-3,112 ura3-52 trp1 his3 rme1 HML**a** leu
BAS65-1B	TB50a *avo3-1*
SF43-1C	TB50**a** *tor2::kanMX4* / pRS314*3HA-TOR2*
SH121	JK9-3d**a** *tor2::ADE2 ade2* / YCplac111::*tor2-21^{ts}*
SH229	JK9-3da ade2 his3 HIS4 tor1::HIS3 tor2::ADE2 / YCplac111::tor2-29
SW70	TB50**a** *3HA-TOR2*
RS38-4C	TB50**a** bit2::KanMX bit61::KanMX
RS88-6D	TB50**a** avo2::NAT bit2::KanMX bit61::KanMX

PART III

Conclusions and Perspectives

Cell growth of yeast, or any organism, is an essential process for its life. Therefore, understanding the mechanisms behind cell growth leads to better comprehension of life, one of the ultimate objectives of biology (and human beings). In addition, cell growth is a critical step of cancer proliferation. Many genes that encode proteins mediating cell growth are potential oncogenes, and thereby are possible candidates for drug discovery. In spite of intensive efforts to identify and characterize growth-related genes and proteins, we still do not yet have a satisfactory explanation for several fundamental questions of cell growth (Broach 2005). Cell growth is defined as accumulation of cell mass, but how does mass accumulation translate into information which cells can interpret, or how does a cell sense its size? Nutrient limitation specifically blocks G_1/S transition, but how do cells sense nutrient availability in either the intercellular or the extracellular environments? And how do cells translate such information into cell cycle progression? Is cell growth controlled by a central processing pathway, or by multiple signaling cascades? Understanding of cell growth is still its way.

Here, we describe a functional analysis of TORC2 signaling pathway, and reveal that TORC2, but not TORC1, mediates sphingolipid biosynthesis. However, the control mechanism has not been elucidated. Our findings include two major indications. One is a crosstalk between TOR and lipid biosyntheses, and the other one is the downstream networks of TORC2.

TORC2 and lipid biosynthesis

Cell growth implicates mass accumulation and surface expansion. Therefore, cell has to coordinate both processes during cell growth. Lipid synthesis, degradation, recycling, and transport have to be regulated in response to growth. As a spatial controller of cell growth, TORC2 function on sphingolipid biosynthesis fits the idea. Then, does TORC2 mediate the other lipid biosyntheses?

For this, it is not clear answer yet. However, our lipid profiling results of TORC2 deficient cells showed reduction of phosphatidylserine (PS) levels. This is the first indication that TORC2 might mediate phospholipid biogenesis. PS is a membrane phospholipid that is ubiquitously present in membranes of eukaryotic and prokaryotic cells. Unlike mammals, PS in yeast is the major phospholipid, comprising 34% of total phospholipid (Zinser, Sperka-Gottlieb et al. 1991). Reduction of PS in TORC2 deficient cells indicates downregulation of PS synthesis and/or up-regulation of PS lipase. PS synthesis are carried out by PS synthase, which is one of the most highly regulated enzymes of phospholipid metabolism (Carman and Henry 1999). So far, we can found several reports that mention regulation of PS synthase by transcriptional level (Carman, Iwata et al. 1982; Poole, Homann et al. 1986; Bailis, Poole et al. 1987; Homann, Bailis et al. 1987), by phosphorylation by protein kinase A (Kinney and Carman 1988), by the other phospholipids (Bae-Lee and Carman 1990), by sphingoid bases (Wu, McDonough et al. 1995), by inositol (Kelley, Bailis et al. 1988), by CTP (McDonough, Buxeda et al. 1995), and the availability of zinc (Iwanyshyn, Han et al. 2004). In construct, degradation of PS in yeast has been not well-studied. Phospholipases, PLB1, PLB2, and PLB3 can hydrolyze PS, but also phosphatidylinositol, which does not significantly change both in steady-state level and two hour labeling by [^{3}H]-inositol (Figure). It remains to elucidate whether these factors are the link between PS synthase activity and TORC2. We should note that although reduction of PS is observed, level of phosphatidylcholine (PC) is just slightly decreased. Because PC is produced from PS, reduction of PC could be expected. This gap of two phospholipid species is also to be uncovered.

The other lipid species found in yeast is ergosterol, yeast equivalent to cholesterol of mammals. There is little evidence that indicate connection between TORC2 and ergosterol. However, the genetic interactions of ergosterol genes and sphingolipid genes result in a requirement for both lipids for lipid raft formation, and therefore changes in composition of each lipid species should affect raft properties.

, has strong genetic interactions with sphingolipid biosynthesis. However, several reports have proposed that So there is a possibility that TOR mediate ergsterol biosynthesis via sphingolipid. The genetic interaction of ergosterol genes and

sphingolipid genes probably result in that both lipids are required for the lipid raft formation and thereby changes in composition of each lipid species affect the raft property. An *erg26* temperature sensitive mutant that does not synthesize ergosterol at non-permissive temperature is defective in complex sphingolipid synthesis and hydroxylation of ceramide which SCS7 catalyzes (Swain, E sterol). Another connection of sterol and sphingolipid has been shown in an *erg24* mutant. Although, this mutant is lethal, the additional deletion of the fatty acid elongase *ELO3* can suppress (Veen, M 2005, ergosterol). In contrast to the *erg24* mutant, an *erg6* deletion mutant is viable, but is synthetic lethal with an *ELO3* deletion mutant (Eisnkolb, M 2002). A coordination of sphingolipid and sterol biosynthesis has also been described that down regulation of ergosterol biosynthesis pathway by azole anti-fungicides demonstrates significant reduction of expression of *SUR2* and *LCB1* (Bammert, GF 2000, ergosterol).

The TORC2 downstream networks

Our findings indicate that sphingolipid biosynthesis is a novel downstream target of the TORC2 signaling pathway. Interestingly, the other TORC2 readout, actin cytoskeleton organization and endocytosis, are also mediated by sphingolipids, specifically sphingoid bases, via the mammalian PDK1 homolog PKH proteins (Friant, Lombardi et al. 2001; deHart, Schnell et al. 2002). Furthermore, two known substrates of TORC2, YPK kinases and SLM proteins, are involved in actin cytoskeleton organization and endocytosis (Chen, Zheng et al. 1995; Roelants, Torrance et al. 2002; Schmelzle, Helliwell et al. 2002; Audhya, Loewith et al. 2004; Fadri, Daquinag et al. 2005; Kamada, Fujioka et al. 2005). Although we did not see an endocytosis defect of *tor2*^{ts} and *AVO3-1* in our strain background, the piled evidences for a functional connection between actin and endocytosis and downstream networks of TORC2 (Figure) point out that TORC2 is a controller of endocytosis.

There are several evidence that support involvement of TORC2 in regulation of endocytosis. Genetic and microscopic analyses have indicated that the transient formation of actin patches at the cell cortex is an important step in endocytosis. Screening mutants that are defective in α-factor uptake has resulted in isolation of

several genes related to actin cytoskeleton; *ACT1*, the only yeast actin gene; *SAC6*, encoding the F-actin cross-linking protein fimbrin, and *SLA2*, encoding an actin-binding protein important for polarization of the actin cytoskeleton (Kubler and Riezman 1993; Raths, Rohrer et al. 1993; Munn, Stevenson et al. 1995). Deficiency of the Arp2/3 complex, a highly conserved actin nucleation center for the motility and integrity of actin patches (Machesky and Gould 1999), shows abnormal and depolarized actin patches. Temperature sensitive mutants of components of the Arp2/3 complex, either *ARP2* (*arp2-1*) or *ARC35* (*end9*), are also defective in uptake of uracil permease and α-factor, respectively, indicating that the Arp2/3 complex is also required for endocytic internalization (Munn and Riezman 1994; Moreau, Madania et al. 1996; Moreau, Galan et al. 1997). There is strong correlation between genes whose mutation displays in actin cytoskeleton defect, i.e., depolarized actin patches, and genes whose mutations show endocytic phenotype (Engqvist-Goldstein and Drubin 2003). These findings indicate that actin cytoskeleton organization is tightly connected to endocytosis; therefore it is very likely that TORC2 is involved in endocytosis event as well as actin cytoskeleton organization.

So, if TORC2 indeed participates in endocytosis, which step of endocytosis could be regulated by TORC2?

Endocytosis in yeast is supposed to occur at static sites marked by PIL1, LSP1, and SUR7 (Walther, Brickner et al. 2006). It is unknown mechanism, but the early markers of endocytosis, clathrin, AP180, EDE1, ENT1, and ENT2 accumulates the site where PIL1 localize. The next step in endocytosis is the recruitment of additional endocytic coat proteins SLA1, PAN1, END3, and SLA2, as well as the Arp2/3 activating protein, LAS17. Up to this step, these protein markers are non-motile (Kaksonen, Sun et al. 2003). Consequently, LAS17 promotes actin polymerization at the plasma membrane, which is also regulated by type I myosin MYO5 and MYO3, and BBC1 from the perspective of (Anderson, Boldogh et al. 1998; Evangelista, Klebl et al. 2000; Mochida, Yamamoto et al. 2002; Soulard, Lechler et al. 2002; Soulard, Friant et al. 2005). During this step, invagination of membrane is supposed to occur, and proteins show slow movement. The amphiphysin-like protein RVS161 and RVS167 are proposed to pinch off the endocytic vesicle (Breton, Schaeffer et al. 2001) and then actin

polymerization drives the vesicle inward. This is followed by disassembly of the endocytic coat and associated proteins. In this period, proteins are in fast movement, and the patches are moving away from their initial cortical position, toward the cell center (Kaksonen, Sun et al. 2003).

TORC2 deficient cells display actin depolarization, indicating that actin assembly at the endocytosis site already begins. Therefore TORC2 should mediate a step before the actin assembly. A potential target of TORC2 is myosins I, encoded by MYO5 and MYO3. Myosins I regulates actin assembly (Anderson, Boldogh et al. 1998), and is phosphorylated by YPK2 (Grosshans, Grotsch et al. 2006), which is a substrate of TORC2 (Kamada, Fujioka et al. 2005). In this context, TORC2 activates MYO5 via YPK2, and progress endocytosis. In contrast, TORC2 deficient cells could loose its kinase activity, and thereby be unable to activate myosins I to sustain endocytosis. Consistent with this, *MYO5* temperature-sensitive allele shows a strong defect in the internalization step of endocytosis (Geli and Riezman 1996). Another potential target of TORC2 is PIL1 and LSP1. The proteins are involved in an immobile protein complex that localize at the cell periphery (Walther, Brickner et al. 2006). These proteins are supposed to mark a site of endocytosis, and known to negatively regulate PKH and down stream pathways PKC1 and YPK1 (Zhang, Lester et al. 2004).

If TORC2 is indeed required for endocytosis, it is possible that one of the upstream signals toward TORC2 is a cell cycle dependent, since endocytosis i.e., actin cytoskeleton are organized in a cell cycle dependent manner. However, further studies on TORC2 would provide useful insights to answer the fundamental questions of cell growth, shown in the beginning of this session.

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Appendix

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Publications

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