INTRODUCTION

During cell growth and division, many physiological processes need to be coordinated and modified according to nutrient availability. Signaling through target of rapamycin (TOR) kinases plays a central role in this regulation. Even though TOR kinases are related to phosphoinositide lipid kinases, they are Ser/Thr protein kinases, with a small number of known targets that regulate many cellular processes. Together, the diverse TOR signaling outputs regulate cell growth, both spatially and temporally (De Virgilio and Loewith, 2006; Reiling and Sabatini, 2006; Wullschleger et al., 2006).

The characterization of TOR signaling was greatly aided by the discovery of the antifungal and immunosuppressant macrocyclic lactone rapamycin. The search for targets of this drug in the yeast *Saccharomyces cerevisiae* lead to the discovery of the TOR kinases and subsequently helped to identify their molecular function (Heitman et al., 1991).

The rapamycin-sensitive branch of TOR signaling regulates processes that collectively modulate the rate of cell growth. TOR signaling is active under conditions of excess nutrients, blocking catabolic processes, such as autophagy and stress responses. In contrast, exposure to rapamycin or withdrawal of nitrogen or carbon sources lead to inactivation of TOR signaling and down-regulation of anabolic processes, such as protein synthesis. Together these processes coordinate cell growth with nutrient availability (De Virgilio and Loewith, 2006; Wullschleger et al., 2006). This function of TOR signaling is performed by TOR complex 1 (TORC1) that consists of the kinase plus three additional subunits in yeast, named Lst8, Kog1, and Tco89 or two subunits named raptor and mLst8 in mammals. Its molecular targets include translation initiation factors (e.g., eukaryotic initiation factor 4E, eIF4G, and eIF2) and the Sch9-kinase (homologue of PKB/Akt and p70S6 in mammals), which are thought to mediate its effect on translation. Sch9 is a member of the AGC-family of protein kinases (protein kinases A, G, and C) that form one level of the signaling network downstream of TOR kinases.

In addition, TOR kinase and Lst8 are present in a second complex that is not responsive to rapamycin and contains several different subunits, encoded by *AVO1, AVO2, AVO3*, and *BIT61* in yeast (Loewith et al., 2002; Wedaman et al., 2003; Reinke et al., 2004). Similarly to TORC1, the general architecture of this complex is conserved in evolution, and homologous subunits corresponding to Avo1 and Avo3 have been characterized in mammals and were named hSin1 and rictor, respectively (Jacinto et al., 2004; Sarbassov et al., 2004). Less is known about the regulation and targets of this second TOR complex, named TOR complex 2 (TORC2). In yeast, it is required for actin organization, efficient endocytosis and normal cell polarization (Schmidt et al., 1997, 1998; Loewith et al., 2002; deHart et al., 2003; Aronova et al., 2008). The effect on actin organization is conserved in mammals (Jacinto et al., 2004). In addition, TORC2 participates in the regulation of sphingolipid metabolism as functional TORC2 is required for maintaining normal ceramide levels in yeast (Beeler et al., 1998; Tabuchi et al., 2006; Aronova et al., 2008). This regulatory pathway is a component of an intricate signaling network emanating from TORC2 and the sphingolipid responsive Pkh-kinases (homologues of the mammalian PDK1) that regulate many aspects of cellular physiology through the combinatorial phosphorylation of AGC kinases, such as Ypk1/2 or Sch9 in yeast and Akt in mammals, respectively. Phosphorylation by both the TORC2 branch and the Pkh-kinases is required for full activation of AGC-
MATERIALS AND METHODS

Yeast Strains

All yeast strains and their genotypes used in this study are listed in Supplemental Table I. AVO1-GFP::HIS, AVO2-GFP::HIS, AVO3-GFP::KAN, BAP1-GFP::HIS, KOG1-GFP::HIS, and KOG1-GFP::KAN strains were obtained from W303 wild-type strain TWY138 by homologous recombination of polymerase II promoters and tagging of LSP1, with a RFpmars::NAT tagged fragment in TW680, TW701, and TW969 strains, respectively. Analogously, TWY815, TW969, and TW780 strains were generated by transforming SLA1-RFPmars, Aph1-RFPmars, and CRX1-RFPmars fragments with NAT” marker in TW680. TW980 was generated by transforming PMRA1-RFPmars::NAT in TW696. AVO1-GFP::KAN” was transformed in TWY779 to yield TWY808. TW722 was similarly generated by transformation and homologous recombination of AVO3-GFP::KAN” in the EDE1-RFP::hanging string strain TW367, which was described previously (Toshima et al., 2006).

The wild-type strains TWY318 and TWY319 were crossed, and zygotes were pulled to obtain the diploid wild-type strain TW806. TW825 was generated by homologous recombination of the PCR fragment of avo1-C::GFP::HIS in the diploid wild type. TW680 was crossed with the wild-type strain TWY319 to obtain TWY849, in which a avo1-C::Cam::X::NAT fragment was transformed (TWY860). Sporulation and dissection yielded TWY870. This strain was then crossed with TW779, and zygotes were pulled to obtain TWY882. Dissection of the latter yielded TWY891. TWY918 was generated by transformation and homologous recombination of NAT“::GAL-GFP::avol-C-term in TWY806.

Yeast Culture

Yeast strains were grown according to standard procedures. For growth curves, cells were diluted in 200 μl of YPD to OD600 = 0.1, and OD600 was measured every 20 min at 30°C under constant shaking (Bioscreen: Lab-“off, Oulu, Finland). Yeast spotting was performed on YPD plates, which were incubated at 24, 30, and 37°C, respectively. For vacuole membrane staining, cells were incubated 15 min on ice with 10 μM N-[3-triethylammoniumpropyl]-[6-[diethylaminophenylhexatrienyl]pyridinium dibromide (diTAMU-A364, 10 μM), washed three times with media, and incubated at 30°C for 2 h. For actin disassembly, cells were incubated 10 min at 30°C with 10 μM latrunculin A (LAT-A).

Microscopy

For fluorescence microscopy, cells were grown in synthetic media to OD600 = 0.6. Cells were then mounted on coverslips coated with concanavalin A. Cells were imaged with a laser-based spinning disk confocal microscope (Andor Technology, Belfast, Northern Ireland), built from an IMC body (TiLL/Agilent, Graefelfing, Germany). Filtered images (Samrock emission filters) were acquired with a 100xTIRFM objective with a numerical aperture of 1.45 (Olympus, Japan) and an Andor iMIC body (TiLL/Agilent, Amersfoort, The Netherlands). Lipids were dissolved in 150 mM NaCl, 50 mM Tris, pH 8.0, and 10% glycerol. The N-terminal His-Tagged avo1-C-term was expressed and purified by nickel-nitritotriacetic acid chromatography (Qiagen, Hilden, Germany), followed by buffer exchange using an HiTrap desalting column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The protein was stored in buffer containing 50 mM NaCl, 50 mM Tris, pH 8.0, and 10% glycerol.

Liposome Preparation

Liposomes were prepared by rehydration of 100% dioleoyl phosphatidylcholine (DOPC, Avanti Polar Lipids), or DOPE combined in a 97:3 molar ratio with dioleoyl phosphatidylserine (DOPE, Avanti Polar Lipids), dipalmitoyl phosphatidylinositol-4-phosphate (DPP4IP), dipalmitoyl phosphatidylinositol-3-phosphate (DPPI3P), dipalmitoyl phosphatidylinositol-3,4-phosphate (DPP34P) and dipalmitoyl phosphatidylinositol-4,5-phosphate (DPP45P) (all Matreya, State College, PA), respectively. Lipids were dissolved in CHCl3:ethanol (1:1, vol/vol) with 0.1% HCl and vortexed in a round-bottomed glass flask. Lipids were mixed according to the above-stated molar ratio to a final concentration of 25 mM. The solvent was evaporated under constant stream of argon gas for 10 min and subsequently dried in a desiccator for 4 h. Dried lipids were hydrated in 150 mM NaCl 50 mM Tris, pH 8.0, and 10% glycerol and vortexed extensively until solution was clear. After three freeze thaw cycles in liquid nitrogen, lipids were extracted (Mini extruder; Avanti Polar Lipids) through a 100 nm filter (Whatman, Maidstone, United Kingdom) and kept at 4°C.

Liposome Floitation Assay

Purified His6-avol-C-term (10 μM) was incubated in the absence or presence of 150 μM lipids in liposomes in a final volume of 50 μl for 1 h at 4°C. The liposome flotation assay was performed in a discontinuous sucrose gradient as described previously (Narayan and Lemmon, 2006). After incubation, 50 μl of 80% sucrose in 150 mM NaCl, 50 mM Tris pH 8.0, and 10% glycerol was added to the middle of the gradient.
added to the samples, mixed, and overlaid with 300 μl of 30% sucrose in the same buffer. The gradient was centrifuged at 55,000 rpm for 1 h at 4°C. Fractions of 50 μl were taken from the top and the bottom and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (4–12% gradient NuPAGE; Invitrogen), Coomassie staining, and silver staining.

RESULTS

TORC1 and TORC2 Complexes Localize to Distinct Cellular Compartments

Previous experiments investigating the subcellular localization of TOR complexes have relied on immune detection of tagged components after subcellular fractionation or electron microscopy and indirect immune fluorescence (Cardenas and Heitman, 1995; Kunz et al., 2000; Chen and Kaiser, 2003; Wedaman et al., 2003; Aronova et al., 2007). Because these techniques do not allow the visualization of protein localization with respect to other organelles in live cells, we aimed to fluorescently tag components of TORC1 and TORC2. We chose the yeast S. cerevisiae as a model system because genomic tagging in this organism allows one to express only the fluorescently labeled protein from their endogenous promoter. To follow the localization of both TOR complexes independently, we fused GFP tags to the exclusive subunits of TORC2 (Avo1, Avo2, Avo3, and Bit61), the TORC1-specific Kog1, and the shared subunit Lst8. The resulting strains expressing C-terminally GFP-tagged TOR complex components all grew normally at 30°C (Figure 1a, left). In contrast, C-terminally tagged alleles of TOR2 that can be found in both complexes were not viable (data not shown), consistent with a recent report (Sturgill et al., 2008).

Because TOR kinases react to stress, we also tested whether the tagged TOR complex subunits support growth at elevated temperature. Under heat stress at 37°C, all cells harboring tagged subunits of the TORC2 grew as well as the wild-type strain, but mutants carrying Kog1-GFP or Lst8-GFP did not grow (Figure 1a, right). This might indicate that the GFP interferes with the essential interaction of either Lst8 or Kog1 with the TOR kinase (Kim et al., 2003; Wullschleger et al., 2005).

In summary, GFP-tagged versions of essential Avo1 and Avo3 are fully functional, whereas KOG1-GFP and LST8-GFP are novel temperature-sensitive alleles of the TORC1 complex and both TOR complexes, respectively.

Initial imaging of TOR complexes on a conventional epifluorescence microscope showed only dim and barely detectable signals. To analyze the subcellular localization of TOR complexes, we therefore made use of recent advances in fluorescence microscopy, by using a highly sensitive electron multiplying charge-coupled device (EM-CCD) camera (99% quantum yield) combined with a spinning disk confocal microscope, a high numerical aperture objective (1.45 numerical aperture), and high optical magnification (200× after all components; see Materials and Methods).

Images of exponentially growing cells expressing Kog1-GFP or Bit61-GFP revealed distinct localizations of both TOR complexes. Whereas TORC1 marked by Kog1-GFP localized exclusively to one or a few central rings in all cells
(100% of cells; n > 200), which we suspected were vacuoles (Figure 1b, left), TORC2 labeled by Bit61-GFP localized in a peculiar, punctate pattern at the cell surface forming small foci in all cells observed (100%, n > 200; Figure 1b, right). The localization of neither TORC1 nor TORC2 components was dependent on growth conditions (Berchtold and Walther, unpublished observation). Consistent with its localization in both TOR complexes, Lst8-GFP was found in a pattern overlaying the two localizations at the cell periphery and the central rings (Figure 1b, middle). However, fewer foci were found at the cell periphery in Lst8-GFP compared with Bit61-GFP–expressing cells. The formation of fewer spots at the cell periphery might indicate that the GFP-tagged Lst8 protein does not get incorporated into TORC2 as efficiently as the wild type, consistent with the reduced functionality of LST8-GFP seen in growth assays at 37°C.

**TORC1 Localizes to the Yeast Vacuole**
To determine to which site in the cell the TOR complexes are localized, we performed double labeling experiments with different organelle markers. Because the TORC1 component Kog1-GFP localized in one or a few rings in the cell interior, suggesting a vacuolar localization, we tested whether the Kog1-GFP signal was located at the vacuolar delimiting membrane. Indeed, the Kog1-GFP signal colocalized completely with the vital dye FM4-64 used to label the vacuolar membrane (Figure 1c), confirming previous findings (Araki et al., 2005; Huh et al., 2003). Similarly, we observed a precise overlay for the ring-like Lst8-GFP signal in the center of the cells and vacuolar FM4-64 (data not shown), as it was proposed previously (Chen and Kaiser, 2003). This confirms that TORC1 indeed localizes to the vacuolar membrane in live cells, a finding supported by a report that the nonconserved TORC1 subunit Tco89 is also localized to the vacuolar membrane (Reinke et al., 2004; Urban et al., 2007).

**TORC2 Localizes to the Plasma Membrane**
TORC2 foci marked by Bit61-GFP localize exclusively in the cell periphery (Figure 1b, right). To test whether Bit61 localizes to the plasma membrane, we expressed a red fluorescently tagged plasma membrane protein Pma1-RFPmar in Bit61-GFP–expressing cells and imaged them by fluorescence microscopy. Midsection images show that Bit61-GFP foci completely overlap with the plasma membrane signal of Pma1-RFPmar (Figure 1d), confirming that Bit61-GFP is a plasma membrane protein. From these data, we conclude that the two TOR complexes have distinct and mutually exclusive localizations in the cell.

**All TORC2 Components Localize As Oligomeric Foci to the Plasma Membrane**
To investigate whether the other subunits of the TORC2 complex are localized in a pattern equivalent to Bit61, we analyzed fluorescence images of all tagged members of TORC2. Midsection (Figure 2a, left) and cell surface images (Figure 2a, right) revealed that they all localize to the plasma membrane in small foci of identical appearance.

To estimate the amount of each protein in a TORC2 focus, we related the GFP signal from single foci to a GFP standard. For this, we first analyzed the fluorescence intensity signal from GFP molecules bound to the Lac-operator in the nucleus that form one spot (Brickner and Walter, 2004). For each of the components, we observed a very similar median fluorescence. In summary, for all components, this value corresponds to 3.8 ± 0.4 GFPs (Figure 2b). In histograms of fluorescence intensities, we observed a trail in the distribution toward higher fluorescence intensities that might indicate formation of complexes with more subunits (see Supplemental Figure 1, A–F). To confirm these conclusion, we also GFP-tagged kinetochore proteins Cse4 and Spc105 as markers to quantify in vivo protein numbers (Joglekar et al., 2008). One kinetochore cluster of Cse4-GFP represents the fluorescence of 32 GFP molecules, whereas a kinetochore cluster of Spc105-GFP represents 80 GFP molecules. From this experiment, the median fluorescence of the four measured TORC2 components corresponded to 3.3 ± 0.4 GFPs (see Supplemental Figures 2 and 3, a–h), consistent with our measurements using the Lac-operator. Together, these data suggest that TORC2 foci at the plasma membrane represent higher order oligomers, with two to six copies of each subunit present in each focus.

**Plasma Membrane TORC2 Is Highly Dynamic**
During our analysis of TORC2 foci, we observed that their plasma membrane pattern is highly dynamic. To characterize this behavior in more detail, we recorded time-lapse movies of cells expressing Avo3-GFP. The movies showed...
Tracks of three different TORC2 foci from the first frame to bleached area. Bar, 5 min (right) is shown. Bottom panels show higher magnification of the image before the bleach (left), right after the bleach (middle), and after 6 one-half of a cell and followed TORC2 foci over time. A representative To confirm the appearance of TORC2 foci independently, we bleached surface and time. (f) TORC2 foci form in photo bleached regions of the cell. that within 10 s, the surface pattern of TORC2 changes almost completely (Figure 3a and Supplemental Movie 1). Further inspection of movies revealed that TORC2 complexes display random short lateral movements. We analyzed this movement and tracked individual TORC2 foci by using movies with 3-s intervals (Supplemental Movie 2). Figure 3b shows three such tracks from the first frame of the movies to the disappearance of the foci. Individual foci have quite a different overall behavior, but similar speeds of their movement, with an average of 0.039 μm/s (quantitated from n = 141 tracks). We only observed movement in the plane of the membrane and never detected detachment of a TORC2 focus from the plasma membrane. Besides the lateral movement four additional events contribute to the highly dynamic pattern: 1) appearance, 2) disappearance, 3) fusion, and 4) splitting of foci (Figure 3d and Supplemental Movies 3–6). Fusion was accompanied by doubling of fluorescence intensity, whereas splitting of foci resulted in a decrease of fluorescence (Figure 3d). To quantitate the behavior, we counted the number of spots occurring per time and found that TORC2 foci formed with a rate of 0.61 per μm² membrane surface and minute. Consistent, but slightly higher, results were obtained for the rate of foci disappearance, possibly due to bleaching of some spots (data not shown). In contrast, fusion and splitting of the foci were much less frequent and only occurred in a minority of image sequences.

To confirm the appearance and disappearance of TORC2 foci independently, we bleached one-half of the cell and followed fluorescence recovery over time. In optical midsections (Figure 3f and Supplemental Movie 7) and top sections (data not shown), TORC2 foci reappeared in a previously bleached area. In few cases, these could be attributed to migration of foci from the unbleached area of the cell. In most cases, however, the TORC2 focus occurred de novo. This basic characterization demonstrates that TORC2 focus formation is dynamic. To begin to analyze the molecular requirements for TORC2 localization, we performed analogous analysis of cells expressing Avo3-GFP in which we deleted either AVO2 or BIT61. TORC2 plasma membrane signals were not changed as measured by the density, velocity, and intensity of Avo3 foci, showing that these non-essential subunits are not required for TORC2 localization and dynamics. For bit61Δ cells, the rate of appearance of the TORC2 foci was elevated slightly, maybe indicating that Bit61 stabilizes the interaction with the plasma membrane (Supplemental Figure 4).

**Figure 3.** TORC2 localization at the plasma membrane is highly dynamic. (a) The TORC2 pattern at the plasma membrane changes rapidly. Surface images of cells expressing Avo3-GFP were collected in 400-ms intervals. The image t = 0 s is shown in green (left); the image t = 10 s is shown in red (middle). Overlay of the images shows that the pattern is highly dynamic. Bar, 2.5 μm. (b) TORC2 foci move in the plane of the membrane. Tracks of three different TORC2 foci from the first frame to their disappearance are shown. Markers indicate the position of the foci at 3-s intervals. (c) TORC2 foci move with an average speed of 0.039 μm s⁻¹. Tracks (141) such as shown in b were used to calculate the average speed of foci. (d) TORC2 foci undergo fusion, fission, and they appear and disappear. Analogously to a, movies with 3-s intervals were collected. Panels show representative time-lapse sequences of appearance, disappearance, fusion, and fission of foci. (e) On average, 0.61 TORC2 foci occur per minute on a square micrometer of cell surface. Appearances were counted in image sequences such as used for b and related to the cell surface and time. (f) TORC2 foci form in photo bleached regions of the cell. To confirm the appearance of TORC2 foci independently, we bleached one-half of a cell and followed TORC2 foci over time. A representative image before the bleach (left), right after the bleach (middle), and after 6 min (right) is shown. Bottom panels show higher magnification of the bleached area. Bar, 5 μm.

**TORC2 Foci at the Plasma Membrane Are Distinct from Actin Patches**

TORC2 regulates actin in both yeast and mammalian systems. In yeast, actin localizes in patches at the cell cortex and forms actin cables, found mostly in the cell interior. Actin patches mediate at least a subset of endocytic events. Because TORC2 is also required for efficient endocytosis (de-Hart et al., 2003), we hypothesized that TORC2 foci are part of actin patches. To test this, we analyzed the localization of Avo3-GFP in cells expressing different fluorescently labeled actin patch proteins. Because actin patches are highly dynamic structures of changing protein composition, we selected several proteins that are recruited at the early (Ede1, Sla1), later (Crt1), and at final stage (Abp1) of an actin patch assembly cycle (Kaksonen et al., 2003, 2005). Consistent with previous findings by Wedaman et al. (2003), we did not find any colocalization of Avo3-GFP with any of these proteins, either in surface snapshots (Figure 4a) or when we followed actin patches over time (Supplemental Movies 8 and 9). Furthermore, TORC2 did not localize at the sites of actin patch formation before or after their residence at the plasma membrane (Supplemental Movies 8 and 9). Thus, TORC2 is not a component of actin patches.

To determine whether the movement of TORC2 at the plasma membrane is dependent on actin, we incubated cells with the actin depolymerizing drug latrunculin A. This...
Figure 4. TORC2 foci are different from actin patches. (a) TORC2 foci do not colocalize with actin patches. Avo3-GFP (green) was expressed in cells expressing either Sla1, Ede1, Crn1, or Abp1 tagged with the RFPmars fluorophore (red). Representative surface images are shown; insets show parts of the cell surface in higher magnification. Bar, 5 µm. (b) TORC2 lateral mobility is not dependent on actin. Cells expressing Abp1-RFPmars (red) and Avo3-GFP (green) were treated with latrunculin A [+ (LAT)-A] for 10 min (right). This lead to complete disassembly of Abp1 patches. Kymographs of representative movies show Avo3-GFP signal along the perimeter of control and LAT-A treated cells for a period of 90 s (bottom).

treatment efficiently disassembled actin patches as expected. For example, Abp1-RFPmars lost its normal patch localization and was present in a diffuse cytoplasmic signal (Figure 4b, top right). When we investigated the motility of TORC2 under these conditions, we did not detect any changes compared with untreated controls, i.e., neither TORC2 localization at the plasma membrane nor its dynamics were significantly changed compared with untreated controls (Figure 4b, bottom). From these results, we conclude that the TORC2 foci are independent from actin patches and that actin is not required for TORC2 motility.

TORC2 Localizes in a Distinct Plasma Membrane Domain

To further characterize the membrane environment of TORC2, we asked whether it localizes to a specific membrane subcompartment. The plasma membrane of *S. cerevisiae* is organized in two mutually exclusive membrane domains that are composed of different lipids and proteins. One of these domains is organized by large cytosolic protein complexes termed eisosomes and harbors several transporter proteins, such as Can1 (Malinska et al., 2003; Malinska et al., 2004); Walther et al., 2006; (Grossmann et al., 2007). Accordingly, it was termed membrane compartment occupied by Can1 (MCC). The MCC is organized by large protein complexes termed eisosomes that localize there beneath the plasma membrane and consist of two major subunits, Pil1 and Lsp1. Deletion of Pil1 results in the collapse of the MCC to one or a few clusters at the plasma membrane (Grossmann et al., 2006; Walther et al., 2006). Besides several specific proteins, the MCC also has a distinct lipid composition, as visualized by accumulation of the sterol binding fluorescent dye filipin (Grossmann et al., 2007). A mutually exclusive domain is characterized by the presence of Pma1 and was therefore named membrane compartment occupied by Pma1 (MCP).

To determine to which of these plasma membrane domains TORC2 localizes, we imaged surfaces of cells expressing one of the TORC2-specific subunits fused to GFP and the RFPmars-tagged eisosome component Lsp1. We never observed colocalization between the two proteins (Figure 5a). This is readily apparent in fluorescence intensity line profiles showing clearly separated peaks for each channel (Figure 5b). Simultaneous imaging of GFP-tagged TORC2 subunits and Lsp1-RFPmars over time clearly shows that TORC2 foci do not overlap with eisosomes during the course of the experiment (Supplemental Movie 10). Consistent with these observations, TORC2 localizes normally in *pil1Δ* cells, where the MCC collapses to one or a few remnants per cell (Supplemental Figure 5; Walther et al., 2006; Grossmann et al., 2007).

Because the MCC is mutually exclusive with MCP, our data predicted that TORC2 would occupy the latter plasma membrane domain. To test this directly, we analyzed the localization of Bit61-GFP in cells expressing Pma1-RFPmars. To our surprise, we found that TORC2 marked by Bit61-GFP localized in areas excluding Pma1 (Figure 5c). This is particularly evident in fluorescence intensity line profiles through the cell surface (Figure 5d). Instead of being a component of MCP, TORC2 occupied domains that were devoid of Pma1 (Figure 5c, top, see magnified inset). In addition, we always observed plasma membrane regions devoid of fluorescence signal from either Pma1 or TORC2, and thus presumably representing MCC (Figure 5c, bottom, see magnified inset). TORC2 therefore defines a previously unrecognized plasma membrane compartment that we name membrane compartment containing TORC2 (MCT).

Plasma Membrane Targeting of TORC2 through the C-Terminal Domain of Avo1 Is Essential

To investigate the function of TORC2 plasma membrane binding and to begin analyzing the mechanism of TORC2 membrane binding, we wanted to identify its membrane anchor. From sequence inspection, homology modeling, and previous studies in mammalian systems, we suspected that
the C terminus of Avo1 could potentially form a Pleckstrin homology (PH)-like domain (Schroder et al., 2007; and Ziolkowska, Berchtold, and Walther, unpublished observation). PH domains most often bind phosphoinositide-(4,5)-bisphosphate [PI(4,5)P$_2$], a plasma membrane-specific lipid in yeast (Lemmon, 2008; Odorizzi et al., 2000). Therefore, the C terminus of Avo1 was a good candidate to mediate TORC2 plasma membrane anchoring.

To test whether the C terminus of Avo1 is required for localization and/or function of TORC2, we deleted the last 117 amino acids of Avo1 in one of the genomic copies of diploid cells. Analysis of the haploid progeny from a sporulation of these heterozygous cells showed that only spores with the wild-type allele of AVO1 were viable, whereas the avo1-C/H9004 allele was lethal (Figure 6a). The C terminus of Avo1 is therefore essential for yeast viability.

The requirement for the C terminus could be explained by at least three alternative hypotheses: it could be necessary for stability or folding of Avo1, its stable incorporation into TORC2, or if it indeed formed a PH-domain, for lipid binding and its localization to the plasma membrane. To distinguish between these possibilities, we tested whether replacing the C terminus by a heterologous plasma membrane targeting signal was sufficient to support yeast growth. To achieve this, we replaced the C terminus of Avo1 with a CaaX-motif, which gets enzymatically lipidated (Onken et al., 2006). Surprisingly, given that the Avo1 C terminus is required for viability, cells harboring the deletion allele replaced by a short CaaX-box had no discernible growth defect under any condition investigated, as seen for example in solid media growth assays (Figure 6b) or in growth curves obtained from liquid cultures (data not shown).

To examine the dynamics of artificially plasma membrane targeted TORC2 complexes, we investigated avo1-C/H9004-CaaX cells with GFP-tagged Avo3. To our surprise, the localization and dynamics of TORC2 complexes targeted to the plasma membrane by the CaaX-box membrane anchor was indistinguishable from wild type, both in the pattern of the foci (Figure 6c), their size (Figure 6d), their density on the plasma membrane and their dynamics of movement (Figure 6f), and turnover (Figure 6g). From these data, we conclude that the C-terminal domain of Avo1 mediates the essential plasma membrane targeting of TORC2.

To further determine whether the Avo1 C-terminal domain is sufficient for plasma membrane targeting in vivo, we fused the avo1 C-term sequence in frame with the C terminus of GFP and expressed it from the GAL promoter. After induction of GFP-avo1 C-term expression, we detected cytoplasmic GFP fluorescence and accumulation of GFP signal at the plasma membrane (Figure 7a, left). This was corroborated by line scans through midsections of confocal images (Figure 7a, right).

We next asked whether the C-terminal sequence of Avo1 directly mediates lipid binding, as its similarity to a PH-domain suggests. Because PH-domains often bind PI(4,5)P$_2$ or other phosphoinositides, we focused on these lipids. To investigate a possible lipid interaction, we expressed the

Figure 5. TORC2 foci localize to distinct plasma membrane domains. (a) TORC2 foci localize to a plasma membrane domain distinct from MCC/eisosomes. The indicated subunits of TORC2 were expressed as GFP fusion proteins (green) in cells also expressing a RFPmars-tagged eisosome component (red). Insets show a higher magnification of the area indicated. Bar, 5 μm. (b) Line scan of the indicated region of a Bit61-GFP/Lsp1-RFPmars image obtained as in a. (c) TORC2 foci localize to a plasma membrane compartment different from MCP. Bit61-GFP (green) was expressed in cells also harboring the MCP marker Pma1-RFPmars (red). Representative surface images are shown. Insets show a higher magnification of the area indicated. Bar, 2.5 μm. (d) Line scan of the indicated region of a Bit61-GFP/Pma1-RFPmars image analogous to c.
Avo1 C-terminal domain (amino acids 1054–1176) as a 6xHis-fusion in *E. coli*, purified it to apparent homogeneity, and performed liposome flotation experiments. Figure 7b shows that Avo1 C-terminal domain floated to the top of a sucrose gradient (fraction T) bound to phosphatidylcholine (PC) liposomes containing 3% PI(4,5)P\(_2\), and to a much lesser extent to liposomes containing 3% PI(3,4)P\(_2\). No flotation of the Avo1 C-terminal domain was observed in reactions containing no liposomes or liposomes containing PC, PC/3% phosphatidylinositol (PI), PC/3% phosphatidylserine (PS), PC/3% phosphatidylinositol (3) phosphate [PI(3)P], or PC/3% phosphatidylinositol (4) phosphate [PI(4)P] (Figure 7b). Similar results were obtained with an independently generated glutathione transferase-tagged construct (Berchtold and Walther, data not shown).

Together, our in vivo data, biochemical observations, and the structural homology strongly suggest that the Avo1 C terminus forms a PH-like domain that can bind PI(4,5)P\(_2\) and is sufficient and necessary for plasma membrane targeting of TORC2.

**DISCUSSION**

TOR kinases are important regulators of cellular physiology and exist in two distinct complexes. We show that TORC2 localizes exclusively at the plasma membrane, segregated from TORC1, which is found only at the vacuolar membrane. TORC2 did not colocalize with previously known functional membrane compartments and instead defines a distinct compartment that we name the MCT.

The spatial segregation of TOR kinases in different complexes is conceptually similar to the targeting of protein kinase A (PKA) to a variety of subcellular localizations.
TORC2 Plasma Membrane Localization

(Smith et al., 2006). In that case, the same PKA is recruited by A-kinase anchoring proteins, which are in turn bound to different organelles or the cytoskeleton. This segregation helps to provide specificity for G protein-coupled signals that relay information for example on growth factors availability via cAMP to PKA. Analogously, one could see Avol as a TOR plasma membrane anchoring protein within TORC2. Similarly, TORC1 must contain (a) targeting subunit(s) for the vacuole. Because Kog1 is the only TORC1-specific component that is conserved through evolution, it is a good candidate for such an anchor.

Scaffolding and subcellular segregation of kinase complexes is a recurrent theme in signal transduction. In yeast, also the mitogen-activated kinase MAKK Ste11 is present in two complexes that mediate the response to pheromone and changes in osmolarity, respectively. Again, this segregation is important for signaling specificity and to prevent cross talk between pathways (Schwartz and Madhani, 2006). From these examples, it seems likely that one function of TOR anchoring to the plasma membrane and the vacuole is to separate the output of the kinases. Consistent with this notion, the TORC2 target Ypk1 is also localized to the plasma membrane, but not to the vacuolar membrane (Berchtold and Walther, unpublished observation; Sun et al., 2000). Conversely, the TORC1 target Sch9 is localized exclusively to the vacuolar surface (Jorgensen et al., 2004; Urban et al., 2007). Because both of these target kinases are quite similar (44% identity) and could therefore be recognized by each TORC, their separation is likely required to ensure signaling fidelity.

In addition, TOR localization might be important for the detection of input signals. Consistent with its function in nutrient sensing, we found TORC1 localized exclusively to the yeast vacuole, which is a major nutrient reservoir in yeast. This is in agreement with previous data, reporting Tor2 kinase (a member of both TORC1 and TORC2) localization at the vacuolar membrane (Cardenas and Heitman, 1995). Indeed, several studies underscore a prominent role for vacuolar functions in TORC1 signaling (Aronova et al., 2007; Zurita-Martinez et al., 2007; Puria et al., 2008). Contrary to previous reports, we did not detect Kog1-GFP in the nucleus or the plasma membrane (Aronova et al., 2007; Tsang and Zheng, 2007). However, our results do not exclude the possibility of a small nondetectable, but biologically significant pool of Kog1 at those locations or that TORC1 gets targeted to other locations after altering conditions. At the vacuolar membrane, TORC1 patches are less pronounced than those of TORC2. Together with the high mobility of yeast vacuoles and the lack of spatial landmarks in the vacuolar membrane, this precludes further analysis of TORC1 dynamics.

Compared with TORC1, far less is known about the input signals of TORC2. Because it is required for maintaining normal ceramide levels (Beeler et al., 1998; Tabuchi et al., 2006; Aronova et al., 2008), one model suggests that TORC2 senses ceramide levels to maintain their level in a feedback loop. In this scenario, its localization at the plasma membrane where most cellular ceramides are localized could be important for sensing. However, ceramides are mostly present in the outer leaflet of the plasma membrane (van Meer and Lisman, 2002), so it is unlikely that TORC2 bound to the cytoplasmic face is regulated by directly binding ceramides. Ceramides are the major class of sphingolipids in yeast and are thought to form specialized liquid ordered plasma membrane domains together with ergosterol, the main yeast sterol (Simons and Ikonen, 1997; Bagnat and Simons, 2002). TORC2 could therefore indirectly sense sphingolipid levels by responding to alterations in plasma membrane structure.

At the plasma membrane, TORC2 localizes to neither of the previously recognized plasma membrane domains (Malinska et al., 2003, 2004; Grossmann et al., 2006; Walther et al., 2006) but instead occupies a distinct compartment that we name MCT. The TORC2 foci at the plasma membrane represent higher oligomeric forms of the complex. One possible model of TORC2 assembly and dynamics is that individual TORC2 complexes are bound to the plasma membrane where they interact to form dynamic higher order complexes. This model is supported by our observation that TORC2 foci are formed, fall apart, and can fuse and segregate.

The formation of TORC2 foci might be important for the regulation of its activity because multimerization was correlated previously with kinase activity biochemically (Wullschleger et al., 2005). TORC2 foci formation in the MCT is probably mediated by a mechanism distinct from its membrane anchoring by P(T)xy, because targeting to the plasma membrane by a heterologous lipid anchor also results in MCT localization.

How many proteins are exclusively targeted to MCT, MCP, or MCC and whether these domains are really segregated or represent examples from a continuous spectrum of behaviors of plasma membrane proteins are not yet clear. It is also not clear whether MCC, MCP, and MCT have distinct lipid compositions. The MCC was shown previously to be enriched in ergosterol, as visualized by the fluorescent sterol-binding dye filipin (Grossmann et al., 2007). Also, the MCP might be rich in these lipids as its highly abundant Pma1 component is used as a marker for lipid rafts, lateral membrane domains rich in ergosterol and sphingolipids (Bagnat et al., 2000; Lee et al., 2002). It is therefore possible that only the MCT represents the nonraft plasma membrane domain in yeast, enriched in phospholipids, but poor in sterols and sphingolipids. Consistent with this notion, proteins lipidated at a CaaX-box similar to the one targeting TORC2 to the MCT, were found previously in such raft-deficient membrane domains (Zacharias et al., 2002).

CaaX-box anchored TORC2 was constitutively bound to the membrane, presumably due to embedding of the lipid moiety in the bilayer. This artificially anchored TORC2 is fully functional and its dynamics are indistinguishable from the wild type, arguing that TORC2 does not have to go through association/dissociation cycles with the plasma membrane for its function. Consistently, we never observed dissociation of wild-type TORC2 foci from the membrane into the cytoplasm.

Downstream of TORC2 at least some signals are transduced to the conserved Ypk-kinases, which also localize to the plasma membrane (Kamada et al., 2005; Berchtold and Walther, unpublished observation). For full activation, Ypk-kinases need both phosphorylations by TORC2 at the conserved HM-site and by Pkh-kinases at the activation loop (Kamada et al., 2005). Even though both TORC2 and Pkh-kinases localize to the cell cortex, they are in different compartments: Pkh-kinases were previously found in the MCC/eisosomes, separated from the MCT (Walther et al., 2007). This sequestration of kinases of the same signaling network into distinct plasma membrane compartments provides opportunity for an additional level of regulation. It also necessitates communication between compartments to achieve complete phosphorylation of targets such as Ypk2. In the case of the TORC2/Pkh-signaling network, this regulation could be mediated at least in part by the Slm-proteins that are targets of both TORC2 and Pkh-signaling (Audhya et al.,...
2004; Fadri et al., 2005; Daquinag et al., 2007). Sm-proteins are required for some of the output of the network, e.g., actin polarization, placing them downstream of TORC2/Pkh-kinases in the network (Audhya et al., 2004; Daquinag et al., 2007). The notion that Sm-proteins mediate between the TORC2 and Pkh-branch of the signaling network is supported by their localization in both the MCC/eisosomes and also outside these domains in an MCT pattern (Berchtold and Walther, unpublished observation). Further work will be needed to determine their role in TORC2/Pkh signaling.

Interestingly, the Pkh-kinases are regulated by sphingolipids (specifically sphingoid long chain bases (Friant et al., 2001; Liu et al., 2005). This suggests that the TORC2/Pkh-kinase network integrates information on sphingolipid levels and transduces the information via Ypk-kinases to regulate many cellular functions, such as lipid metabolism, cell wall integrity, and actin polarization. Ceramides and long-chain bases often have opposing effects on cells. The balance of their amounts is therefore critical and was termed the ceramide/long chain base rheostat. The physical basis of this control mechanism might be the TORC2/Pkh-kinase signaling module.

The overall features of this signaling network and the TOR complex architecture are evolutionary conserved (Casamayor et al., 1999; Jacinto et al., 2004; Sarbassov et al., 2004). Therefore, it is likely that our findings are also relevant for higher eukaryotes. Indeed, it was observed that TORC2 can localize to the plasma membrane in human embryonic kidney 293 cells (Schoroder et al., 2007). Whether and how the homologous mTORC2 and PDK1 signaling complexes are organized spatially at the plasma membrane, particularly in situations relevant to disease, such as insulin signaling, remains a fascinating topic for future research.

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REFERENCES


Supplementary Figure 1: Histograms of fluorescence intensities of TORC2 foci. Fluorescence intensities of TORC2 foci or a nuclear spot containing 128 GFPs were measured on images from strains expressing the indicated GFP tagged TORC2 components. Histograms of the measurements are shown either alone (a-e, h) or combined (f). To test the linearity of detection, fluorophores were serially diluted over the range of measurement of TORC2 complexes and 128 GFP nuclear foci and the resulting fluorescence intensity was plotted against the concentration of the fluorophore (g).

Supplementary Figure 2: Plasma membrane foci of TORC2 subunits have a similar median fluorescence. Each TORC2 focus contains 2-6 copies of each subunit. GFP signals measured from unprocessed cell surface images were quantitated and related to the fluorescence intensity of two GFP-tagged kinetochore cluster proteins, Cse4 and Spc105. The median of the signal from more than 200 measurements is shown as a red diamond, the mean is shown as a white line in a grey box, outlines of the box indicate the 0.25 and the 0.75 quartiles. Error bars are 1.5 inter-quartile ranges from the mean. For details see Supplementary Figure 3 and materials and methods.

Supplementary Figure 3: Histograms of fluorescence intensities of TORC2 foci. Fluorescence intensities of the plasma membrane foci of the indicated GFP tagged TORC2 components or the kinetochore cluster of Cse4-GFP and Spc105-GFP, respectively, were measured as described in Materials and Methods. Histograms of the measurements are shown either alone (a-d, f-h) or combined (e).

Supplementary Figure 4: AVO2 and BIT61 are not required for normal TORC2 localization and dynamics. (a) Avo3-GFP was expressed in either wt, avo2Δ or bit61Δ cells and representative confocal midsections and transmitted light images are shown. (b) TORC2 foci have normal intensity in avo2Δ or bit61Δ cells. (c) TORC2 foci have the same density in wildtype and avo2Δ or
*bit61Δ* cells. Surface images were used to calculate the number of foci per $\mu$m$^2$.

(d) TORC2 foci have the same velocity in wildtype and in *avo2Δ* or *bit61Δ* cells. Velocities in the plane of the membrane were calculated as in Figure 3c. (e) Rate of TORC2 foci appearance in wildtype and *avo2Δ* or *bit61Δ* cells. Rates were calculated as in Figure 3e.

**Supplementary Figure 5:** *PIL1* is not required for TORC2 localization in plasma membrane foci. Avo3-GFP was expressed in either wild type or *pil1Δ* cells. Fluorescent images representing midsections (right panel) or transmitted light images (left panels) are shown.

**Supplementary Table 1:** Genotypes of all strains used in this study are listed, together with their name and reference to their first publication.

**Supplementary Movie 1:** Representative surface movie of cells expressing Avo3-GFP. Images were recorded with 400 ms intervals.

**Supplementary Movie 2:** Representative surface movie of cells expressing Avo3-GFP. Images were recorded with 3 seconds intervals. A single Avo3-GFP focus was manually tracked over a time period of 105 seconds. Line (green) indicates the covered distance of the focus.

**Supplementary Movie 3:** Representative surface movie of cells expressing Avo3-GFP, which demonstrates the appearance of two Avo3-GFP foci in frame 3 and 9, respectively. Images were recorded with 3 seconds intervals.

**Supplementary Movie 4:** Representative surface movie of cells expressing Avo3-GFP, which demonstrates the disappearance of an Avo3-GFP focus in frame 8. Images were recorded with 3 seconds intervals.
**Supplementary Movie 5:** Representative surface movie of cells expressing Avo3-GFP, which demonstrates the fusion of Avo3-GFP foci. Images were recorded with 3 seconds intervals.

**Supplementary Movie 6:** Representative surface movie of cells expressing Avo3-GFP, which demonstrates the fission of an Avo3-GFP focus. Images were recorded with 3 seconds intervals.

**Supplementary Movie 7:** Representative midsection movie of cells expressing Avo3-GFP. Images were recorded with 30 seconds intervals. The indicated region was bleached in the third frame.

**Supplementary Movie 8:** Representative surface movie of cells expressing Avo3-GFP (green) and Sla1-RFPmars (red). Images were recorded with 3 seconds intervals.

**Supplementary Movie 9:** Representative surface movie of cells expressing Avo3-GFP (green) and Abp1-RFPmars (red). Images were recorded with 3 seconds intervals.

**Supplementary Movie 10:** Representative surface movie of cells expressing Avo3-GFP (green) and Lsp1-RFPmars (red). Images were recorded with 3 seconds intervals.
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Alexa 488 concentration [nM]    fluorescence intensity [a.u.]

Avo1-GFP

frequency [%]

fluorescence intensity [a.u.]

0 50 100 150 200

Avo2-GFP

frequency [%]

fluorescence intensity [a.u.]

0 50 100 150 200

Avo3-GFP

frequency [%]

fluorescence intensity [a.u.]

0 50 100 150 200

Bit61-GFP

frequency [%]

fluorescence intensity [a.u.]

0 50 100 150 200

combined histogram of TORC2 foci

Avo1

Avo2

Avo3

Bit61

fluorescence intensity [a.u.]

0 50 100 150 200

128 GFPs

frequency [%]

fluorescence intensity [a.u.]

0 1500 3000 4500 6000

Avo3-GFP avo1-C Δ-CaaX

fluorescence intensity [a.u.]

0 50 100 150 200

flourescence intensity [a.u.]

Alexa 488 concentration [nM]

0 10 10 100 1000 10000 100000

0 1 10 100 1000 10000 100000

Berchtold and Walther 2008, Supplementary Figure 1
Berchtold and Walther 2008,  Supplementary Figure 2

Fluorescence intensity [a.u.]

- Avo1
- Avo2
- Avo3
- Bit61
- Avo3
- avo1-CΔ-CaaX
- 1 GFP (Cse4)
- 1 GFP (Spc105)
Wt
avo2Δ
bit61Δ

transmitted
Avo3-GFP

Fluorescence intensity [a.u.]

Fluorescence intensity [a.u.]
wt  avo2Δ  bit61Δ

Density of foci [µm²]
wt  avo2Δ  bit61Δ

Appearance velocity [µm sec⁻¹]
wt  avo2Δ  bit61Δ

Transmitted Avo3-GFP

Berchtold and Walther 2008, Supplementary Figure 4
Berchtold and Walther 2008, Supplementary Figure 5
transmitted  Kog1-GFP  transmitted  Lst8-GFP

24 °C

10 min 37 °C

30 min 37 °C

1 h 37 °C

2 h 37 °C