TORC2 Plasma Membrane Localization Is Essential for Cell Viability and Restricted to a Distinct Domain

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The conserved target of rapamycin (TOR) kinases regulate many aspects of cellular physiology. They exist in two distinct complexes, termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2), that possess both overlapping and distinct components. TORC1 and TORC2 respond differently to the drug rapamycin and have different cellular functions: whereas the rapamycin-sensitive TORC1 controls many aspects of cell growth and has been characterized in great detail, the TOR complex 2 is less understood and regulates actin polymerization, cell polarity, and ceramide metabolism. How signaling specificity and discrimination between different input signals for the two kinase complexes is achieved is not understood. Here, we show that TORC1 and TORC2 have different localizations in *Saccharomyces cerevisiae*. TORC1 is localized exclusively to the vacuolar membrane, whereas TORC2 is localized dynamically in a previously unrecognized plasma membrane domain, which we term *membrane compartment containing TORC2* (MCT). We find that plasma membrane localization of TORC2 is essential for viability and mediated by lipid binding of the C-terminal domain of the Avo1 subunit. From these data, we suggest that the TOR complexes are spatially separated to determine downstream signaling specificity and their responsiveness to different inputs.

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* led 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 [eIF]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-

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kinases (Inagaki *et al.*, 1999; Kamada *et al.*, 2005; Lee *et al.*, 2005; Sarbassov *et al.*, 2005; Urban *et al.*, 2007).

The importance of both TORC1 and TORC2 for cellular physiology is highlighted by the fact that several components of each complex (Kog1, Lst8, Avo1, Avo3, and Tor2) are essential for viability of yeast.

How the two different TOR complexes achieve their different essential functions is not known. It is also unclear how cross-talk from the identical kinases is prevented and how specific signaling in response to signals for either complex is achieved. To begin to address these fundamental questions, we investigated the subcellular localization and dynamics of the TOR complexes *in vivo* by using *S. cerevisiae* as a model.

MATERIALS AND METHODS

Yeast Strains

All yeast strains and their genotypes used in this study are listed in Supplemental Table 1. *AVO1-GFP::HIS*, *AVO2-GFP::HIS*, *AVO3-GFP::KAN^R*, *BIT61-GFP::HIS*, *LST8-GFP::HIS*, and *KOG1-GFP::HIS* were generated in the W303 wild-type strain TWY138 by homologous recombination of polymerase chain reaction (PCR)-generated fragments as described previously (Janke *et al.*, 2004). TWY776, TWY777, and TWY778 strains were generated by PCR-mediated tagging of *LSP1*, with a *RFPmars::NAT^R* fragment in TWY680, TWY701, and TWY696 strains, respectively. Analogously, TWY815, TWY699, and TWY780 strains were generated by transforming *SLA1-RFPmars*, *Abp1-RFPmars*, and *CRN1-RFPmars* fragments with *NAT^R* marker in TWY680. TWY801 was generated by transforming *PMA1-RFPmars::NAT^R* in TWY696. *AVO1-GFP::KAN^R* was transformed in TWY779 to yield TWY808. TWY722 was similarly generated by transformation and homologous recombination of *AVO3-GFP::KAN^R* in the *EDE1-RFP*-harboring strain TWY367, which was described previously (Toshima *et al.*, 2006).

The wild-type strains TWY138 and TWY139 were crossed, and zygotes were pulled to obtain the diploid wild-type strain TWY806. TWY825 was generated by homologous recombination of the PCR fragment of *avo1-CA-GFP::HIS* in the diploid wild type. TWY680 was crossed with the wild-type strain TWY139 to obtain TWY849, in which a *avo1-CA-CaaX::NAT^R* fragment was transformed (TWY860). Sporulation and dissection yielded TWY877. This strain was then crossed with TWY779, and zygotes were pulled to obtain TWY882. Dissection of the latter yielded TWY891. TWY918 was generated by transformation and homologous recombination of *NAT^R::GAL::GFP-avo1-C-term* in TWY806.

Yeast Culture

Yeast strains were grown according to standard procedures. For growth curves, cells were diluted in 200 μ l of YPD to OD₆₀₀ = 0.1, and OD₆₀₀ was measured every 20 min at 30°C under constant shaking (Bioscreen; Lab-systems, Helsinki, 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 μ M N-[3-triethylammoniumpropyl]-4-[p-diethylaminophenyl]hexatrienyl]pyridinium dibromide (FM4-64) (in dimethyl sulfoxide), 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 μ M 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 iMIC body (TiLL/Agilent), a CSU22 spinning disk (Yokogawa, Amersfoort, The Netherlands), diode pumped solid state lasers (488 and 561 nm), and a FRAPPA module (TiLL/Agilent, Gräfelfing, Germany). Filtered images (Sammrock emission filters in a Sutter filter wheel) were recorded with a D-977 iXon EMCCD+ camera (Andor Technology) after twofold magnification (Andor Technology) by using a 100 $\times$ TIRFM objective with a numerical aperture of 1.45 (Olympus, Tokyo, Japan), resulting in a measured pixel size of 0,086 μ m at a magnification of 200 $\times$.

For surface and midsection snapshots, single frames with exposure times ranging between 400 and 500 ms were averaged four times. To examine the dynamic behavior of TORC2 components, exposure time and laser intensity were reduced to a minimum, and only two times averaging of single frames was applied. Time-lapse movies were recorded with variable delays of 1–30 s between exposures.

For quantification of the green fluorescent protein (GFP) foci size of all TORC2 components, snaps without or with two times averaging were taken from the cell surface. Identical settings were used to obtain images of the nuclear spot of 128 GFPs (TWY804) and the kinetochore cluster of Cse4-GFP

and Spc105-GFP, respectively. Next, we tested whether our microscopy setup linearly records fluorescence intensity over the measurement range by determining fluorescence intensities of serial dilutions of a fluorophore with similar absorbance and emission spectra as GFP. To this end, we imaged serial dilutions of the fluorescent dye Alexa488 (Invitrogen, Carlsbad, CA) two times each. The mean fluorescence intensity of each acquired image was measured, background intensity (water sample) was subtracted, and values of the duplicate sample were averaged. Intensity values were plotted against the concentration of Alexa488 by using logarithmic scales, and a power trend line was added (Supplemental Figure 1G).

We found that the measured fluorescence intensity was linearly proportional to the amount of fluorophores. From these experiments, we calculated an arbitrary GFP fluorescence unit and related these measurements to the intensities observed for individual TORC2 foci labeled with each of the specific subunits.

Image Processing and Calculation

Images were processed using ImageJ software (<http://rsbweb.nih.gov/ij/>) and the MBF ImageJ for Microscopy collection of plug-ins (<http://www.microphtonics.ca/imagej/>). Single channels were separately processed using a median filter with 1-pixel radius and adjusting brightness and contrast. For time-lapse series and stacks, a median 3D filter was applied.

For quantification of the GFP foci size of all TORC2 components, the mean fluorescence intensity of each single detectable GFP foci was measured in a region of 4 $\times$ 4 pixels. The same region of interest was used to obtain mean values of the background and the nuclear background, respectively. Mean background values were averaged and subtracted from each measured value of the foci. Histograms for all values of each TORC2 component, 128 GFPs, Cse4-GFP, and Spc105-GFP were compiled using Excel (Microsoft, Redmond, WA). To get an approximate fluorescence intensity value for a single GFP, the average intensity of the 128 GFP spot was divided by 128, the average value for a Cse4-GFP cluster was divided by 32, and the average intensity of a Spc105-GFP kinetochore cluster was divided by 80. Box plots were used for comparison of the signal intensity of GFP foci (of 4 different TORC2 components) with the approximate value of a single GFP.

For quantification of the dynamic behavior of TORC2 foci, time-lapse movies (10 frames; 3-s intervals) were recorded, and the background was subtracted. For every single cell, a region as small as possible containing all Avo3-GFP foci was designed, and the area was measured. All foci were tracked manually over time using the Manual Tracking Plugin (ImageJ). Obtained data were used for further analysis of density and velocity of foci and their direction of movement using Excel (Microsoft).

Protein Expression and Purification

For bacterial expression of the HIS⁶-tagged *avo1* C terminus, the sequence coding for the last 123 amino acids of *AVO1* were amplified by polymerase chain reaction (PCR) from purified yeast genomic DNA and cloned into a pETM14-cdB vector. Positive plasmids were identified by sequencing and transformed in *Escherichia coli* BL21 (DE3) Rosetta.

The N-terminal HIS⁶-tagged *avo1* C-term was expressed and purified by nickel-nitrilotriacetic acid chromatography (QIAGEN, Hilden, Germany), followed by a buffer exchange step using HiTrap desalting column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The protein was stored in buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, and 10% glycerol.

Liposome Preparation

Liposomes were made of either 100% dioleoyl phosphatidylcholine (DOPC, Avanti Polar Lipids), or DOPC combined in a 97:3 molar ratio with dioleoyl phosphatidylserine (DOPS, Avanti Polar Lipids), dipalmitoyl phosphatidylinositol (DPPi), dipalmitoyl phosphatidylinositol-3-phosphate (DPPI(3)P), dipalmitoyl phosphatidylinositol-(4)-phosphate (DPPI(4)P), dipalmitoyl phosphatidylinositol-3,4-phosphate (DPPI(3,4)P₂) and dipalmitoyl phosphatidylinositol-4,5-phosphate (DPPI(4,5)P₂) (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-mentioned 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 extruded (Mini extruder; Avanti Polar Lipids) 15 times through a 100-nm filter (Whatman, Maidstone, United Kingdom) and kept at 4°C.

Liposome Flotation Assay

Purified HIS⁶-*avo1* C-term (10 μ M) was incubated in the absence or presence of 4 mM lipids in liposomes in a final volume of 50 μ l 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 μ l of 80% sucrose in 150 mM NaCl, 50 mM Tris pH 8.0, and 10% glycerol was

Figure 1. Functional GFP-tagged TORC1 and TORC2 subunits localize to the vacuolar and the plasma membrane, respectively. (a) GFP-tagged TORC2 subunits Avo1, Avo2, Avo3, and Bit61 support normal yeast growth, whereas *LST8-GFP* and *KOG1-GFP* are temperature-sensitive alleles. The indicated subunits of TOR complexes were tagged with GFP in their endogenous locus (see *Materials and Methods*). The resulting strains were grown in Log-phase, serially diluted, spotted on YPD medium and grown at 30°C (left) and under heat shock conditions at 37°C (right). (b) Kog1-GFP, Lst8-GFP, and Bit61-GFP localize to distinct places. Cells expressing the indicated GFP fusions were analyzed by confocal microscopy. Representative fluorescent images (bottom) and transmitted light images (top) are shown. Bar, 5 μm. (c) Kog1-GFP localizes to the vacuolar membrane. Kog1-GFP (left) localization was analyzed by fluorescence microscopy in cells with FM4-64 marked vacuoles (middle). The right panel shows an overlay of the channels. Representative confocal midsections are shown. Insets indicate higher

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

magnification of the indicated part of the image. Bar, 5 μm. (d) Bit61-GFP localizes to the plasma membrane. Bit61-GFP (left) localization was analyzed by fluorescence microscopy in cells expressing Pma1-RFPmars (middle). The right panel shows an overlay of the channels. Representative confocal midsections are shown. Insets indicate higher magnification of the indicated part of the image. Bar, 5 μm.

(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-RFPmars 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-RFPmars (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 indi-

Figure 2. All subunits of TORC2 localize to the plasma membrane in a similar pattern. (a) All exclusive TORC2 subunits show a similar pattern at the plasma membrane. Strains carrying the indicated subunits of TORC2 as the only allele of the corresponding gene were analyzed by confocal microscopy. Representative midsections (left) and top sections (right) are shown. White lines delineate the perimeter of the cell. Bar, 5 μ m. (b) Each TORC2 focus contains two to six copies of each subunit. GFP signals measured from unprocessed cell surface images were quantitated and related to a GFP standard. The median of the signal from >100 measurements is shown as a red diamond, the mean is shown as a white line in a gray box, outlines of the box indicate the 0.25 and the 0.75 quartiles. Error bars are 1.5 interquartile ranges from the mean. For details, see Supplemental Figure 1 and *Materials and Methods*.

cate 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

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 $0.039 \mu\text{m s}^{-1}$. 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 .

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-range 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 $0.039 \mu\text{m/s}$ (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 μm^2 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 *bit61* Δ 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).

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 (Crn1), 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 μ m. (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 led 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 50 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 *pil1* Δ 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

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

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)P₂], 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-CΔ* 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-CΔ-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)P₂ or other phosphoinositides, we focused on these lipids. To investigate a possible lipid interaction, we expressed the

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.

Figure 6. Plasma membrane localization of TORC2 is essential. (a) The C-terminal domain of Avo1 is essential. The C-terminal 117 amino acids of Avo1 were deleted in a diploid strain; the resulting heterozygote sporulated and the progeny were dissected. A representative dissection is shown. Marker analysis revealed that progeny with the C-terminal part of Avo1 deleted are not viable. (b) A heterologous CaaX-box lipidation signal can rescue the deletion of the Avo1 C terminus. The C-terminal 117 amino acids of Avo1 were replaced by a CaaX box in a diploid strain. The resulting cells were sporulated and dissected. The progeny showed no growth phenotype on either a spotting plate (b) or in liquid culture as analyzed by optical density measurements at 600 nm over time. (c) The CaaX box replacing the C terminus of Avo1 is sufficient to mediate normal plasma membrane targeting of TORC2. *avo1-Δ-CaaX* was expressed in cells harboring a fluorescently tagged Avo3 (green) and Lsp1-RFPmars (red). Cells were imaged by fluorescence microscopy and representative midsections (top), top sections (middle), and higher magnification inserts (bottom) are shown. Bar, 5 μm . (d) TORC2 foci have normal size in *avo1-Δ-CaaX* cells. Foci from images as shown in c were analyzed as in Figure 2b. (e) TORC2 foci have the same density in wild-type and *avo1-Δ-CaaX* cells. Surface images were used to calculate the number of foci per square micrometer. (f) TORC2 foci have the same velocity in wild-type and *avo1-Δ-CaaX* cells. Velocities in the plane of the membrane were calculated as in Figure 3c. (g) TORC2 foci have the same rate of appearance in wild-type and *avo1-Δ-CaaX* cells. Rates were calculated as in Figure 3e.

Figure 7. The C-terminal domain of Avo1 binds to plasma membrane PI(4,5)P₂. (a) The C-terminal domain of Avo1 is sufficient to mediate plasma membrane targeting. The C-terminal 117 amino acids of Avo1 were fused to GFP and expressed from the Gal promoter. A representative midsection is shown (left). The right panel shows a line profile through the midsection of the cell, as indicated in the left panel. Bar, 5 μm . (b) The C-terminal domain of Avo1 binds to PI(4,5)P₂. Amino acids 1054–1176 of Avo1 were fused to a 6xHis-Tag, expressed in *E. coli*, and purified by affinity chromatography. The pure protein was incubated 1 h with liposomes containing only PC, PC with 3% PS, PC with 3% PI, PC with 3% PI(3)P, PC with 3% PI(4)P, PC with 3% PI(3,4)P₂, or PC with 3% PI(4,5)P₂. Subsequently, the samples were loaded onto a sucrose gradient, spun, and the floating fraction (T) was analyzed in comparison with the fraction remaining at the bottom of the gradient (B) by SDS-PAGE.

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)P₂, and to a much lesser extent to liposomes containing 3% PI(3,4)P₂. No flotation of the Avo1 C-terminal domain was observed in reactions containing no liposomes or liposomes containing only PC, PC/3% phosphatidylinositol (PI), PC/3% phosphatidylserine (PS), PC/3% phosphatidylinositide (3) phosphate [PI(3)P], or PC/3% phosphatidylinositide (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)P₂ 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

(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 Avo1 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 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 (Wullschlegel *et al.*, 2005). TORC2 foci formation in the MCT is probably mediated by a mechanism distinct from its membrane anchoring by PI(4,5)P₂, 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). Slm-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 Slm-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 (Schroder *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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