TORC2 and eisosomes are spatially interdependent, requiring optimal level of phosphatidylinositol 4, 5-bisphosphate for their integrity

KATELYN BARTLETT, SHIVA KUMAR GOU D GADILA, BRANDON TENAY, HYOEUN MCDERMOTT, BRETT ALCOX and KYOUNGTAE KIM

Department of Biology, Missouri State University, 901 S National Ave, Springfield, MO 65807, USA

*Corresponding author (Fax, +1-417-836-5126; Email, kkim@missouristate.edu)

The elucidation of the organization and maintenance of the plasma membrane has been sought due to its numerous roles in cellular function. In the budding yeast Saccharomyces cerevisiae, a novel paradigm has begun to emerge in the understanding of the distribution of plasma membrane microdomains and how they are regulated. We aimed to investigate the dynamic interdependence between the protein complexes eisosome and TORC2, representing microdomains MCC and MCT, respectively. In this study, we reveal that the eisosome organizer Pil1 colocalizes with the MCT marker Avo2. Furthermore, we provide evidence that the formation of MCT is dependent on both eisosome integrity and adequate levels of the plasma membrane phosphoinositide PI(4,5)P₂. Taken together, our findings indicate that TORC2, eisosomes, and PI(4,5)P₂ exist in an interconnected relationship, which supports the emerging model of the plasma membrane.

1. Introduction

The plasma membrane is essential for diverse cellular functions, including endocytosis, exocytosis, cell-to-cell communication, and signal transduction pathways. Its organization and maintenance is imperative for optimal biochemical activity. Many elements contribute to the makeup and management of the plasma membrane, most notably sterols, sphingolipids, phosphoinositides, and transmembrane and peripheral membrane proteins. The plasma membrane of eukaryotic cells harbours aggregates of specific lipids and proteins called lipid rafts, or microdomains (Simons and Sampaio 2011; Chiantia and London 2013). The budding yeast Saccharomyces cerevisiae is an excellent model organism for the study of microdomains, since mammalian microdomains are significantly smaller and more transient (Pike 2006; Klotzsch and Schutz 2013), deeming them more difficult to study. Yeast cells exhibit three microdomains: membrane compartment containing Can1 (MCC), membrane compartment containing Pma1 (MCP), and membrane compartment containing Tor2 (MCT) (Malinska et al. 2003; Berchtold and Walther 2009; Malinsky et al. 2010). MCP exists in a network and has been established as the primary site for endocytosis (Grossmann et al. 2008; Brach et al. 2011; Spira et al. 2012). MCC has attracted much attention due to its characteristic 50 nm-deep invagination and its tight association with the eisosome (Stradalova et al. 2009). Eisosomes are large, immobile protein complexes and were initially thought to mark sites of endocytosis (Walther et al. 2006), but more recent studies have confirmed that the
majority of endocytosis occurs at MCP (Grossmann et al. 2008; Brach et al. 2011). However, Murphy et al. provided evidence that the eisosome contributes to the maintenance of proper endocytosis due to the observation that loss of the eisosome organizer protein Pil1 leads to aberrant recruitment of endocytic proteins, and therefore inefficient endocytosis (Murphy et al. 2011). Nevertheless, the primary function of the eisosome has been difficult to define since its discovery. MCT is the most recently identified microdomain and is marked by the presence of the protein Tor2 and its corresponding complex target of rapamycin complex 2 (TORC2). TORC2 consists of the proteins Tor2, Lst8, Avo1, Avo2, Avo3, and Bit61 (Loewith et al. 2002; Wullschleger et al. 2005). Tor2, the main organizer of TORC2, is a highly conserved kinase that has proved to be an important regulator of multiple cellular functions including cell growth, actin polymerization, endocytosis, and sphingolipid synthesis (Cybulski and Hall 2009; Bartlett and Kim 2014). Despite significant advancements, the full understanding of the Tor2 signalling networks is incomplete. However, it has been an important topic of research for many years due to the implications of mTor in human diseases, such as cancer (Cornu et al. 2013; Shimohayashi and Hall 2014).

Previously, the model of the plasma membrane depicted microdomains as distinct and non-overlapping (Malinska et al. 2003; Grossmann et al. 2007; Malinsky et al. 2010). However, new evidences have begun to create a paradigm shift in the understanding of the organization of the plasma membrane and its functions, suggesting that microdomains exist in a more interdependent relationship than formerly suspected. One example of this was mentioned above in regard to the MCC-associated eisosome and endocytosis, a process that occurs at the MCP (Murphy et al. 2011). Furthermore, a recent study by the same group provided evidence that the loss of Tor2 function leads to a similar phenotype in which less synaptojanins and amphiphysins were observed at the endocytic site, leading to inefficient scission (Tenay et al. 2013). These two reports indicate that MCC/eisosome and MCT proteins influence a cellular task that takes place at the MCP. This concept along with many other evidences has influenced the adoption of a new model of the plasma membrane that is still being elucidated.

We sought to further characterize the organization and preservation of microdomains in budding yeast. Here, we present evidence that proteins of the eisosome and MCT do partially colocalize and play particular roles to regulate their localization. Furthermore, we investigated whether other components of the plasma membrane, such as sterols and phosphoinositides might also contribute to microdomains maintenance. Indeed, we observed that PI(4,5)P₂ is essential for normal assembly of MCT.

2. Materials and methods

2.1 Yeast strain construction and media

Yeast strains used in this study are listed in table 1. Strains expressing GFP- and RFP-fused proteins were constructed by integrating respective GFP or RFP sequence at the 3’ end of the gene of interest as described previously (Longtine et al. 1998; Campbell et al. 2002; Kim et al. 2006; Nannapaneni et al. 2010). To generate gene deletion mutants, wild-type cells were transformed with PCR products carrying a disruption construct as described before (Longtine et al. 1998). Resulting transformants on selective plates were screened by a confocal or conventional fluorescence microscope (Olympus IX81 ZCD2) and colony PCR to verify and collect those that correctly expressed the gene of interest. The mutant strains tor2Δ, in which TOR1 locus is replaced by a LEU2 cassette (HellIWell et al. 1994), and tor2Δts (a Tor2 PI kinase temperature-sensitive mutant harbouring the tor2Δts-allele) (Schmidt et al. 1996) were obtained from Michael Hall’s lab. TOR2 is an essential gene; therefore Kunz et al. replaced TOR2 with ADE2 and introduced a plasmid with a mutation in the kinase domain of Tor2 to produce the tor2Δts strain (Kunz et al. 1993). The TOR2-CEN-URA plasmid (table 2) was also introduced into Pil1-GFP tor2Δts (KKY 697) strain expressing Pil1-GFP via one-step transformation protocol (Chen et al. 1992). mss2Δ (KKY 413) was generously given by Scott Emr, and was made as previously described (Stefan et al. 2002). Yeast strains were grown in their respective selective media or in standard yeast peptone dextrose (YPD) media and cultured at either 30°C or 37°C.

2.2 Fluorescence microscopy

Still images of GFP and/or RFP labeled cells were made with a spinning disk confocal system that includes an inverted Olympus IX81 microscope, a Yokogawa CSUX1 spinning disk head, a 100× magnification, numerical aperture (NA) 1.4, PlanApo oil objective, and an Electron Amplified CCD (ImageEM, Hamamatsu). All images were obtained using confocal microscopy, unless otherwise indicated. Simultaneous two-color imaging was done using an image splitter to separate red and green emission signals. Conventional microscopy was used for strains KKY 1259, KKY 1269, KKY 0234, KKY 1334, KKY 1336, KKY 1366, KKY 1356, and KKY 1361. The temperature of the specimen and stage was maintained at 30°C or at 37°C as required. The image was focused at the equatorial plane of the cells.

2.3 Quantitation and statistical analysis

Images of GFP- and RFP-tagged membrane proteins were visualized using Slidebook (v5), and the spatial relationship....
<table>
<thead>
<tr>
<th>Strain Number</th>
<th>Strain Name</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>KKY 0002</td>
<td>Wt, Yeast Gene Collections (BY4741)</td>
<td>(Schmidt et al. 1996)</td>
<td>MATα his3Δ1 leu2Δ met15Δ ura3Δ</td>
</tr>
<tr>
<td>KKY 0234</td>
<td>Pil1-GFP</td>
<td>(Murphy et al. 2011)</td>
<td>Mat a his3Δ1 leu2Δ ura3Δ met15Δ PIL1-GFP-HIS</td>
</tr>
<tr>
<td>KKY 0423</td>
<td>mss4Δ</td>
<td>(Stefan et al. 2002)</td>
<td>MAT alpha leu2-3,112 ura3-2 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9, mss4Δ::HIS3 [CEN LEU2 mss4Δ]</td>
</tr>
<tr>
<td>KKY 0613</td>
<td>Pil1-GFP mss4Δ</td>
<td>This study</td>
<td>MAT alpha leu2-3,112 ura3-2 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9, mss4Δ::HIS3 [CEN LEU2 mss4Δ] PIL1-GFP-TRP</td>
</tr>
<tr>
<td>KKY 0812</td>
<td>Pil1-GFP erg4Δ</td>
<td>This study</td>
<td>Mat alpha his3ΔαΔleuΔtrpΔlysΔ ERG4::HIS3, PIL1-GFP-TRP1</td>
</tr>
<tr>
<td>KKY 0907</td>
<td>Pil1-GFP erg3Δ</td>
<td>This study</td>
<td>MATα his3Δ1 leu2Δ met15Δ ura3Δ PIL1-GFP-HISαΔ erg3Δ::KanMX6</td>
</tr>
<tr>
<td>KKY 0917</td>
<td>tor1Δ</td>
<td>(Schmidt et al. 1996)</td>
<td>Mat a leu2-3,112 ura3-2 rme1 trp1 his4 GAL+ HMLα tor1::LEU2-4</td>
</tr>
<tr>
<td>KKY 0918</td>
<td>tor2Δ</td>
<td>(Schmidt et al. 1996)</td>
<td>Mat a Leu2-3,112 ura3-2 rme1 trp1 his4 GAL+ HMLα ade2Δ tor2::ADE2-3 / YCplac111::tor2-21ts</td>
</tr>
<tr>
<td>KKY 0964</td>
<td>Pil1-GFP tor1Δ</td>
<td>This study</td>
<td>Mat a leu2-3,112 ura3-2 rme1 trp1 his4 GAL+ HMLα tor1::LEU2-4 Pil1-GFP-Trp</td>
</tr>
<tr>
<td>KKY 0967</td>
<td>Pil1-GFP tor2Δ</td>
<td>This study</td>
<td>Mat a Leu2-3,112 ura3-2 rme1 trp1 his4 GAL+ HMLα ade2Δ tor2::ADE2-3 / YCplac111::tor2-21ts Pil1-GFP-Trp</td>
</tr>
<tr>
<td>KKY 0990</td>
<td>Pil1-GFP avo2Δ</td>
<td>This study</td>
<td>Mat a his3Δ1 leu2Δ ura3Δ met15Δ PIL1-GFP-HIS Avo2::Kan</td>
</tr>
<tr>
<td>KKY 0991</td>
<td>Pil1-GFP bit61Δ</td>
<td>This study</td>
<td>Mat a his3Δ1 leu2Δ ura3Δ met15Δ PIL1-GFP-HIS Bit61::Kan</td>
</tr>
<tr>
<td>KKY 1202</td>
<td>Bit61-GFP pil1Δ</td>
<td>This study</td>
<td>MATα his3ΔαΔleuΔtrpΔlysΔ PIL1::KanMX6 (194) Bit61-GFP-TRP</td>
</tr>
<tr>
<td>KKY 1227</td>
<td>Avo2-GFP pil1Δ</td>
<td>This study</td>
<td>MATα his3ΔαΔleuΔtrpΔlysΔ PIL1::KanMX6 (194) Avo2-GFP-HIS</td>
</tr>
<tr>
<td>KKY 1238</td>
<td>Pil1-GFP tor2Δ</td>
<td>This study</td>
<td>Mat a Leu2-3,112 ura3-2 rme1 trp1 his4 GAL+ HMLα ade2Δ tor2::ADE2-3 / YCplac111::tor2-21ts Pil1-GFP-Trp Tor2-Cen-Ura</td>
</tr>
<tr>
<td>KKY 1259</td>
<td>Avo2-GFP</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Avo2-GFP-HIS</td>
</tr>
<tr>
<td>KKY 1261</td>
<td>Bit61-GFP</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Bit61-GFP-HIS</td>
</tr>
<tr>
<td>KKY 1269</td>
<td>Slm1-RFP Avo2-GFP</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Slm1-RFP-KanMX6 Avo2-GFP-HIS</td>
</tr>
<tr>
<td>KKY 1289</td>
<td>Pil1-RFP Avo2-GFP</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Slm1-RFP-KanMX6 Avo2-GFP-HIS</td>
</tr>
<tr>
<td>KKY 1334</td>
<td>Slm1-RFP Avo2-GFP erg3Δ</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Slm1-RFP-KanMX6 Avo2-GFP-HIS Erg3::Trp</td>
</tr>
<tr>
<td>KKY 1336</td>
<td>Slm1-RFP Avo2-GFP erg4Δ</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Slm1-RFP-KanMX6 Avo2-GFP-HIS Erg4::Trp</td>
</tr>
<tr>
<td>KKY 1356</td>
<td>Avo2-GFP erg4Δ</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Avo2-GFP-HIS Erg4::Trp</td>
</tr>
<tr>
<td>KKY 1361</td>
<td>Avo2-GFP mss4Δ</td>
<td>This study</td>
<td>MAT alpha leu2-3,112 ura3-2 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9, mss4Δ::HIS3 [CEN LEU2 mss4Δ] Avo2-GFP-TRP</td>
</tr>
<tr>
<td>KKY 1366</td>
<td>Avo2-GFP erg3Δ</td>
<td>This study</td>
<td>Mat alpha his3ΔuraΔneuΔtrpΔlysΔ Avo2-GFP-HIS Erg3::Trp</td>
</tr>
</tbody>
</table>
between the membrane proteins was quantified. For this, the percent of colocalization between them was determined by dividing the number of Avo2 puncta colocalizing with eisosome puncta by the total number of Avo2 puncta (n>50 cells), then multiplying by 100. The experiment was carried out three times, and then the average and standard deviation were found. The measurement of Pil1-GFP fluorescence intensity in wild-type and tor\(^{2ts}\) cells was obtained by using GraphPad Prism 5 software. Fluorescence intensity values of >50 cells per time point were used to create an XY scatterplot, and then average peak intensity was quantified by finding the area under the curve. Values between the peaks were used to represent the intensity in the cytoplasm. Values obtained from wild-type Pil1-GFP were normalized to 100%, to which all other values were standardized.

2.4 Immunofluorescence

Cells were grown overnight in YPD medium to OD 0.6, then fixed with formaldehyde (4% final concentration) directly to the media for 10 min, then permeabilized by sonication for 30 s. Cells were collected by centrifugation and resuspended in 1 ml milk washing three times with blocking buffer (Tris pH 7, 1M NaCl, 0.2% Tween, 10 units zymolase) and incubated for 45 min at 37°C. Cells were then collected by centrifugation and washed with buffer (phosphate buffer pH 7, sorbitol, β-mercaptoethanol, and 10 units zymolase) and incubated for 45 min at 37°C. Cells were then collected by centrifugation and washed with buffer (phosphate buffer pH 7, sorbitol) twice. Cells were collected by centrifugation and resuspended in milk blocking buffer (Tris pH 7, 1M NaCl, 0.2% Tween, 1% dry milk, 10 μg/ml BSA) and blocked for 20 min. Cells were centrifuged and resuspended in 1 ml milk blocking buffer and incubated for 1 hour with primary antibody (rabbit polyclonal Tor2 antibody: sc-33627, Santa Cruz Biotech, Santa Cruz, USA) using the recommended 1:50 dilution. Cell were centrifuged and washed three times with blocking buffer (Tris pH 7, 1M NaCl, 0.2% Tween, 10 μg/ml BSA). Cells were resuspended in 1 ml blocking buffer and incubated for 1 hour with secondary antibody conjugated to Texas Red (goat anti-rabbit IgG: sc-2780, Santa Cruz Biotech) using the recommended 1:100 dilution. Cells were centrifuged and washed with blocking buffer four times, then resuspended in blocking buffer and viewed on the spinning disk confocal system described above.

2.5 RT-qPCR

Wild-type (KKY 0002), wild-type Pil1-GFP (KKY 0234), and Pil1-GFP tor\(^{2ts}\) (KKY 0967) cells were grown to OD 0.5 at 30°C, then incubated at 37°C for 30 min. Total RNA was isolated using the NucleoSpin RNA II kit from Clontech (Mountain View, CA). RNA concentrations were set to equal concentrations and confirmed by 1% agarose gel electrophoresis. 0.5 μg total RNA was converted to cDNA using the Verso cDNA kit (Thermo Scientific, Pittsburg) according to the manufacturer’s protocol. RNA was heated to 70°C for 5 min, then quick-cooled to remove secondary structure. The RNA was then subjected to reverse transcription at 50°C for 60 min using a 3:1 mix of random primers and oligo-dT primers, followed by enzyme inactivation at 95°C for 2 min. Reaction products were diluted with nuclelease-free water to 100 μl for use as template in PCR.

Relative gene expression was measured using SYBR green-based real-time/quantitative PCR (qPCR). Relative expression values for the gene of interest were determined after normalization to expression levels of the endogenous reference gene Alg9 using the 2\(^{−}\Delta\Delta C_{t}\) method of relative quantification (Pfaf1 2001). PCR reactions were performed in duplicate with a final reaction volume of 25 μl consisting of 1 μl GoTaq qPCR master mix (Promega, Madison, USA), 10 μl cDNA template (50 ng), and 0.5 μl PCR primers (final concentration of 200 nM each). PCR was performed using an Mx3005P QPCR system (Agilent Technologies). PCR amplification was as follows: 95°C (10 min), followed by 40 cycles of 95°C (30 s), 60°C (60 s), and a final dissociation curve segment.

PCR primer pairs were designed using the SGD Gene/Sequence Resources and oligonucleotides were obtained from ITD (Integrated DNA Technologies). Primer pairs were designed with the following parameters: primer length of 25 nt, primer melting temperature in the range of 52–68°C, and amplicon length in the range of 150-200 bp. Gene of interest Pil1 primer sequence: F: 5′-GGTCTTGGAACAAGAATGTTGCGT-3′; R: 5′-AGCAGGCCCTGTTTCACTGGAGTG-3′. Reference gene Alg9 primer sequence: F: 5′-CATTTGCTGATGTGTTTCACCACTGACAG-3′; R: 5′-TGATGCCCATAATGGCCATAATCTC-3′.

2.6 Actinomycin D treatment

Logarithmically growing yeast cells expressing Pil1 (KKY 0234 and KKY 0967) were treated with 10μg/ml...
Microdomains and eisosome

303

actinomycin D for 6 h. Next, RNA extraction and RT-qPCR quantitation experiments were performed as described above.

2.7 Filipin III Staining/sterol fluorescence microscopy

Logarithmically growing yeast cells (WT, erg3Δ, and erg4Δ) were treated with formaldehyde (working concentration of 3.75%) for 20 min. The fixed cells were washed with water and treated with actinomycin-D (0.12 mg/ml culture) for 15 min in dark at room temperature, followed by microscopic analyses.

3. Results

3.1 Avo2 and Pil1 partially colocalize

Microdomains were previously described to be distinct and non-overlapping (Malinska et al. 2003; Grossmann et al. 2007; Malinsky et al. 2010). However, due to the dynamic nature of the plasma membrane, we hypothesized that colocalization might occur between proteins of different microdomains. To test this, we examined the spatial relationship between proteins of MCT and MCC/eisosome under wild-type conditions. We constructed a yeast strain that expresses Avo2-GFP and Slm1-RFP and observed that 19.5 ±6.5% of Avo2-GFP patches partially colocalize with Slm1-RFP under normal conditions (figure 1A, B). Avo2-GFP patches also exhibited 20.7±3.5% colocalization with the primary eisosome organizer Pil1 (figure 1B, C). Consistently, our colocalization experiment between Pil1-GFP and Tor2 by an immunofluorescence approach confirmed a spatial overlap between the eisosome and MCT (figure 1D).

Taken together, the results presented here and published data (Berchtold et al. 2012) indicate that the eisosome proteins Slm1 and Pil1 colocalize with TORC2.

3.2 Eisosome integrity is required for the proper organization of MCT

Other groups have shown that loss of Pil1 compromises eisosome integrity, which leads to the formation of fewer and larger eisosome remnants containing other eisosome and MCC factors (Walther et al. 2006; Grossmann et al. 2008). In light of finding that MCT is spatially overlapped with eisosome, we hypothesized that the integrity of MCT may be compromised upon the loss of normal eisosome assembly. To test this possibility, we examined the localization of Avo2- and Bit61-GFP in wild-type cells and pil1Δ cells. As expected, Avo2- and Bit61-GFP in pil1Δ cells were localized to pools reminiscent of eisosome remnants, while those fusion proteins in wild-type cells were located at the plasma membrane in punctated patches representing the MCT (figure 2A). The amount of Avo2-GFP and Bit61-GFP fluorescence in the cytoplasm was not significantly different in wild-type and the mutant cells (figure 2A, B). These results raised the question of whether a loss of TORC2 components may affect eisosome. Our fluorescence analysis shows that Pil1’s localization was unaffected by the loss of Avo2 or Bit61 (figure 2C).

3.3 Presence of Pil1 protein is increased in the absence of Tor2 activity

Next we investigated the effect of compromised Tor2 kinase activity on the localization of Pil1 using a tor2Δ strain harbouring a temperature sensitive mutation in TOR2. After shift-up from the permissive (30°C) to the non-permissive temperature (37°C), tor2Δ cells were collected and visualized at various time points (30 min, 2 h, and 5 h) to determine Pil1-GFP localization. Punctated Pil1-GFP pattern at the plasma membrane in both wild-type and tor2Δ mutant cells was not altered, but the cellular fluorescent intensity of the fusion protein was robustly elevated (figure 3A). In particular, the cytoplasm of tor2Δ mutant cells showed high Pil1-GFP fluorescence intensity when compared with wild-type cells. Our fluorescence intensity quantitation of Pil1-GFP at the plasma membrane and in the cytoplasm using GraphPad Prism 5 supports our observations that significantly more Pil1-GFP was found in the cytoplasm in tor2Δ mutant cells than in wild-type at each time point (figure 3B and figure legend). Tor1, a non-essential kinase of TORC1, functions at the vacuole (Loewith et al. 2002; Sturgill et al. 2008; Berchtold and Walther 2009). As expected, no alteration of Pil1-GFP was observed in cells lacking Tor1 (figure 3A). Although the presence of Pil1-GFP at the plasma membrane increased when tor2Δ mutant cell culture was shifted to the non-permissive temperature, its level gradually decreased over time, as is the case for wild-type cells. In order to examine whether the observed hyper-intensity of Pil1-GFP in the cytoplasm of tor2Δ cells was mainly due to the loss of Tor2 activity, we performed a complementation assay in which a plasmid harbouring full-length Tor2 was introduced to tor2Δ cells. As shown in figure 3A, the phenotype was partially rescued by the introduction of Tor2. This observation implies the possibility that another gene might be indirectly affected downstream by the loss of Tor2 activity, leading to an adverse effect on Pil1. These data indicate that upon loss of Tor2 kinase activity, the overall presence of Pil1 proteins is increased. To test whether Pil1 mRNAs is being more highly transcribed in the absence of active Tor2, we quantified the abundance of Pil1 mRNA using quantitative reverse transcriptase PCR (qRT-PCR).
Total RNA was isolated from wild-type cells, Pil1-GFP wild-type cells, and Pil1-GFP tor2 \(^{ts}\) cells after 30-minute incubation in the non-permissive temperature. Our results show that overall RNA expression of Pil1 is not significantly changed in tor2 \(^{ts}\) mutant cells (Figure 3 C), indicating that Tor2 does not notably affect Pil1 at the level of transcription. Our result raised a question of whether Pil1 mRNA stability or lifespan in tor2 \(^{ts}\) mutant cells is less affected negatively over time. To test our hypothesis, we treated wild-type and tor2 \(^{ts}\) cells with the mRNA synthesis blocker actinomycin D and measured Pil1 mRNA levels. After 6 h of treatment, Pil1 mRNA levels decreased rapidly in wild-type cells, but upon treatment of actinomycin D, more degradation of Pil1 mRNA levels was observed compared to Pil1 mRNA levels of non-treated wild-type cells. In tor2 \(^{ts}\) cells, after 6 h the levels of Pil1 mRNA even in the presence of actinomycin D was higher when compared with wild-type (supplementary figure 1). This experiment suggests that Pil1 mRNA in tor2 \(^{ts}\) mutant cells is more stable by unknown reasons, and this result is consistent with the finding in which Pil1-GFP fluorescence intensity is higher in tor2 \(^{ts}\) cells at the non-permissive temperature.
3.4 PI(4,5)P₂ is required for proper MCT organization

In addition to proteins, the plasma membrane consists of three lipid components: sphingolipids, sterols, and phosphoinositides. An interconnection between microdomain proteins and sphingolipids has been well documented (Aguilar et al. 2010; Dickson 2010; Mollinedo 2012; Niles et al. 2014). Ergosterol, the yeast counterpart of mammalian cholesterol, localizes to the MCC (Grossmann et al. 2007). In order to investigate whether ergosterol affects MCT stability, we generated ergosterol-mutant cells (erg3Δ and erg4Δ) that express Avo2-GFP. No apparent difference was observed compared to wild-type (figure 4A). As a control experiment, we also check the levels of sterols in WT and erg mutant cells by treating Filipin III (0.12 mg/ml culture) and by visualizing the fluorescence intensity of Filipin with fluorescence microscopy. As shown in the supplementary figure 2, sterol fluorescence intensity difference between WT and erg mutant cells was minimal. In good agreement with the unpublished data from Fröhlich and coworkers (Frohlich et al. 2009), Pil1-GFP localization was not disturbed in either erg mutants. However, an increase in cytoplasmic Pil1 was observed in cells lacking Erg4 (figure 4A). No noticeable difference in cytoplasmic Avo2-GFP fluorescence intensity was observed when compared to wild-type strains, most likely due to the characteristic high background of Avo2-GFP cells.

To test whether the lack of ergosterol affects the partial colocalization between eisosome and MCT, we examined the extent of spatial overlapping of Slm1-RFP with Avo2-GFP in cells lacking Erg3 or Erg4 (figure 4B). As shown in figure 4B, 22.6±0.86 % of Avo2-GFP patches colocalized with Slm1-RFP patches in erg3Δ cells and 25.9±2.24% in erg4Δ cells (figure 4B). The levels of colocalization between them were not significantly different than the observed colocalization levels in wild-type cells (figure 4B), suggesting that the presence of ergosterol holds little significance on MCT localization and its spatial organization with eisosome. We then wished to investigate the potential effect of

Figure 2. Pil1 is required for the formation of MCT. (A) Representative images of Avo2-GFP and Bit61-GFP in wild-type (KKY 1259 and KKY 1261, respectively) and in pil1Δ (KKY 1227 and KKY 1202, respectively) cells. Avo2 and Bit61 localized to pools reminiscent of eisosome remnants indicated by arrowheads. Scale bar represents 1 μm. (B) Fluorescence intensity quantitation of Avo2- and Bit61-GFP in WT and pil1Δ cells. (C) Representative images of Pil1-GFP in wild-type (KKY 0234), avo2Δ (KKY 0990), and bit61Δ (KKY 0991) cells. Scale bar represents 1 μm.
(A) WT  tor2ts  tor2ts + Tor2  tor1Δ

Time (minutes)
0  30  120  300

(B) Fluorescence Intensity in the Cytoplasm over Time

Fluorescence Intensity at the Plasma Membrane over Time

(C) Pil1 mRNA Expression

J. Biosci. 40(2), June 2015
phosphoinositide depletion on TORC2. The most abundant phosphoinositide in the plasma membrane is PI(4,5)P₂, which has been confirmed to play an important role in the assembly of eisosomes (Karotki et al. 2011; Kabeche et al. 2014). It has been shown that PI(4,5)P₂ level is decreased by 90% in cells lacking Mss4, which is an essential enzyme that converts PI(4)P to PI(4,5)P₂ (Desrivières et al. 1998). Notably, the level of PI(4,5)P₂ is unchanged in wild-type and mss4Δ cells at the permissive temperature (supplementary figure 3A, B). Consistent with previous findings (Karotki et al. 2011), mss4Δ cells in the non-permissive temperature exhibited Pil1-GFP puncta mislocalized to the cytoplasm (figure 4C). We examined Avo2-GFP localization in the mss4Δ cells both in the permissive and non-permissive conditions and found that Avo2-GFP puncta disappeared from the plasma membrane not only in the nonpermissive (figure 4C), but also in permissive (30°C) conditions (supplementary figure 4), suggesting that the mutant Mss4 protein was not fully functional even at the permissive temperature. Taken together, our observations indicate a vital role for PI(4,5)P₂ in the organization of MCT in addition to the MCC/eisosome.

4. Discussion

A long-standing membrane model in yeast proposes that the plasma membrane harbours 3 functionally and spatially distinctive microdomains: MCC, MCT and MCP (Malinska et al. 2003; Stradalova et al. 2009; Malinsky et al. 2010). However, lines of recent evidence have pushed back against the current model. One of the first lines of evidence came from the observations made by the Loewith group; they found that Slm1-2xRFPmars colocalizes with Avo3-GFP and that the extent of their colocalization is increased upon sphingolipid depletion by aureobasidin A treatment, while the presence of Slm1 at the eisosome decreases drastically (Berchtold et al. 2012). The studies presented here extend these earlier observations and demonstrate that another TORC2 component Avo2 exhibits a spatial overlap with the main MCC/eisosome organizer Pil1, making a strong case that the spatial correlation between MCC/eisosome and MCT exists. Based on these findings, including data from Loewith group, one can speculate that proteins of three microdomains (MCC, MCP, and MCT) can partially colocalize with one another in physiological conditions to form a more interconnected network between microdomains than previously thought. In support of this notion, Spira et al. recently reported the colocalization between the MCC protein Can1 and the MCP protein Pma1 (Spira et al. 2012). Although the significance of colocalizations between proteins of different microdomains remains elusive, one can postulate that the spatial correlation between them might lead to a functional connection between microdomains, by which a chemical signalling network is established. In this scenario, a loss of an important network-building protein may affect the structural integrity of neighboring microdomains and in turn their functional capacity is compromised at least in part, if not wholly. In agreement with this idea, loss of the main MCC/eisosome organizer Pil1 (Walther et al. 2006; Olivera-Couto et al. 2011) led to a severe defect in MCC formation, manifested by a drastic reduction in the number of MCC/eisosome (Walther et al. 2006; Grossmann et al. 2008; Stradalova et al. 2009). More importantly, we found that the disruption of MCC/eisosome negatively affects the integrity of MCT, based on the observation that the localization of two-GFP fused MCT proteins, Avo2-GFP and Bit61-GFP, were severely disrupted in pil1Δ cells. Interestingly, it appears that a loss of nonessential MCT component (Avo2 or Bit61) and a mutation in Tor2-kinase activity resulted in no major defects in Pil1 localization to MCC/eisosomes, suggesting that MCC/eisosome assembly may not heavily depend on the presence of MCT. It is therefore logical to speculate that MCC and MCT are interconnected, but clearly MCT is more labile and transient than MCC. However, the validity of this speculation and the full understanding of microdomain formation at each location and the formation of network between them are yet to be elucidated.

Although the instability of MCT due to a loss of Tor2-kinase activity did not alter Pil1’s membrane localization, we observed a robust increase in Pil1-GFP fluorescence intensity in the cytoplasm when Tor2 activity is lost. Given that Pil1 mRNA levels remain unchanged in tor2Δ cells at the non-permissive temperature, one possible explanation for our observation is that Pil1 mRNA might be more stable or its degradation may be limited, allowing for more mRNA to
be translated into Pil1 protein. Another possibility is that Pil1 protein, after translation, may also be stable in tor2Δ cells. These results indicate that Tor2 is a putative negative regulator for the production of Pil1. It has been known that cells with decreased Pil1 expression exhibited few eisosomes of normal size, while cells with more Pil1 had larger eisosomes, but at a fixed density of eisosomes, implying that a maximum eisosome threshold exists (Moreira et al. 2009). Based on these data, our best explanation for the increased Pil1-GFP cytoplasmic intensity is that the eisosome density
threshold is reached in \textit{tor}^{\text{ts}} mutant cells due to increase in the level of Pil1, forcing excess Pil1 to reside in the cytoplasm rather than at eisosomes. However, further investigation will be required to elucidate the direct mechanism by which this phenomenon occurs.

How important are the three major types of plasma membrane lipids (sphingolipids, phospholipids, and ergosterol) in the organization of microdomains? First, it appears that sphingolipid long-chain bases and PHS (phytosphingosine) play a critical role in the establishment and turnover of eisosomes and MCC (Walther et al. 2007; Luo et al. 2008) by regulating the phosphorylation activity of Pkh1 and Pkh2 (Sun et al. 2000; Friant et al. 2001; Liu et al. 2005). The Pkh1/2-mediated phosphorylation of Pil1 leads to eisosome disassembly, while eisosomes assembled upon Pil1 hypophosphorylation or reduction of Pkh1 kinase activity (Walther et al. 2007). However, the tentative sphingolipid role in MCC organization remain poorly understood at that moment. Second, it was found that proper amount of mature ergosterol is necessary for the structural integrity of MCC according to a genome-wide screen of \textit{erg} mutants (Grossmann et al. 2008). The possibility of ergosterol affecting the integrity of MCC was tested for the first time in the present study, and our observations revealed that both MCC/eisosome and MCC displayed normal phenotype in the absence of \textit{erg3} and \textit{erg4}, consistent with unpublished results by Frohlich et al. (2009). These findings reflect the minor or insignificance role that ergosterol plays in the homeostasis of MCC and MCT, yet providing one of the first clues for the relationship between TORC2-carrying MCC and ergosterol in yeast. A group of investigators recently showed that mTOR (mammalian Tor kinase) regulates cholesterol synthesis (Laplanne and Sabatini 2009). However, the question of whether yeast Tor2 signalling affects synthesis of ergosterol or its physiological function is of interest to be explored. Third, the phosphoinositide PI(4,5)P$_2$ is an essential component of the plasma membrane (Delage et al. 2013) that interacts with the MCC/eisosome proteins Slm1 and Slm2 to signal downstream to the actin cytoskeleton in conjunction with the Slm proteins’ activator TORC2 (Audhya et al. 2004; Fadri et al. 2005). Karotki et al previously showed that the significance of PI(4,5)P$_2$ for the integrity of MCC, with the finding that MCC organization was severely compromised upon the disruption of PI(4,5)P$_2$ (Karotki et al. 2011). In addition, Kabche et al. recently revealed that PI(4,5)P$_2$ defects lead to flaws in fission yeast MCC/eisosomes (Kabche et al. 2014), indicating that the role of PI(4,5)P$_2$ is conserved among yeast. Whether the presence of PI(4,5)P$_2$ affects TORC2 localization and establishment of MCT had not yet been investigated. Here, we showed for the first time that the presence of PI(4,5)P$_2$ is imperative for MCT recruitment and organization. We suggest that since Avo2 and Bit61 are severely mislocalized in cells lacking eisosome and MCC integrity, disruptions in eisosome organization due to PI(4,5)P$_2$ inhibition also lead to aberrant Avo2 localization. These results and previous evidences point to a central role for PI(4,5)P$_2$ in the organization of both MCC/eisosome and MCT.

Acknowledgements

We would like to thank Michael Hall (University of Basel) for providing tor mutant strains (\textit{tor1}^{\Delta}, and \textit{tor2}^{\Delta}); Scott Emr (Cornell University) for mutant strain (\textit{mss4}^{\Delta}); Paul Schweiger for his assistance with statistical analysis; Leah Cardwell for assistance with RTq-PCR; and Michelle Williams for helpful discussion. This work was supported by a National Scientific Foundation Grant 0923024 (to KK) and by thesis funding from Missouri State University (KB).

References


\textit{J. Biosci.} 40(2), June 2015


Mollinedo F 2012 Lipid raft involvement in yeast cell growth and death. *Front Oncol.* **2** 140


Niles BJ, Joslin AC, Fresques T and Powers T 2014 TOR complex 2-Ypk1 signaling maintains sphingolipid homeostasis by sensing and regulating ROS accumulation. *Cell Rep.* **6** 541–552

Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29** e45


*MS received 05 August 2014; accepted 31 March 2015*

Corresponding editor: *Amit CHATTOPADHYAY*