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Accessibility
A role for eisosomes in maintenance of plasma membrane phosphoinositide levels

Florian Fröhlich, Romain Christiano, Daniel K. Olson, Abel Alcazar-Roman, Pietro DeCamilli, and Tobias C. Walther

ABSTRACT The plasma membrane delineates the cell and mediates its communication and material exchange with the environment. Many processes of the plasma membrane occur through interactions of proteins with phosphatidylinositol(4,5)-bisphosphate (PI(4,5)P₂), which is highly enriched in this membrane and is a key determinant of its identity. Eisosomes function in lateral organization of the plasma membrane, but the molecular function of their major protein subunits, the BAR domain–containing proteins Pil1 and Lsp1, is poorly understood. Here we show that eisosomes interact with the PI(4,5)P₂ phosphatase Inp51/Sjl1, thereby recruiting it to the plasma membrane. Pil1 is essential for plasma membrane localization and function of Inp51 but not for the homologous phosphatidylinositol bisphosphate phosphatases Inp52/Sjl2 and Inp53/Sjl3. Consistent with this, absence of Pil1 increases total and available PI(4,5)P₂ levels at the plasma membrane. On the basis of these findings, we propose a model in which the eisosomes function in maintaining PI(4,5)P₂ levels by Inp51/Sjl1 recruitment.

INTRODUCTION The plasma membrane forms the boundary of cells. It mediates all communication and transport in and out of cells. To perform the many cellular processes mediating these functions, the composition of the plasma membrane is distinct from that of other cellular membranes and is regulated during changing conditions. For example, sphingolipids and sterols are predominantly present in the outer leaflet of the plasma membrane, where they are believed to provide a tight membrane seal. In the cytoplasmic leaflet of the plasma membrane, phosphatidylinositol(4,5)-bisphosphate (PI(4,5)P₂) is highly enriched compared with other cellular membranes and interacts with a set of plasma membrane–specific proteins. These interactions provide spatial specificity for many biochemical reactions that occur at the plasma membrane, such as endocytosis, exocytosis, and cell signaling.

PI(4,5)P₂ is generated at the plasma membrane by phosphorylation of phosphatidylinositol. The kinase reactions are counteracted by phosphoinositide phosphatases. Mechanisms that control the localization of these enzymes play a major role in achieving PI(4,5)P₂ homeostasis. Mammalian cells express nine PI(4,5)P₂ phosphatases, which have different tissue distributions (Pirruccello and De Camilli, 2012). Whereas numerous studies have addressed specific functions of these enzymes, elucidation of basic principles, such as their shared and/or specific functions, have been complicated by their large number. The genome of the yeast Saccharomyces cerevisiae encodes only three such enzymes—Inp51/Sjl1, Inp52/Sjl2, and Inp53/Sjl3 (Srinivasan et al., 1997; Singer-Kruger et al., 1998; Stolz et al., 1998)—whose roles in maintaining appropriate PI(4,5)P₂ levels remain largely unknown. Analysis of one such isoform, Inp52, revealed an important function specifically during actin patch–mediated endocytosis (Singer-Kruger et al., 1998; Stefan et al., 2005; Sun et al., 2007), suggesting that phosphatases might play specialized roles in different plasma membrane processes. It is expected that...
further analysis of the function of these enzymes in yeast may help to shed light on fundamental PI(4,5)P2 metabolism.

The plasma membrane is not only distinct in its composition from other membranes, but it is also organized into lateral compartments of distinct protein and lipid composition. In the yeast Saccharomyces cerevisiae, the plasma membrane contains a number of domains that appear either as punctate foci or networks of percolating proteins (Malinska et al., 2003, 2004; Berchtold and Walther, 2009; Ziolkowska et al., 2012). Staining with lipid-binding dyes, such as filipin, further suggests that lipids, such as ergosterol, are unevenly distributed between the domains (Grossmann et al., 2007).

One prominent plasma membrane domain is the membrane compartment containing Can1 (MCC), which ultrastructurally is defined by distributed membrane furrows directed toward the cytoplasm (Stradalova et al., 2009; Karotki et al., 2011; Moreira et al., 2012). MCC domains are formed by large protein complexes peripherally associated with the furrows, termed eisosomes (Walther et al., 2006; Karotki et al., 2011). Eisosomes consist primarily of two homologous, highly abundant core subunits, Pil1 and Lsp1, both present at an abundance of ~100,000 copies/cell, as well as a host of substoichiometric proteins of mostly unclear function (Aguilar et al., 2010; Moreira et al., 2012). Pil1 and Lsp1 contain Bin1, amphiphysin, Rvs161/167 (BAR) domains (Olivera-Couto et al., 2011; Ziolkowska et al., 2011) and assemble into helical half-cylinders at the plasma membrane, molding the membrane into an ~50-nm-deep, ~200- to 300-nm-long furrow (Stradalova et al., 2009; Karotki et al., 2011).

Among eisosome proteins, Pil1 is particularly important for the architecture of the complex. In its absence, the normally distributed eisosome pattern collapses, and MCC and eisosome proteins localize to one or a few remnants that represent large, aberrant plasma membrane invaginations (Walther et al., 2006; Stradalova et al., 2009).

Membrane interactions of Pil1 and Lsp1 are likely mediated by a short N-terminal protein segment and a patch of positively charged amino acids in their BAR domains, which specifically interact with negatively charged lipid head groups, for example, of PI(4,5)P2. Consequently, gross alterations of PI(4,5)P2 levels—for example, due to inactivation of the only yeast plasma membrane phosphatidylinositol 4-phosphate (P(4)P) kinase, Mss4—reduce plasma membrane association of Pil1 and Lsp1 (Karotki et al., 2011).

Membrane organization by eisosomes is important for control of cell signaling. Studies of sphingolipid signaling revealed that the organization of the plasma membrane into eisosomes and domains containing the target of rapamycin complex 2 (TORC2) kinase is crucial for detection of membrane stress and alteration of sphingolipid levels (Berchtold and Walther, 2009; Berchtold et al., 2012; Frohlich et al., 2009).

Here we analyze genetic interactions of eisosome core components to reveal a specific connection of Pil1 with the PI(4,5)P2 phosphatase Inp51, also known as synaptojanin-like protein 1 (Sjl1). We find that Pil1 specifically recruits Inp51 to the plasma membrane and is crucial for maintaining normal plasma membrane phosphatidylinositol levels and availability.

RESULTS

The phosphatidylinositol bisphosphate phosphatases Inp51, Inp52, and Inp53 have distinct cellular functions

To investigate the physiological function of Pil1 in membrane organization, we analyzed its genetic interactions, systematically measured in an epistatic mini array profile (E-MAP; Schuldiner et al., 2005, 2006; Collins et al., 2006; Breslow et al., 2008; Aguilar et al., 2010; Hoppins et al., 2011). Specifically, we used an E-MAP data set containing ~700 genes involved in lipid metabolism and membrane trafficking, as well as the gene encoding the eisosome component Pil1 (Surma et al., 2013).

First, we analyzed correlations of genetic interaction profiles in the E-MAP. If two mutations have similar physiological functions, they likely share suppressing and aggravating genetic interactions with other mutations, resulting in highly similar genetic interaction profiles. We find that PIL1 and INP51 have the highest correlation with each other in the lipid E-MAP (Figure 1, a and b; correlation coefficient [CC] = 0.54), suggesting that these genes function together or in similar processes. In contrast, we did not find significant correlations between PIL1 and the other two PI(4,5)P2 phosphatase genes, INP52 and INP53 (Figure 1, c and d; CC = 0.10 and 0.06, respectively). Similar correlations between PIL1 and INP51 are present in another E-MAP focusing on genes implicated in plasma membrane function (F.F. and T.C.W., unpublished data; Aguilar et al., 2010; Karotki et al., 2011).

INP52 has the highest correlations with genes encoding endocytic actin patch proteins, such as ABP1 or SLA1 (Figure 1, a and c; CC = 0.39 and 0.34). Consistent with previous results characterizing Inp52 function, these data highlight its function in actin-dependent endocytosis (Singer-Kruger et al., 1998; Stefan et al., 2005; Sun et al., 2007). Genetic interactions of INP53 correlate well with the profiles of VPS30 and VPS38, both encoding subunits of phosphatidylinositol 3-kinase. This complex is involved in endosomal and autophagy trafficking (Figure 1, a and d; VPS30 CC = 0.59 and VPS38 CC = 0.58), suggesting Inp53 functions in these processes. The correlations of Inp51, Inp52, and Inp53 phosphoinositide phosphatases with actin patches, eisosomes, or endosomal trafficking are specific, as there is little cross-correlation between each particular phosphoinositide phosphatase (Figure 1e) and the processes related to any of their homologues (Figure 1, b–d).

Inp51 localizes to eisosomes

Genetic interactions suggest that Inp51, Inp52, and Inp53 phosphoinositide phosphatases function in different processes, predicting potentially different subcellular localizations. To test this hypothesis directly for phosphoinositide phosphatase function at the plasma membrane, we investigated the localization of Inp51, Inp52, and Inp53 with respect to eisosomes and actin patches. We inserted the sequence encoding the green fluorescent protein (GFP) fluorophore at the 3′ end of the open reading frame of INP51, INP52, or INP53 at their respective genomic loci, driving their expression from their endogenous promoters. Analysis by confocal fluorescence microscopy shows that Inp51 localizes in a punctate pattern at the plasma membrane. Inp51 spots colocalize with eisosomes, marked with red fluorescently tagged Lsp1 (Figure 2a, left; quantification in Figure 3c) but was excluded from actin patches marked with Abp1 fused to red fluorescent protein (Figure 2a, right). Localization of Inp51 at static eisosomes is highly dynamic (Figure 3a and Supplemental Movie S1). The residence time of an Inp51 focus at a particular eisosome varied greatly, ranging from <2 to >30 s (Figure 3b).

Similarly, Inp52 localized in a punctate pattern at the plasma membrane. However, we did not observe colocalization with eisosomes (Figure 2b, left) but instead with a subset of endocytic actin patches (Figure 2b, right), as observed previously (Stefan et al., 2005; Sun et al., 2007).

Compared with Inp51 and Inp52 localization, Inp53 showed a distinct pattern with little or no signal at the plasma membrane and did not colocalize with either Lsp1 or Abp1 (Figure 2c, left and right). Instead, it formed larger foci within the cytoplasm (Figure 2c, right). Because the genetic interaction analyses suggest a function of Inp53 in endosomal trafficking, this signal might correspond to endosomes.
Inp51 physically interacts with eisosomes

Our data reveal specialized functions for phosphoinositide phosphatases, as well as spatial segregation of Inp51 and Inp52 at the plasma membrane. Localization of Inp51 to eisosomes and its genetic correlation and suppression of genetic interaction with Pil1 (Figures 1 and 2 and Supplemental Figure S1) suggest that eisosomes may physically interact with Inp51. To test this hypothesis, we immunopurified GFP-tagged Inp51 from cells metabolically labeled with “heavy,” nonradioactive lysine 8 (stable isotope labeling with amino acids in cell culture [SILAC]; Ong et al., 2002). In parallel, we performed a mock purification from wild-type (WT) cells labeled with normal (“light”) lysine. We mixed eluates from both purifications and analyzed them by high-resolution mass spectrometry–based proteomics (Walther and Mann, 2010). Peptides from proteins containing the “heavy” isotope–labeled lysine are shifted right in the spectra compared with the same peptide from unlabeled protein, allowing quantitation of abundance ratios for each detected peptide and protein from the eluates.

In these experiments, we identified Inp51 as the most enriched protein in eluates from strains expressing the GFP-tagged form compared with controls. We also detected the known regulator Irs4 as a specific interactor of Inp51 (Morales-Johansson et al., 2004). In addition, we identified several known eisosome proteins, including the eisosome core proteins Pil1 and Lsp1, as well as Eis1, Seg1, Msc3, and Ygr130C as highly specific interactors of Inp51 (Figure 4a; Aguilar et al., 2010; Moreira et al., 2012).

To assay independently for the interaction of Inp51 and eisosomes, we also purified GFP-tagged Pil1 from “heavy”-labeled yeast cells and compared it with a mock purification from “light”-labeled WT cells. We identified all known eisosome interacting proteins—Lsp1, Eis1, Seg1, Ygr130C, Msc3, Phk1, and Ykl105C. In addition, we identified Inp51 as a significantly enriched protein (Figure 4b).

The specificity of the interaction between Inp51 and eisosomes is particularly apparent when the experiments are compared with one another. We plotted the heavy/light ratios of proteins from the Inp51 pull down versus the ratios of proteins from the Pil1 pull down. As expected, the majority of proteins detected are contaminants not enriched in both experiments. The specific interactors from both pull downs localize to the top right quadrant of the plot and show all known eisome components (Pil1, Lsp1, Seg1, Ykl105C, Eis1, Ygr130C, and Msc3), as well as Inp51 and its known regulator, Irs4 (Figure 4c). We did not find evidence for the interaction of

Inp51, Inp52, and Inp53 do not correlate with each other.
Inp51 localization to the plasma membrane is Pil1 dependent

Motivated by the genetic, cell biological, and biochemical data, we next tested whether eisosomes recruit Inp51 to the plasma membrane. We analyzed Inp51 localization in *pil1Δ* cells by fluorescence microscopy. We did not observe Inp51 at the plasma membrane in a *pil1Δ* strain, compared with many foci in WT cells (Figure 5a, Supplemental Figure S2, and Supplemental Movie S2). To test whether Inp51 is mislocalized or degraded in *pil1Δ* cells, we analyzed Inp51-GFP levels in WT and *pil1Δ* cells after immunoprecipitation. We did not observe any differences in protein levels, suggesting that Inp51 is not degraded but fails to target the plasma membrane in the absence of Pil1 (Supplemental Figure S2b). Of importance, the effect of *PIL1* deletion was specific to Inp51 localization, as we did not observe any significant changes in Inp52 and Inp53 localization in this strain (Figure 5, b and c). Eisosome-dependent recruitment of Inp51 to the plasma membrane required specifically Pil1, as deletion of the highly homologous Lsp1 subunit did not have an effect on Inp51 localization (Supplemental Figure S3).

To further test whether deletion of *PIL1* interferes with Inp51 function at the plasma membrane, we tested for genetic interactions of *pil1Δ* with *inp52Δ inp53Δ*. Synaptotagmin triple-knockout mutations are lethal (Stolz et al., 1998; Figure 6a). As expected if Inp51 function is dependent on recruitment to the plasma membrane by Pil1, deletion of *PIL1* in an *inp52Δ inp53Δ* strain yielded a strong synthetic growth defect phenocopying the effects of a synaptotagmin triple mutant (Figure 6b).

Capitalizing on this genetic assay, we tested whether other known eisosome components are required for normal Inp51 function. We did not detect genetic interactions between any of the known eisosome components, *LSP1, EIS1, YGR130C, SEG1, YKL105C*, or *MSC3*, and *inp52Δ inp53Δ*. Thus specifically Pil1 is required for Inp51 function (Figure 6c and Supplemental Figure S4).

The genetic interaction with *inp52Δ inp53Δ* allowed us to test requirements of specific Pil1 features, such as the C-terminal extension from the BAR domain (Ziolkowska et al., 2011) or phosphorylation sites, for Inp51 function. In summary, these experiments showed that the BAR domain of Pil1 is sufficient to rescue synthetic lethality when combined with *inp52Δ inp53Δ* mutants and that phosphorylation at the Pkc1-dependent phosphorylation sites (S230 and T233; Mascaraque et al., 2013) is not required (Supplemental Figure S5).

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**FIGURE 2:** The three yeast phosphatidylinositol bisphosphate phosphatases localize to different compartments within cells. (a) Colocalization of GFP-tagged Inp51 with RFPmars-tagged Lsp1 (left) and RFPmars-tagged Abp1 (right). Representative confocal midsections. The graphs show the intensity profiles for both channels along the perimeter of the cell. (b) Colocalization of GFP-tagged Inp52 with RFPmars-tagged Lsp1 (left) and RFP-mars-tagged Abp1 (right). Representative confocal midsections. The graphs show the intensity profiles for both channels along the perimeter of the cell. (c) Colocalization of GFP-tagged Inp53 with RFPmars-tagged Lsp1 (left) and RFP-mars-tagged Abp1 (right). Representative confocal midsections. The graphs show the intensity profiles for both channels along the perimeter of the cell. Scale bar, 2.5 μm.
Pi1 controls phosphatidylinositol bisphosphate plasma membrane levels by recruitment of Inp51

It is unknown how plasma membrane phosphoinositol levels are regulated in yeast. Recruitment of Inp51 to eisosomes suggests that these plasma membrane domains may have an important function in lipid homeostasis. To test this hypothesis, we assayed phosphoinositol levels in pil1Δ, inp51Δ, and pil1Δinp51Δ double-mutant cells compared with WT cells. We found that total cellular levels of phosphatidylinositol 3-phosphate (PI(3)P) were not significantly changed in mutants compared with WT cells. As expected, there was a robust increase in PI(4,5)P2 levels (1.6 times) with a concomitant decrease in PI(4)P levels in inp51Δ cells (Figure 7a). We also observed a statistically significant increase in PI(4,5)P2 levels in cells lacking Pil1 (1.3 times). Of interest, PI(4,5)P2 levels of a pil1Δinp51Δ double knockout were the same as in a pil1Δ single-mutant strain.

To determine PI(4,5)P2 localization in the different mutant strains, we integrated a construct containing two pleckstrin homology (PH) domains of mammalian phospholipase Cδ (PLCδ) fused to GFP under the control of the CPY promoter (GFP-2xPH<sub>PLCδ</sub>) into the genome of WT, pil1Δ, inp51Δ, or pil1Δinp51Δ cells and measured GFP fluorescence intensity at the plasma membrane and in the cytoplasm. We found a small but statistically significant (p < 0.01) 20% increase of GFP signal from our PI(4,5)P2 reporter construct at the plasma membrane in inp51Δ cells compared with WT cells.

Surprisingly, we observed an even larger signal increase at the plasma membrane of pil1Δ, as well as pil1Δinp51Δ cells (1.9- and 1.5-fold, respectively; Figure 7b; quantitation in Figure 7, c and d). We obtained similar results when expressing the PH domain of Slm1, whose localization in our hands is highly sensitive to plasma membrane PI(4,5)P2 levels in pil1Δ cells (Supplemental Figure S6). These data suggest that free PI(4,5)P2 amounts available for protein binding are increased even more than the total cell levels of this lipid. Total protein levels of the GFP-2xPH<sub>PLCδ</sub> reporter construct were elevated in pil1Δ and pil1Δinp51Δ cells, likely indicating that PI(4,5)P2-bound reporter is protected from turnover (Figure 7, e and f).

**DISCUSSION**

We systematically analyzed the role of the different yeast phosphoinositide phosphatases. Our data and previous reports taken together suggest that Inp53 functions at endosomes. Of the two plasma membrane–localized phosphoinositide phosphatases, Inp52 is a component of endocytic actin patches, with a distinct time point during the biogenesis of the patch when it is recruited and leaves, suggesting it is part of the core machinery executing clathrin-mediated endocytosis (Singer-Kruger et al., 1998; Stefan et al., 2005; Sun et al., 2007). This function is very similar to the one proposed for synaptojanin in clathrin-mediated endocytosis in mammalian cells (Cremona et al., 1999; Di Paolo and De Camilli, 2006).

In contrast, Inp51 is excluded from actin patches and localizes in a highly dynamic manner to eisosomes instead. Pi1 is required for Inp51 recruitment to eisosomes, and the proteins physically interact with each other. We observe Inp51 localization as clearly distinct from actin patches and do not observe Inp52 or Inp53 on eisosomes. In addition, the different genetic interaction profiles of Inp51 and Inp52 support the notion of different functions of the two phosphatases. Therefore our data indicate a specific function for Pil1 and Inp52 in recruiting several phosphoinositide phosphatases (Murphy et al., 2011).

Thus Pi1 and Inp51 functioning together presents another instance of a specific BAR domain protein and a synaptojanin–like phosphatase acting together. This is similar to the cooperation of Rvs161/Rvs167 with Inp52 in endocytic actin patches and endophilin with synaptojanin in metazoan cells (Milosevic et al., 2011). This likely highlights the generality and ancient origin of this function of BAR domain–containing proteins.

How eisosomes recruit Inp51 mechanistically remains unknown. Our analyses of synthetic phenotypes of eisosome components with
inp52Δinp53Δ show that specifically Pil1, but none of the other known eisosome components, is required to maintain Inp51 function. Consistent with this model, Inp51 does not localize to eisosome remnants containing the remaining components of the complex in pil1Δ cells. We thus posit that Pil1 might directly contact Inp51 or a protein with which it is in complex, such as Irs4. Pil1 assembles into a semicylindrical protein coat with Lsp1 in eisosomes. Crystallographic and electron tomographic data (Karotki et al., 2011; Ziolkowska et al., 2011) show that the flexible C-terminal tail of Pil1 points from this structure toward the cytoplasm. However, our genetic interactions show that this Pil1 tail is not required for Inp51 function at the plasma membrane. Instead, a Pil1-fourth helix of the Pil1 BAR domain not found in other domains of this family was required for the interaction. However, in the absence of this helix, eisosomes fail to assemble normally, complicating the interpretation of this mutation (unpublished data).

Pil1 and Lsp1 are heavily phosphorylated. In Pil1 there are at least 11 phosphorylation sites that are targets of the Pkh1/2 and Pkc1 pathways, which mediate signaling in response to lipid and plasma membrane changes (Luo et al., 2008; Frohlich et al., 2009; Mascaraque et al., 2013). Some of these phosphorylation sites regulate Pil1 and Lsp1 assembly (Walther et al., 2007; Luo et al., 2008). Another phosphorylation site (S59) lies within a pocket of the Pil1 and Lsp1 BAR domains facing the plasma membrane and is required for P(4,5)P2 binding (Ziolkowska et al., 2011). Still other phosphorylation sites are found on the BAR domain or in the C-terminal region outside of the BAR domain. We ruled out in genetic experiments for some of these sites that they are important for Inp51 recruitment (S230 and T233). Other sites might regulate Inp51 recruitment, which might help coordinate the need for P(4,5)P2 at the plasma membrane.

We find that the total cellular amount of P(4,5)P2, as well as the amount available for protein binding, is increased in pil1Δ mutants. Because endocytosis and actin are regulated by plasma membrane P(4,5)P2, this increase may explain at least some of the phenotypes observed on actin patches and the endocytic efficiencies for a variety of cargoes in pil1Δ cells (Walther et al., 2006; Grossmann et al., 2008; Brach et al., 2011; Murphy et al., 2011).

What is the function of eisosomes and their recruitment of Inp51/Slj1 in phosphoinositide metabolism? Our working model is that Pil1 and Inp51 function to maintain adequate levels of P(4,5)P2 at the plasma membrane. Pil1 (and Lsp1) BAR domains each contain a binding site for P(4,5)P2. Their assembly in an eisosome, which contains hundreds of each of the proteins, thus likely recruits and clusters these lipids in the plasma membrane. Consistent with this notion, we observe that the absence of Pil1 leads to increased P(4,5)P2 available for binding and a more modest increase of total levels. In contrast, deletion of INP51 results in a larger amount of total P(4,5)P2 levels, with a mild increase in the P(4,5)P2 levels available for binding. The hundreds of binding sites on Pil1 and Lsp1 might thus normally bind a large pool of available P(4,5)P2 to buffer for fluctuations in availability of this lipid by binding or releasing it at

![Figure 4](image_url)
available as a substrate for Inp52 or Inp53. The sequestering of PI(4,5)P$_2$ by Pil1 binding might operate in concert with recruitment of Inp51 to eisosomes to regulate PI(4,5)P$_2$ availability and turnover. In our model, these mechanisms together maintain normal plasma membrane phosphoinositide levels.

**MATERIALS AND METHODS**

**Strains and plasmids**

All yeast strains used in this study and their genotypes are listed in Supplemental Table S1, as are all plasmids used in this study. Standard yeast manipulations, including transformation, homologous recombination of PCR-generated fragments, and tetrad dissections, were performed as described previously (Janke et al., 2004; Berchtold and Walther, 2009; Frohlich et al., 2009).

The CPY$_{promotor}$-2xGFP$_{PLC\delta}$ plasmid was created by cloning this construct from pRS426GFP-2xPH$_{PLC\delta}$ (Stefan et al., 2002) into the NotI and HindIII sites of pRS306.

**Yeast culture**

All experiments were performed on yeast grown at 30°C. For microscopy, cells were grown in synthetic complete medium and bound to concanavalin A–treated coverslips.

**FIGURE 5:** PIL1 is required for normal localization of Inp51.
(a) Inp51-GFP was expressed and imaged either in WT or pil1Δ cells. Representative confocal midsections. Pil1 is not required for regular distribution of Inp52 (b) or Inp53 (c). Scale bar, 2.5 μm.

**FIGURE 6:** PIL1 genetically interacts with INP52 and INP53. (a) Tetrad analysis of inp51Δ mutants crossed with inp52Δinp53Δ. (b) Tetrad analysis of pil1Δ mutants crossed with inp52Δinp53Δ. (c) Tetrad analysis of lsp1Δ mutants crossed with inp52Δinp53Δ.
For SILAC labeling, the lysine prototroph yeast strains W303 WT, W303 PIL1-GFP, and W303 INP51-GFP were grown according to the protocol for native SILAC (Frohlich et al., 2013) in the presence of normal l-lysine or l-lysine-U-13C6,15N2 (Cambridge Isotope Labs, Tewksbury, MA).

Fluorescence microscopy

Yeast cells from cultures grown to OD600 ≈ 0.5 were mounted with concanavalin A in growth medium, and images were collected on a DeltaVision workstation (Applied Precision, Issaquah, WA) based on an inverted microscope (IX-70; Olympus, Tokyo, Japan) using a 100×/1.4 numerical aperture (NA) oil immersion lens. Images were captured at 24°C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics, Tucson, AZ) and deconvolved using the iterative-constrained algorithm and the measured point spread function.

Alternatively, cells were grown in synthetic medium containing raffinose as carbon source and switched to galactose-containing medium to induce protein expression from a GAL promoter for 2 h. Cells were mounted with concanavalin A and imaged with a spinning-disk confocal microscope (TILL iMIC CSU22; Andor, Belfast, UK) using a back-illuminated electron-multiplying charge-coupled device camera (iXonEM 897; Andor) and a 100×/1.4 NA oil immersion objective (Olympus). From this setup, 16-bit images were collected using Image iQ (version 1.9; Andor) in the linear range of the camera. For presentation, images were converted to 8-bit images and cropped using ImageJ software (National Institutes of Health, Bethesda, MD).

For quantification of GFP_2xPH<sub>PLC</sub> signals, images were all acquired with the same settings. The intensity at the plasma membrane and in the cytoplasm was measured using ImageJ. For quantification of the plasma membrane to cytoplasmic signal, the two measured values for each cell were divided.

Affinity purification and mass spectrometry

Inp51-GFP cells or Pil1-GFP cells were grown in the presence of “heavy” lysine (L-lysine-U-13C6,15N2), and WT cells were grown in the presence of normal, “light” lysine. We harvested 500 OD600 units of cells by centrifugation and resuspended them in 500 μl of lysis buffer (150 mM KOAc, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 10% glycerol, and complete protease inhibitor cocktail [Roche, Basel, Switzerland]). Zirconia beads (500 μl, 0.1-mm diameter; BioSpec Products, Bartlesville, OK) were added, and cells were lysed using a FASTPREP (MP Biomedicals, Solon, OH) for 60 s at 4°C. Beads were removed by centrifugation, and Triton X-100 was added to a final concentration of 1%. After a 30-min incubation at 4°C, lysates were cleared by centrifugation for 10 min at 10000 × g. Equivalent amounts of “light”-labeled control and “heavy”-labeled PIL1-GFP–containing lysates or “light”-labeled control and “heavy”-labeled INP51-GFP–containing lysates were incubated (separately) with GFP-Trap agarose beads (Allele Biotechnology, San Diego, CA) for 30 min at 4°C. Beads were washed three times with brightfield images. Scale bar, 2.5 μm. (c) Quantification of b. The average intensity of the plasma membrane signal of the GFP_2xPH<sub>PLC</sub> is plotted. Error bars represent SDs. n = 39 (WT), 39 (pil1Δ), and 39 (inp51Δ), and 39 (inp1Δinp51Δ). (d) Quantification of b. Ratio of plasma membrane–bound to cytoplasmic signal of the GFP_2xPH<sub>PLC</sub> domain is plotted. Error bars represent SDs. n = 39 (WT), 39 (pil1Δ), and 39 (inp51Δ), and 39 (inp1Δinp51Δ). (e) Yeast lysates of WT, pil1Δ, inp51Δ, and pil1Δinp51Δ cells expressing GFP_2xPH<sub>PLC</sub> and control cells were blotted and probed with antibodies against GFP, Pil1, and Pgk1. (f) Quantification of the GFP signal from e normalized to WT levels.

**FIGURE 7**: *PIL1* is required for normal PI(4,5)P<sub>2</sub> levels at the plasma membrane. (a) Cellular levels of PI(3)P (left), PI(4)P (middle), and PI(4,5)P<sub>2</sub> (right) as percentage of total phosphatidylinositolates in WT cells (white bars), pil1Δ cells (light gray bars), inp51Δ cells (dark gray bars), and pil1Δinp51Δ cells (black bars). Error bars represent SDs, n = 3. (b) WT (left), pil1Δ (middle left), inp51Δ (middle right), and pil1Δinp51Δ cells expressing two PH domains of phospholipase Cδ under the control of a CPY promoter (top). Bottom, corresponding averages for each cell were divided.
lysis buffer and three times with wash buffer (150 mM NaCl, 20 mM HEPES, pH 7.4). Beads from INPS1-GFP pull downs and control pull downs or PIL1-GFP and control pull downs were combined in 100 μl of denaturation buffer (8 M urea, 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol) and incubated for 30 min. Proteins were alkylated by the addition of 5.5 mM iodoacetamide for 20 min in the dark and digested with the endoprotease LysC overnight at 37°C. The resulting peptide mixture was removed from the beads and desalted following the protocol for StageTip purification (Rappsilber et al., 2003). Peptides were subjected to reversed-phase chromatography on a Thermo Easy nLC system connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) through a nano-electrospray ion source, as described previously (Colombi et al., 2013). The resulting mass spectroscopy (MS) and MS/MS spectra were analyzed using MaxQuant (version 1.4.0.8, www.maxquant.org; Cox and Mann, 2008; Cox et al., 2011) as described previously (Frohlich et al., 2013). All calculations and plots were performed with the R software package (www.r-project.org/).

Genetic interaction data
Data sets for the analysis of E-MAP data were derived from Surma et al. (2013).

Quantification of phosphoinositide levels by high-performance liquid chromatography analysis
Labeling of cells and extraction of phosphoinositides were done as previously described (Audhya and Emr, 2002). Briefly, cells were grown in synthetic medium overnight and kept in log phase. We incubated 5 OD units of cells in inositol-free medium for 15 min and labeled them with 50 μCi of [3H]myo-inositol (MP Biomedical) for 1 h at room temperature. Cells were then lysed by vortexing samples with glass beads in ice-cold 4.5% perchloric acid for 15 min. Lysates were extracted and spun, and resulting pellets were washed twice with 0.1 M EDTA. Samples were then deacylated and separated by high-performance liquid chromatography (Shimadzu Scientific Instruments, Kyoto, Japan) and phosphoinositides identified using deacylated 2P standards and an online flow scintillation analyzer (B-RAM; IN/US, Conquer Scientific, San Diego, CA) as described (Devereaux and Di Paolo, 2013).

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