A Role for Eisosomes in Maintenance of Plasma Membrane Phosphoinositide Levels

Florian Fröhlich1,2*, Romain Christiano1,2, Daniel K. Olson1,2, Abel Alcazar-Roman1,4, Pietro DeCamilli1,4 and Tobias C. Walther1,2,3*

1 Department of Cell Biology, Yale School of Medicine, New Haven, USA
2 Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, USA
3 Department of Cell Biology, Harvard Medical School, Boston, USA
4 Howard Hughes Medical Institute and Program in Cellular Neurosciences, Neurodegeneration and Repair, Yale School of Medicine, New Haven, CT 06510, USA

*Correspondence should be sent to:

Tobias Walther
Harvard School of Public Health
Department of Genetics and Complex Diseases
677 Huntington Avenue
Boston, MA 02115
twalther@hsph.harvard.edu

or

Florian Fröhlich
Harvard School of Public Health
Department of Genetics and Complex Diseases
677 Huntington Avenue
Boston, MA 02115
froehlic@googlemail.com

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 PIP₂ 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. Based on these findings, we propose a model in which the eisosome 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 other cellular membranes and regulated during changing conditions. For example, sphingolipids and sterols are predominantly present in the outer leaflet of the plasma membrane, where they are thought 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). While 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 (Singer-Kruger et al., 1998; Srinivasan et al., 1997; 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 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)P$_2$ metabolism.

The plasma membrane is not only distinct in its composition from other membranes, but 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 (Berchtold and Walther, 2009; Malinska et al., 2003, 2004; Ziólkowska 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 towards the cytoplasm (Karotki et al., 2011; Moreira et al., 2012; Stradalova et al., 2009). MCC domains are formed by large protein complexes peripherally associated with the furrows, termed eisosomes (Karotki et al., 2011; Walther et al., 2006). Eisosomes consist primarily of two homologous, highly abundant core subunits Pil1 and Lsp1, both present at an abundance of ~100,000 copies per 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 BAR (Bin1, amphiphysin, Rvs161/167) domains (Olivera-Couto et al., 2011; Ziólkowska et al., 2011) and assemble into helical half-cylinders at the plasma membrane, molding the membrane into a ~50nm deep and ~200-300 nm long furrow (Karotki et al., 2011; Stradalova et al., 2009). 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 (Stradalova et al., 2009; Walther et al., 2006).
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, e.g., 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 PI(4)P-kinase Mss4, diminish 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 et al., 2012; Berchtold and Walther, 2009; Frohlich et al., 2009).

Here, we analyzed genetic interactions of eisosome core components to reveal a specific connection of Pil1 with the PI(4,5)P₂ phosphatase Inp51, also known as synaptotagmin-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 PIP₂ 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; Aguilar et al., 2010; Breslow et al., 2008; Collins et al., 2006; Hoppins et al., 2011; Schuldiner et al., 2005; Schuldiner et al., 2006). Specifically, we used an E-MAP dataset containing roughly 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 (Fig. 1a, 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)P$_2$ phosphatase genes \(INP52\) and \(INP53\) (Fig. 1c,d; CC=0.10 and CC=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., data not shown and Aguilar et al., 2010; Karotki et al., 2011).

\(INP52\) has the highest correlations with genes encoding endocytic actin patch proteins, such as \(ABP1\) or \(SLA1\) (Fig. 1a,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 (Fig. 1a,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 (Fig. 1e) and the processes related to any of their homologs (Fig. 1b,c,d).

**Inp51 Localizes to Eisosomes**

Genetic interactions suggest 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 in
respect to eisosomes and actin patches. We inserted the sequence encoding the 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 (Fig. 2a, left panel and quantification in Fig. 3c), but was excluded from actin patches marked with Abp1 fused to red fluorescent protein (Fig. 2a, right panel). Localization of Inp51 at static eisosomes is highly dynamic (Fig. 3a and suppl. movie 1). The residence time of an Inp51 focus at a particular eisosome varied largely, ranging from less than two seconds to more than 30 seconds (Fig. 3b).

Similarly, Inp52 localized in a punctate pattern at the plasma membrane. However, we did not observe any colocalization with eisosomes (Fig. 2b, left panel), but instead with a subset of endocytic actin patches (Fig. 2b, right panel), as was 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 (Fig. 2c, left and right panel). Instead, it formed larger foci within the cytoplasm (Fig. 2c, right panel). As 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 suppressing genetic interaction with Pil1 (Fig. 1, 2 and suppl. Fig 1) suggests eisosomes may physically interact with Inp51. To test this hypothesis, we immunopurified GFP-tagged Inp51 from cells metabolically labeled with “heavy”, non-radioactive lysine 8 (stable isotope labeling with amino acids in cell culture, (SILAC, Ong et al., 2002). In parallel, we performed a mock purification
from 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 (Fig.4a; Aguilar et al., 2010; Moreira et al., 2012).

To independently assay 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, Pkh1 and Ykl105C. In addition, we identified Inp51 as a significantly enriched protein (Fig. 4b).

The specificity of the interaction between Inp51 and eisosomes is particularly apparent when both experiments are compared to one another. We plotted the heavy/light ratios of proteins from the Inp51 pull-down versus the ratios of proteins from the Pil1 pull-downs. 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 eisosome components (Pil1, Lsp1, Seg1, Ykl105c, Eis1, Ygr130C, Msc3) as well as Inp51 and its known regulator Irs4 (Fig. 4c). We did not find evidence for the interaction of eisosome components with Inp52 or Inp53, suggesting the interaction of eisosomes with Inp51 is specific for this phosphoinositide phosphatase.
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 fluorescent microscopy. We did not observe Inp51 at the plasma membrane in a pil1Δ strain, compared with many foci in WT cells (Fig. 5a, suppl. Fig. 2 and suppl. movie 2). To test whether Inp51 is miss-localized 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 (suppl. Fig 2b). Importantly, 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 (Fig. 5b and Fig. 5c). 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 (suppl. Fig. 3).

To further test whether deletion of PIL1 interferes with Inp51 function at the plasma membrane we tested for genetic interactions of pil1Δ with inp52Δinp53Δ. Synaptojanin triple knockout mutations are lethal (Stolz et al., 1998 and Fig. 6a). As expected if Inp51 function is dependent upon recruitment to the plasma membrane by Pil1, deletion of PIL1 in an inp52Δinp53Δ strain yielded a strong synthetic growth defect pheno-copying the effects of a synaptojanin triple mutant (Fig. 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 inp52Δinp53Δ. Thus, specifically Pil1 is required for Inp51 function (Fig. 6c and suppl. Fig. 4).

The genetic interaction with inp52Δinp53Δ 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 \textit{inp52\_inp53\_mutant}s and that phosphorylation at the Pkc1 dependent phosphorylation sites (S230; T233; Mascaraque et al., 2013) is not required (suppl. Fig. 5).

**Pil1 controls PIP2 plasma membrane levels by recruitment of Inp51**

Currently, it is unknown how plasma membrane phosphoinositide 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 phosphoinositide levels in \textit{pil1\_mutant}s, \textit{inp51\_mutant}s and \textit{pil1\_inp51\_mutant} double mutant cells compared with WT cells. We found that total cellular levels of PI(3)P were not significantly changed in mutants compared to WT cells. As expected, there was a robust increase in PI(4,5)P$_2$ levels (1.6x) with a concomitant decrease in PI(4)P levels in \textit{inp51\_mutant}s (Fig. 7a). We also observed a statistically significant increase in PI(4,5)P$_2$ levels in cells lacking \textit{PIL1} (1.3x). Interestingly, PI(4,5)P$_2$ levels of a \textit{pil1\_inp51\_mutant} double knockout where the same as in a \textit{pil1\_mutant} single mutant strain.

To determine PI(4,5)P$_2$ localization in the different mutant strains, we integrated a construct containing two PH domains of mammalian phospholipase δ fused to GFP under the control of the CPY promoter (GFP-2xPH$_{PLC}$) into the genome of WT, \textit{pil1\_mutant}s, \textit{inp51\_mutant}s or \textit{pil1\_inp51\_mutant}s 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)P$_2$ reporter construct at the plasma membrane in \textit{inp51\_mutant}s compared with WT cells.

Surprisingly, we observed an even larger signal increase at the plasma membrane of \textit{pil1\_mutant}s as well as \textit{pil1\_inp51\_mutant}s cells (1.9 fold and 1.5 fold, respectively; Fig. 7b; quantitation in c and d). We obtained similar results when expressing the PH-domain of S1m1, whose localization in our hands is highly sensitive to plasma membrane PI(4,5)P$_2$ levels in \textit{pil1\_mutant}s (suppl. Fig. 6). These data suggest that free PI(4,5)P$_2$ amounts available for protein binding are increased even more than the total cell levels
of this lipid. Total protein levels of the GFP-2xPH$_{PLC}$ reporter construct were elevated in pil1Δ cells and pil1Δinp51Δ cells, likely indicating that PI(4,5)P$_2$ bound reporter is protected from turnover (Fig. 7e and f).

**Discussion**

We have systematically analyzed the role of the different yeast phosphoinositide phosphatases. Our data and previous reports 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 fashion to eisosomes instead. Pil1 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 we 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 with Inp51, rather than a suggested more general role of Pil1 in recruiting several phosphoinositide phosphatases (Murphy et al., 2011).

Thus, Pil1 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 $inp52\Delta inp53\Delta$ 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 $pil1\Delta$ cells. We thus posit that Pil1 might directly contact Inp51 or a protein it is in complex with, such as Irs4. Pil1 assembles into a semi-cylindrical 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 towards 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 (data not shown).

Pil1 and Lsp1 are heavily phosphorylated. In Pil1 there are at least eleven phosphorylation sites that are targets of the Pkh1/2 and Pkc1 pathways, which mediate signaling in response to lipid and plasma membrane changes (Frohlich et al., 2009; Luo et al., 2008; Mascaraque et al., 2013). Some of these phosphorylation sites regulate Pil1 and Lsp1 assembly (Luo et al., 2008; Walther et al., 2007). Another phosphorylation site (S59) lies within a pocket of the Pil1 and Lsp1 BAR domains facing the plasma membrane and is required for PI(4,5)P$_2$ 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 have 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 PI(4,5)P$_2$ at the plasma membrane.

We find that the total cellular amount of PI(4,5)P$_2$, as well as the amount available for protein binding is increased in $pil1\Delta$ mutants. Since endocytosis and actin are regulated by plasma membrane PI(4,5)P$_2$, 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 (Brach et al., 2011; Grossmann et al., 2008; Murphy et al., 2011; Walther et al., 2006).

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 PI(4,5)P2 at the plasma membrane. Pil1 (and Lsp1) BAR domains each contain a binding site for PI(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 PI(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 PI(4,5)P2 levels with a mild increase in the PI(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 PI(4,5)P2 to buffer for fluctuations in availability of this lipid by binding or releasing it at concentrations around the Kd of the interaction. In the absence of Pil1, this PI(4,5)P2 is free in the plasma membrane and can be bound by the reporter. It is possible that this free pool of PI(4,5)P2 becomes available as a substrate for Inp52 or Inp53. The sequestering of PI(4,5)P2 by Pil1 binding might operate in concert with recruitment of Inp51 to eisosomes to regulate PI(4,5)P2 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 suppl. table 1. All plasmids used in this study are listed in suppl. table 1. Standard yeast manipulations including transformation, homologous recombination of PCR-generated fragments and tetrad dissections were performed as described previously (Berchtold and Walther, 2009; Frohlich et al., 2009; Janke et al., 2004).
The \( \text{CPY}_{\text{promotor-2xGFP}}_{\text{PLCδ}} \) plasmid was created by cloning this construct from \( \text{pRS426GFP-2xPH}_{\text{PLCδ}} \) (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.

For SILAC labeling the lysine prototroph yeast strains W303 WT, W303 PIL1-GFP or 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-\(^{13}\)C\(_6\),\(^{15}\)N\(_2\) (Cambridge isotope labs).

**Fluorescence Microscopy**

Yeast cells from cultures grown to \( \text{OD}_{600} \approx 0.5 \) were mounted with concavalin A in growth medium, and images were collected on a DeltaVision workstation (Applied Precision) based on an inverted microscope (IX-70; Olympus) using a 100×1.4NA oil immersion lens. Images were captured at 24°C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics) 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 glucose containing medium to induce protein expression from a GAL promotor for 2h. Cells were mounted with concavalin A and imaged with a spinning-disk confocal microscope (TiLL iMIC CSU22; Andor) using a back-illuminated EM 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 (http://rsbweb.nih.gov/ij/).

For quantification of GFP_2xPH\(_{\text{PLCδ}}\) 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-\(^{13}\)C\(_6\),\(^{15}\)N\(_2\)) and WT cells were grown in the presence of normal, “light” lysine. 500 OD\(_{600}\) units of cells were harvested by centrifugation and re-suspended in 500 µl of lysis buffer (150 mM KOAc, 20 mM Hepes, pH 7.4, 10% glycerol, and complete protease inhibitor cocktail [Roche]). 500 µl of zirconia beads (0.1 mm diameter, BioSpec Products Inc.) were added and cells were lysed using a FASTPREP (MP Biomedicals) for 60s 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 1000 x 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) for 30 min at 4°C. Beads were washed three times with lysis buffer and three times with wash buffer (150mM NaCl, 20mM Hepes pH 7.4). Beads from INP51-GFP pulldowns and control pulldowns or PIL1-GFP and control pulldowns were combined in 100µl of denaturation buffer (8M Urea, 50mM Tris-HCl pH=8, 1mM DTT) and incubated for 30 min. Proteins were alkylated by addition of 5.5 mM Iodoacetamide (IAA) for 20 min in the dark and digested with the endoproteinase 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) through a nano-electrospray ion source, as described previously (Colombi et al., 2013). The resulting MS and MS/MS spectra were analyzed using MaxQuant (version 1.4.0.8, http://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 (http://www.r-project.org/).

Genetic Interaction data
Datasets for the analysis of EMAP data were derived from (Surma et al., 2013).

Quantification of phosphoinositide levels by HPLC 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. 5 ODs of cells were incubated in inositol-free medium for 15 minutes and labeled with 50 µCi ³H-myoinositol (MP Biomedicals) for 1hr at room temperature. Cells were then lysed by vortexing samples with glass beads in ice-cold 4.5% perchloric acid for 15 minutes. Lysates were extracted, spun and resulting pellets washed twice with 0.1mM EDTA. Samples were then deacylated and separated by high performance liquid chromatography (Shimadzu Scientific Instruments) and phosphoinositides identified using deacylated 32P-standards and an online flow scintillation analyzer (B-RAM, IN/US) as described (Devereaux and Di Paolo, 2013).

Acknowledgments
We thank Drs. Christopher G. Burd and Richard J. Chi for critical comments on our manuscript. This work was supported by the Yale Top scholar award (to T.C.W.) and by NIH grant R01GM095982 (to T.C.W.).

References:


The EMBO journal 26, 1-8.


Figure 1: Global analysis of all phosphoinositide phosphatase and PIL1 genetic interactions. (a) Histograms of correlation coefficients generated by comparing the profiles of genetic interactions for each phosphoinositide phosphatase and Pil1 to all other profiles in the E-MAP analysis. PIL1 and INP51 display strongest correlation to each other. INP52 shows correlation with genes encoding actin patch proteins. INP53 is
correlated with genes encoding the endosomal PI(3,5)P kinase. (b) Genes with correlating genetic profiles are shared between INP51 and PIL1 but not INP51 and ABP1 or VPS30. Correlation coefficients between the genetic profile of INP51 and each of the other profiles in the E-MAP are plotted on the x axis. On the y axis, either the similar set of values for the PIL1 profile with all other profiles (dark grey), those for ABP1 with all other profiles (grey) or those for VPS30 with all other profiles (light grey). CC values in blue and red and green indicate the correlation coefficients for the full set of red, blue or green points plotted. (c) Genes with correlating genetic profiles are shared between INP52 and ABP1 but not INP52 and PIL1 or VPS30. (d) Genes with correlating genetic profiles are shared between INP53 and VPS30, but not INP52 and PIL1 or ABP1. (e) INP51, INP52 and INP53 do not correlate with each other.
Figure 2: The three yeast PIP2 phosphatases localize to different compartments within cells. (a) Colocalization of GFP-tagged Inp51 with RFPmars-tagged Lsp1 (left panels), RFPmars-tagged Abp1 (right panels). Representative confocal midsections are shown. 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 panels) and RFP-mars-tagged Abp1 (right panels. Representative confocal midsections
are shown. 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 panels) and RFP-mars-tagged Abp1 (right panels). Representative confocal midsections are shown. The graphs show the intensity profiles for both channels along the perimeter of the cell. Scale bar 2.5µm.
Figure 3: Inp51 localization at the plasma membrane is dynamic. (a) The Inp51 pattern at the plasma membrane changes rapidly. Images of cells expressing Inp51-GFP and Lsp1-RFPmars were collected in 2s intervals for 30s. The upper panel shows the summed intensities of the whole time-course for Lsp1-RFPmars (left), Inp51-GFP (middle) and the merged channels (right). The white dotted arrow indicates start and endpoints for the kymographs in the lower panels. The middle panels show...
representative images of the time-course at 12 seconds and 22 seconds. Arrows highlight specific foci in the images and the kymographs. (b) Quantification of Inp51-GFP resident time at the plasma membrane. The histogram shows the residence time of Inp51 foci (n=63). Average residence time of the foci is 4s as shown in the density dot plot. Scale bar 2.5µm (c) Quantification of Inp51-GFP spots colocalizing with eisosomes in percent from total.
Fröhlich et al Figure 4
**Figure 4: Inp51 physically interacts with Pil1.** (a) Affinity purification and MS analysis of “heavy” labeled cells expressing GFP-tagged Inp51 and untagged control cells. Intensities are plotted against normalized heavy/light SILAC ratios. Significant outliers (P < 1e-11) are colored in red, orange (P < 0.0001) or light blue (P < 0.05); other identified proteins are shown in dark blue. (b) Affinity purification and MS analysis of “heavy” labeled cells expressing GFP-tagged Pil1 and untagged, “light” labeled control cells. Intensities are plotted against normalized heavy/light SILAC ratios. Significant outliers (P < 1e-11) are colored in red, orange (P < 0.0001) or light blue (P < 0.05); other identified proteins are shown in dark blue. (c) Proteins identified in both, the Inp51-GFP and the Pil1-GFP pull-down are plotted against each other. Color coding is according to density with darker colors showing an enrichment of spots. Outliers that are significant in both pull-downs are labeled.
Fröhlich et al Figure 5
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 are shown. Pil1 is not required for regular distribution of Inp52 (b) or Inp53 (c). Scale bar 2.5µm
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*. 
Fröhlich et al. Figure 7
Figure 7: *PIL1* is required for normal phosphatidylinositol(4,5)-phosphate levels at the plasma membrane. (a) Cellular levels of PtdIns(3)P (left panel), PtdIns(4)P (middle panel) and PtdIns(4,5)P$_2$ (right panel) as percentages of total phosphatidylinositides in WT cells (white bars), *pil1*Δ cells (light grey bars), *inp51*Δ cells (dark grey bars) and *pil1*Δ*inp51*Δ cells (black bars) are shown. Error bars represent standard deviations, n=3. (b) WT cells (left panels), *pil1*Δ (middle left panels) cells, *inp51*Δ cells (middle right panels) and *pil1*Δ*inp51*Δ cells (right panels) (expressing two PH domains of PLC$\delta$ under the control of a CPY promotor (upper panels). Corresponding brightfield images are shown in the lower panels. Scale bar=2.5µM (c) Quantification of (b). The average intensity of the plasma membrane signal of the GFP-2xPH$_{PLC\delta}$ is plotted. Error bars represent standard deviations. n=39 (WT) and n=39 (*pil1*Δ) n=36 (*inp51*Δ), n= 39 (*pil1*Δ*inp51*Δ) (d) Quantification of (b) Ratio of plasma membrane bound to cytoplasmic signal of the GFP-2xPH$_{PLC\delta}$ domain is plotted. Error bars represent standard deviations. n=39 (WT) and n=39 (*pil1*Δ) n=36 (*inp51*Δ), n= 39 (*pil1*Δ*inp51*Δ). (e) Yeast lysates of WT cells, *pil1*Δ cells, *inp51*Δ cells, *pil1*Δ*inp51*Δ cells expressing GFP-2xPH$_{PLC\delta}$ 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.