

Stability of Phosphatidylserine in the Membrane of Yeast

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Abstract

Phosphatidylserine (PS) is a phospholipid that is commonly enriched in the inner leaflet of the plasma membrane of yeast and other eukaryotes. PS is produced in the endoplasmic reticulum and is trafficked to the plasma membrane or used to make other phospholipids. Although there have been some advances in understanding PS trafficking, little is known about the duration of PS at the plasma membrane. Preliminary work involving extracellular addition of lyso-PS (PS with one fatty acid tail) indicated a >3 hour duration of PS at the plasma membrane. To further study this, we engineered a strain of *S. cerevisiae* where the PS synthase gene (*CHO1*) is controlled by the repressible galactose promoter (P_{GAL1}). In the presence of galactose, Cho1p enzyme is produced, which allows synthesis of PS. However, when glucose is present in the medium, P_{GAL1} is repressed, in turn repressing the expression of Cho1p and thus PS production. In addition, we introduced a fluorescent, PS-specific probe (GFP-Lact-C2) to this strain. This probe allows studies of the localization of PS within yeast via fluorescent microscopy. In this study, we combined these two tools in order to study PS localization over time. We found that glucose repression of *CHO1* via P_{GAL1} caused a diffusion of GFP-Lact-C2 away from the membrane, indicating a decrease in PS levels. In addition, we tested the effects of membrane-perturbing agents on PS localization and found that some compounds do seem to cause a global redistribution of PS within yeast. Future work includes the adaptation of these tools into *Candida glabrata* to allow for similar studies within a relevant pathogen.

Introduction

Phosphatidylserine (PS) is a phospholipid in the plasma membrane that plays a significant role in membrane structure and cell signaling.⁵ PS is produced in the endoplasmic reticulum (ER) by the PS synthase enzyme Cho1p (Figure 1). After production, PS can either be trafficked to the plasma membrane or used to make phosphatidylethanolamine (PE) in the mitochondria or endosome. While some studies have been performed on the trafficking of PS to the mitochondria or to the plasma membrane, little is known about the dynamics of PS once it reaches these locations¹⁴. Our research focuses on studying the duration of PS at the plasma membrane in the model organism *Saccharomyces cerevisiae*.

In order to observe the movement of PS, we constructed a version of the *CHO1* gene which is under the control of a repressible promoter. The galactose promoter (P_{GAL1}) allows expression of a particular gene in the presence of galactose and represses the expression of the gene in the presence of glucose. By inserting the P_{GAL1} in place of the native *CHO1* promoter (P_{GAL1} -*CHO1*), we are able to control the expression of Cho1p, and thus the production of PS in the cell. Thus, when grown in medium containing galactose, *CHO1* is turned on, allowing the production of PS. Alternatively, when in the presence of glucose, *CHO1* is shut off, halting the production of PS.

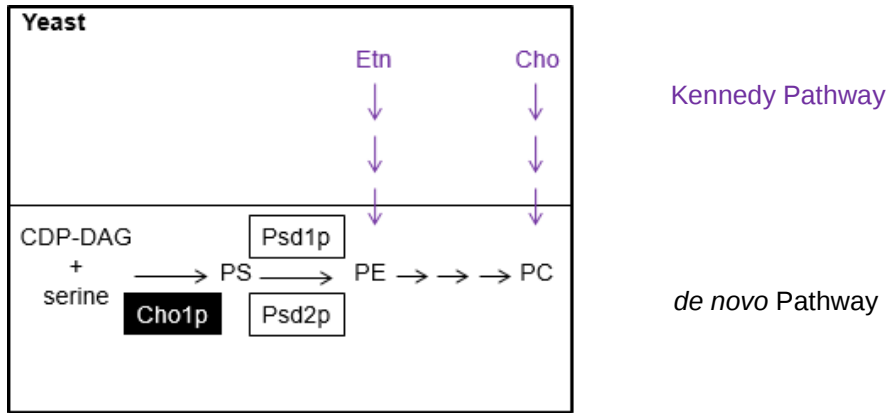


Figure 1: Phospholipid Biosynthesis in Yeast

Cytidyldiphosphate diacylglycerol (CDP-DAG) and serine are used as substrates by Cho1p to make Phosphatidylserine (PS). Phosphatidylethanolamine (PE) is subsequently produced from PS via Psd1p or Psd2p. Phosphatidylcholine (PC) is further produced. In addition, exogenous ethanolamine (Etn) and choline (Cho) can be taken up by the cells to produce PE and PC via an alternate pathway. Figure adapted from ³.

In order to visualize PS localization, we utilized a fluorescent, PS-specific probe. Lactadherin-C2 (Lact-C2) is a bovine protein that binds specifically to PS. Fluorescent probes were fused to Lact-C2 (GFP-Lact-C2 and mCherry-Lact-C2) to allow for the observation of PS in the cell through fluorescent microscopy.¹²

These approaches will allow us not only to control the production of PS, but also to watch the movement of PS overtime within the cell. Findings from our experiments will provide a greater understanding of PS dynamics and help shed light on an important aspect of cellular metabolism. In addition, previous studies in the Reynolds lab showed that PS is important for virulence of *C. albicans*³. The tools described in this project can easily be adapted into *Candida glabrata*, a related fungal pathogen of increasing importance¹⁵, and can ultimately aid in antifungal discovery and the general understanding of fungal pathogens.

Materials and Methods

Table 1. Strains produced in this study.

Strain	Organism	Genotype	Source
Σ (TRY 181)	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ</i>	Lab Strain
BMY2	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ cho1 Δ</i>	Lab Strain
RF01	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ pGFP-Lact-C2</i>	This study

RF04	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ cho1Δ pGFP-Lact-C2</i>	This study
RF09	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ pmCherry-Lact-C2</i>	This study
RF10	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ cho1 Δ pmCherry-LactC2</i>	This study
RF21	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ pmCherry-Lact-C2 pGFP-2x Ph(PLCδ)</i>	This study
RF18	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ cho1Δ pmCherry-Lact-C2 pGFP-2x Ph(PLCδ)</i>	This study
RF25	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R</i>	This study
RF26	<i>S. cerevisiae</i>	<i>ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R pGFP-Lact-C2</i>	This study
Cg 27	<i>C. glabrata</i>	<i>ura3Δ his3Δ leu2Δ tryp1Δ</i>	Lab Strain
RF29	<i>C. glabrata</i>	<i>ura3Δ his3Δ leu2Δ tryp1Δ pGFP-Lact-C2</i>	This study

Table 2. Plasmids used in this study.

Plasmid	Inserts	Source
p200	GFP-Lact-C2, <i>URA3</i> , Cen/Ars, Amp ^R	Yeung <i>et al.</i> , 2008
pBS34	mCherry, <i>URA3</i> , Amp ^R	Shaner <i>et al.</i> , 2004
pGPD416-mCherry-Lact-C2	mCherry-Lact-C2, <i>URA3</i> , Amp ^R	Fairn <i>et al.</i> , 2011
pRS423	<i>HIS3</i> , Amp ^R	Lab Strain
pRS426GFP-2xPH(PLCδ)	GFP-2x Ph(PLCδ), <i>URA3</i> , Amp ^R	Addgene plasmid # 36092
pRF1	GFP-2x Ph(PLCδ), <i>HIS3</i> , Amp ^R	This study
p73	<i>P_{GAL1}</i> , G418 ^R , Amp ^R	Lab Strain
EBp42	NAT ^R , Amp ^R	Lab Strain

Strain Production

Wild-type *S. cerevisiae* and a *CHO1* deletion mutant (*cho1Δ*), which is unable to produce PS, were used. Using a standard lithium acetate transformation method we created the strains used in this study.⁷ **Transformation of GFP-Lact-C2 into WT and *cho1Δ* *S. cerevisiae*:** GFP-Lact-C2 (p200) was transformed into wild-type and *cho1Δ* using the *URA3* marker to produce *ura3Δ his3Δ pGFP-Lact-C2* and *ura3Δ his3Δ cho1Δ pGFP-Lact-C2*, respectively. **Transformation of mCherry-Lact-C2 into WT and *cho1Δ* *S. cerevisiae*:** mCherry-Lact-C2 (pGPD416-mCherry-Lact-C2) was transformed into wild-type and *cho1Δ* using the *URA3* marker to produce *ura3Δ his3Δ pmCherry-Lact-C2* and *ura3Δ his3Δ cho1Δ pmCherry-LactC2*, respectively.

Transformation of GFP-2x Ph(PLCδ) into *ura3Δ his3Δ pmCherry-Lact-C2* and *ura3Δ his3Δ cho1 Δ pmCherry-LactC2 S. cerevisiae*: The GFP-2x Ph(PLCδ) was cut from pRS426GFP-

2×PH(PLC δ) and ligated into pRS423 to produce pRF1. pRS426GFP-2×PH(PLC δ) (pRF1) was transformed into *ura3 Δ his3 Δ pmCherry-Lact-C2* and *ura3 Δ his3 Δ cho1 Δ pmCherry-LactC2* using the HIS3 marker to produce *ura3 Δ his3 Δ pmCherry-Lact-C2 GFP-2x Ph(PLC δ)* and *ura3 Δ his3 Δ cho1 Δ pmCherry-Lact-C2 GFP-2x Ph(PLC δ)*, respectively. **Transformation of P_{GAL1} into WT and cho1 Δ *S. cerevisiae*:** P_{GAL1} was amplified from p73 with primers containing overhangs of the native CHO1 promoter. The PCR product was transformed into wild-type and *cho1 Δ* using the G418/Kanamycin resistance marker to produce *ura3 Δ his3 Δ cho1 Δ ::P_{GAL1}-CHO1 G418/Kan^R*. **Transformation of GFP-Lact-C2 into *ura3 Δ his3 Δ cho1 Δ ::P_{GAL1}-CHO1 G418/Kan^R *S. cerevisiae*:*** GFP-Lact-C2 (p200) was transformed into *ura3 Δ his3 Δ cho1 Δ ::P_{GAL1}-CHO1 G418/Kan^R* using the URA3 marker to produce *ura3 Δ his3 Δ cho1 Δ ::P_{GAL1}-CHO1 G418/Kan^R GFP-Lact-C2*.

All strains and plasmids used in this study can be found in Tables 1 and 2, respectively.

Fluorescent Microscopy

To confirm the presence of GFP-Lact-C2, overnight cultures were assessed via fluorescent microscope (Leica) during stationary phase. In addition, to assess log phase, overnight cultures were diluted back to 0.1 or 0.2 OD₆₀₀ and allowed to grow for 2-4 hours before fluorescent microscopy was again performed.

For quantification of localized, partially localized, and diffuse GFP-Lact-C2, 80 total frames were assessed. The percentages of each category in each frame was calculated and then averaged with the rest of the frames for each carbon source.

All images were analyzed using Leica Application Suite 4.4.

Serial Dilution Plating

Overnight cultures of *ura3 Δ his3 Δ cho1 Δ ::P_{GAL1}-CHO1 G418/Kan^R* were diluted to 0.1, 0.2, 4 x 10⁻³, 8 x 10⁻⁴, and 1.6 x 10⁻⁶ OD₆₀₀. 10 μ l aliquots were spotted onto minimal medium plates supplemented with galactose or glucose, +/- 1 mM ethanolamine. Plates were incubated at 30°C and growth was observed following overnight growth.

Lipid Extraction and Thin Layer Chromatography

The *ura3 Δ his3 Δ cho1 Δ ::P_{GAL1}-CHO1 G418/Kan^R* strain was grown in minimal medium supplemented with either glucose or galactose overnight. Lipids were extracted using a standard hot ethanol extraction⁶. Isolated lipids and lipid standards (Avanti) were spotted on thin layer chromatography plates (Milipore) and separated with chloroform:ethanol:water:trimethylamine (35:30:7:35) solvent system. Lipids were imaged using primulin dye and UV-light exposure.

Lyso PS Experiment

The Lyso-PS protocol from Moser von Filseck *et al.* in 2015 was used in this experiment.² Overnight cultures of *ura3 Δ his3 Δ cho1 Δ pGFP-Lact-C2* were either used directly (stationary phase study) or diluted to 0.1 and allowed to grow for 2 hours (log phase study). Cells were washed with cold water, followed by lyso-PS (Avanti) addition and a 10 minute incubation on ice. Tubes were then incubated at 30°C and at select time points, a 10 μ L sample of cells was

removed and mounted onto slides. Prolong Gold (Life Technologies) was used to preserve samples until microscopy could be performed. Stationary phase time points: 0, 15, 30, and 60 minutes. Log phase time points: 0, 1, 2, and 3 hours.

Compound Experiment

The *ura3Δ his3Δ pGFP-Lact-C2* and *ura3Δ his3Δ cho1Δ pGFP-Lact-C2* strains were used to test the effects of Papuamide-A (Pap-A) (Flintbox), SB-224289 (Tocris), and Staurosporine (CGP 41251) (SelleckChem) on the PS localization. Overnight cultures were diluted to 0.2 OD₆₀₀ and allowed to grow for 3-4 hours. Cultures were then diluted to 0.1 OD₆₀₀ and 200 μL of the *ura3Δ his3Δ pGFP-Lact-C2* were either treated with 50 μg/mL Pap-A, 4.5 μg/mL staurosporine, 250 μM SB-224289, or just the solvent (water:methanol (2:1)) for the Pap-A and DMSO for SB-224289 and staurosporine) as negative controls. *ura3Δ his3Δ cho1Δ pGFP-Lact-C2* was used as a positive control for PS mislocalization. All samples were then incubated at 30°C for 1 hour and 20 minutes followed by fluorescent microscopy. A second trial was performed with a decreased concentration of SB-224289 (100 μM) and cell plating for viability.

Results

Phosphatidylserine is produced in the endoplasmic reticulum, and can either be trafficked to the membrane or sent to the mitochondria where it is used to produce phosphatidylethanolamine (PE). Some studies have provided a greater understanding of PS trafficking to these areas, however; the duration of PS at the plasma membrane is currently unknown. In this work, we undertook experimentation to determine the length of time that PS remains at the membrane in the model yeast *Saccharomyces cerevisiae*.

In order to perform these studies, we began by creating strains harboring GFP-Lact-C2.¹ GFP-Lact-C2 binds to PS in the cell and emits a green fluorescence, allowing us to visualize PS localization within cells

Transformation of GFP-Lact-C2 (p200) into wild-type and *cho1Δ S. cerevisiae*

Figure 2 shows micrographs of *ura3Δ his3Δ pGFP-Lact-C2* and *ura3Δ his3Δ cho1Δ pGFP-Lact-C2*. GFP-Lact-C2 localizes to the membrane in *ura3Δ his3Δ pGFP-Lact-C2* where PS is enriched. In the *ura3Δ his3Δ cho1Δ pGFP-Lact-C2*, which has no PS, GFP-Lact-C2 is diffuse throughout the cell. In order to analyze PS localization in actively growing cells, we performed fluorescent microscopy on the same strains during log phase growth. Figure 3 demonstrates that cells in log phase show a greater distinction between localized and diffuse GFP-Lact-C2.

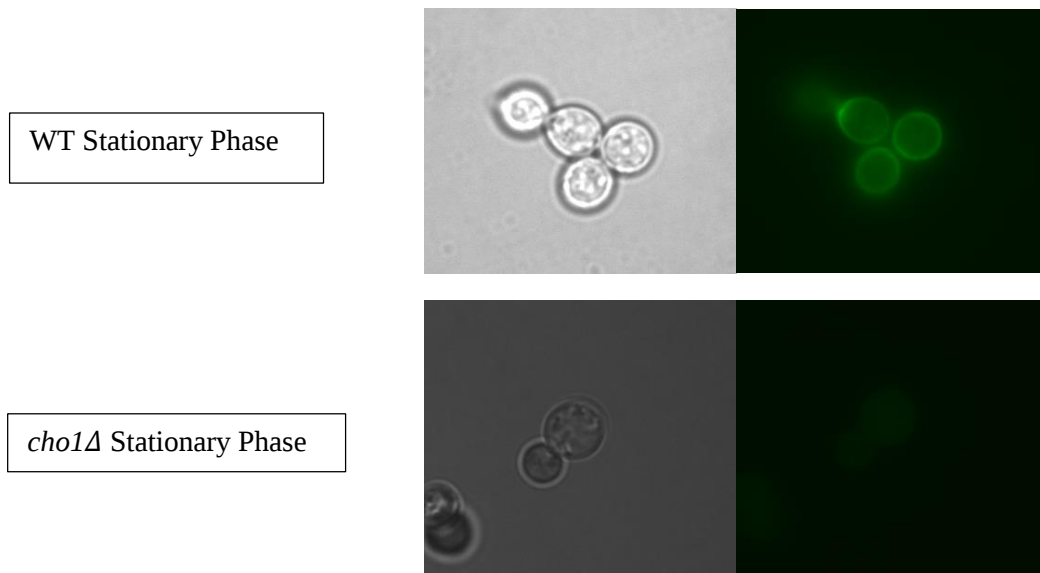


Figure 2. Fluorescent microscopy of *ura3Δ his3Δ pGFP-Lact-C2* and *ura3Δ his3Δ cho1Δ pGFP-Lact-C2* in stationary phase.

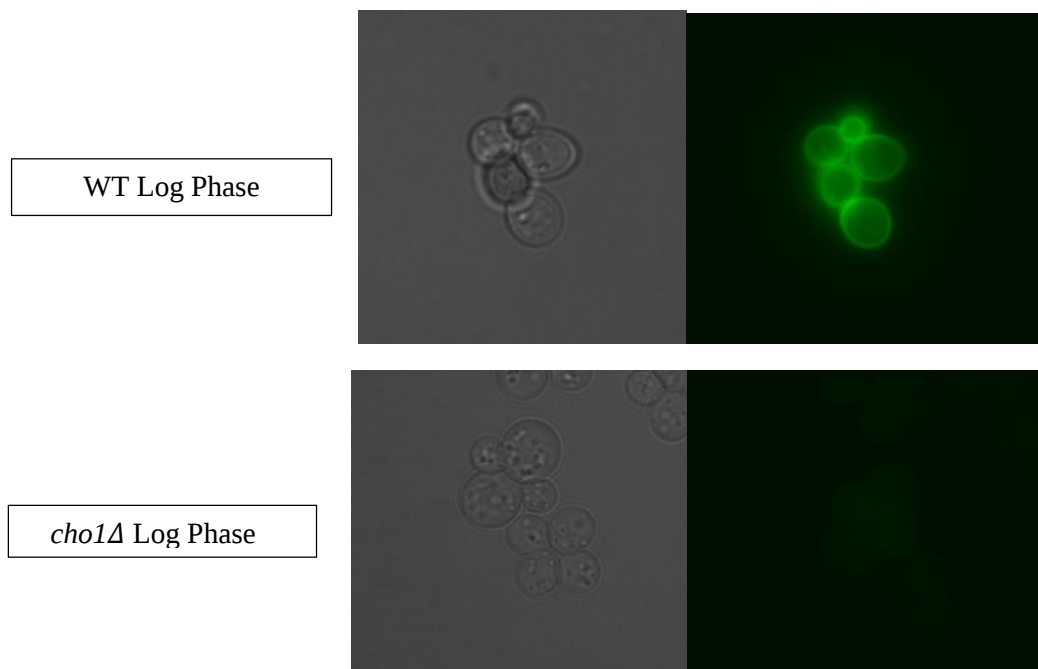


Figure 3. Fluorescent microscopy of *ura3Δ his3Δ pGFP-Lact-C2* and *ura3Δ his3Δ cho1Δ pGFP-Lact-C2* in log phase.

Transformation of mCherry-Lact-C2 (pGPD416-mCherry-Lact-C2) into WT and *cho1Δ* *S. cerevisiae*

We further wanted to determine if the probe bound to the fluorophore mCherry might offer even clearer signal. Figure 4 shows micrographs of *ura3Δ his3Δ pmCherry-Lact-C2* and *ura3Δ his3Δ cho1Δ pmCherry-Lact-C2* grown to log phase. As was shown with GFP-Lact-C2, we see localization of mCherry-Lact-C2 at the membrane in *ura3Δ his3Δ pmCherry-Lact-C2* whereas diffuse signal in *ura3Δ his3Δ cho1Δ pmCherry-Lact-C2*. However, although overall we saw diffusion in *ura3Δ his3Δ cho1Δ pmCherry-Lact-C2*, we did see some cells with slight localization at the membrane.

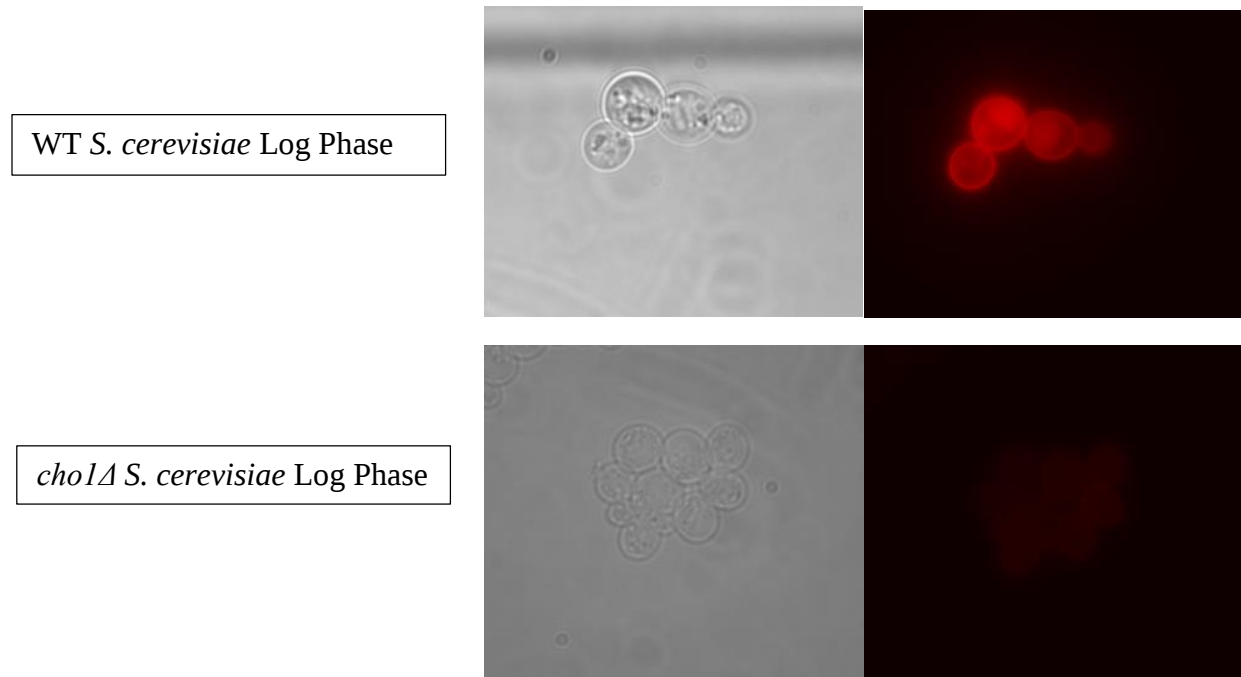


Figure 4. Fluorescent microscopy of *ura3Δ his3Δ pmCherry-Lact-C2* and *ura3Δ his3Δ cho1Δ pmCherry-Lact-C2* in log phase.

Transformation of GFP-2x Ph(PLCδ) (pRS426GFP-2×PH(PLCδ)) into WT and *cho1Δ S. cerevisiae*

In order to better create a distinction between membrane-bound and diffuse Lact-C2 probe, we wished to incorporate a second, plasma membrane positive marker. The Plekstrin homology domain (PH domain) is a domain of approximately 120 amino acids, and it binds to PI4,5P2 in the membrane of *S. cerevisiae*.⁴ We hypothesized that localization of the PH domain fused with GFP (GFP-2x Ph(PLCδ)) to PI4,5P2 could be used as a positive marker for the plasma membrane. This, combined with the Lact-C2 mCherry, would then allow us to monitor the precise location of PS in the cell.

Under this model, mCherry-Lact-C2 and GFP-2x Ph(PLCδ) would overlap (forming a yellow signal) in the wild-type organism. In the *cho1Δ*, the membrane would be green (GFP-2x Ph(PLCδ)) and the cytoplasm would be red (diffuse mCherry-Lact-C2 signal). We further hypothesized that, should this tool be useful, we could add a third component to control PS production. Thus, PS should start out located at the membrane, but upon the shut off of PS production, it should diffuse into the cytoplasm. Resulting overlay images of the red and green

fluorescence under normal conditions, should show a yellow color around the membrane. When the expression of *CHO1* is turned off, there should be a separation of colors as the red begins to diffuse into the cytoplasm, while the green stays at the membrane.

GFP-2x Ph(PLC δ) was transformed into *ura3 Δ his3 Δ pmCherry-Lact-C2* and *ura3 Δ his3 Δ cho1 Δ pmCherry-Lact-C2* to produce *ura3 Δ his3 Δ pmCherry-Lact-C2 GFP-2x Ph(PLC δ)* and *ura3 Δ his3 Δ cho1 Δ pmCherry-Lact-C2 GFP-2x Ph(PLC δ)*. As expected, In *ura3 Δ his3 Δ pmCherry-Lact-C2 GFP-2x Ph(PLC δ)*, there is localization of the fluorescence under both green and red fluorescence (Fig 5C). In addition, as shown in Figure 5A, *ura3 Δ his3 Δ cho1 Δ pmCherry-Lact-C2 GFP-2x Ph(PLC δ)* under the green fluorescence display green (GFP-2x Ph(PLC δ)) localized at the plasma membrane. When under the red fluorescence, the mCherry-Lact-C2 signal is diffuse throughout the cell. However, the results were inconsistent throughout the cultures with some showing localization of mCherry-Lact-C2, and still others showing low mCherry-Lact-C2 signal all together (Fig 5B). As a result, we began to focus efforts on the use of GFP-Lact-C2 alone.

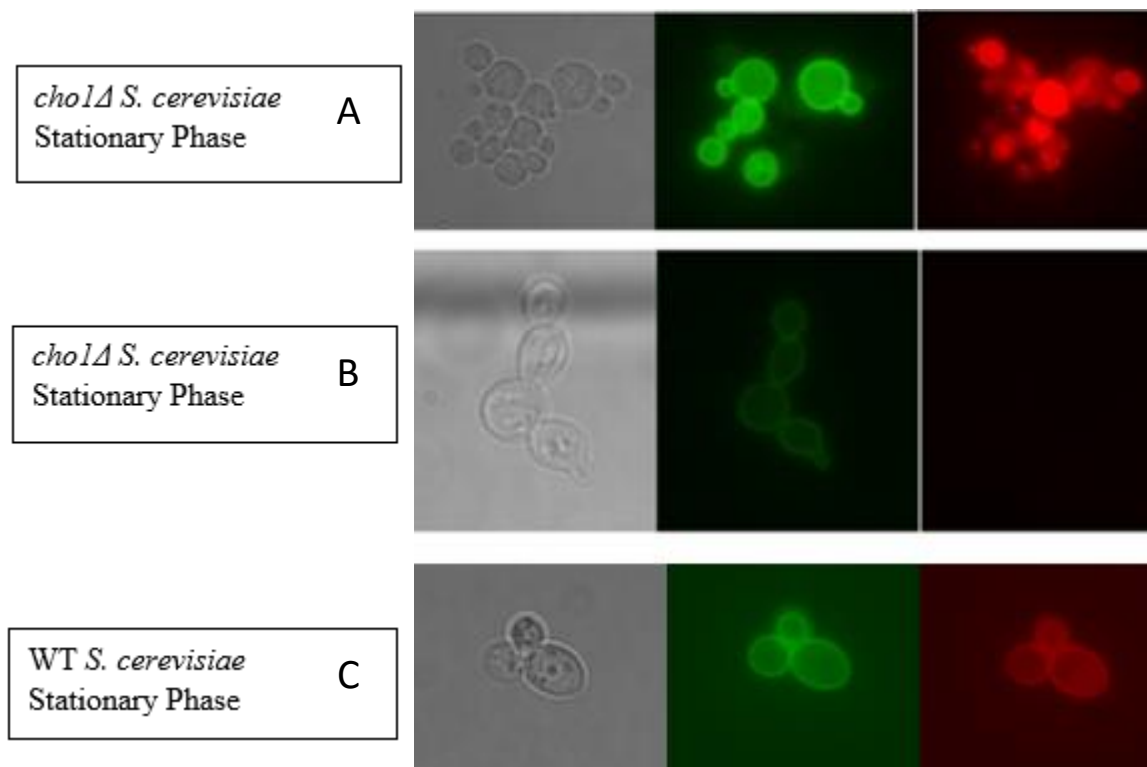


Figure 5. shows the *cho1 Δ S. cerevisiae* with the PH domain GFP and Lact-C2 mCherry under red and green fluorescence in stationary phase.

Transformation of *P_{GAL1}*, G418^R, Amp^R (p73) into WT *S. cerevisiae*

Next, in order to study the length of time that PS remains at the membrane, we wished to create a strain where the PS synthase gene (*CHO1*) was under a repressible promoter. We engineered the strain *ura3 Δ his3 Δ cho1 Δ ::P_{GAL1}-CHO1 G418^R* which allows PS production when the strain is grown in galactose medium, and inhibits PS production when the strain is grown in glucose.

We performed two experiments to validate *ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R*. First, the strain was grown on minimal medium with: 1) histidine and uracil to account for the auxotrophies of the parent strain, 2) galactose or glucose to either repress or allow PS production, and 3) +/- 1 mM ethanolamine to account for any auxotrophy produced by PS decreases.¹³

As shown in Figure 6, in the presence of galactose, the *P_{GAL1}* is turned on, allowing for the expression of *CHO1* and hence the production of PS (Fig 6A). In the presence of glucose the *P_{GAL1}* is shut off, turning off the expression of *CHO1* and preventing PS from being made (Fig 6B). Addition of ethanolamine allows for the production of PE in the absence of PS via the Kennedy Pathway (Fig 1), and allows growth (Fig 6D). However, in the presence of glucose without ethanolamine, the organism should be unable to grow. Unfortunately, though there is a decrease in growth, the fact that the organism is still able to grow (Fig 6C) is an indication that there is an incomplete shut off of *P_{GAL1}*. In this case, we hypothesize that some PS is still being produced in the cell to the extent that PE can be made in the absence of PS.

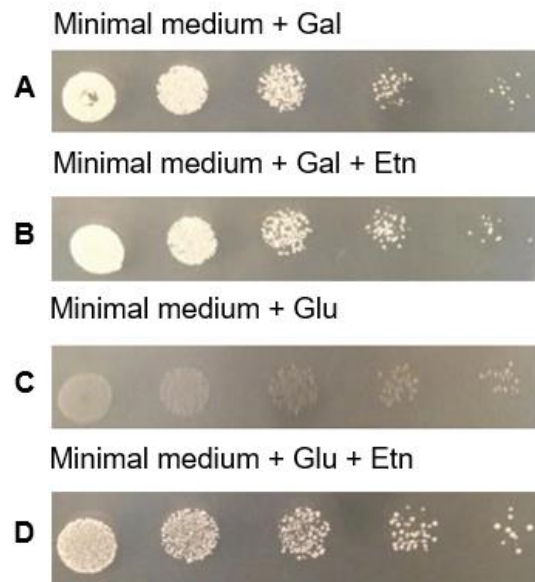


Figure 6. Serial dilutions of the *P_{GAL1}-CHO1* strain in media supplemented with glucose (Glu) or galactose (Gal) and (+/-) ethanolamine (Etn). Results show decreased growth on medium + Glu without Etn where both *de novo* and Kennedy PE synthesis are inhibited (Figure 1).

In order to determine the effects of this incomplete shut-off on PS levels, we isolated lipids from strains grown in all conditions mentioned above. We then performed thin layer chromatography to qualitatively measure PS levels within each condition. As shown in Figure 7, there is PS in *ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R* grown in galactose and *ura3Δ his3Δ* (WT) negative control, because the *CHO1* gene is being expressed. Inversely, there is no PS in

ura3Δ his3Δ cho1Δ positive control. However, as suggested by the growth of glucose-grown *ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R* in figure 6C, there is some PS in the strain grown in glucose, further displaying the incomplete shut off of *P_{GAL1}*.

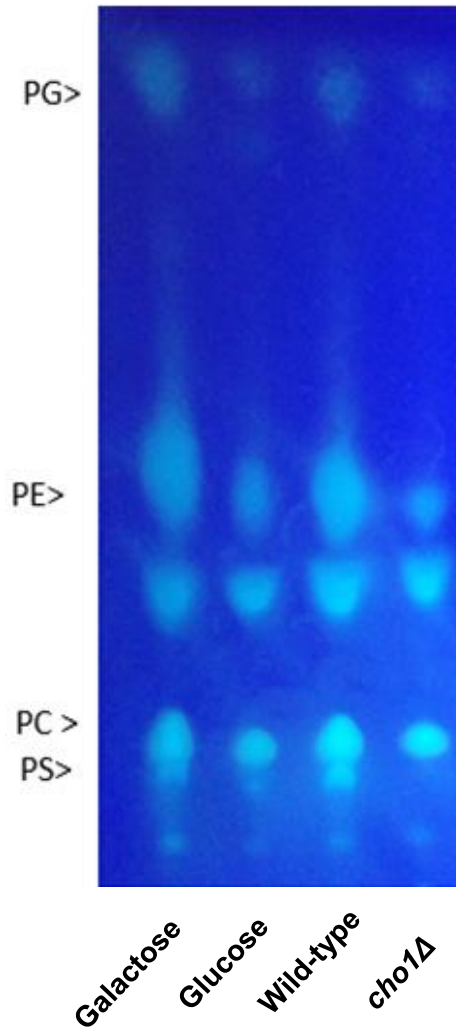


Figure 7. *P_{GAL1}-CHO1* shows decreased, but not complete loss of, PS in presence of glucose as compared to strain grown in galactose, wild-type, and the *cho1Δ* negative control. These results indicate an incomplete shut off of the *CHO1* gene.

Despite these data (Fig 6 and 7), we were interested in performing microscopy in an effort to identify any GFP-Lact-C2 localization differences in this strain. We inserted GFP-Lact-C2 within *ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R* and created *ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1G418^R pGFP-Lact-C2*. We then performed microscopy to determine the influence of growth in glucose or galactose on PS localization. Figure 8A shows *ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R pGFP-Lact-C2* grown in galactose. As expected, GFP-Lact-C2 localized around the cell membrane. Figure 8B, *ura3Δ his3Δ cho1Δ::P_{GAL1}-CHO1 G418^R pGFP-Lact-C2* is grown in glucose, shutting off *P_{GAL1}*, and hence repressing the expression of the *CHO1* gene. PS is not being produced, so the GFP-Lact-C2 is diffuse in the cytoplasm.

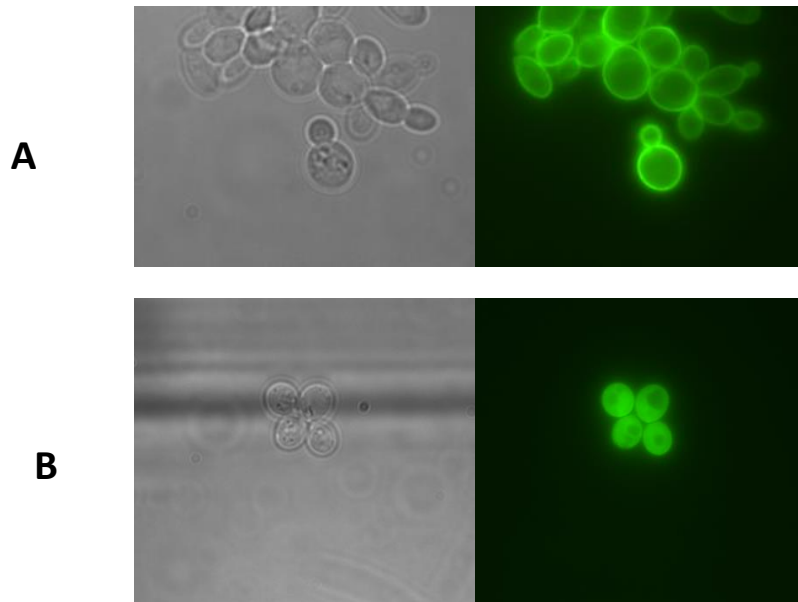
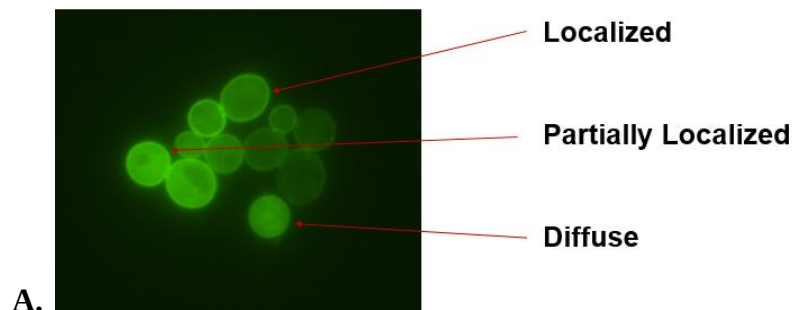


Figure 8. Panel A shows the P_{GAL1} -CHO1::GFP-Lact-C2 *S. cerevisiae* grown in galactose medium, with localized PS at the membrane. Panel B shows the strain grown in glucose and the diffusion of Lact-C2-GFP into the cytoplasm.

Unfortunately, these results were not consistent and we met with high variability when viewing the cells in the fluorescent microscope. As shown in Figure 9A, three localization signatures were seen: 1) distinct localization with the PS localized mainly at the membrane, 2) diffuse with no signs of fluorescence at the membrane, and 3) partial localization with some fluorescence diffuse and localized at the membrane. This further indicated that P_{GAL1} produced an incomplete shutoff and that PS was still being produced to some extent in some cells.



B.

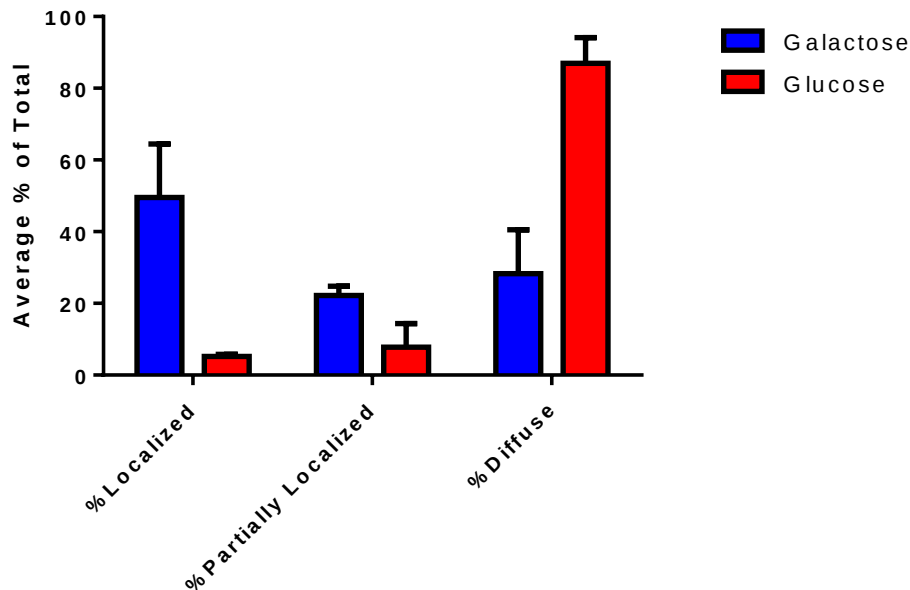


Figure 9. A) Examples of cells localized, partially localized, and diffuse. B) Quantification of GFP-Lact-C2 localization reveals that shut off of Cho1p (i.e. cells grown in glucose) causes quantitative decreases in plasma membrane localization of the probe, but that the result is not consistent. Galactose grown cells showed less growth and weaker fluorescence due to the nature of the galactose carbon source compared to glucose. Localized, partially localized, and diffuse fluorescing cells (Fig 5C) are calculated as a percent of total cells counted . n = 1669 fluorescing cells.

To quantify these findings, we counted the cells falling into the three signature fluorescence types. Figure 9B shows the quantification of 80 frames of fluorescing cells (n=1669 fluorescing cells). Localized, partially localized, and diffuse fluorescing cells were calculated as percentages of each frame, then averaged to produce an overall percentage. Although there is a significant increase in the mislocalization when the strain is grown in glucose, the frames are not completely uniform, and the variability was judged as too high to continue these experiments. Additionally, since galactose is not as good of a carbon source as glucose, the cells showed less growth, and also a weaker fluorescence signal.

Exogenous Addition of Lyso PS

Previous studies found that addition of lyso PS (PS with one fatty acid chain) to *cho1Δ* strain which produces no PS caused the cells to uptake the lyso PS and recycle it in the cell as PS. The lyso PS is converted to PS and transferred to the membrane from the ER.²

We wished to reproduce this effect by adding lyso-PS to *ura3Δ his3Δ cho1Δ pGFP-Lact-C2*. Then we monitored the movement of PS through the cell over a time course via fluorescent microscopy. We added lyso PS to cultures for 10 minutes at room temperature to allow for uptake. Tubes were then placed in the 30°C water bath to allow for trafficking within the cells.

Under the green fluorescence, the cells showed diffuse GFP-Lact-C2 signal for the 0 and 15 minute time points (Fig 10). We hypothesize that lyso PS is uptaken here, and in the process of being repackaged in the ER. The cells begin to show localization around the 30 minute time point where the cells have likely sent re-modeled PS to the plasma membrane. At 60 minutes, the PS is more localized at the membrane. It remains localized at 120 and 180 minute time points, though some of the signal begins to diffuse back in to the cytoplasm.

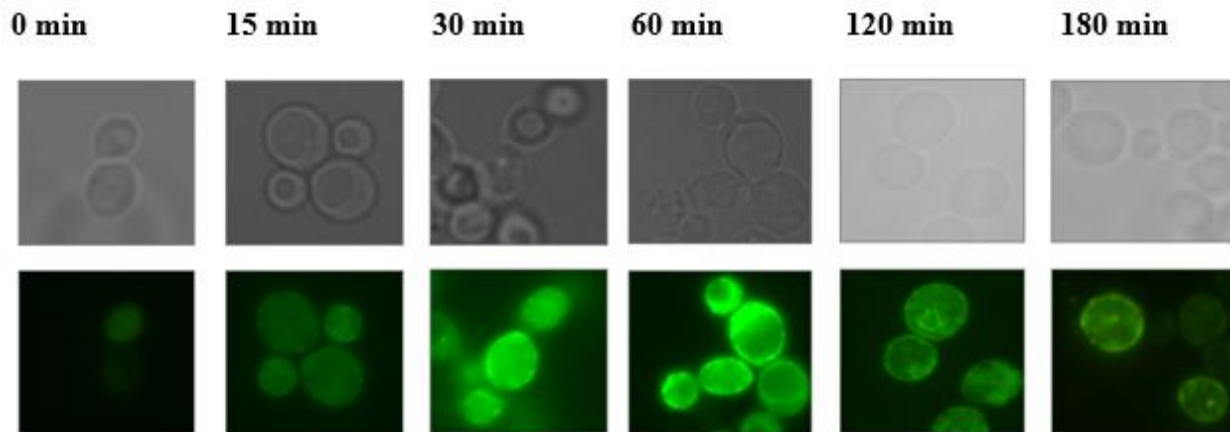


Figure 10. shows the *cho1Δ S. cerevisiae* with Lact-C2 GFP at time points in the Lyso PS experiment. (Photo panel by Chelsi Cassilly)

Compound Experiment

After having engineered the *ura3Δ his3Δ pGFP-Lact-C2* and *ura3Δ his3Δ cho1Δ pGFP-Lact-C2* strains, we were interested in using this tool (GFP-Lact-C2) to determine effects of membrane-perturbing agents on PS localization. Papuamide-A, SB-224289, and staurosporine were chosen, because previous experiments have been performed that indicate that these compounds either disrupt PS trafficking or plasma membrane structure in *Candida albicans*.^{8,11} Papuamide-A is a compound that binds to PS in the plasma membrane and causes the cell lysis and death⁹. Staurosporine blocks endosomal sorting and recycling of PS, and hence inhibits the trafficking of PS to the membrane⁸. SB-224289 is 5-HT_{1B} receptor antagonist that came up as a possible Cho1p inhibitor in a compound screen performed previously in the Reynolds Lab.¹¹

To test the effects of these compounds on PS localization, we treated log phase *ura3Δ his3Δ pGFP-Lact-C2* with varying concentrations of each compound and examined the GFP-Lact-C2 probe signal via fluorescent microscopy. All data was compared to an untreated control and *ura3Δ his3Δ cho1Δ pGFP-Lact-C2*. As shown in Figure 11B, when *ura3Δ his3Δ pGFP-Lact-C2* were treated with Pap-A, accumulation of fluorescence inside the cells was observed, as well as a decrease in cell size, indicating the process of cell death. This was further demonstrated by plating the treated cell sample on medium. Samples treated with Pap-A were unable to grow, whereas untreated cells grew. When treated with Staurosporine, which is supposed inhibit the

trafficking of PS to the membrane, PS was seen trapped in punctuate spots within the cytoplasm. Last, cells treated with SB-224289 showed accumulation of GFP-Lact-C2 in the cytoplasm, and a decrease in signal at the membrane. This result indicates a possible disruption in PS trafficking. Viability plating of Staurosporine and SB-224289-treated cells indicated no signs of cell toxicity via a fungicidal mechanism.

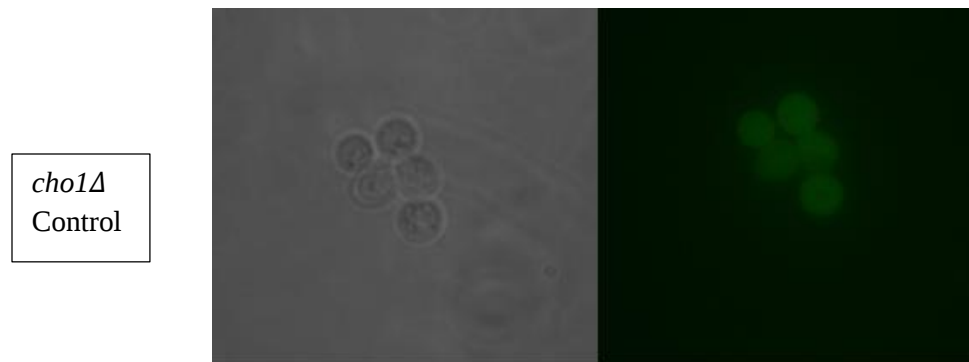


Figure 11. shows *ura3Δ his3Δ cho1Δ pGFP-Lact-C2* control.

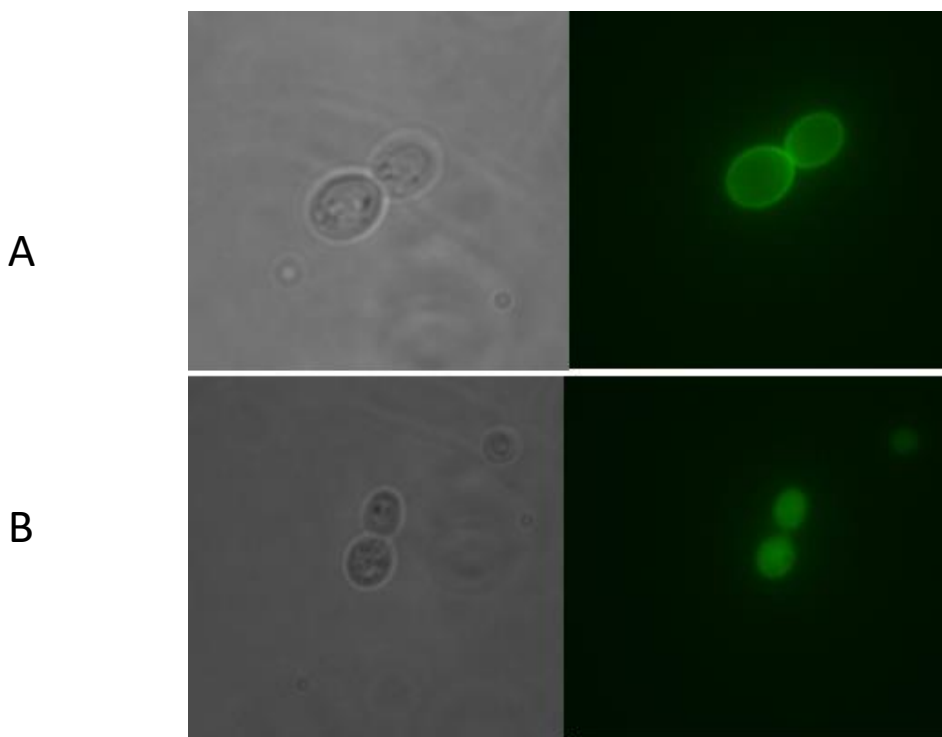
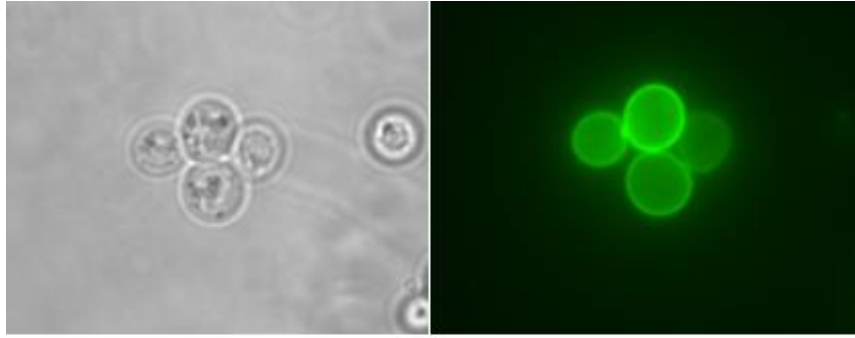


Figure 12. Panel A shows *ura3Δ his3Δ pGFP-Lact-C2* untreated with a methanol:water solution, while panel B shows the strain treated with 50 μg/mL Pap-A.

A



B

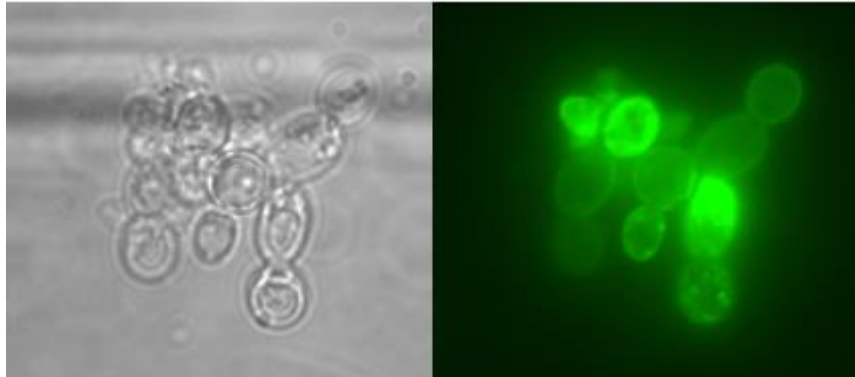
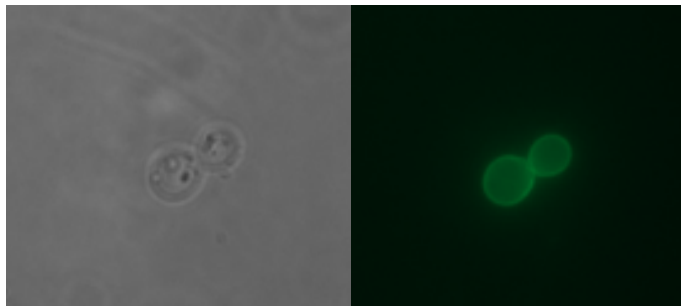


Figure 13. Panel A shows *ura3Δ his3Δ pGFP-Lact-C2* untreated with DMSO, while Panel B shows the strain treated with 4.5μg/mL Staurosporine.

A



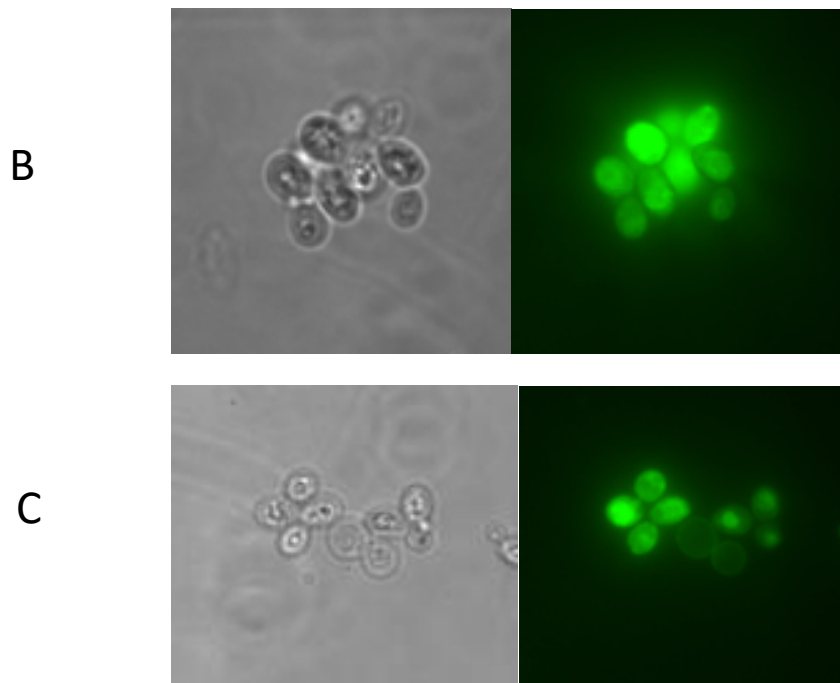


Figure 14. Panel A shows *ura3Δ his3Δ pGFP-Lact-C2* untreated with DMSO, while panel B shows the strain treated with SB-224289 at 250μM. Panel C shows the strain treated with SB-224289 at 100μM.

Discussion

In this research project, we have introduced PS specific probes, GFP-Lact-C2 and mCherry-Lact-C2 into wild-type and *cho1Δ* strains of *S. cerevisiae*. In both instances, we observed probe localization to the membrane in wild-type cells where PS is enriched. Alternatively, in *cho1Δ* strains, we saw diffuse signal since PS is not present in these strains.

After introducing these probes to *S. cerevisiae*, we decided to go one step further and utilize a membrane specific probe. This would allow us to monitor the movement of PS through the cell based on a separation of colors in overlapped pictures of the two probes under either red or green fluorescence. Upon the introduction of this probe, the PH domain, we found inconsistencies in the cells when shown under the red fluorescence. Most of the cells that showed clear localization around the membrane under the green fluorescence, show low or undetectable signal under the red fluorescence. The localization at the membrane under the green fluorescence demonstrated the localization of GFP-PH Domain bound to the membrane, while the low signal under the red fluorescence demonstrates the diffusion of the mCherry-Lact-C2 probe into the cytoplasm. The mCherry-Lact-C2 probe was diffuse in the *cho1Δ*, because PS is not being produced. Although the cells might have shown diffuse red fluorescence as predicted, the signal was bleached soon after the fluorescence was switched to the red color. This would have presented a challenge in performing time-lapse experiments with the two colors, because if the pictures are overlapped and the mCherry-Lact-C2 does not fluoresce as brightly as the PH domain-GFP, we would not be able to sufficiently observe a separation of colors. As a result, although elegant in theory, this project proved insufficient for our purposes, and we focused efforts elsewhere.

In the WT strain, *ura3Δ his3Δ pmCherry-Lact-C2 GFP-2x Ph(PLCδ)*, the localization is seen under both red and green fluorescence. This is expected, because there is PS being produced and localized in the membrane. Therefore, the mcherry-Lact-C2 would be bound to the PS at the membrane, while the PH domain fused with GFP would remain at the membrane.

However, due to inconsistent results, this method of viewing the trafficking of PS was deemed insufficient for the time-lapse experiments. The variability of the fluorescence of two colors presented too much of a challenge to viewing the movement of PS in the time-lapse experiments.

We next focused efforts on controlling PS synthesis *in vivo*. We utilized the *P_{GAL1}* promoter which should shut off *P_{GAL1}* in the presence of glucose, suppressing the *CHO1* gene. We expected no growth as a result of ethanolamine auxotrophy, but found that the strain grown in glucose was able to grow, though at a lesser degree than the strain grown in galactose. This finding was further supported by thin layer chromatography. In this experiment, we saw a low level of PS visible in the lipids extracted from the strain grown in glucose.

Despite this issue, we still wanted to determine if shutting of *CHO1*—albeit incompletely—might have an effect on PS localization. We inserted the GFP-Lact-C2 probe into the *P_{GAL1}* strain, then then viewed it under a fluorescent microscope. We saw an increase in mislocalization when the strain was grown in glucose, showing more diffuse signal than when grown in galactose media. However, some frames showed cells with localized GFP-Lact-C2 signal, while some showed diffusion more diffuse signal. In addition, there were many cells that showed partial localization, indicating that some PS was still being made within the population of cells.

Due to the incomplete shutoff, the *P_{GAL1}-CHO1* strain was deemed insufficient for the time-lapse experiments in monitoring the trafficking of PS. Additional production of PS when the promoter is turned off created complications in viewing where the PS at the membrane goes and how long it stays there.

In an effort to rectify the problems seen in the described experiments, a doxycycline repressible *CHO1* will be made in order to control the *CHO1* gene in *Candida glabrata*. *Candida glabrata* is a yeast that is similar to *S. cerevisiae*, but is also an opportunistic pathogen. The *CHO1* gene will be knocked out of the wild-type Cg27 strain of *Candida glabrata*. Then, the p200 plasmid with GFP-Lact-C2 will be transformed into Cg27 and *cho1Δ* strain. The doxycycline repressible *CHO1* will be produced in a series of transformations: one transforming the tetracycline promoter into the strain, and another transforming the tetracycline repressor into the strain of *C. glabrata*. If this is successful, time-lapse experiments will be performed to observe the dynamics of PS at the membrane.

Lyso PS Experiment

In addition to creating probes for visualizing PS localization, we were able to utilize these strains in experiments to determine the effects of specific treatments on probe localization. First, we

treated *ura3Δ his3Δ cho1Δ pGFP-Lact-C2* with lyso PS to monitor the movement of PS into the cells. After performing the lyso PS experiment several times, it was seen that the PS was more localized after the first two time points. Around the 30 minute time point, the localization around the membrane could be seen, because the lyso PS was being converted into PS, and hence was being transported from the rough ER to the plasma membrane. Once it was at the plasma membrane, the PS remained for until the 60 minute time point, but eventually some began to diffuse back into the cytoplasm, where the localization was not as clear in the last two time points shown in Figure 10. Although this experiment showed that PS mostly remains at the membrane for at least 30 minutes, it is not representative of the biological process that PS is produced in cells. This experiment depends on the cell taking up the lyso PS from the media, and forming PS from it, instead of producing PS in the ER and trafficking it to the membrane.

Compound Experiments

Last, we chose to test the effects of membrane-perturbing agents on GFP-Lact-C2 localization. In Figure 12, the cells treated with Pap-A showed signs of cell death. This result was expected, because Pap-A punctures the cell membranes of cells, causing them to lyse. As a result, we see cells that are dead or dying, with PS accumulated in the cytoplasm. Cells treated with staurosporine also had expected accumulation of PS in punctuate dots in the cytoplasm. Cells treated with SB-224289 at both 250 μ M and 100 μ M, showed a similar accumulation of PS in the cells which might show signs of inhibiting the trafficking of PS (Figure 14). Therefore, SB-224289 might have a role in killing *S. cerevisiae* cells or inhibiting their survival. In future experiment, SB-224289 will be tested at varying concentrations. In future experiments, the effects of PaP-A, Staurosporine, and SB-224289 on PS can to further aid us in observing the dynamics of PS at the cell membrane when faced with other variables, such as these compounds.

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