Phosphatidylinositol 3,5-bisphosphate regulates the transition between trans-SNARE complex formation and vacuole membrane fusion

Gregory E. Miner1, Katherine D. Sullivan1, Annie Guo1, Brandon C. Jones1, Logan R. Hurst1, Ez C. Ellis2, Matthew L. Starr2, and Rutilio A. Fratti1,3,*

1Department of Biochemistry and 2Center for Biophysics and Quantitative Biology, University of Illinois at Urbana–Champaign, Urbana, IL 61801

ABSTRACT Phosphoinositides (PIs) regulate a myriad of cellular functions including membrane fusion, as exemplified by the yeast vacuole, which uses various PIs at different stages of fusion. In light of this, the effect of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) on vacuole fusion remains unknown. PI(3,5)P2 is made by the PI3P 5-kinase Fab1 and has been characterized as a regulator of vacuole fusion during hyperosmotic shock, where it interacts with the TRP Ca2+ channel Yvc1. Here we demonstrate that exogenously added dioctanoyl (C8) PI(3,5)P2 abolishes homotypic vacuole fusion. This effect was not linked to Yvc1, as fusion was equally affected using yvc1Δ vacuoles. Thus, the effects of C8-PI(3,5)P2 on fusion and fission operate through distinct mechanisms. Further testing showed that C8-PI(3,5)P2 inhibited vacuole fusion after trans-SNARE pairing. Although SNARE complex formation was unaffected, we found that C8-PI(3,5)P2 blocked outer leaflet lipid mixing. Overproduction of endogenous PI(3,5)P2 by the fab1T2250A hyperactive kinase mutant also inhibited the lipid mixing stage, bolstering the model in which PI(3,5)P2 inhibits fusion when present at elevated levels. Taken together, this work identifies a novel function for PI(3,5)P2 as a regulator of vacuolar fusion. Moreover, it suggests that this lipid acts as a molecular switch between fission and fusion.

INTRODUCTION

The ability of lipids, and specifically phosphoinositides (PIs), to regulate membrane homeostasis has been well established. Classically, PI-dependent regulation is associated with the recruitment of soluble proteins to membranes to promote the proteins’ function. However, a growing body of work now shows that PIs and other regulatory lipids contribute to a wide array of functions including transcriptional regulation (Carman and Han, 2009), protein sequestering to inhibit function (Starr et al., 2016), microdomain formation (Simons and Toomre, 2000; Fratti et al., 2004), modulating the physical properties of membranes (Andersen and Koepepe, 2007; Zick et al., 2014), and regulating the activity of membrane-anchored proteins (Dong et al., 2010; Li et al., 2014; Kiontke et al., 2017). The rapid modification of lipids by specific kinases, phosphatases, and lipases adds an additional layer of complexity by affecting the spatiotemporal control of cellular events. In the context of Saccharomyces cerevisiae vacuole homeostasis, two of the important PIs are PI3P and PI(3,5)P2. These lipids appear to play opposing roles, as PI3P is characterized as a positive regulator of fusion (Boeddinghaus et al., 2002; Fratti et al., 2004; Karunakaran and Fratti, 2013; Lawrence et al., 2014), whereas PI(3,5)P2 promotes vacuolar fission (Gary et al., 1998; Bonangelino et al., 2002).

PI(3,5)P2 is found predominantly in the late endosome and vacuole, where it is made exclusively by the PI3P 5-kinase Fab1 (Yamamoto et al., 1995; Gary et al., 1998). Deletion of Fab1 results in a complete loss of PI(3,5)P2, as well as a drastic enlargement of the vacuole. PI(3,5)P2 can be dephosphorylated to PI3P by the PI(3,5)P2 5-phosphatase Fig4 (Gary et al., 2002), yet the deletion of Fig4 leads to an overall decrease in PI(3,5)P2 (Duex et al., 2006a,b; Efe et al., 2007). This is due to the required presence of Fig4 along
made under isotonic conditions, albeit at lower concentrations (Bonangelino et al., 2002; Duex et al., 2006b). Although much is known about PI(3,5)P₂, its effect during vacuole fusion is unclear. Here we tested the effects of adding exogenous dioctanoyl (C8) PI(3,5)P₂ to in vitro vacuole homotypic fusion reactions. We found that C8-PI(3,5)P₂ inhibited vacuole fusion in a dose-dependent manner with an IC₅₀ of ~100 µM (Figure 1A). The apparent IC₅₀ includes the large fraction of the lipid that does not incorporate into the membrane. Others have found that 340 µM C8-PI(4,5)P₂ is needed to reach 4 mol% incorporation into liposomes (Collins and Gordon, 2013). Thus, we can roughly estimate that 100 µM C8-PI(3,5)P₂ would translate to 1.2 mol% incorporated into vacuoles.

During hyperosmotic shock, PI(3,5)P₂ activates the TRP Ca²⁺ channel Yvc1. To test whether the inhibitory effect of PI(3,5)P₂ was due to Yvc1 activation, we used vacuoles from yvc1Δ yeast. The addition of C8-PI(3,5)P₂ to yvc1Δ vacuoles had an inhibitory effect on fusion nearly identical to that of WT vacuoles (Figure 1B). Thus, we can conclude that the effect of C8-PI(3,5)P₂ is independent of activating Yvc1 function. As a control we used the PI(3,5)P₂ precursor PI3P. WT vacuoles were treated with a curve of C8-PI3P and tested for fusion. Unlike the inhibitory effects of PI(3,5)P₂, there was no significant reduction in fusion with C8-PI3P (Figure 1C). To see whether the inhibitory effect of PI(3,5)P₂ was related to the presence of two phosphates on the head group, we used C8-PI(4,5)P₂ in fusion assays. We found that C8-PI(4,5)P₂ also inhibited vacuole fusion, albeit at a significantly higher concentration (IC₅₀ > 300 µM) relative to C8-PI(3,5)P₂ (Figure 1D). The difference in IC₅₀ values was likely due to nonoverlapping sets of interacting partners. For instance PI(4,5)P₂ interacts with twinfilin (Palmgren et al., 2001), coflin (Ojala et al., 2001), and Spo14 (Sciolla et al., 2002), while C8-PI(5)P₂ interacts with Vph1 (Li et al., 2014), Ivy1 (Malia et al., 2018), Atg18 (Dove et al., 2004), and Yvc1 (Dong et al., 2010).

C8-PI(3,5)P₂ inhibits vacuole fusion at the docking stage

Previously, we found that adding C8-phosphatidic acid (PA) inhibited vacuole fusion at the priming stage by preventing the recruitment of Sec18 to cis-SNARE complexes (Starr et al., 2016). To begin to determine at which stage PI(3,5)P₂ inhibited fusion, we first tested SNARE priming. When Sec18 is recruited to inactive SNAREs for priming, it associates with cis-SNARE complexes through its interactions with Sec17. Once Sec18 hydrolyzes ATP to dissociate SNAREs, Sec17 is released from the membrane (Mayer et al., 1996). Thus, priming can be measured by the loss of Sec17 from the membrane. Here, fusion reactions were incubated with reaction buffer, C8-PI(3,5)P₂, or N-ethylmaleimide (NEM), a known inhibitor of priming (Paumet et al., 2000). Individual reactions were incubated for specific times after which they were centrifuged to separate the membrane-bound (pellet) and solubilized (supernatant) fractions of Sec17. These experiments showed that PI(3,5)P₂ did not negatively affect SNARE priming, as levels of Sec17 were released similar to

RESULTS AND DISCUSSION

Dioctanoyl PI(3,5)P₂ inhibits vacuole homotypic fusion

Yeast vacuole fusion is promoted by a group of regulatory lipids including PI3P, PI4P, PI(4,5)P₂, ergosterol, and diacylglycerol, whereas vacuole fusion during hyperosmotic shock is regulated by the PI(3,5)P₂ (Dove et al., 1997; Bonangelino et al., 2002; Duex et al., 2006b). PI(3,5)P₂ is made sparingly and turned over rapidly, a likely indication that its prolonged presence or elevated concentration could have a negative impact on vacuole homeostasis. PI(3,5)P₂ is also

FIGURE 1: Dioctanoyl lipids and vacuole homotypic fusion. WT (A) or yvc1Δ (B) vacuoles were tested for fusion with buffer alone or a dose-response curve of C8-PI(3,5)P₂. (C) WT vacuole fusion in the presence of C8-PI3P. (D) WT vacuole fusion in the presence of C8-PI(3,5)P₂ or C8-PI(4,5)P₂. Error bars are SEM (n = 3). **p < 0.01, ***p < 0.001 (unpaired t-test).

with Vac7, Vac14, and Atg18 in the Fab1 complex to yield maximum activity (Sbrissa et al., 2007; Botelho et al., 2008; Jin et al., 2008).

The enlarged vacuolar phenotype seen with the loss of Fab1 suggested a role for PI(3,5)P₂ in membrane fission. Further work showed that hypertonic conditions, which induce vacuolar fission, lead to a rapid increase in PI(3,5)P₂ levels (Dove et al., 1997; Bonangelino et al., 2002; Duex et al., 2006b). The rise in PI(3,5)P₂ triggers a release of Ca²⁺ through the exporter Yvc1 (Dong et al., 2010). Deletion of either Fab1 or Yvc1 attenuates the ability of hypertonic conditions to cause membrane fission, indicating that the increase in PI(3,5)P₂ and subsequent release of Ca²⁺ are essential for membrane fission. PI(3,5)P₂ also binds directly to the vacuole-specific V-ATPase V₀ subunit Vph1 to trigger V-ATPase assembly (Li et al., 2014). Vacuolar acidification itself has been shown to be an essential component of membrane fission, as inhibition of V-ATPase H⁺ pumping activity leads to fusion defects (Baars et al., 2007). In this study we examined the ability of PI(3,5)P₂ to regulate membrane fusion.
Inhibitors were added to individual reactions at different time points throughout the incubation period. Fusion reactions become resistant to a reagent once the target of the inhibitor completes its function. Thus, inhibitors of early events (e.g., priming) would lose their effects before inhibitors of later stages (e.g., hemifusion). Through this approach, we found that PI(3,5)P$_2$ inhibited fusion late in the docking stage, as its resistance curve was after the anti-Sec18 immunoglobulin G (IgG) priming inhibition curve and the tethering inhibitor GDI (Figure 2, C and D). GDI extracts the Rab Ypt7 during fusion to prevent tethering and prevents the formation of trans-SNARE pairs (Haas et al., 1995). The uninhibited control was normalized to 1 for each time point. These data indicate that PI(3,5)P$_2$ inhibits vacuole fusion after Ypt7-dependent vacuole association.

**C8-PI(3,5)P$_2$ does not inhibit trans-SNARE complex formation**

Owing to the indication that PI(3,5)P$_2$ inhibited vacuole fusion after tethering, we next examined whether SNARE complex formation occurred. For this, we used our GST–Vam7 bypass assay, where fusion reactions are blocked at the priming stage by anti-Sec17 IgG (Karunakaran and Fratti, 2013; Miner et al., 2016). The added GST–Vam7 forms new trans complexes with free SNAREs (Merz and Wickner, 2004; Thorngren et al., 2004). Between the addition of anti-Sec17 and Vam7, we further treated individual reactions with GDI or PI(3,5)P$_2$. Once GST–Vam7 was added, the reactions were incubated at 27°C or on ice as indicated. After incubation, the reactions were placed on ice and solubilized as described in Materials and Methods. GST–Vam7 SNARE complexes were isolated with reduced glutathione beads and SNARE complexes were detected by immunoblotting. Figure 2E shows that GST–Vam7 formed complexes with its cognate SNAREs Vam3 and Nvy1 when incubated at 27°C, whereas remaining on ice prevented complex formation. While GDI reduced SNARE complex formation, the addition of PI(3,5)P$_2$ had no effect on the interactions of these proteins. Quantitation of multiple experiments showed that PI(3,5)P$_2$ indeed had no significant effect on SNARE pair formation (Figure 2F).

**Lipid mixing is blocked by C8-PI(3,5)P$_2$**

Previous studies have shown that lysophosphatidylcholine (LPC) blocked fusion late in the pathway, with a gain-of-resistance curve similar to what we saw with PI(3,5)P$_2$ (Reese and Mayer, 2005). While LPC permitted SNARE complex assembly, it was shown that LPC blocked hemifusion (outer leaflet lipid mixing) in a dose-dependent manner. We next examined whether PI(3,5)P$_2$ had an effect similar to that of LPC in blocking outer leaflet lipid

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**FIGURE 2:** C8-PI(3,5)P$_2$ inhibits vacuole fusion at the docking stage. (A) WT vacuoles were monitored for Sec17 release of upon SNARE priming. Fusion reactions containing 3 µg of vacuoles were incubated with reaction buffer, 232 µM C8-PI(3,5)P$_2$, or 1 mM NEM. Vacuoles were incubated at 27°C for the indicated times, after which the organelles were pelleted by centrifugation. Released proteins in the supernatant were separated from the membrane-bound fraction. The membrane pellets were resuspended in volumes of buffer equal to the supernatant. Sec17 was detected by immunoblotting and the amount released was calculated by densitometry. (B) Normalized values were averaged and plotted over time of incubation. (C) Gain-of-resistance kinetic vacuole-fusion assays were performed in the presence of reaction buffer, 70 µg/ml anti-Sec18 IgG, 2 µM GDI, or C8-PI(3,5)P$_2$. Reactions were incubated at 27°C. Reactions were treated with reagents or shifted to ice at the indicated time points. Fusion inhibition was normalized to the reactions receiving buffer alone. (D) Calculated half-times of resistance from assays in C. (E) Vacuole fusion reactions were incubated with anti-Sec17 IgG to block SNARE priming. After incubation for 15 min at 27°C, select reactions were further treated with either reaction buffer, GDI, or C8-PI(3,5)P$_2$ and incubated for an additional 70 min. One reaction remained on ice for the duration of the assay. Reactions were then processed for glutathione pull down of GST–Vam7 protein complexes. Isolated protein complexes were immunoblotted for the presence of the SNAREs Vam3 and Nvy1. (F) Quantitation of SNARE complex formation in the presence or absence of C8-PI(3,5)P$_2$. Error bars are SEM (n = 3).
mixing after allowing trans-SNARE complex formation. Lipid mixing was measured by the quenching of Rhodamine-PE (Rh-PE) on the outer leaflets of vacuoles. Rh-PE-labeled vacuoles were incubated with an excess of unlabeled vacuoles. Upon the fusion of the outer leaflets, Rh-PE was diluted to restore fluorescence. Our results showed that PI(3,5)P₂ potently blocked lipid mixing, similarly to what was seen with LPC (Figure 3, A and B). As a negative control, we used GDI to block fusion at the tethering stage. These data indicate that PI(3,5)P₂ blocks fusion at a stage after trans-SNARE pairing but before lipid mixing can occur.

### Fab1 mutants affect lipid mixing

To verify that an excess of PI(3,5)P₂ inhibits lipid mixing, we used vacuoles from yeast expressing the hyperactive kinase mutation fab1⁰²⁵⁰Δ (Lang et al., 2017). As predicted, fab1⁰²⁵⁰Δ vacuoles failed to undergo lipid mixing (Figure 4, A and B). This was attributed to the increased production of PI(3,5)P₂ and is consistent with our data using exogenous lipid. This posed the question of whether lowering free PI(3,5)P₂ on fab1⁰²⁵⁰Δ vacuoles would restore lipid mixing. To this aim we used the PI(3,5)P₂ probe ML1-N, a polypeptide region at the N-terminus of the endosomal protein TRPML Ca²⁺ channel (Dong et al., 2010). We found that added 50 nM ML1-N partially restored lipid mixing (Figure 4J). This further supports the model in which an excess of free PI(3,5)P₂ negatively regulates vacuole fusion.

We next tested whether the lack of PI(3,5)P₂ would have the opposite effect on lipid mixing. To check this, we used vacuoles from yeast with the kinase dead mutant fab1¹⁴¹⁴EE (Li et al., 2014). Although we expected to find elevated lipid mixing relative to WT vacuoles, we found that fab1¹⁴¹⁴EE vacuoles were blocked for lipid mixing (Figure 4C and D). This suggests that some PI(3,5)P₂ production is needed for fusion, but an excess is inhibitory, which is consistent with a biphasic role for the lipid in vacuole fusion. Biphasic roles for lipids are not unusual. For instance, PI(4,5)P₂ is needed for fusion, yet an excess of the lipid has an inhibitory effect. Another example lies in the turnover of PI(4,5)P₂ by phospholipase C (PLC) to diacylglycerol. It was shown that while low levels of PLC1 enhanced fusion, excess PLC1 blocked fusion altogether (Jun et al., 2004).

Finally, we used fig4Δ vacuoles, which produce reduced amounts of PI(3,5)P₂ in comparison with WT cells. We found that fig4Δ vacuoles showed a modest reduction in lipid mixing (Figure 4, E and F). This suggests that the reduced PI(3,5)P₂ levels associated with fig4Δ cells are sufficient to support lipid mixing, albeit at reduced efficiency. Together, these data suggest that the transition from docking to the hemifusion stage requires some Fab1 activity, but augmented PI(3,5)P₂ production is inhibitory.

To further test the effects of modulating PI(3,5)P₂ concentrations on lipid mixing, we asked whether adding C8-PI(3,5)P₂ to fab1⁰EE or fig4Δ vacuoles would restore lipid mixing. When fig4Δ vacuoles were treated with 25, 50, or 100 µM C8-PI(3,5)P₂, we found that lipid mixing was inhibited. However, when they were compared with WT, we discovered that the inhibitory power of C8-PI(3,5)P₂ was not as potent with fig4Δ vacuoles (Figure 4G). Comparison of the changes in lipid mixing due to 100 µM C8-PI(3,5)P₂ showed an ~50% reduction in lipid mixing inhibition of fig4Δ vacuoles (Figure 4H). This was not due to complete blocking of lipid mixing, as it can be further inhibited at higher C8-PI(3,5)P₂ concentrations (Figure 3). We attribute the reduced inhibition of fig4Δ vacuole lipid mixing to the reduced endogenous levels of PI(3,5)P₂ associated with the deletion. Thus, the combined amount of endogenous and C8-PI(3,5)P₂ in fig4Δ vacuoles was less than ino WT vacuoles, resulting in different effects. This also shows that sufficient endogenous PI(3,5)P₂ is present on isolated vacuoles to affect fusion efficiency.

In parallel, we found that adding C8-PI(3,5)P₂ had no effect on fab1⁰EE lipid mixing (Figure 4J). The lack of any rescue suggested that other factors could be affected by the lack of Fab1 activity. Western blotting of known fusion factors on isolated vacuoles showed that Sec17 and Mon1 were reduced on both fab1⁰EE and fab1¹²²⁵⁰Δ vacuoles (Figure 4K). Sec17 is needed for SNARE priming (Mayer et al., 1996), while Mon1 is the guanine nucleotide exchange factor or the Rab Ypt7 (Nordmann et al., 2010). In addition, fab1⁰EE vacuoles contained reduced levels of Yct1, an ABC transporter needed for efficient vacuole fusion (Sasser et al., 2013). A reduction in any of these factors could be expected to affect lipid mixing negatively. Other key factors such as SNAREs, HOPS, or Ypt7 were not altered on these vacuoles, and the normal amount of Ypt7 suggested that sufficient Mon1 was present to recruit the Rab. It should be noted that these blots were not comprehensive and there could be further changes in protein sorting in fab1⁰EE and fab1¹²²⁵⁰Δ that could alter vacuole homeostasis.

### Endogenous PI(3,5)P₂ on isolated vacuoles and fusion

Thus far, our studies show that while high levels of PI(3,5)P₂ potently inhibit fusion, lower concentrations are in fact beneficial for fusion. To further investigate this, we used the PI(3,5)P₂ ligand ML1-N described above. We added a curve of purified GST-ML1-N to fusion reactions and found that fusion was completely blocked (Figure 5A). This suggests that the endogenous vacuolar PI(3,5)P₂ plays a positive role in the fusion pathway.

To visualize endogenous levels of vacuolar PI(3,5)P₂, we used Cy3-labeled GST-ML1-N to perform docking experiments. Here we used WT, fab1¹⁴¹⁴EE, and fab1¹²²⁵⁰Δ vacuoles. Cy3-ML1-N was added to vacuoles incubated for 20 min at 27°C to allow docking. After incubation, vacuoles were stained with MDY-64 and examined by
fluorescence microscopy. Equal exposure times were used for each strain. Z-stacks of images were taken for ten fields per strain and deconvolved using Zeiss Axiovision software. Figure 5B shows equatorial images of docked vacuoles. WT vacuoles were stained with Cy3-ML1, which clustered at the junctions where vacuoles come into contact, similarly to the localization of other regulatory lipids as previously shown (Fratti et al., 2004). Fab1EEE vacuoles showed no staining whatsoever, in keeping with the lack of kinase activity. This also illustrates the specificity of Cy3-ML1 for PI(3,5)P2, as other PIs such as PI3P and PI(4,5)P2 are present in the absence of

FIGURE 4: Fab1 mutants block lipid mixing. Lipid-mixing assays were performed with WT, fab1T2250A (A, B), fab1EEE (C, D), or fig4Δ (E, F) vacuoles. Reactions were started with the addition of ATP after 5 min and incubated for 60 min. Fluorescence was measured every 60 s. B, D, and F show quantitation of average maximum Rh-PE fluorescence. (G) WT and fig4Δ vacuoles were treated with C8-PI(3,5)P2 and tested for lipid mixing. (H) Comparison of the inhibitory effects of C8-PI(3,5)P2 on lipid mixing. The inhibition of WT vacuole lipid mixing by C8-PI(3,5)P2 was normalized to 1 and compared with the relative inhibition of fig4Δ vacuole lipid mixing. (I) fab1EEE vacuoles were treated with C8-PI(3,5)P2 and tested for lipid mixing. The amount of lipid mixing at the end of 60 min in the absence of C8-PI(3,5)P2 was normalized to 1 and compared with the relative results in the presence of C8-PI(3,5)P2 at the indicated concentrations. Data are represented as whisker plots. (J) fab1T2250A vacuoles were treated with ML1-N and tested for lipid mixing. The terminal level of lipid mixing in the untreated sample was normalized to 1 and compared with the relative results in the presence of ML1-N. (K) Analysis of fusion factors on purified vacuoles (5 µg protein) from WT, fab1EEE, or fab1T2250A yeast. Antibodies against the indicated proteins were used for immunoblotting. Error bars are SEM (n = 3). *p < 0.5.
206 | G. E. Miner et al.

Molecular Biology of the Cell

...the direct interactions of the V₀ subunit Vph1 and PI(3,5)P₂, which promote binding of the V₁ complex. The increased assembly of the V₁–V₀ holoenzyme would in turn deplete free V₀ complexes on the vacuole membrane. The significance of altering the levels of free V₀ complexes and vacuole fusion lies in the ability of V₀ complexes to interact in trans between apposed membranes. Mayer and colleagues originally found that the formation of trans V₀–V₀ complexes plays a role in membrane fusion (Peters et al., 2001). Since their discovery, several other groups using various systems found that V₀ plays important noncanonical roles in membrane fusion, including the release of insulin (Sun-Wada et al., 2006), phagosome–lysosome fusion (Peri and Nusslein-Volhard, 2008), and the release of exosomes through the fusion of multivesicular bodies with the plasma membrane (Liégeois et al., 2006). The c-rings of the V₀ also interact in trans between membranes to form conductance channels through their proteolipid pores (Couoh-Cardel et al., 2016). Although PI(3,5)P₂ has not been shown to reduce V₀–V₀ trans complexes directly, it stands to reason that the increase in V₁–V₀ complexes would in turn diminish the former and potentially alter fusion efficiency.

Another potential target for PI(3,5)P₂ could involve altering Ca²⁺ transport across the vacuole membrane. During salt shock, PI(3,5)P₂ is rapidly synthesized and interacts with the TRP channel Yvc1 to release vacuolar Ca²⁺ stores. Because vacuolar Ca²⁺ stores are also released in response to the formation of trans-SNARE pairs (Merz and Wickner, 2004), we initially hypothesized that PI(3,5)P₂ could affect fusion through interactions with Yvc1. However, vacuoles from yvc1Δ yeast were equally susceptible to PI(3,5)P₂ relative to the WT parents, suggesting that PI(3,5)P₂ has a different target for the inhibition of fusion. Taken together with the studies on PI(3,5)P₂ and vacuole fission, we propose that this lipid performs two opposing functions as a molecular switch to go between fission and fusion.

**FIGURE 5: Endogenous PI(3,5)P₂ on isolated vacuoles. (A) WT vacuoles were incubated with ML1-N to bind PI(3,5)P₂. (B) Docking reactions of vacuoles incubated with Cy3-ML1-N. Vacuoles were counterstained with the MDY-64 to label the entirety of the membranes. Representative vacuole clusters are shown for qualitative assessment of PI(3,5)P₂ distribution.**

Fab1 activity (Gary et al., 1998). It is also important to note that fab1ΔΔΔ vacuoles were commonly enlarged and deformed, as exemplified by the selected cluster (asterisk). This is in agreement with the enlarged vacuoles seen in fab1Δ cells. Finally, we observed increased Cy3-ML1 staining on fab1ΔΔΔ vacuoles, as expected in the presence of elevated PI(3,5)P₂. Interestingly, we also observed an increase in small vesicles with increased Cy3-ML1-N staining tightly apposed to larger vacuoles. Although further detailed testing would be required to verify it, these small vesicles could be the result of vacuole fission linked to the hyperactive nature of this mutant.

Taken together, the results of this study have demonstrated that while PI(3,5)P₂ is needed for vacuole fusion, an excess of the lipid strongly blocks the system. This leaves us with the question of how PI(3,5)P₂ directly affects fusion. One possible mechanism for the inhibition of fusion could be linked to the vacuolar ATPase. Others have reported that PI(3,5)P₂ stabilizes the interactions of the V₁ and V₀ components of the V-ATPase (Li et al., 2014). This occurs through

**MATERIALS AND METHODS**

**Reagents**

Soluble reagents were dissolved in PIPES–sorbitol (PS) buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol) with 125 mM KCl unless indicated otherwise. Anti-Sec17 IgG, anti-Sec18 and Pbs2 (Protease B inhibitor) were prepared as described previously (Haas and Wickner, 1996; Mayer et al., 1996; Slusarewicz et al., 1997). C8-Pi3P (1,2-dioctanoyl-phosphatidylinositol 3-phosphate) and C8-Pi(4,5)P₂ (1,2-dioctanoyl-phosphatidylinositol 3,5-bisphosphate) were purchased from Echelon. Recombinant GST–Vam7 and GDI were prepared as previously described (Fratti et al., 2007; Fratti and Wickner, 2007; Starai et al., 2007). Plasmid to produce recombinant GST-ML1-N was a gift from H. Xu (University of Michigan). GST-ML1-N was prepared as described and dialyzed against PS buffer with 125 mM KCl (Dong et al., 2010). Lissamine Rhodamine (Rh-PE) was from ThermoFisher. NEM (N-ethylmaleimide) was from Sigma.

**Strains**

BJ3505 (Jones et al., 1982) and DKY6281 (Haas et al., 1994) were used for fusion assays (Table S1 in the Supplemental Material). YVC1 was deleted by homologous recombination using PCR products amplified from pFA6-KanMX6 with the primers 5’-YVC1-KO and 3’-YVC1-KO with homology flanking the YVC1 coding sequence (Table S2 in the Supplemental Material). The PCR product was transformed into BJ3505 and DKY6281 yeast by standard lithium acetate methods and plated on YPD media containing G418 (250 µg/ml) to generate BJ3505 yvc1Δ::kanMX6 (RFY74) and DKY6281 yvc1Δ::kanMX6 (RFY75). Similarly, FAB1 was deleted by recombination using the primers 5’-FAB1-KO and 3’-FAB1-KO to
generate BJ3505 fab1Δ::kanMX6 (RFY76). RFY76 was transformed with a plasmid encoding the hyperactive kinase mutant fab1\textsuperscript{1725SA} (pRS416-FAB1-T2250A) and grown in selective media lacking uracil to make RFY78 (Lang et al., 2017). Similarly, a plasmid encoding the kinase–dead mutant fab1\textsuperscript{T2250A} (pRS416-FAB1-E1EE) was transformed into RFY76 to make RFY80 (Li et al., 2014). Plasmids harboring Fab1 mutants were a gift from Lois Weisman (University of Michigan).

FIG4 was deleted through recombination as described above, using PCR products amplified from pAG32 with the primers 5′-FIG4-KO and 3′-FIG4-KO to generate BJ3505 fig4Δ::hphMX4 (RFY82). Transformants were grown in YPD with hygromycin (250 µg/ml).

**Vacuole isolation and in vitro fusion assay**

Vacuoles were isolated as described (Haas et al., 1994). In vitro fusion reactions (30 µl) contained 3 µg each of vacuoles from BJ3505 (PHO8 pep4Δ) and DKY6281 (pho83 PEPA4) backgrounds, reaction buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl\(_2\)), ATP regenerating system (1 mM ATP, 0.1 mg/ml creatine kinase, 29 mM creatine phosphate), 10 µM CoA, and 283 nM Pbi2. Fusion was determined by the processing of pro-Pho8 by direct activation of mucolipin Ca\(_{2+}\)-channel function: an energetic perspective. Annu Rev Biophys Biomol Struct 36, 107–130.

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**Lipid mixing**

Lipid mixing assays were conducted using rhodamine B DHPE (Rh-}

**Vacuole docking**

Docking reactions (30 µl) containing 6 µg of vacuoles from WT, fab1\textsuperscript{1725SA}, or fig4Δ yeast were incubated in docking buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 100 mM KCl, 0.5 mM MgCl\(_2\)), ATP regenerating system (0.3 mM ATP, 0.7 µg/ml creatine kinase, 6 mM creatine phosphate), 20 µM CoA, and 283 nM Pbi2 (Fratti et al., 2004). Pl(3,5)P\(_2\) was labeled with 2.5 µM Cy3-GST-ML1-N. Reactions were incubated at 27°C for 20 min. After incubation, reaction tubes were placed on ice and vacuoles were stained with 1 µM MDY-64. Reactions were next mixed with 50 µl of 0.6% low-melt agarose (in PBS buffer), vortexed to disrupt nonspecific clustering, and mounted on slides for observation by fluorescence microscopy. Images were acquired using a Zeiss Axio Observer Z1 inverted microscope equipped with an X-Cite 120XL light source, a Plan Apochromat 63X oil objective (NA 1.4), and an AxioCam CCD camera.

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