

# Regulation of Fab1 Phosphatidylinositol 3-Phosphate 5-Kinase Pathway by Vac7 Protein and Fig4, a Polyphosphoinositide Phosphatase Family Member

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The *Saccharomyces cerevisiae* *FAB1* gene encodes the sole phosphatidylinositol 3-phosphate [PtdIns(3)P] 5-kinase responsible for synthesis of the polyphosphoinositide PtdIns(3,5)P<sub>2</sub>. *VAC7* encodes a 128-kDa transmembrane protein that localizes to vacuolar membranes. Both *vac7* and *fab1* null mutants have dramatically enlarged vacuoles and cannot grow at elevated temperatures. Additionally, *vac7Δ* mutants have nearly undetectable levels of PtdIns(3,5)P<sub>2</sub>, suggesting that *Vac7* functions to regulate Fab1 kinase activity. To test this hypothesis, we isolated a *fab1* mutant allele that bypasses the requirement for *Vac7* in PtdIns(3,5)P<sub>2</sub> production. Expression of this *fab1* allele in *vac7Δ* mutant cells suppresses the temperature sensitivity, vacuolar morphology, and PtdIns(3,5)P<sub>2</sub> defects normally exhibited by *vac7Δ* mutants. We also identified a mutant allele of *FIG4*, whose gene product contains a *Sac1* polyphosphoinositide phosphatase domain, which suppresses *vac7Δ* mutant phenotypes. Deletion of *FIG4* in *vac7Δ* mutant cells suppresses the temperature sensitivity and vacuolar morphology defects, and dramatically restores PtdIns(3,5)P<sub>2</sub> levels. These results suggest that generation of PtdIns(3,5)P<sub>2</sub> by the Fab1 lipid kinase is regulated by *Vac7*, whereas turnover of PtdIns(3,5)P<sub>2</sub> is mediated in part by the *Sac1* polyphosphoinositide phosphatase family member Fig4.

## INTRODUCTION

The various intracellular compartments located along the secretory and endocytic pathways in eukaryotic cells are maintained by highly efficient and accurate sorting systems. Cargo proteins are first selected and packaged into vesicular carriers that bud and ultimately fuse with the proper target membrane (reviewed in Jahn and Sudhof, 1999). These sorting mechanisms maintain the unique composition of proteins and lipids present in each organelle. Besides the protein machinery that regulates selective sorting and trafficking of cargo, several phospholipids, particularly the phosphorylated phosphatidylinositol (PtdIns) derivatives, also have critical roles in this process (reviewed in De Camilli *et al.*, 1996; Odorizzi *et al.*, 2000). The ability of this class of lipids to serve as a regulator in these complex pathways

derives from the differential and combinatorial phosphorylation of the inositol headgroup.

Members of both the D-3 and D-4 phosphorylated PtdIns derivatives have been implicated in the regulation of membrane trafficking events (De Camilli *et al.*, 1996). In yeast, PtdIns(4)P synthesis is required for protein secretion (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). The observation that D-3 PtdIns derivatives play distinct roles in intracellular trafficking events was determined by the requirement for the PtdIns 3-kinase Vps34 in yeast vacuolar protein sorting (Schu *et al.*, 1993). *vps34* mutants do not produce PtdIns(3)P and have defects in protein transport from the *trans*-Golgi to the vacuole. Additional phosphorylation of PtdIns(3)P by the yeast PtdIns(3)P 5-kinase Fab1 results in the generation of PtdIns(3,5)P<sub>2</sub> (Cooke *et al.*, 1998; Gary *et al.*, 1998). This lipid, although not required for general anterograde transport to the vacuole, is involved in regulating vacuolar homeostasis in yeast (Gary *et al.*, 1998), perhaps through its roles in two novel sorting pathways. Evidence suggests that PtdIns(3,5)P<sub>2</sub> is required for inclusion of the vacuolar hydrolase carboxypeptidase S into vesicles that invaginate into the lumen of an endosomal com-

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partment, forming multivesicular bodies (MVBs) (Odorizzi *et al.*, 1998). Additionally, data also suggest that PtdIns(3,5)P<sub>2</sub> is required for the recycling of membrane proteins through retrograde transport from the vacuole to earlier compartments (Bryant *et al.*, 1998). The inability to generate PtdIns(3,5)P<sub>2</sub> may prevent MVB formation, leading to increased endosomal membrane delivered to the limiting membrane of the vacuole. Likewise, a block in membrane recycling from the vacuole could also result in formation of the grossly enlarged vacuoles in *fab1* mutants (Gary *et al.*, 1998).

Downstream of the lipid kinases, effector proteins bind specific PtdIns derivatives to mediate downstream biological signaling events. The PH, PX, FYVE, and ENTH domains are four well-characterized lipid-binding domains commonly present in these effectors (reviewed in Hurley and Meyer, 2001; Sato *et al.*, 2001; Wishart *et al.*, 2001). PH domains have been predicted in >200 human proteins and have been found to interact with a number of phosphoinositides (Toker and Cantley, 1997; Schultz *et al.*, 2000). Proteins containing FYVE (Wurmser *et al.*, 1999) and PX (Cheever *et al.*, 2001; Xu *et al.*, 2001; Yu and Lemmon, 2001) domains have been directly implicated in protein trafficking by specifically binding to PtdIns(3)P. Last, ENTH domains specifically bind PtdIns(4,5)P<sub>2</sub> to facilitate clathrin-mediated endocytosis (Ford *et al.*, 2001; Itoh *et al.*, 2001). Through protein-lipid interactions, these domains function in targeting proteins to membrane compartments.

The determination that PtdIns derivatives act as signaling molecules to maintain proper membrane trafficking events in yeast suggests that their synthesis and turnover are highly regulated. Indeed, genetic and biochemical studies in yeast have identified upstream activators for some of these PtdIns kinases. The calmodulin-related calcium-binding protein Frq1 interacts with the PtdIns 4-kinase Pik1 to stimulate synthesis of PtdIns(4)P (Hendricks *et al.*, 1999). The recruitment and activation of the Vps34 PtdIns 3-kinase to membranes is dependent upon direct interactions with the membrane-associated protein kinase Vps15 (Stack *et al.*, 1993, 1995). Last, overexpression of Fab1 does not result in increased generation of PtdIns(3,5)P<sub>2</sub> (Gary *et al.*, 1998), suggesting that Fab1 PtdIns(3)P 5-kinase activity is regulated by a limiting activator. Both *fab1* and *vac7* mutants share similar phenotypes, including temperature-sensitive growth, an enlarged vacuole morphology, and severely reduced levels of PtdIns(3,5)P<sub>2</sub> (Bonangelino *et al.*, 1997; Gary *et al.*, 1998). Furthermore, both Fab1 and the integral membrane protein Vac7 localize to vacuolar membranes. These similarities suggest that Vac7 may function in the upstream regulation of Fab1 kinase activity.

There are several turnover pathways for the degradation of PtdIns derivatives in yeast. Phospholipase C hydrolyzes PtdIns(4,5)P<sub>2</sub> into the secondary signaling molecules diacylglycerol and the soluble Ins(1,4,5)P<sub>3</sub> (Rebecchi and Pentylala, 2000). PtdIns(4,5)P<sub>2</sub> can also be dephosphorylated by one of four inositol polyphosphate 5-phosphatases: Inp51, Inp52, Inp53, and Inp54 (Stolz *et al.*, 1998a,b; Guo *et al.*, 1999; Wiradajaja *et al.*, 2001). Inp51–53, also known as the synaptotjanin-like proteins Sjl1, Sjl2, and Sjl3, respectively, and Inp54 all contain a PtdIns(4,5)P<sub>2</sub> 5-phosphatase domain at their carboxy termini (Srinivasan *et al.*, 1997; Guo *et al.*, 1999). Sjl2/Inp52 and Sjl3/Inp53, as well as two additional pro-

teins *Sac1* and *Fig4*, also contain *Sac1* domains at their amino termini. The *Sac1* domains of *Sac1* and Inp53/Sjl3 have been shown to catalyze the dephosphorylation of PtdIns(3)P, PtdIns(4)P, and PtdIns(3,5)P<sub>2</sub> in vitro (Guo *et al.*, 1999; Hughes *et al.*, 2000). Furthermore, *sac1* mutations result in large accumulations of PtdIns(4)P, as well as increased levels of PtdIns(3)P and PtdIns(3,5)P<sub>2</sub> in vivo (Guo *et al.*, 1999). Last, PtdIns(3)P turnover in vivo occurs through multiple mechanisms. PtdIns(3)P on endosomal membranes can be internalized into MVBs, which are degraded by hydrolases upon delivery to the vacuole (Wurmser and Emr, 1998; Gillooly *et al.*, 2000). In addition, PtdIns(3)P that remains on the limiting membrane can be degraded by the myotubularin homolog (Taylor *et al.*, 2000) Ymr1, or phosphorylated by Fab1 (Cooke *et al.*, 1998; Gary *et al.*, 1998).

In this report, we identify genes in yeast that are required for the maintenance of PtdIns(3,5)P<sub>2</sub> levels. We isolated a mutant *fab1* allele (*fab1-5*) that bypasses the requirement for Vac7 function. The identification of a *fab1* mutant that can bypass the requirement for Vac7 is consistent with Vac7 functioning as a positive regulator of Fab1 kinase activity. Additionally, a genetic screen to isolate mutants that bypass the *vac7Δ* temperature sensitivity (*bvs*) identified a mutant allele of *FIG4*, which contains a *Sac1*-like polyphosphoinositide phosphatase domain. Expression of the *fig4-1* mutant allele or deletion of *FIG4* in *vac7Δ* mutant cells rescued the temperature-sensitive growth defects, aberrant vacuolar morphology, and restored PtdIns(3,5)P<sub>2</sub> levels. Together, these results suggest that Vac7 functions as an upstream regulator of the Fab1 lipid kinase activity, whereas the *Sac1* lipid phosphatase family member *Fig4* mediates turnover of PtdIns(3,5)P<sub>2</sub>.

## MATERIALS AND METHODS

### Strains and Media

The *Escherichia coli* strain used for cloning and plasmid propagation was XL1-Blue (supE44 thi-1 lac endA1 gyrA96 hsdR17 relA1 F' proAB LacIq ΔM15). This bacterial strain was grown in standard LB media. The *Saccharomyces cerevisiae* strains used in this study (Table 1) were grown in standard YPD or SD minimal media with the addition of necessary auxotrophic supplements.

### Genetic and DNA Manipulations

Restriction enzymes (Roche Applied Sciences, Indianapolis, IN), T4 DNA ligase, synthetic oligonucleotides, and dNTPs (Invitrogen, Carlsbad, CA) were used according to company specifications. Standard molecular biology techniques were used for all other DNA-based protocols (Maniatis *et al.*, 1982). Yeast transformations and isolation of yeast genomic DNA are described in Gary *et al.* (1998). The chromosomal deletion of *VAC7* in SEY6210 and JGY133 strains was accomplished using the constructs and protocols previously described (Bonangelino *et al.*, 1997). *FIG4* was polymerase chain reaction (PCR) amplified from genomic DNA by using the primers 500 base pairs 5' and 3' from the open reading frame (ORF). The resulting PCR product was digested with *Bam*HI and *Kpn*II ~370 base pairs 5' and 460 base pairs 3' of the *FIG4*, respectively, and cloned into similarly digested pRS416 vector (Sikorski and Hieter, 1989). An identical method was used to clone the *bvs16* allele (*fig4-1*) from strain JGY136.

The *FIG4* ORF was PCR cloned from genomic DNA by using the primers incorporating a unique *Sal*I and *Eag*I restriction sites 300 base pairs 5' from the start codon and *Eag*I 200 base pairs 3' from the stop codon, respectively. This PCR product was digested with *Sal*I/

**Table 1.** *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
SEY6210	<i>MAT<math>\alpha</math> leu2-3, 112 ura3-52 his3-<math>\Delta</math> 200 trp1-<math>\Delta</math> 901 lys2-801 suc2-<math>\Delta</math> 9</i>	Robinson <i>et al.</i> , 1988
SEY6211	<i>MAT<math>\alpha</math> leu2-3, 112 ura3-52 his3-<math>\Delta</math> 200 trp1-<math>\Delta</math> 901 ade2-101 suc2-<math>\Delta</math> 9</i>	Robinson <i>et al.</i> , 1988
JGY132	SEY6210; <i>sjl2<math>\Delta</math> ::HIS3 sjl3<math>\Delta</math> ::TRP1</i>	Foti <i>et al.</i> , 2001
JGY133	SEY6210; <i>fab1<math>\Delta</math> ::LEU2</i>	Gary <i>et al.</i> , 1998
JGY134	SEY6210; <i>vac7<math>\Delta</math> ::HIS3</i>	This study
JGY135	SEY6210; <i>fab1<math>\Delta</math> ::LEU2 vac7<math>\Delta</math> ::HIS3</i>	This study
JGY136	SEY6210; <i>vac7<math>\Delta</math> ::HIS3 bvs16</i>	This study
JGY137	SEY6210; <i>fig4<math>\Delta</math> ::LEU2 vac7<math>\Delta</math> ::HIS3</i>	This study
JGY138	SEY6210; <i>fig4<math>\Delta</math> ::LEU2</i>	This study
JGY139	SEY6210; <i>vac7<math>\Delta</math> ::HIS3 sac1<math>\Delta</math> ::TRP1</i>	This study
JGY140	SEY6210; <i>vac7<math>\Delta</math> ::HIS3 sjl2<math>\Delta</math> ::HIS3</i>	This study
JGY141	SEY6210; <i>vac7<math>\Delta</math> ::HIS3 sjl3<math>\Delta</math> ::TRP1</i>	This study
JGY142	SEY6210; <i>FIG4-HA ::HIS3MX6</i>	This study
JGY143	SEY6211; <i>vac7<math>\Delta</math> ::HIS3</i>	This study
JGY144	SEY6211; <i>vac7<math>\Delta</math> ::HIS3 bvs16</i>	This study
YCS191	SEY6210; <i>fig4<math>\Delta</math> ::LEU2 sjl2<math>\Delta</math> ::HIS3 sjl3<math>\Delta</math> ::TRP1</i>	This study

*EagI* and cloned into *SalI/NotI* digested pBluescriptII (KS<sup>-</sup>) (Stratagene, La Jolla, CA), generating pBS-FIG4. The construct used for deletion of the *FIG4* gene pBS-FIG4::LEU2 was made by digesting pBS-FIG4 with *XbaI/Nru I* (removing 1.8 kb of *FIG4*) and ligating to a similarly digested 2.2-kb fragment that included the *LEU2* gene. The chromosomal *FIG4* gene was replaced with the *fig4 $\Delta$ ::LEU2* construct by linearizing pBS-FIG4::LEU2 with *BglI* and transforming it into SEY6210 or JGY134. Chromosomal insertion of the *fig4 $\Delta$ ::LEU2* disruption was confirmed by PCR analysis. The genomic integration construct was PCR amplified from the pFA6a-3HA-His3MX6 template described by Longtine *et al.* (1998). The resulting 1.7-kb PCR product was then transformed into SEY6210. Chromosomal integration was confirmed by PCR analysis and expression of Fig4-HA was verified by Western blotting.

To mutagenize *FAB1*, a 4.8-kb fragment of *FAB1* was PCR amplified from the plasmid pAY60 (Yamamoto *et al.*, 1995) by using 0.3 $\times$  dATP and 150  $\mu$ M MnCl<sub>2</sub>. The primers corresponded to sites 550 base pairs upstream of the 5' *Sfo I* site and 550 base pairs downstream of the *Nru I* site, respectively. Mutagenized PCR products were then cotransformed into JGY135 with a pAY60 fragment that had been gapped by a *Sfo I/Nru I* digestion. The transformed cells were then plated on selective media and incubated at 38°C. Plasmids from mutants viable at 38°C were isolated and retested for plasmid linkage.

### FM4-64 Labeling of Yeast Vacuoles

Yeast were harvested at an OD<sub>600</sub> reading of 0.6–0.8. Approximately 1 OD<sub>600</sub> of cells was labeled with FM4-64 (Molecular Probes, Eugene, OR) as previously described (Vida and Emr, 1995). Labeled cells were then observed by Nomarski optics and fluorescence (rhodamine channel) as described previously (Wendland *et al.*, 1996).

### Steady-State In Vivo Analysis of Phosphoinositides

The labeling of cells with myo-[2-<sup>3</sup>H]inositol (Amersham Biosciences, Piscataway, NJ) was done in SD-inositol as described (Gary *et al.*, 1998; Bonangelino and Weisman, unpublished data). For this study however, the 10-min 0.9 M NaCl shock was omitted from the labeling protocol and lipid extraction was conducted by a perchloric acid, precipitation-based procedure (Whiteford *et al.*, 1996, 1997). The subsequent high-performance liquid chromatography (HPLC) analysis of deacylated phosphoinositides was done as previously described (Gary *et al.*, 1998). A total of 3.5 million cpm of deacylated lipid extract was injected for each analysis. To more

accurately compare gPtdIns(3,5)P<sub>2</sub> levels between experiments, the levels of each of the lipids from a single HPLC run were normalized based on the integration of the gPtdIns(4,5)P<sub>2</sub> peak being 30,000 cpm. Despite the variation in the raw cpm data from many wild-type labelings, the ratio of gPtdIns(4,5)P<sub>2</sub> to gPtdIns(3,5)P<sub>2</sub> always remained constant.

### Mutagenic Screen for vac7 $\Delta$ Bypass Mutants

Ethyl methanesulfonate mutagenesis of the JGY134 strain was performed as described (Wendland *et al.*, 1996). The treatment was titrated to allow ~40% viability when grown at the permissive temperature of 26°C. After mutagenesis, cells were diluted and plated at 38°C for selection of suppressors. The original isolate, JGY136, was backcrossed to strain JGY143 three times. The final isolate, JGY144, was then transformed with a *LEU2*-CEN *S. cerevisiae* genomic library (Rose and Broach, 1991). Transformants were plated onto SD-LEU-ADE plates at 26°C supplemented with 5.4  $\mu$ g/ml adenine. After 6 d, white colonies were selected and re-screened on identical plates. The library plasmid (pBVS16-107) was isolated and retransformed into JGY136 to verify for plasmid-dependent temperature sensitivity.

### Analysis of Fig4-HA Membrane Association

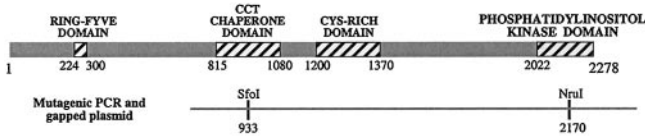
Subcellular fractionation and immunoblot analyses were performed as previously described (Gary *et al.*, 1998). Monoclonal antibody against the hemagglutinin (HA) epitope (Roche Applied Sciences) was used at a 1:1500 dilution. The sucrose density gradients were done as previously described (Babst *et al.*, 1998).

## RESULTS

### Isolation of a *fab1* Mutant That Suppresses *vac7 $\Delta$*

*VAC7* and *FAB1* were each isolated in a screen for mutants defective in vacuolar inheritance (Weisman *et al.*, 1990). Both *vac7* and *fab1* mutants share a common set of phenotypes, including temperature-sensitive growth, dramatically enlarged vacuoles (Wang *et al.*, 1996), and a severe reduction in steady-state PtdIns(3,5)P<sub>2</sub> levels (Gary *et al.*, 1998). These data raise the possibility that Vac7 and Fab1 function in a common pathway, with Vac7 playing an important regulatory role in activation of the Fab1 kinase. A prediction from

## Fab1



## Vac7



**Figure 1.** Schematic diagram of the *S. cerevisiae* Fab1 and Vac7 proteins. Four domains identified within Fab1 are indicated by the hatched boxes and their amino acid positions are noted. The PCR-mutagenized region of *FAB1* is denoted by the gray line and restriction sites used in gapped plasmid repair are also indicated. Vac7 contains a transmembrane domain (TMD) at its carboxy terminus (amino acids 919–941).

this hypothesis is that mutant *fab1* alleles may be capable of suppressing the defects associated with the loss of Vac7 function. We tested this hypothesis by identifying *fab1* mutant alleles that bypassed the requirement for VAC7 function. Accordingly, a 4.8-kb fragment of *FAB1* was randomly mutagenized by error-prone PCR (Figure 1). This region of Fab1 includes the lipid kinase domain as well as the CCT chaperone and cysteine-rich domains conserved in Fab1 homologs (Schultz *et al.*, 2000). Mutagenized *fab1* was then transformed into the *fab1Δ vac7Δ* double mutant strain and transformants were screened for growth at 38°C, the restrictive temperature for the *vac7Δ* strain.

Of the ~10,000 colonies screened, seven were found to grow at 38°C. Next, the vacuole morphology of these *fab1* mutants was assessed using the vital, lipophilic dye FM4-64 (Vida and Emr, 1995). One allele, *fab1-5*, completely suppressed the temperature sensitivity and the enlarged vacuole morphology of the *vac7Δ fab1Δ* double mutant (Table 2 and Figure 2A). The vacuoles in *vac7Δ fab1-5* double mutant cells appear similar in size and number to the vacuoles found in wild-type cells, despite the absence of the VAC7

**Table 2.** Summary of growth phenotypes

Strain	Growth at 38°C
Wild-type	++
<i>vac7Δ</i>	–
<i>vac7Δ fab1-5</i>	++
<i>vac7Δ bvs16</i>	++
<i>vac7Δ bvs16 + FIG4</i>	–
<i>vac7Δ fig4Δ</i>	+
<i>vac7Δ fig4-1</i>	++
<i>vac7Δ sac1Δ</i>	++
<i>vac7Δ sjl2Δ</i>	–
<i>vac7Δ sjl3Δ</i>	–

++, strong growth.  
+, moderate growth.  
–, no growth.

gene product. With pulse-chase labeling followed by immunoprecipitation experiments, we did not observe any difference in protein amount or stability between wild-type Fab1 and the Fab1-5 mutant protein (our unpublished data), suggesting that suppression of the mutant phenotypes by the *fab1-5* allele was not due to an increase in Fab1 stability or expression.

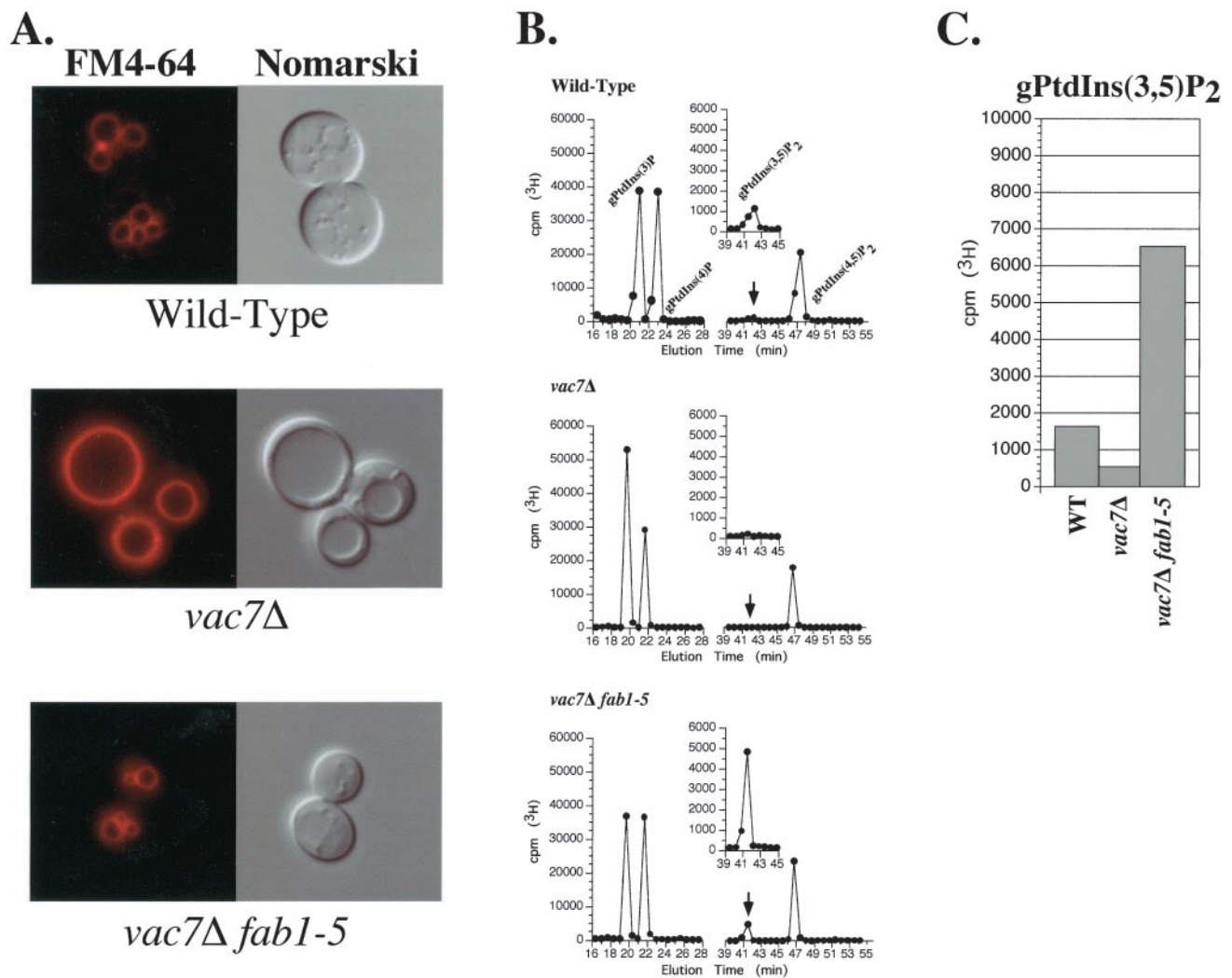
### *fab1-5* Mutation Results in Dramatic Increase in PtdIns(3,5)P<sub>2</sub> Levels

We determined whether suppression of the *vac7Δ* mutant phenotypes by the *fab1-5* mutation was due to a restoration of PtdIns(3,5)P<sub>2</sub> levels by measuring in vivo steady-state phosphoinositide levels. PtdIns derivatives were isolated from myo-[2-<sup>3</sup>H]inositol-labeled cells by lipid precipitation (Whiteford *et al.*, 1996, 1997; Bonangelino and Weisman, unpublished data) and chemical deacylation. Deacylated phosphoinositides were separated by HPLC and quantified by <sup>3</sup>H detection. Wild-type yeast cells produced detectable quantities of four glycerophosphoinositide derivatives corresponding to PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P<sub>2</sub>, and PtdIns(4,5)P<sub>2</sub> (Figure 2B). Compared with wild-type cells, *vac7Δ* cells contained nearly undetectable levels of PtdIns(3,5)P<sub>2</sub> (100–200 cpm above background) and an increased amount of PtdIns(3)P, which is consistent with this lipid being a precursor of PtdIns(3,5)P<sub>2</sub> (Figure 2, B and C). Furthermore, *vac7Δ* mutant cells hyperosmotically shocked in 0.9 M NaCl did not display detectable levels of PtdIns(3,5)P<sub>2</sub> by this analysis. This is similar to results from a previous study in which *fab1Δ* mutants also lacked detectable levels of PtdIns(3,5)P<sub>2</sub> under hyperosmotic conditions (Cooke *et al.*, 1998).

In striking contrast, we found that expression of the *fab1-5* allele in *vac7Δ fab1Δ* double mutant cells resulted in a 40-fold increase in the steady-state levels of PtdIns(3,5)P<sub>2</sub> compared with *vac7Δ* cells expressing wild-type Fab1 (Figure 2, B and C). In addition, *vac7Δ fab1-5* double mutant cells produced approximately fourfold more PtdIns(3,5)P<sub>2</sub> than even wild-type cells. Expression of the *fab1-5* mutation, however, did not significantly alter the levels of the other PtdIns derivatives (Figure 2, B and C). Furthermore, expression of the *fab1-5* allele in wild-type cells also results in a similar increase in PtdIns(3,5)P<sub>2</sub> levels (our unpublished data), indicating that the *fab1-5* mutation is dominant. This dramatic increase in PtdIns(3,5)P<sub>2</sub> indicates that the *fab1-5* mutation restores PtdIns(3,5)P<sub>2</sub> levels in the absence of VAC7, suggesting that this mutation allows the bypass of Vac7 function required for the generation of PtdIns(3,5)P<sub>2</sub>. We have attempted to map the mutation within the *fab1-5* allele that confers the suppression of the *vac7Δ* phenotype and have found that this effect is dependent upon multiple mutations in distinct regions of the *FAB1* gene (our unpublished data).

### Isolation of Other Mutants That Bypass the Requirement for Vac7

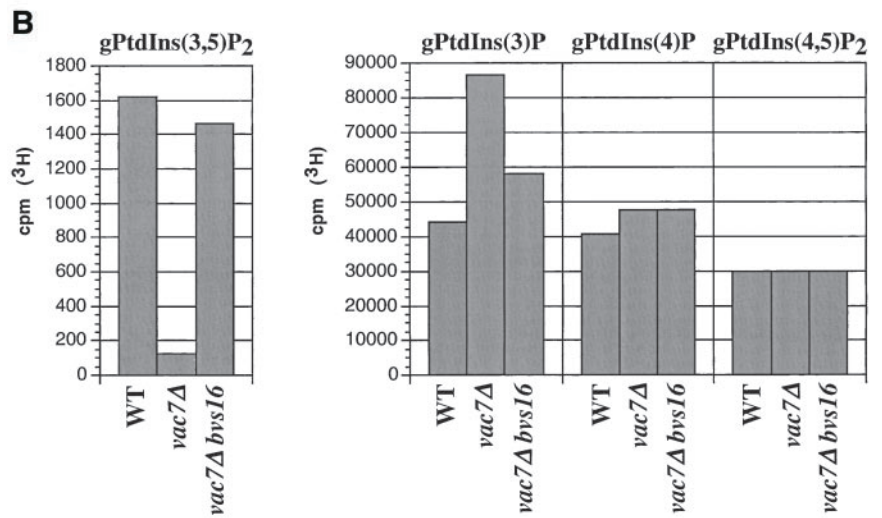
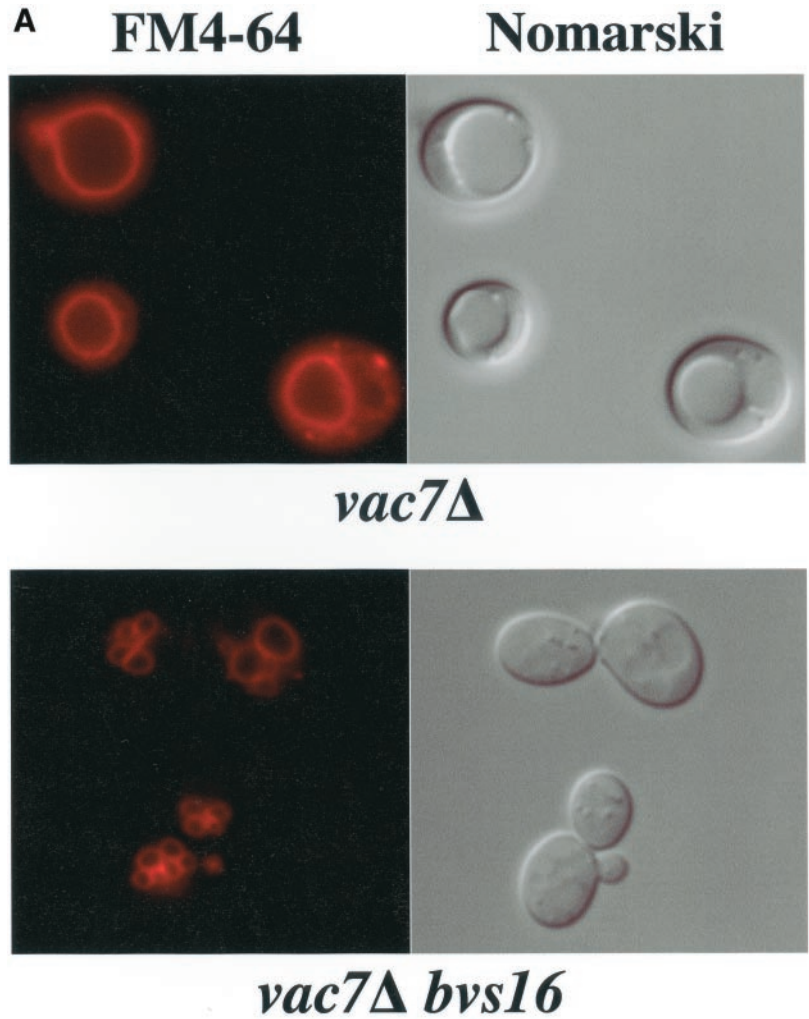
Previously, we proposed that PtdIns(3)P 5-kinase activity was regulated, because overexpression of Fab1 alone did not lead to an increase in PtdIns(3,5)P<sub>2</sub> levels (Gary *et al.*, 1998). Furthermore, the identification of the gain-of-function *fab1-5* allele that suppresses the mutant phenotypes associated with the *vac7Δ* strain suggests that Fab1 activity is indeed



**Figure 2.** Expression of the *fab1-5* mutant allele suppresses the vacuolar morphology defects and restores PtdIns(3,5)P<sub>2</sub> levels in *fab1Δ vac7Δ* double mutant cells. (A) Visualization of vacuolar morphology in wild-type, *vac7Δ*, and *vac7Δ fab1-5* cells. Vacuoles of the indicated strains were visualized by staining with FM 4-64 (left) or by Nomarski optics (right). Cells grown to mid-log phase were labeled with FM 4-64 for 10 min and chased with media for 1 h at 26°C as described in MATERIALS AND METHODS. (B) Representative HPLC profile of the four known PtdIns derivatives from wild-type, *vac7Δ*, and *vac7Δ fab1-5* cells. Cells were labeled with myo-[2-<sup>3</sup>H]inositol for 14 h at 26°C. After precipitation of crude cell lysates with perchloric acid, the total cellular lipids were deacylated as described in MATERIALS AND METHODS. Deacylated phosphoinositides were separated by HPLC analysis and measured by radiolabel detection. The positions of the deacylated PtdIns derivatives are indicated. The peak corresponding to  $\text{gPtdIns(3,5)P}_2$  is highlighted by an arrow and shown in the inset. (C) Bar graph representing the quantified levels of  $\text{gPtdIns(3,5)P}_2$  from the indicated strains. The height of each bar represents the normalized average of three or more independent labeling and HPLC experiments with SD <10%.

regulated, possibly through an interaction with Vac7. We attempted to identify additional genes involved in regulating cellular PtdIns(3,5)P<sub>2</sub> levels by screening ethyl methanesulfonate-mutagenized *vac7Δ* cells for the restoration of growth at 38°C. We reasoned that mutations in at least three classes of genes might be identified: 1) positive and negative regulators of the Fab1 kinase activity; 2) downstream factors responsible for PtdIns(3,5)P<sub>2</sub> degradation, such as PtdIns phosphatases; and 3) genes encoding PtdIns(3,5)P<sub>2</sub> binding effectors, in which mutations would suppress the temperature-sensitivity phenotype but not affect PtdIns(3,5)P<sub>2</sub> synthesis.

Of the 50,000 colonies screened, we isolated 17 mutants that were able to bypass the *vac7Δ* temperature sensitivity (*bvs*) phenotype. These *bvs* mutants were then assessed for suppression of the enlarged vacuole morphology phenotype by FM4-64. All of the *bvs* isolates had morphologies that differed to some degree from the parental *vac7Δ* strain. We focused our attention on the *vac7Δ bvs16* double mutant, which grew at 38°C (Table 2) and displayed a near wild-type vacuolar morphology (Figure 3A). Lipid labeling of the *vac7Δ bvs16* double mutant with myo-[2-<sup>3</sup>H]inositol and subsequent HPLC analysis revealed that this double mutant had restored



**Figure 3.** *bvs16* mutation suppresses the mutant phenotypes associated with *vac7Δ* mutants. (A) Vacuolar morphology of *vac7Δ* *bvs16* double mutants. As in Figure 2, vacuoles from *vac7Δ* or *vac7Δ bvs16* mutant cells were visualized by fluorescent staining with FM 4-64 (left) or Nomarski optics (right). (B) Quantification of PtdIns derivatives in *vac7Δ* *bvs16* mutants. The levels of radiolabeled PtdIns derivatives from the indicated strains were isolated, separated, and measured as described in MATERIALS AND METHODS. The values of each bar represents the normalized average of three or more experiments with SD <15%.

the steady-state level of PtdIns(3,5)P<sub>2</sub> (Figure 3B). The *vac7Δ bvs16* double mutant strain maintained near wild-type levels of PtdIns(3,5)P<sub>2</sub>, >18-fold more PtdIns(3,5)P<sub>2</sub> than the *vac7Δ* strain. The relative levels of other PtdIns derivatives were slightly altered in this mutant. PtdIns(4)P and PtdIns(3)P levels were 1.2- and 1.3-fold, respectively, above wild-type levels. Thus, the *bvs16* mutation restored the steady-state PtdIns(3,5)P<sub>2</sub> level and suppressed the mutant phenotypes in the *vac7Δ* mutant strain.

### The *bvs16* Mutation Is Allelic to *FIG4*

The diploid strain resulting from crossing the *vac7Δ bvs16* isolate to the parental *vac7Δ* strain displayed the identical mutant phenotypes of the *vac7Δ/vac7Δ* diploid strain (our unpublished data). In addition, suppression of the mutant phenotypes segregated with a 2:2 ratio in the haploid progeny from this cross, indicating that the *bvs16* allele results from a single recessive mutation (our unpublished data). To clone the gene allelic to *bvs16*, we generated a *vac7Δ bvs16 ade2* triple mutant that, due to the *ade2* mutation, accumulated a red pigment in its vacuoles that cause colonies to appear red (Wada *et al.*, 1992). The color change is dependent upon vacuolar acidification. Thus, *vac7Δ ade2* double mutant cells, which do not contain acidified vacuoles, appear white. Therefore, we reasoned that we would be able to identify the gene allelic to *bvs16* by transforming the *vac7Δ bvs16 ade2* triple mutant with a *S. cerevisiae* genomic library and screening for white colonies.

From this screen, we isolated one transformant that displayed the enlarged vacuole phenotype of *vac7Δ* mutants and was no longer able to grow at 38°C. The library plasmid pBVS16-107 from this transformant was then rescued and sequenced, revealing a 10.8-kb genomic DNA insert containing six ORFs, which included the *FIG4* gene. Additional complementation analysis determined that transformation of the *FIG4* gene alone into the *vac7Δ bvs16* double mutant strain was sufficient for reversion to *vac7Δ* mutant phenotypes (Figure 4A and Table 2). Furthermore, we found that deletion of the *FIG4* gene in *vac7Δ* cells suppressed the *vac7Δ* temperature sensitivity and vacuole morphology defects (Table 2 and Figure 4A). Quantification of the PtdIns derivatives in the *vac7Δ Fig4Δ* double mutant strain showed that steady-state PtdIns(3,5)P<sub>2</sub> levels increased sevenfold compared with the *vac7Δ* alone (Figure 4B). Slight changes in the other phosphorylated forms were also observed relative to *vac7Δ* (Figure 4B); PtdIns(3)P and PtdIns(4)P levels decreased 40 and 55%, respectively.

### *Fig4* Contains a Polyphosphoinositide Phosphatase Domain

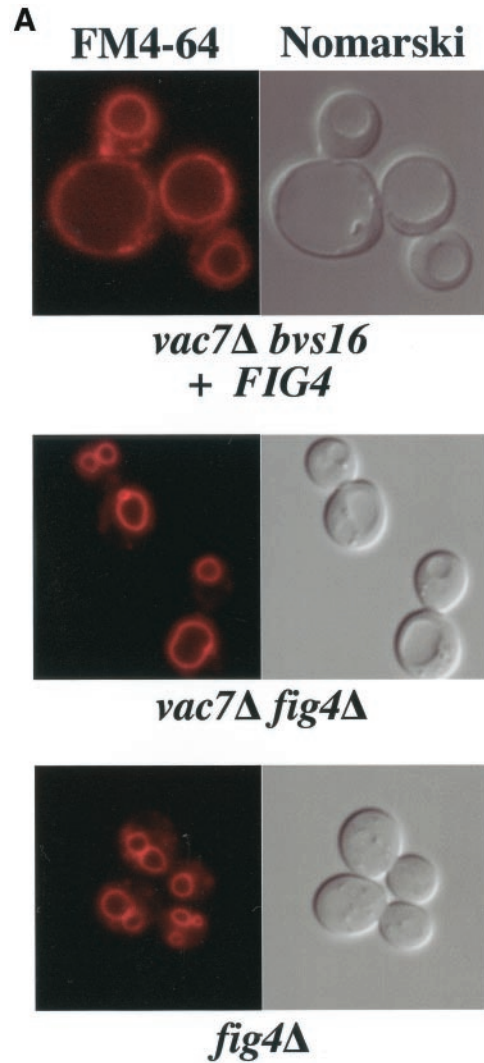
*Fig4* is one of four proteins in yeast that contains a polyphosphoinositide phosphatase domain called the *Sac1* domain (Figure 5A; Guo *et al.*, 1999). Other representatives include *Sac1* and the inositide polyphosphate 5-phosphatases Sjl2/Inp52 and Sjl3/Inp53 (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998a,b; Guo *et al.*, 1999). In vitro, the *Sac1* domains from *Sac1* and Sjl3 are able to dephosphorylate PtdIns(3,5)P<sub>2</sub>, PtdIns(3)P, and PtdIns(4)P but do not appear to recognize PtdIns(4,5)P<sub>2</sub> (Guo *et al.*, 1999; Hughes *et al.*, 2000). Furthermore, *sac1* mutants accumulate ~2.5-fold higher levels of PtdIns(3,5)P<sub>2</sub> than wild-type cells (Guo *et al.*,

1999). Because evidence suggests that PtdIns(3,5)P<sub>2</sub> may be a substrate for the *Sac1* domains of *Sac1* and Sjl3, we addressed the specificity of *fig4* inactivation to mediate bypass of *VAC7* function by assessing whether the loss of other *Sac1* domain-containing proteins similarly affected *vac7Δ* mutants. The *sac1Δ vac7Δ* double mutant strain was able to grow at 38°C, whereas *sjl2Δ vac7Δ* and *sil3Δ vac7Δ* double mutant strains were not able to grow (Table 2). As expected, neither deletion of *SJL2* nor *SJL3* was able to rescue PtdIns(3,5)P<sub>2</sub> levels in *vac7Δ* mutant cells. In contrast, the PtdIns(3,5)P<sub>2</sub> levels in the *vac7Δ sac1Δ* double mutant strain were higher than in *vac7Δ fig4Δ* double mutant cells (Figure 5B). However, in addition to the elevation in the level of PtdIns(3,5)P<sub>2</sub>, the level of PtdIns(4)P also was dramatically elevated, ~16-fold more than in wild-type or *vac7Δ* cells (Figures 4B and 5B). Furthermore, transformation of the *SAC1* gene in the remaining *bvs* mutants did not reverse the *vac7Δ* suppression, suggesting that mutations in *SAC1* were not isolated. *Sac1* is an integral membrane protein localized to the endoplasmic reticulum in yeast that primarily dephosphorylates PtdIns(4)P in vivo (Guo *et al.*, 1999; Foti *et al.*, 2001). These results suggest that inhibition of PtdIns(3,5)P<sub>2</sub> turnover by inactivation of *Fig4* or *Sac1* can restore PtdIns(3,5)P<sub>2</sub> levels to allow the bypass of *Vac7* function.

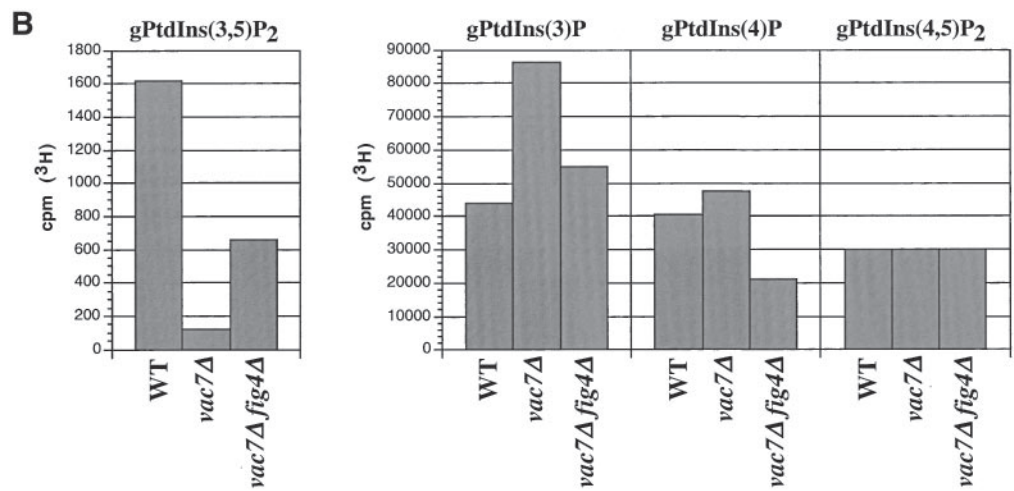
### Identification of *fig4-1* Mutation

Our genetic evidence and the presence of a *Sac1* domain suggests that *Fig4* is a lipid phosphatase that regulates the turnover of PtdIns(3,5)P<sub>2</sub>. Surprisingly, however, analysis of steady-state lipid levels revealed that *fig4Δ* cells did not contain significantly higher levels of PtdIns(3,5)P<sub>2</sub> compared with wild-type cells (Figure 6A). In sharp contrast, *fig4-1* mutant cells had almost threefold more PtdIns(3,5)P<sub>2</sub> than wild-type and *fig4Δ* cells. Similarly, the level of PtdIns(3,5)P<sub>2</sub> in *vac7Δ fig4-1* double mutants was almost 2.5-fold greater than in *vac7Δ fig4Δ* double mutant cells (compare Figure 3B with 4B). These observations suggest that the *fig4-1* mutation has a greater effect on the restoration of PtdIns(3,5)P<sub>2</sub> levels than does deletion of *FIG4* itself. To understand the nature of the *fig4-1* mutation, we sequenced the *fig4-1* allele and identified a single mutation that resulted in the exchange of glycine at position 519 for arginine (G519R; Figure 6B). This amino acid change occurred ~50 amino acids outside of the *Sac1* catalytic domain (RXNCXDCLDRTN) and is conserved in both Sjl2/Inp52 and Sjl3/Inp53 (Figure 6B). *Sac1* does not have a conserved glycine at this position, however, a mutation that changes the corresponding alanine inactivates *Sac1* phosphatase activity (Whitters *et al.*, 1993).

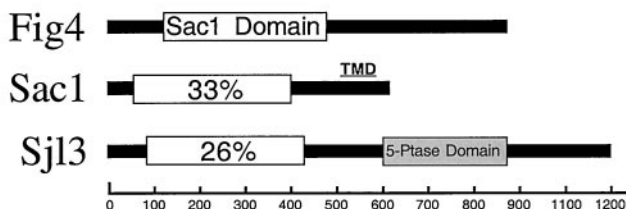
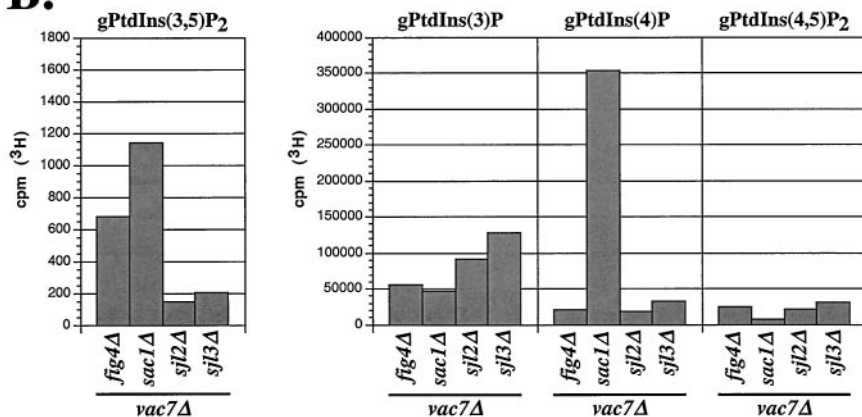
A possible explanation for the lack of elevated PtdIns(3,5)P<sub>2</sub> levels in *fig4Δ* cells may be due to other *Sac1* domain-containing lipid phosphatases, which compensate for the loss in *Fig4* activity. Consistent with earlier work (Guo *et al.*, 1999), we found that *sjl2Δ sjl3Δ* double mutant cells maintain increased cellular levels of PtdIns(3,5)P<sub>2</sub> compared with wild-type cells (Figure 6C). This suggests that both Sjl2 and Sjl3 may be responsible for dephosphorylating PtdIns(3,5)P<sub>2</sub> in *fig4Δ* mutant cells. Indeed, in *fig4Δ sjl2Δ sjl3Δ* triple mutant cells, the PtdIns(3,5)P<sub>2</sub> level was more than fivefold greater than in wild-type cells with modest increases in both PtdIns(4)P and PtdIns(4,5)P<sub>2</sub> (Figure 6C). These results indicate that Sjl2, Sjl3, and *Fig4* may have



**Figure 4.** Deletion of *FIG4* suppresses the mutant phenotypes of *vac7Δ* mutants. (A) Vacuole morphology of the indicated strains was visualized by labeling with FM 4-64 (left) or Nomarski optics (right). (B) Quantification of radiolabeled PtdIns derivatives from wild-type, *vac7Δ*, and *vac7Δ fig4Δ* mutant strains. The normalized average of deacylated PtdIns derivatives from the indicated strains were quantified as described in MATERIALS AND METHODS. The values of each bar represents the average of three or more independent labeling experiments with SD <10%.





**A.****B.**

**Figure 5.** Fig4 contains a *Sac1* polyphosphoinositide phosphatase domain. (A) Schematic diagram indicating the positions of *Sac1* domains (open rectangle) within Fig4, *Sac1*, and Sjl3/Inp53. The *Sac1* domain of Fig4 is 33 and 25% identical to the *Sac1* domains of *Sac1* and Sjl3, respectively. In addition, *Sac1* contains a predicted transmembrane domain (TMD) at its carboxy terminus, whereas Sjl3 also contains an inositol polyphosphate 5-phosphatase domain. (B) Quantification of radiolabeled PtdIns derivatives from *vac7Δ fig 4Δ*, *vac7Δ sac1Δ*, *vac7Δ sjl2Δ*, and *vac7Δ sjl3Δ* double mutants. Radiolabeled PtdIns derivatives were isolated and quantified from the indicated mutant strains as described in MATERIALS AND METHODS. The values of each bar represents the average of three or more independent labeling experiments with SD <15%.

overlapping functions in regulating PtdIns(3,5)P<sub>2</sub> homeostasis in vivo.

### Fig4 Is a Peripheral Membrane Protein

Because our data suggest that Fig4 is a lipid phosphatase, we expected that Fig4 would associate with membrane compartments. To determine whether Fig4 associates with membranes, we constructed a strain that expressed a Fig4-HA tag fusion and used differential centrifugation to generate two membrane-enriched fractions. The Fig4-HA fusion protein was equally enriched in both the 13,000 × *g* (P13) and 100,000 × *g* (P100) pelletable fractions (Figure 7A), indicating that Fig4 may associate with distinct compartments. Furthermore, the vacuolar transmembrane protein Vam3 was found exclusively in the P13 fraction (Figure 7A), suggesting that a fraction of Fig4 associates with vacuolar membranes. Alternatively, the P13 fraction of Fig4-HA may result from an insoluble aggregation or association with cytoskeletal elements. We addressed these possibilities by loading the P13 fraction at the bottom (fraction 5) of a sucrose density step gradient. During a 20-h centrifugation at 200,000 × *g*, the low-density membranes, such as vacuolar membranes, migrate up toward the top of the gradient into fractions 1–3, whereas insoluble proteins are found at the bottom (fraction 5 and pellet). After centrifugation, Fig4-HA appeared primarily in the second fraction of the gradient, as did Vam3 (Figure 7B). These results are consistent with an association of Fig4 with membrane compartments.

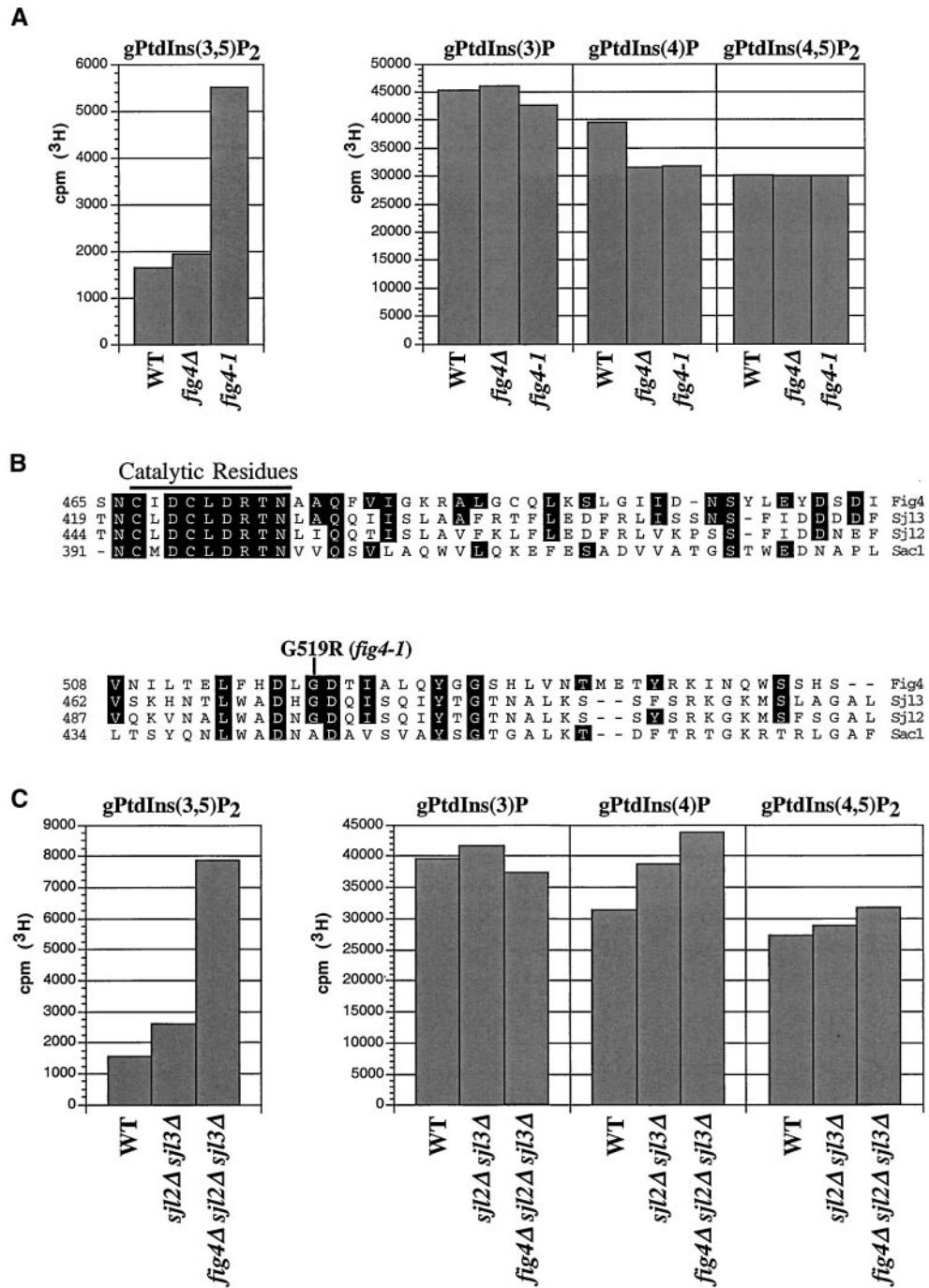
Although Fig4 appears to associate with membranes, sequence analysis failed to detect any membrane-spanning

domains. We attempted to define the nature of the Fig4 membrane association by determining the conditions in which Fig4 could be extracted from membrane fractions. Lysates from yeast cells expressing Fig4-HA were incubated with buffer alone, 1 M NaCl, 0.1 M Na<sub>2</sub>CO<sub>3</sub> pH 11, or 1% SDS. After incubation and centrifugation at 100,000 × *g*, Fig4-HA was found to redistribute into the soluble S100 fraction after treatment with NaCl, Na<sub>2</sub>CO<sub>3</sub>, or SDS (Figure 7C). As expected, the transmembrane protein Vam3 shifted into the S100 fraction only upon treatment with the detergent SDS (our unpublished data). Consistent with the absence of any identifiable transmembrane domains, these results indicate that Fig4 is a peripheral membrane protein.

## DISCUSSION

### Regulation of Fab1 Lipid Kinase by Vac7

Both *vac7* and *fab1* mutants contain dramatically enlarged vacuoles and are temperature sensitive for growth at 38°C (Bonangelino *et al.*, 1997; Gary *et al.*, 1998). Mutations that inactivate the PtdIns(3)P 5-kinase Fab1 result in an inability to generate the polyphosphoinositide PtdIns(3,5)P<sub>2</sub> (Gary *et al.*, 1998). Like *fab1* mutants, *vac7* mutants also lack PtdIns(3,5)P<sub>2</sub>. Based on these observations, it was proposed that Vac7 functions upstream of Fab1, possibly through direct activation of the lipid kinase. Consistent with Vac7 functioning as an activator of Fab1 activity, we found that Vac7 is necessary for the generation of high levels of PtdIns(3,5)P<sub>2</sub> upon hyperosmotic shock. Fab1 has been previously shown to generate the large amounts PtdIns(3,5)P<sub>2</sub>

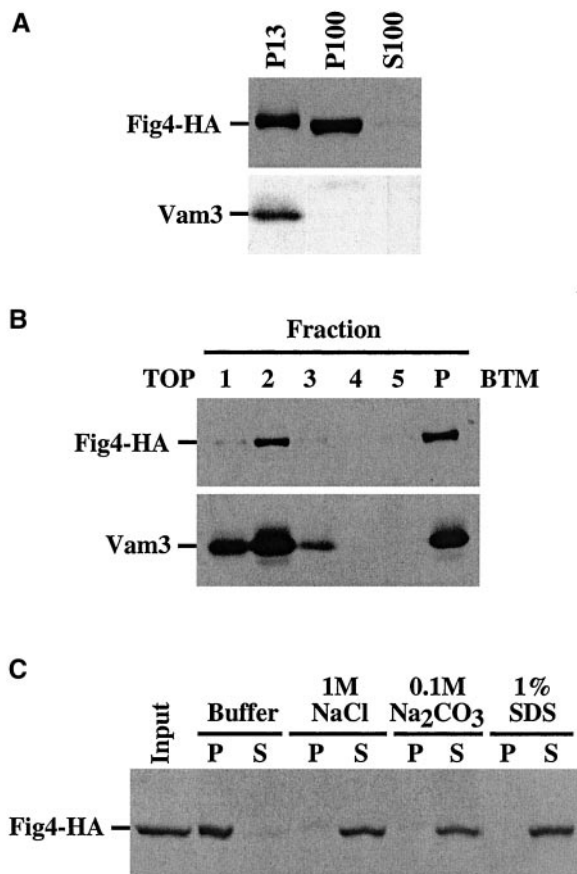


**Figure 6.** *fig4-1* mutants accumulate high levels of PtdIns(3,5)P<sub>2</sub>. (A) Steady-state quantification of PtdIns derivatives from wild-type, *fig4Δ*, and *fig4-1* cells. Radiolabeled PtdIns derivatives were isolated and quantified from the indicated strains as described in MATERIALS AND METHODS. The height of each bar represents the normalized average of three or more experiments with a SD <15%. (B) Quantification of radiolabeled PtdIns derivatives from wild-type, *sjl2Δ sjl3Δ*, and *fig4Δ sjl2Δ sjl3Δ* mutant cells. Radiolabeled PtdIns derivatives were isolated and quantified from the indicated strains as described in MATERIALS AND METHODS. The height of each bar represents the normalized average of three or more experiments with SD <10%. (C) Protein sequence alignment of a region of the *Sac1* domain in Fig4, Sjl3/Inp53, Sjl2/Inp52, and *Sac1*. The lipid phosphatase catalytic motif is denoted by the black line. Boxed amino acids identify residues that are conserved between Fig4 and other *Sac1* domain family members. The glycine at position 519 changed to an arginine in the *fig4-1* mutant is indicated.

within yeast cells in hyperosmotic conditions (Cooke *et al.*, 1998; Gary *et al.*, 1998). Similarly, *vac7Δ* mutants did not generate detectable amounts of PtdIns(3,5)P<sub>2</sub> in 0.9 M NaCl. This type of relationship between positive regulators and lipid kinases has previously been observed in yeast. The PtdIns 4-kinase *Pik1* is activated by the calcium-binding protein *Frq1* (Hendricks *et al.*, 1999), whereas activation of the PtdIns 3-kinase *Vps34* requires the protein kinase *Vps15*

(Stack *et al.*, 1993, 1995). We have attempted to detect physical interactions between *Vac7* and *Fab1* by native coimmunoprecipitation experiments; however, because the *Vac7* protein is unstable in cell extracts (our unpublished data), it was not possible to perform these experiments.

In a genetic approach to identify regulatory interactions between *Vac7* and *Fab1*, we isolated a mutant *fab1* allele that can bypass the requirement for *VAC7*. Expression of the



**Figure 7.** Characterization of Fig4-HA membrane association. (A) Subcellular fractionation of Fig4-HA. Lysate from wild-type yeast cells expressing Fig4-HA was fractionated by centrifugation at  $13,000 \times g$  to generate the P13 pellet and S13 soluble fractions. The S13 soluble fraction was further centrifuged at  $100,000 \times g$  to generate the P100 pellet and the S100 soluble fractions. Proteins in these fractions were precipitated, resolved by SDS-PAGE, and detected by Western blotting with anti-HA or anti-Vam3 antibodies, respectively. Each lane represents lysate from 1 OD<sub>600</sub> equivalent of cells. (B) Sucrose gradient analysis of Fig4-HA is described in MATERIALS AND METHODS. Briefly, the P13 pellet from the FIG4-HA strain was generated as explained in A and resuspended in 60% sucrose. The resuspended pellet was then loaded beneath a two-step gradient containing 55 and 35% sucrose. After 20-h centrifugation at  $200,000 \times g$ , fractions from the gradient were collected, and proteins were precipitated and resolved by SDS-PAGE. Fig4-HA and Vam3 were visualized by Western blotting with anti-HA and anti-Vam3 antibodies, respectively. Fractions are numbered from the top of the gradient (1) down to the pellet material (P). Fraction 5 corresponds to the initial position of the loaded P13 material. (C) Membrane association of Fig4-HA. Lysate from cells expressing Fig4-HA was centrifuged at  $3000 \times g$ . The soluble fraction (S3) was then incubated on ice in the indicated buffer conditions for 30 min. After the incubation, samples were then centrifuged at  $100,000 \times g$  to generate the pelletable (P) and soluble (S) fractions. Both fractions were precipitated and Fig4-HA was visualized by Western blotting with anti-HA antibody.

mutant *fab1-5* gene product in *vac7Δ fab1Δ* double mutant cells results in suppression of the *vac7Δ* growth and vacuolar morphology phenotypes (Table 2 and Figure 2A) and re-

sulted in  $\sim 40$ -fold higher PtdIns(3,5)P<sub>2</sub> levels than in *vac7Δ* cells (Figure 2, B and C). This suggests that the *fab1-5* mutation results in Vac7-independent activation of the Fab1 kinase. Furthermore, these results are consistent with Vac7 functioning as an upstream regulator of Fab1 kinase activity. However, additional experiments are necessary to determine a role for Vac7 in directly activating Fab1.

### Putative Polyphosphoinositide Phosphatase Fig4 Regulates PtdIns(3,5)P<sub>2</sub> Levels

In a genetic screen to identify additional regulators of PtdIns(3,5)P<sub>2</sub> levels, we identified a mutant allele of FIG4 whose gene product contains a conserved *Sac1* lipid phosphatase domain, as a suppressor of the *vac7Δ* temperature sensitivity. Expression of the *fig4-1* mutant allele in *vac7Δ* cells restored growth at 38°C and suppressed the enlarged vacuole morphology (Table 2 and Figure 3A). Bypass of *vac7Δ* phenotype was due to a restoration of PtdIns(3,5)P<sub>2</sub> levels. In *vac7Δ fig4-1* double mutants, PtdIns(3,5)P<sub>2</sub> levels were almost equal to that in wild-type cells (18-fold greater than in *vac7Δ* cells; Figure 3C). Similarly, deletion of the FIG4 gene also bypassed the *vac7Δ* mutant phenotypes (Figure 4, A and B, and Table 2). Thus, even in the absence of Fab1 activation by Vac7, mutations in Fig4 may prevent the turnover of residual PtdIns(3,5)P<sub>2</sub> and allow for the accumulation of PtdIns(3,5)P<sub>2</sub> to levels that restore vacuolar homeostasis and function.

*Sac1* domains were initially identified in the yeast proteins *Sac1* and Fig4, and the synaptojanin-like inositol polyphosphate 5-phosphatases Sjl2/Inp52 and Sjl3/Inp53 (Guo *et al.*, 1999). The *Sac1* domains from *Sac1* and Sjl3 have been determined to dephosphorylate PtdIns(3)P, PtdIns(4)P, and PtdIns(3,5)P<sub>2</sub> in vitro (Guo *et al.*, 1999; Hughes *et al.*, 2000). We are currently attempting to purify and test the *Sac1* domain of Fig4 for phosphatase activity in vitro. In addition, we found that deletion of *SAC1* suppressed the phenotypes of *vac7Δ* mutants, whereas deletion of *SJL2* or *SJL3* did not (Table 2 and Figure 5B). In *vac7Δ sac1Δ* double mutant cells, PtdIns(3,5)P<sub>2</sub> levels were  $\sim 1.7$ -fold greater than *vac7Δ fig4Δ* cells (Figure 5B). However, as previously reported for *sac1Δ* mutants (Guo *et al.*, 1999), the levels of PtdIns(4)P in *vac7Δ sac1Δ* double mutants were dramatically elevated,  $>17$ -fold greater than *vac7Δ fig4Δ* mutant cells. Recently, characterization of a temperature-conditional *sac1<sup>tsf</sup>* mutant determined that PtdIns(4)P is the primary substrate of *Sac1* in vivo (Foti *et al.*, 2001). Inactivation of the *sac1<sup>tsf</sup>* mutant resulted in a sevenfold increase in PtdIns(4)P levels, with only modest changes in other phosphoinositides.

Our biochemical data indicate that Fig4 is a peripheral membrane protein (Figure 7, A–C). A portion of Fig4 cofractionates with membranes enriched in vacuoles, which is consistent with the previously observed vacuolar localization of both Vac7 and Fab1 (Bonangelino *et al.*, 1997; Gary *et al.*, 1998). These results suggest the possibility that PtdIns(3,5)P<sub>2</sub> levels on the vacuolar membrane may be regulated through Fab1-mediated synthesis and Fig4-dependent degradation. In contrast, *Sac1* has been shown to primarily localize to the endoplasmic reticulum (Whitters *et al.*, 1993; Foti *et al.*, 2001), raising the possibility that the restoration of PtdIns(3,5)P<sub>2</sub> levels in *vac7Δ sac1Δ* mutants may be indirect. The large excess pool of PtdIns(4)P present in *sac1Δ*

mutant cells may compete with PtdIns(3,5)P<sub>2</sub> for Fig4 activity. However, we cannot rule out the possibility that *Sac1* may also play a role in the dephosphorylation of a pool of PtdIns(3,5)P<sub>2</sub> in endoplasmic reticulum membranes or an additional compensatory role in PtdIns(3,5)P<sub>2</sub> dephosphorylation on other membranes.

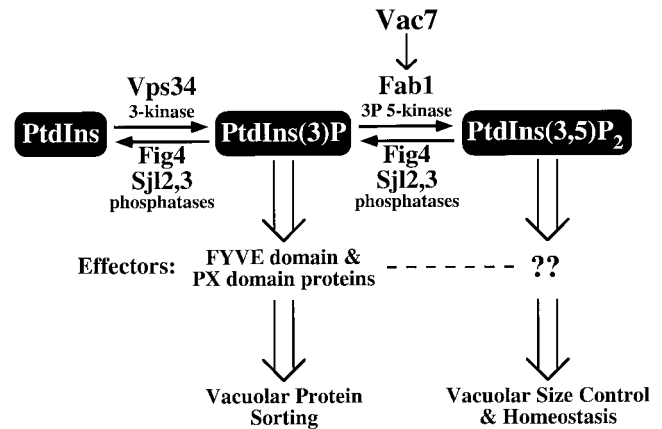
### *fig4-1* Mutation Dramatically Stabilizes PtdIns(3,5)P<sub>2</sub> Levels

Surprisingly, deletion of the *FIG4* gene did not result in a dramatic increase in PtdIns(3,5)P<sub>2</sub> levels; the PtdIns(3,5)P<sub>2</sub> levels in *fig4Δ* mutant cells were ~20% greater than in wild-type cells (Figure 6A). This suggests that Fig4 is not the only PtdIns(3,5)P<sub>2</sub> lipid phosphatase in yeast. Consistent with this hypothesis, we found that PtdIns(3,5)P<sub>2</sub> levels were significantly greater in *fig4Δ sjl2Δ sjl3Δ* triple mutant cells (Figure 6C) over *fig4Δ* (Figure 6A) and *sjl2Δ sjl3Δ* mutant cells (Figure 6C). Thus, Sjl2 and Sjl3 may also play some roles in PtdIns(3,5)P<sub>2</sub> turnover and, in the absence of Fig4 activity, they may compensate by dephosphorylating the majority of the excess PtdIns(3,5)P<sub>2</sub>.

Whereas deletion of *FIG4* did not have a pronounced effect, we found that the PtdIns(3,5)P<sub>2</sub> levels in *fig4-1* mutant cells were strikingly higher, almost threefold greater than in wild-type and *fig4Δ* cells (Figure 6A). Mapping of the *fig4-1* mutation identified a substitution of amino acid residue 519 from glycine to arginine just outside of the *Sac1* catalytic domain (Figure 6B). The glycine at this corresponding position is conserved in Sjl2, Sjl3, and Fig4 homologs, whereas *Sac1* contains an alanine. However, mutations that change this alanine in *Sac1* also cause the loss of lipid phosphatase activity (Whitters *et al.*, 1993). Thus, this region of the *Sac1* domain may have a role in regulating the lipid phosphatase activity. The analogous Fig4 mutant may bind and stabilize a pool of PtdIns(3,5)P<sub>2</sub> that is inaccessible to Sjl2 and Sjl3. Alternatively, the subcellular localization of Fig4 may play a critical role in the determining Fig4 substrate specificity. Thus, the *fig4-1* mutation may alter the localization of Fig4 to membranes that do not contain PtdIns(3,5)P<sub>2</sub>. Further biochemical analysis of the *fig4-1* mutant protein will be necessary to discern its effect on lipid phosphatase activity.

### *Vac7* and *Fig4* Function in Regulating PtdIns(3,5)P<sub>2</sub> Levels

Together, our results suggest that both *Vac7* and *Fig4* are key regulators of PtdIns(3,5)P<sub>2</sub> levels in yeast (Figure 8). The synthesis of PtdIns(3,5)P<sub>2</sub> is dependent upon two known lipid kinases. Vps34 is a PtdIns 3-kinase that phosphorylates PtdIns to PtdIns(3)P (Schu *et al.*, 1993), a lipid that has been shown to be required for Golgi-to-vacuole protein sorting through its interactions with proteins containing FYVE (Wurmser *et al.*, 1999) and PX domains (Cheever *et al.*, 2001). Finally, phosphorylation of PtdIns(3)P by the PtdIns(3)P 5-kinase Fab1 results in the generation of PtdIns(3,5)P<sub>2</sub> (Gary *et al.*, 1998). The identification of a mutant *fab1* allele that bypasses the requirement for *Vac7* function, along with the similarities in phenotypes between *vac7* and *fab1* mutants and their localization on the vacuole (Gary *et al.*, 1998), suggest that *Vac7* positively regulates Fab1 kinase activity. The generation of PtdIns(3,5)P<sub>2</sub> is necessary for the recruitment of unknown effector molecules for vacuolar membrane



**Figure 8.** Model for regulation of PtdIns(3,5)P<sub>2</sub> synthesis and degradation. PtdIns is phosphorylated by the PtdIns 3-kinase Vps34 to generate PtdIns(3)P, which is essential for vacuolar protein sorting through interactions with FYVE and PX domain-containing proteins. PtdIns(3)P can be further phosphorylated by the PtdIns(3)P 5-kinase Fab1 to produce PtdIns(3,5)P<sub>2</sub>. Fab1 kinase activity is regulated by the upstream activator *Vac7*. The generation of PtdIns(3,5)P<sub>2</sub> is essential for the maintenance of vacuolar size control and homeostasis through interactions with unknown effectors. Fig4, through its *Sac1* polyphosphoinositide phosphatase domain, functions with Sjl2 and Sjl3, in regulating PtdIns(3,5)P<sub>2</sub> levels by mediating the turnover of this lipid.

homeostasis functions, such as protein sorting at the multivesicular body (Odorizzi *et al.*, 1998) and retrograde transport from the vacuole (Bryant *et al.*, 1998). Furthermore, our data suggest that the *Sac1* domain containing protein Fig4 functions, along with Sjl2 and Sjl3, to mediate the turnover of PtdIns(3,5)P<sub>2</sub>. *Sac1* may also play a role in PtdIns(3,5)P<sub>2</sub> dephosphorylation. The turnover of PtdIns(3,5)P<sub>2</sub> is likely to be essential in attenuating downstream signaling events mediated by PtdIns(3,5)P<sub>2</sub> and its downstream effectors.

Although it is apparent that an equilibrium between PtdIns(3,5)P<sub>2</sub> production and degradation is essential for maintaining normal growth and membrane trafficking in yeast, the mechanism by which PtdIns(3,5)P<sub>2</sub> triggers these downstream signaling events is unknown. Although FYVE and PX domains have been shown to specifically bind to PtdIns(3)P, no PtdIns(3,5)P<sub>2</sub> effectors have been identified. In addition to the *fig4-1* mutation that restores PtdIns(3,5)P<sub>2</sub> levels in *vac7Δ* mutants, we isolated additional mutants (*bvs*) that restored growth at 38°C and vacuolar morphology, but had no effect on steady-state PtdIns(3,5)P<sub>2</sub> levels, suggesting that these mutations occur in genes that function downstream of PtdIns(3,5)P<sub>2</sub>. Further characterization of these mutants may identify novel PtdIns(3,5)P<sub>2</sub> effectors and/or provide insight as to how PtdIns(3,5)P<sub>2</sub> functions in maintaining vacuolar membrane homeostasis.

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