Assembly of a Fab1 Phosphoinositide Kinase Signaling Complex Requires the Fig4 Phosphoinositide Phosphatase

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Phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P₂] regulates several vacuolar functions, including acidification, morphology, and membrane traffic. The lipid kinase Fab1 converts phosphatidylinositol-3-phosphate [PtdIns(3)P] to PtdIns(3,5)P₂. PtdIns(3,5)P₂ levels are controlled by the adaptor-like protein Vac14 and the Fig4 PtdIns(3,5)P₂-specific 5-phosphatase. Interestingly, Vac14 and Fig4 serve a dual function: they are both implicated in the synthesis and turnover of PtdIns(3,5)P₂ by an unknown mechanism. We now show that Fab1, through its chaperonin-like domain, binds to Vac14 and Fig4 and forms a vacuole-associated signaling complex. The Fab1 complex is tethered to the vacuole via an interaction between the FYVE domain in Fab1 and PtdIns(3)P on the vacuole. Moreover, Vac14 and Fig4 bind to each other directly and are mutually dependent for interaction with the Fab1 kinase. Our observations identify a protein complex that incorporates the antagonizing Fab1 lipid kinase and Fig4 lipid phosphatase into a common functional unit. We propose a model explaining the dual roles of Vac14 and Fig4 in the synthesis and turnover of PtdIns(3,5)P₂.

INTRODUCTION

Phosphoinositides (PtdInsPs) are key determinants of the organelle identity code, which establishes and maintains organelle-specific properties such as size, shape, and function. They have important and diverse functions in membrane traffic, including regulation of membrane fission and fusion (Behnia and Munro, 2005; Di Paolo and De Camilli, 2006; Takenawa and Itoh, 2006). There are seven species of PtdInsPs (four in yeast) that are rapidly interconverted through the action of specific PtdIns kinases and phosphatases. Importantly, each PtdInsP has a stereotypical organelle distribution that regulates the localization of cognate effector proteins. PtdInsP recognition is predominantly achieved by short protein modules such as those of the FYVE and PH domain families (Lemmon, 2003; Balla, 2005; Behnia and Munro, 2005; Di Paolo and De Camilli, 2006; Takenawa and Itoh, 2006). Notably, these modules differ significantly in their affinity and specificity for each PtdInsP (Lawe *et al.*, 2000; Balla, 2005). Consequently, PtdInsPs can direct an extensive network of membrane–protein interactions.

Genetic ablation of phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P₂] synthesis in *Saccharomyces cerevisiae* causes numerous vacuolar defects, including nonacidification, im-

paired vacuole-to-endosome retrograde transport, abnormal inheritance, and a prominently enlarged single-lobed vacuole (Efe *et al.*, 2005; Michell *et al.*, 2006). Similarly, PtdIns(3,5)P₂-deficient cells from higher organisms also exhibit enlarged endosomes and impaired membrane traffic (Sbrissa *et al.*, 2000; Nicot *et al.*, 2006; Rusten *et al.*, 2006). Thus, PtdIns(3,5)P₂ governs events associated with endosomal compartments.

In yeast, PtdIns(3,5)P₂ is generated by Fab1, which phosphorylates phosphatidylinositol-3-phosphate [PtdIns(3)P] at the 5-position. Fab1 is a conserved lipid kinase known as PIKfyve in mammals (Cooke *et al.*, 1998; Gary *et al.*, 1998; Sbrissa *et al.*, 1999). Typically, Fab1 and its orthologues share a similar architecture: 1) an N-terminal FYVE domain, 2) a middle region related to the CCT/TCP-1/Cpn60 chaperonins that are involved in productive folding of actin and tubulin, 3) a second middle domain that contains a number of conserved cysteine residues (CCR) unique to the Fab1 family, and 4) a C-terminal lipid kinase domain related to PtdInsP kinases (Efe *et al.*, 2005; Michell *et al.*, 2006). Our understanding of how these domains modulate and coordinate membrane association and kinase activity of Fab1 remains poor. The mammalian PIKfyve depends on its FYVE domain and PtdIns(3)P for association with endosomes (Shishva *et al.*, 1999). However, the functional relevance for the FYVE domain of Fab1 remains unexplored. Similarly, despite being highly conserved, the roles of the CCT and CCR domains are practically uncharacterized (Efe *et al.*, 2005; Michell *et al.*, 2006).

In addition to Fab1, several vacuolar proteins that modulate cellular levels of PtdIns(3,5)P₂ have been identified. These include Vac7, a transmembrane protein with no apparent orthologues outside of Fungi; and the conserved adaptor-like protein Vac14 (Bonangelino *et al.*, 1997; Dove *et al.*, 2002; Gary *et al.*, 2002). In *vac14Δ* and *vac7Δ* cells, PtdIns(3,5)P₂ levels are severely reduced and exhibit a phenotype similar to *fab1Δ* cells (Bonangelino *et al.*, 1997; Dove *et al.*, 2002; Gary *et al.*, 2002). In contrast, deletion of *ATG18*

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Abbreviations used: CCR, conserved cysteine-rich domain; coIP, coimmunoprecipitation; PtdIns(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; HPLC, high-performance liquid chromatography; IP, immunoprecipitation; PtdInsP, phosphoinositide; V/C, vacuole-to-cytosol fluorescence ratio.

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

Strain	Genotype	Reference or source
SEY6210	Mata <i>leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> (1988)
fab1Δ2	SEY6210; <i>fab1Δ::HIS3</i>	Gary <i>et al.</i> (1998)
JGY138	SEY6210; <i>fig4Δ::LEU2</i>	Gary <i>et al.</i> (2002)
JGY145	SEY6210; <i>vac14Δ::TRP1</i>	Gary <i>et al.</i> (2002)
PHY102	SEY6210; <i>ops34Δ::TRP1</i>	Herman and Emr 1990
JGY134	SEY6210; <i>vac7Δ::HIS3</i>	Gary <i>et al.</i> (2002)
SRY10	SEY6210; <i>fab1Δ::HIS3 VAC14-GFP::HIS3</i>	Rudge <i>et al.</i> (2004)
SRY11	SEY6210; <i>fab1Δ::HIS3 FIG4-GFP::HIS3</i>	Rudge <i>et al.</i> (2004)
JEY075	SEY6211; <i>FAB1-Myc::HIS3 ATG18-FLAG::TRP1</i>	This study
JEY53	6210; <i>atg18Δ::HIS3 fig4Δ::LEU2</i>	Efe <i>et al.</i> (2007)
RBV23	SEY6210; <i>fab1Δ::HIS3 VAC14-HA::TRP1</i>	This study
RBV24	SEY6210; <i>vac14Δ::TRP1 FAB1-Myc::HIS3</i>	This study
RBV45	6210.5 <i>FAB1-HA::HIS3 FAB1-Myc::HIS3 ATG18-FLAG::TRP1</i>	This study
RBV47	6210, <i>FAB1-HA::HIS3</i>	This study
RBV52	SEY6211, <i>VAC14-6×HIS-FLAG::TRP1</i>	This study
RBV64	SEY6210; <i>VAC14-HA::TRP1</i>	This study
RBV78	6210, <i>FIG4-Myc::HIS3</i>	This study
RBV80	6211, <i>FIG4-HA::TRP1</i>	This study
RBV93	SEY6210; <i>fig4Δ::LEU2 VAC14-HA::TRP1</i>	This study
RBV94	SEY6210; <i>fig4Δ::LEU2 FAB1-Myc::HIS3 VAC14-HA::TRP1</i>	This study
RBV96	6210.5 <i>FIG4-HA::TRP1/+ FIG4-Myc::HIS3/+</i>	This study
RBV119	SEY6210; <i>FAB1-Myc::HIS3 ATG18-FLAG::TRP1 VAC14-HA::TRP1</i>	This study
RBV129	6210; <i>fab1Δ::HIS3 VAC14-6×HIS-FLAG::TRP1</i>	This study
RBV132	6210; <i>vac7Δ::HIS3 FAB1-Myc::TRP1 ATG18-FLAG::HIS3</i>	This study
RBV133	6210; <i>fab1Δ::HIS3 fig4Δ::HIS3 VAC14-6×HIS-FLAG::TRP1</i>	This study
RBV157	6210; <i>atg18Δ::TRP1 fig4Δ::LEU2 FAB1-Myc::HIS3</i>	This study
RBV160	6210; <i>atg18Δ::TRP1 fig4Δ::LEU2 FAB1-Myc::HIS3 VAC14-HA::TRP1</i>	This study

causes a drastic increase in PtdIns(3,5)P₂ levels (Dove *et al.*, 2004). Atg18 binds PtdIns(3,5)P₂ and is thought to serve dual roles: as an effector of the lipid and as a negative regulator of Fab1 kinase activity (Dove *et al.*, 2004). In addition, Fig4 is a PtdIns(3,5)P₂-specific 5-phosphatase that was identified in a *vac7Δ* suppressor screen that partially rescued the levels of PtdIns(3,5)P₂ (Gary *et al.*, 2002). Therefore, Fig4 was originally proposed to directly counteract Fab1 activity (Gary *et al.*, 2002; Rudge *et al.*, 2004). Interestingly, recent work suggests that Fig4 is also necessary for the synthesis of PtdIns(3,5)P₂; unlike wild-type yeast cells, *fig4Δ* cells do not exhibit a rapid and sharp increase in PtdIns(3,5)P₂ levels upon exposure to high salt (Dove *et al.*, 1997; Duex *et al.*, 2006a,b). Additionally, deletion of *FIG4* prevents the steady-state increase in PtdIns(3,5)P₂ levels in *atg18Δ* cells (Efe *et al.*, 2007). Curiously, Vac14 is also implicated in turnover of PtdIns(3,5)P₂ (Duex *et al.*, 2006b). A key question is how Vac14 and Fig4 carry out their dual roles in PtdIns(3,5)P₂ synthesis and turnover?

The molecular mechanisms that permit the Fab1 kinase and its regulatory proteins to govern PtdIns(3,5)P₂ levels are poorly understood. Conceivably, these proteins form an intricate network of interactions that regulate PtdIns(3,5)P₂. In yeast, the only established interaction as of yet is between Fig4 and Vac14 (Rudge *et al.*, 2004; Duex *et al.*, 2006b), whereas assays testing for Fab1 interaction with its regulators have thus far been unsuccessful (Bonangelino *et al.*, 2002; Rudge *et al.*, 2004). In mammals, it is proposed that PIKfyve forms a ternary complex with ArPIKfyve and hSac3, the Vac14 and Fig4 orthologues, respectively (Sbrissa *et al.*, 2007). However, the functional consequence for this complex remains to be examined. This study provides evidence that Fab1, Vac14, and Fig4 exist in a common vacuole-associated protein complex. Our observations suggest that Vac14 and Fig4 are codependent for interaction with the chaperonin-related domain of Fab1 and suggest a mecha-

nism for the dual role of Vac14 and Fig4 in the synthesis and turnover of PtdIns(3,5)P₂.

MATERIALS AND METHODS

Strains and Media

All *S. cerevisiae* strains used in this study and their genotypes are listed in Table 1. Strains were grown in rich (YPD) or minimal (SD) media supplemented with the appropriate amino acids. Standard growth conditions and manipulations have been described previously (Rose *et al.*, 1990).

Genetic and DNA Manipulation

Restriction enzymes were purchased from New England Biolabs (Ipswich, MA), and polymerase chain reactions (PCRs) were carried out with PfuUltra polymerase (Stratagene, La Jolla, CA) or ExTaq (Takara Mirus Bio, Madison, WI) for cloning and analytical reactions, respectively. Standard molecular biology techniques were used for all DNA manipulations (Maniatis *et al.*, 1992). Yeast transformations were done as described previously (Ito *et al.*, 1983). Yeast genomic preparations were performed with a modified protocol based on the QuickSpin mini-prep kit (QIAGEN, Valencia, CA). Briefly, yeast cell lysis and genomic DNA fragmentation was done by vortexing cells with glass beads in buffer P1 for 10 min. Subsequent steps were done as instructed by the manufacturer, except that DNA was washed once with PB buffer (QIAGEN) after binding to spin-columns.

PCR-amplified fragments were used to make genomic deletions and for epitope tagging as described previously (Longtine *et al.*, 1998). All deletions and integrations were verified by PCR and/or Western blot analysis. Amino acid substitutions were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide sequences are available upon request.

pGEX4T3 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and pET23d(+) (Novagen, Madison, WI) were used to make glutathione transferase (GST) and T7-HIS₆-tagged recombinant proteins, respectively. pJE181 (encoding Atg18), pBP74A, and pRB415A have been described previously (Efe *et al.*, 2007). Constructs expressing *FIG4* and *fig4-1* were described in Gary *et al.* (2002), and pRS416::*FAB1* and pRS416::*FAB1*^{D2134R} were characterized in Gary *et al.* (1998). The methodology used to clone the various constructs used in this study can be found in Supplemental Material.

Growth Assay

Cells were grown in the appropriate medium to OD₆₀₀ ~1 and subsequently diluted to an initial OD₆₀₀ of 0.6 in selective medium. Cultures were then

serially diluted 10-fold, and 2 μ l was spotted onto selective plates and grown at 26 or 38°C for 3 d.

Fluorescence Microscopy and Quantification of Fluorescence Intensity

Vacuoles were labeled with the fluorescent probes *N*-[3-triethylammonium-propyl]-4-[*p*-diethylaminophenyl]hexatrienyl pyridinium dibromide FM4-64 (Invitrogen, Carlsbad, CA) and/or 4-chloromethyl-7-aminocoumarin (Invitrogen) as described previously (Efe *et al.*, 2007). Fluorescence and differential interference contrast (DIC) images of labeled cells and those expressing green fluorescent protein (GFP) fusion proteins were generated with a Delta Vision RT microscopy system (Applied Precision, Issaquah, WA). Specifically, data were acquired using an IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with UV, fluorescein isothiocyanate, and rhodamine filters and coupled to a CoolSNAP HQ camera (Photometrics, Tucson, AZ). Background subtraction and intensity level were linearly adjusted using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Fluorescence intensity of FM4-64 and GFP channels of unprocessed TIFF images was quantified using ImageJ 1.36b (National Institutes of Health, Bethesda, MD). Typically, plot profiles were acquired by drawing a 5-pixel wide by 20- to 40-pixel-long line drawn from the midpoint of the cytosol to the vacuole lumen. Line positioning was randomly selected but avoided punctate structures on the vacuolar membrane. Fluorescence intensity values were exported to Excel 2004 (Microsoft, Redmond, WA). For graphical representation, intensity values were normalized against the highest intensity value to achieve a peak of 1. The vacuolar membrane was defined as the pixels with the highest FM4-64 intensity. A modified method was used to calculate the vacuole-to-cytosol (V/C) fluorescence ratio. A freehand line was drawn over >50% of the vacuolar membrane circumference, defined by the presence of FM4-64, and the average GFP fluorescence intensity was recorded. Mean cytosolic GFP fluorescence intensity was acquired by drawing a freehand area on >25% of the cytosolic area. These two values were then used to calculate the V/C fluorescence ratio. The two-tail, unpaired Student's *t* test was used to check for differences in V/C ratio between samples. Note that the contrast and the brightness of the images shown have been adjusted after acquiring numerical data.

Phosphoinositide Labeling and Analysis

Incorporation of ³H-labeled *myo*-inositol into phosphoinositides was analyzed as described previously by Rudge *et al.* (2004). Briefly, 5 OD₆₀₀ units of cells were labeled with 60 μ Ci of *myo*-[³H]inositol (GE Healthcare) in SD media lacking inositol for 1 h. Phospholipids were then precipitated in 9% perchloric acid for 5 min and washed in 1 ml of 0.1 mM EDTA. Deacylation was done in methylamine reagent (10.7% methylamine, 45.7% methanol, and 11.4% 1-butanol) for 50 min at 53°C, followed by evaporation in a vacuum chamber. Residual methylamine was eliminated by resuspending samples two times in 500 μ l of sterile water and drying. After a third resuspension in water, an equal volume of extraction reagent (1-butanol/ethyl-ether/formic acid ethyl ester at a ratio of 20:4:1) was added, vortexed, and centrifuged. The aqueous phase containing the deacylated phosphoinositides was collected, and the extraction procedure was repeated twice more, followed by a drying step.

For quantitative analyses, dried pellets were resuspended in sterile water and 1 $\times$ 10⁷ cpm were separated on a Partisphere SAX column (Whatman, Florham Park, NJ) attached to a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) and a 610TR on-line radiometric detector (PerkinElmer, Waltham, MA) by using Ultima Flo scintillation fluid (PerkinElmer). The HPLC and on-line detector were controlled with EZStart 7.2.1 and ProFSA 3.3 software, respectively, with final data analysis taking place in the latter.

Immunoprecipitation, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Western Blot Analysis

Yeast cultures were grown to OD₆₀₀ ~0.6, and 20–60 OD₆₀₀ units were used for coimmunoprecipitation (coIP) assays. Cells were spheroplasted and lysed in a 2-ml Dounce homogenizer in HEPES-lysis buffer (20 mM HEPES, pH 7.2, 50 mM potassium acetate, 200 mM sorbitol, and 2 mM EDTA), supplemented with 3 $\times$ Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 0.3 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 3 μ g/ml pepstatin A, 3 μ g/ml leupeptin, 3 mM benzamide, 3 $\times$ chymostatin, and 3 $\times$ aprotinin. Cleared cell lysates were solubilized in 0.25% Tween 20 and detergent-insoluble material spun down at 13,000 $\times$ g. IPs were done against the respective epitopes with 1 μ g/ml monoclonal antibodies: anti-Myc (9E10; Sigma-Aldrich), anti-hemagglutinin (HA) (12CA5; Roche Diagnostics), or anti-FLAG (M2; Sigma-Aldrich) for 1 h at 4°C followed by the addition of GammaBind G-linked Sepharose beads (GE Healthcare) for 1–2 h. Beads were washed three times with detergent-containing HEPES-lysis buffer and two times without detergent. CoIPs were then analyzed by SDS-PAGE and Western blotting. Fig4 was detected with rabbit anti-Fig4 polyclonal antibodies used at a dilution of 1:10,000 (Rudge *et al.*, 2004). Secondary antibodies were horseradish peroxidase linked and detected with ECL reagent (GE Health-

care). Antibody stripping was done with Blot Rejuvenation system (Millipore Bioscience Research Reagents, Temecula, CA).

For the salt-shock experiments, cells were grown as described above and exposed to 0.9 M NaCl for 0, 8, or 25 min. Cells were then quickly spun-down and lysed by vortexing in 300 μ l of lysis buffer supplied with protease inhibitors and 300 μ l of glass beads for 10 min at 4°C. The total volume was then increased to 1.0 ml with lysis buffer and processed as described above.

Subcellular Fractionation by Ultracentrifugation

Cells were lysed as described above and unbroken cells were eliminated by two spins at 500 $\times$ g for 10 min. The supernatant was then centrifuged at 100,000 $\times$ g for 45 min to obtain the cytosolic (supernatant) and the membrane fractions (pellet). Samples were then precipitated with 10% trichloroacetic acid (TCA), washed twice with acetone, and dissolved in sample buffer. Subcellular fractionation was tested with monoclonal anti-Vph1 antibodies (10D7, 1:500; Invitrogen) and rabbit polyclonal anti-glucose-6-phosphate dehydrogenase antibodies (Sigma-Aldrich).

Recombinant Protein Expression, Purification, and In Vitro Binding

BL21 bacteria were used to express recombinant fusion proteins. Cells were grown to OD₆₀₀ 1.0 in SuperBroth media supplemented with ampicillin, and expression was induced with 0.3 mM isopropyl β -D-thiogalactoside overnight at 22°C. Cells were then collected and washed with lysis buffer. To purify GST-fusion proteins, cells were resuspend in 25 ml of GST lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, and 2 mM β -mercaptoethanol, supplemented with protease inhibitors) and lysed with two passes through a French Press. Cell lysates were cleared with two high-speed spins and incubated with 1 ml of packed, washed glutathione-linked agarose beads for 2 h at 4°C. Beads were then washed twice with GST buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, and 2 mM β -mercaptoethanol) and three times with GST buffer B (50 mM Tris, pH 7.5, 200 mM NaCl, and 2 mM β -mercaptoethanol). Proteins were eluted with 20 mM glutathione in GST buffer B, pH 8.0.

To purify HIS₆-tagged proteins, cells were resuspend in 25 ml of HIS-lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 2 mM β -mercaptoethanol, supplemented with protease inhibitors). Lysis and clearing were done as described above. Lysates were incubated with 1 ml of washed Ni²⁺-nitrilotriacetic acid agarose beads for 2 h at 4°C. Beads were then washed five times with His-lysis buffer, and proteins were eluted with His-elution buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 250 mM imidazole, and 2 mM β -mercaptoethanol). All proteins were dialyzed overnight against GST buffer B with 2 mM dithiothreitol (DTT).

For in vitro binding reactions, GST fusion proteins were first immobilized onto 50 μ l of glutathione-linked beads for 1 h in GST buffer B. HIS₆-tagged proteins were then added to a total volume of 500 μ l in GST buffer B + 2 mM DTT and incubated for 2 h. Beads were then washed five times with GST buffer B and eluted with 20 mM glutathione. Samples were concentrated by TCA precipitation and analyzed by Coomassie staining and/or blotting against the N-terminal T7-epitope present in the HIS₆ fusion proteins.

RESULTS

Domain Analysis and Rational Mutant Design

The Fab1 lipid kinase is an ~250-kDa protein made up of several domains (Figure 1A; Efe *et al.*, 2005; Michell *et al.*, 2006). To further understand how these domains contribute to the function of Fab1, we adopted a structural-genetic approach based on rational mutant design and assessed PtdIns(3,5)P₂ levels, growth, and vacuolar morphology.

To perturb the C-terminal lipid kinase domain (KIN), we truncated the entire kinase domain from residues 1652 to 2279 (Fab1^{ΔKIN} encoded by pRB415A::Fab1^{ΔKIN}) or used the Fab1^{D2134R} allele [Fab1^{KIN(D/R)}], a previously characterized mutation that ablates lipid kinase activity (Figure 1A; Gary *et al.*, 1998). The FYVE domain was disrupted by eliminating the first 676 amino acids of Fab1 (Fab1^{ΔHindIII}; encoded by pRB415A::Fab1^{ΔHINDIII}) or more conservatively, by mutating cysteine²⁶² to serine²⁶² [Fab1^{FYVE(C/S)}]. This cysteine is part of a basic cluster found in all FYVE domains that maintains structural integrity by coordinating a Zn²⁺ ion (Stenmark *et al.*, 1996; Burd and Emr, 1998).

The remaining region between residues 800 and 1500 contains the CCT and CCR domains (Efe *et al.*, 2005; Michell *et al.*, 2006). Residues ~800 to ~1050 are related to the CCT chaperonin family, whose members catalyze folding of actin

Figure 1. Characterization of Fab1 point mutants. (A) Schematic representation of the Fab1 domain structure depicting the FYVE domain, the CCT, the CCR domains that together form the GroL region, and the lipid kinase domain. Mutations or truncations were introduced into each domain as indicated. (B) Amino acid sequence alignment of a conserved area in the CCT and CCR domains of several Fab1 orthologues: mm, *Mus musculus*; Hs, *Homo sapiens*; Sc, *S. cerevisiae*; Af, *Aspergillus fumigatus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*. The asterisk (*) denotes conserved residues, whereas the colon (:) and the period (.) indicate near-identical or similar residues, respectively. Enclosed residues were mutated as indicated above. (C) Cellular levels of PtdIns(3,5)P₂ as a percentage of total PtdIns in cells expressing the indicated Fab1 mutants. (D) Temperature-sensitive growth of *fab1Δ* cells expressing Fab1 mutants. Serial dilutions of cultures grown at 26 or 38 °C for three days. (E) Vacuolar morphology of *fab1Δ* cells expressing the designated *fab1* mutants. Cells were labeled with FM4-64 to demarcate the vacuolar limiting membrane. The FM4-64 and DIC images were merged as a grayscale palette. Bar, 5 μm.

and tubulin (Michell *et al.*, 2006). Residues ~1100–1500 contain multiple conserved cysteine and histidine residues and have been referred to as the CCR domain or as the PIP3KII-unique domain (Efe *et al.*, 2005; Michell *et al.*, 2006). Sequence alignment using the conserved domain database places the CCT and CCR domains within the GroL superfamily. Therefore, we refer to the combined CCT and CCR domains as the GroL-related region. To help delineate the function of these two domains, we mutated a conserved motif in the CCT region (T¹⁰¹⁷ILLR to I¹⁰¹⁷LLLA; Fab1^{CCT(T/I)}) and cysteine¹²⁴³ to alanine¹²⁴³ in the CCR domain (Fab1^{CCR(C/A)}); Figure 1, A and B).

Effect of *fab1* Mutations on Incorporation of [³H]Inositol into PtdIns(3,5)P₂ and Vacuole Morphology

We first used HPLC to measure levels of [³H]inositol-labeled PtdIns(3,5)P₂ [³H]PtdIns(3,5)P₂ in *fab1Δ* cells expressing the above-mentioned alleles of *FAB1* (Figure 1C). Cells expressing wild-type Fab1 from a CEN-based vector displayed [³H]PtdIns(3,5)P₂ levels comparable with cells with an intact chromosomal copy of *FAB1*, whereas [³H]PtdIns(3,5)P₂ was virtually undetectable in cells expressing Fab1^{KIN(D/R)} (0.11 ± 0.01 vs. 0%; Figure 1C). In contrast, expression of Fab1^{FYVE(C/S)}, Fab1^{CCT(T/I)}, or Fab1^{CCR(C/A)} produced intermediate levels of [³H]PtdIns(3,5)P₂. Fab1^{CCR(C/A)} showed the greatest decline in lipid levels [0.024 ± 0.01 or 22% of wild-type [³H]PtdIns(3,5)P₂], followed by Fab1^{CCT(T/I)} [0.046 ± 0.01 or 42% of wild-type [³H]PtdIns(3,5)P₂] and then by the FYVE point mutant [0.07 ± 0.01 or 63% of wild-type [³H]PtdIns(3,5)P₂, Figure 1C]. These data suggest that the Fab1 kinase is more sensitive to perturbations in the CCT and CCR regions compared with its FYVE domain, likely because the GroL-like region may regulate Fab1 kinase activity.

We next investigated whether the *fab1* mutants in question could support growth under heat stress (Figure 1D). *fab1Δ* cells are incapable of growing at 38°C and exhibit a growth rate that is 2.5 times slower than wild-type cells at 26°C in rich medium (Yamamoto *et al.*, 1995). Consistent with this, *fab1Δ* cells expressing Fab1^{KIN(D/R)} grew slowly at 26°C and were growth deficient at 38°C (Figure 1D). This was in contrast to cells expressing wild-type Fab1 and Fab1^{FYVE(C/S)}, which grew comparably well at 26 and at 38°C (Figure 1D). *fab1Δ* cells expressing Fab1^{ΔHindIII} also grew similarly well at 38°C, indicating that Fab1^{FYVE(C/S)} is not a hypomorph of the FYVE domain (Supplemental Figure S1A). However, alteration of the GroL-related sequence affected growth under heat stress, whereas expression of both Fab1^{CCT(T/I)} and Fab1^{CCR(C/A)} ameliorated the growth of *fab1Δ* cells at 26°C, these mutant alleles were defective for growth at 38°C (Figure 1D).

Last, we explored the effect of the listed *FAB1* alleles on vacuolar morphology (Figure 1E). Elimination of PtdIns(3,5)P₂ by deletion of *FAB1* or by inactivation of its kinase activity, leads to a conspicuously enlarged, single-lobed vacuole (Figure 1E; Yamamoto *et al.*, 1995; Gary *et al.*, 1998). Similarly, expression of Fab1^{CCR(C/A)} did not rescue vacuolar morphology in a *fab1Δ* strain (Figure 1E). We also compared mutations in cysteine¹²⁴⁷, cysteine¹²⁸⁹, and cysteine¹²⁹² and found they all caused single-lobed, enlarged vacuoles in cells (data not shown). This implies that the cysteines in the CCR region participate in a crucial but uncharacterized function that modulates Fab1 kinase activity. By comparison, expression of Fab1^{CCT(T/I)} and Fab1^{FYVE(C/S)} mutant alleles resulted in vacuoles that were predominantly single-lobed but near wild-type size (Figure 1E), likely because sufficient PtdIns(3,5)P₂ was maintained in these mutants.

Table 2. Characterization of the Fab1 domain point mutants

Fab1 strain	PtdIns(3,5) P ₂ % WT	Vacuole morphology	Growth at 38°C ^a	Localization ^b
Fab1	100		+++	V
Fab1 ^{FYVE(C/S)}	~60		+++	C
Fab1 ^{CCT(T/I)}	~40		–	V-C
Fab1 ^{CCR(C/A)}	~20		–	V
Fab1 ^{KIN(D/R)}	0		–	V

Cells expressing each mutant were scored for PtdIns(3,5)P₂ levels, vacuolar morphology, heat sensitivity, and localization as described in the text.

^a +++, grew well at 38°C; –, failed to grow.

^b V, vacuolar; C, cytosolic; V-C, partially delocalization to the cytosol.

These data indicate that the FYVE domain is largely dispensable for the synthesis of PtdIns(3,5)P₂. Mutating the FYVE domain of Fab1 only abated the ³H-labeled lipid amount by ~40%, and the cells exhibited near-normal vacuolar morphology and survived during heat stress (Table 2). In comparison, perturbing the CCT and CCR regions produced more deleterious effects on the Fab1 lipid kinase activity; consequently, cells displayed larger vacuoles and/or were temperature sensitive for growth (Table 2). This indicates that the CCT and CCR domains are important regulators of the Fab1 kinase, despite the possibility that the applied mutations did not fully incapacitate these domains.

Effect of *fab1* Mutations on Membrane Association

To determine the structural-genetic requirements for Fab1 localization to the vacuole, we visualized GFP-fusion proteins of Fab1 and of its mutant alleles (Figure 2). GFP fusion of wild-type Fab1 rescued vacuolar morphology (Supplemental Figure S1B) and PtdIns(3,5)P₂ levels (data not shown). However, to avoid “dilution” of the fluorescence signal due to vacuole enlargement when expressing certain Fab1 mutants, we expressed GFP-fusion constructs at endogenous levels in wild-type yeast cells. Similar observations were obtained when these GFP-fusion proteins were expressed in a *fab1Δ* strain (Supplemental Figure S1B). The vacuolar limiting membrane was labeled with the FM4-64 dye, and quantification of the fluorescence intensity was done as described previously (Figure 2, A and B; Efe *et al.*, 2007). We used two methods to analyze the behavior of the GFP-fusion proteins: 1) fluorescence line plotting to determine fluorescence correlation between GFP and FM4-64 signal (Figure 2A) and 2) quantification of the vacuole-to-cytosol (V/C) fluorescence ratio of the GFP signal (Figure 2B).

Consistent with Fab1 enrichment on the vacuolar membrane relative to the cytosol, the fluorescence intensity peak of Fab1-GFP correlated well with FM4-64 and exhibited a V/C = 1.78 ± 0.28 (n = 25; Figure 2, A and B). The V/C ratios for Fab1^{CCR(C/A)}-GFP and Fab1^{KIN(D/R)}-GFP were indistinguishable from the wild-type counterpart (1.88 ± 0.34,

Figure 2. Localization of Fab1 mutants. (A) Wild-type 6210 cells or *vps34Δ* cells expressing GFP-tagged Fab1 and its mutant forms at endogenous protein levels. Vacuoles were labeled with FM4-64. FM4-64 and DIC images were merged. Line plot fluorescence measurement of the GFP and FM4-64 signal is shown for each panel. A line 25–40 pixels in length and 5 pixels wide was drawn from the cytosol to the lumen. FM4-64 peak demarcates the vacuole limiting membrane. Fluorescence is normalized for both channels against the highest fluorescence value acquired. C, cytosolic side; V, vacuolar lumen side; dotted line, vacuolar membrane. Bar, 5 μ m. (B) Quantification of the vacuole-to-cytosol ratio (V/C) of the Fab1-GFP fluorescence signal. Fluorescence intensity readings were taken by averaging the signal on >25% of the cytosolic area and >50% of the vacuolar membrane for each cell. The V/C ratio for $n > 20$ and its SD are illustrated. Statistical analysis was performed using unpaired, two-tailed Student's *t* test.

Figure 3. Behavior of the FYVE domain of Fab1. (A) Wild-type (top) or *vps34Δ* cells (bottom) coexpressing GFP-FYVE^{Fab1} and RFP-FYVE^{EEA1}. (B) *fab1Δ* (top) or *vac7Δ* (bottom) cells expressing GFP-FYVE^{Fab1}. Bar, 5 μ m.

$n = 27$ and 1.71 ± 0.19 , $n = 28$, respectively), suggesting that the kinase activity and the CCT region do not impact membrane association (Figure 2B). Mutation of other cysteines in the CCT region did not hinder localization of the lipid kinase as well (data not shown). However, although Fab1^{CCT(T/I)}-GFP bound to the vacuolar membrane, the V/C ratio of this mutant was reduced compared with Fab1-GFP (1.47 ± 0.18 ; $n = 23$ vs. 1.78 ± 0.28 ; $p < 0.05$), implying that the CCT region may have a role in stabilizing Fab1 on the vacuole. We also determined that more drastic alterations to Fab1, such as truncating the CCT domain (GFP-Fab1^{CCTΔ}) or the kinase domain (GFP-Fab1^{KINΔ}), still allowed for at least partial association with the vacuole (Supplemental Figure S2).

In striking contrast, Fab1^{FYVE(C/S)}-GFP and GFP-Fab1^{ΔHindIII} were both displaced to the cytosol (Figure 2A and Supplemental Figure S2). This is corroborated by a low V/C ratio of 1.07 ± 0.08 for Fab1^{FYVE(C/S)}-GFP (Figure 2B; $n = 24$). Moreover, Fab1-GFP was completely cytosolic when expressed in *vps34Δ* cells (Figure 2A), suggesting that the FYVE domain and PtdIns(3)P are required for Fab1 association with the vacuole. Indeed, the FYVE domain of Fab1 (FYVE^{Fab1}) was sufficient for localization to the vacuole (Figure 3A). Its distribution was dissimilar from the endosome-specific FYVE domain of EEA1 (FYVE^{EEA1}) coexpressed in the same cells (Figure 3A). Despite this difference in distribution, they were both cytosolic in *vps34Δ* cells (Figure 3A).

To the best of our knowledge, no reports exist suggesting a lipid partner for the FYVE domain other than PtdIns(3)P. Nonetheless, we postulated that the vacuolar distribution of the FYVE^{Fab1} might occur by binding to PtdIns(3,5)P₂, which is also eliminated in *vps34Δ* cells, or by binding to a vacuolar specific protein partner such as Vac7. Notably, the FYVE^{Fab1} remained bound to the vacuole when expressed in *fab1Δ* or *vac7Δ* cells, excluding PtdIns(3,5)P₂ and Vac7 as determinants of vacuolar localization (Figure 3B). In comparison, GFP fusion of the GroL-like region did not seem to associate with the vacuole and was predominantly cytosolic (Supplemental Figure S2).

Together, these observations suggest that the FYVE domain is the primary membrane targeting mechanism for Fab1 but that the CCT domain may play a role in stabilizing this association. Thus, it is possible that Fab1^{FYVE(C/S)} might retain weak and transient association with the vacuole through a CCT-mediated interaction, explaining its ability to complement *fab1Δ* cells.

The Fab1 Kinase Recruits Vac14 to the Vacuole

Rudge *et al.* (2004) previously showed that Vac14 and Fig4 mislocalize to the cytosol in *fab1Δ* cells. The authors suggested that this was an indirect effect because they could not detect an interaction between Fab1 and Vac14/Fig4. Never-

theless, it remained possible that Fab1 targets Vac14 and Fig4 to the vacuole through protein-protein interactions, or indirectly, through the synthesis of PtdIns(3,5)P₂ or even through protein kinase activity. To distinguish between a role for the Fab1 protein versus its catalytic activity, we used the kinase-dead Fab1 mutant Fab1^{KIN(D/R)} (Gary *et al.*, 1998). We labeled the vacuolar membrane with FM4-64 and acquired paired images of Vac14-GFP and FM4-64 by epifluorescence microscopy and quantified the V/C fluorescence ratio for Vac14-GFP (Figure 4). Vac14 function is not significantly affected by GFP fusion (Rudge *et al.*, 2004).

As expected, the fluorescence peaks of Vac14-GFP and FM4-64 coincided well in *FAB1* cells, but it failed to do so in *fab1Δ* cells (Figure 4A). The V/C ratio for Vac14-GFP in *FAB1* cells (V/C^{FAB1}) was 2.16 ± 0.55 ($n = 20$) versus 0.96 ± 0.2 ($n = 20$) in *fab1Δ* cells ($V/C^{fab1Δ}$; Figure 4B). In comparison, cells expressing *fab1*^{KIN(D/R)} not only exhibited enlarged vacuoles but also the fluorescence profiles for Vac14-GFP and FM4-64 correlated well. Furthermore, the V/C ratio for Vac14-GFP in these cells was statistically indistinguishable from V/C^{FAB1} (1.88 ± 0.43 , $n = 21$; $p > 0.05$; Figure 4). This indicates that Fab1 acts structurally to localize Vac14 to the vacuole and independently of its kinase activity or PtdIns(3,5)P₂. Corroborating this conclusion, Vac14-GFP was displaced from the vacuole in cells expressing the FYVE-point mutant of Fab1, which itself is mislocalized to the cytosol (Figure 2, A and B). The V/C ratio for Vac14-GFP in *fab1Δ* cells and those expressing Fab1^{FYVE(C/S)} was statistically the same (1.07 ± 0.18 ; $n = 20$ vs. 0.96 ± 0.2 ; $p > 0.05$).

We also explored the behavior of Vac14-GFP in cells expressing the Fab1^{CCT(T/I)} and Fab1^{CCR(C/A)} point mutants. The V/C ratio of Vac14-GFP in cells expressing Fab1^{CCT(T/I)} or Fab1^{CCR(C/A)} was 1.21 ± 0.13 ($n = 20$) and 1.19 ± 0.2 ($n = 20$), respectively, which was significantly less than wild-type V/C. Thus, perturbing the CCT and CCR domains of Fab1 led to a measurable dissociation of Vac14-GFP from the vacuole (Figure 4). However, it is important to point out that Vac14-GFP maintained partial association with the vacuole in the GroL-like domain mutants because their V/C ratios were statistically higher than $V/C^{fab1Δ}$ (Figure 4B). This is consistent with the possible hypomorphic nature of these mutants and/or the presence of an additional site on Fab1 may contribute to localization of Vac14 (Figure 1). The localization of Fig4-GFP was similar to that of Vac14-GFP; expression of Fab1^{FYVE(C/S)}, Fab1^{CCT(T/I)} and Fab1^{CCR(C/A)} in *fab1Δ* cells did not rescue Fig4-GFP localization to the vacuole, whereas expression of Fab1 or Fab1^{KIN(D/R)} did (Supplemental Figure S3). Together, these data suggest that Fab1 has a direct role in targeting Vac14 and Fig4 to the vacuole, perhaps via interactions with the CCT and CCR domains.

Figure 4. Localization of Vac14-GFP in cells expressing *fab1* mutants. (A) *fab1*Δ VAC14-GFP cells transformed with vector, *FAB1* or its mutants. Cells were labeled with FM4-64 to denote the vacuolar membrane. FM4-64 is shown overlapped with the corresponding DIC images. Line plotting of Vac14-GFP and FM4-64 fluorescence was done as described in Figure 2. Bar, 5 μm. (B) Quantification of V/C for Vac14-GFP and statistical analysis were as described in Figure 2.

Figure 5. Fab1, Vac14 and Fig4 interact with each other. Monoclonal anti-HA or anti-Myc antibodies were used for IP from solubilized whole cell lysates (A and D) or from cytosolic/membrane fractions (C) as described in *Materials and Methods*. IPs were separated by SDS-PAGE and probed as indicated. Lysates lacking the cognate epitope for IP were used to ensure the specificity of each coIP. Input lanes represent 10% of the total material used for each IP. (A) IP from whole cell lysates with anti-HA (left) or anti-Myc (right) antibodies and probed with the corresponding antibodies as shown. (B) Cell fractionation into a cytosolic (C) and a membrane/pellet fraction (M). (C) Cytosolic (left) and membrane fractions (right) were incubated with anti-HA antibodies for IP. (D) Cells lysates were prepared from diploid cells expressing HA and Myc-tagged alleles of Fig4 (left) or from diploid cells expressing alleles of Fab1 tagged with HA and Myc (middle). IPs were done with anti-Myc antibodies. Whole cell lysates were made from cells expressing Vac14-FLAG and transformed with either empty vector (–) or with HA-Vac14 (+; right). IP was done with anti-HA antibodies.

The Fab1 Kinase Forms a Vacuolar Protein Complex with Vac14 and the Fig4 Phosphatase

Our observations indicated that Fab1, Vac14, and Fig4 might assemble into a single protein complex in yeast cells. It was shown previously that Vac14 and Fig4 coIP and are mutually dependent for association with the vacuolar membrane (Rudge *et al.*, 2004; Duex *et al.*, 2006b). Indeed, we observed coIP of Vac14-HA and Fig4 (Figure 5A). Nevertheless, previous attempts to demonstrate an interaction between Fab1 and Vac14 (or Fig4) were unsuccessful (Bonangelino *et al.*, 2002; Rudge *et al.*, 2004). However, we now demonstrate that Myc-tagged Fab1 was recovered with Vac14-HA (Figure 5A). Most notably, IP of Fab1-Myc retrieved not only Vac14-HA but also the Fig4 phosphatase as well (Figure 5A). Although it is possible that all three proteins interact with each other in pairwise combinations, we interpret these results as support for a protein complex that contains all three proteins together.

We then tested whether this putative Fab1 complex could be detected in both membrane and cytosolic fractions. We first fractionated whole cell lysates into cytosolic and membrane fractions and observed a relative distribution for Fab1-Myc, Vac14-HA, and Fig4 of ~70% versus ~30% between the membrane and cytosolic fractions, respectively (Figure 5B). The absence of the Vph1 vacuole membrane marker from the cytosolic fraction indicates the absence of contaminating membrane (Figure 5B). Additionally, this distribution was comparable with the relative distribution of GFP-fusion proteins by microscopy. We observed that Fab1-Myc and Fig4 were both recovered with Vac14-HA from the membrane fraction (Figure 5C). In contrast, IP of Vac14-HA from the cytosolic fraction recovered Fig4, but not Fab1-Myc (Figure 5C). These data indicate that Vac14 and Fig4 can interact in the cytosol but that association of Fab1 with Vac14 and Fig4 is stimulated and/or stabilized on the vacuolar membrane.

We also tested whether Fab1, Fig4 and Vac14 existed as monomers or whether they possibly displayed higher order

organization. To test this, we simultaneously expressed in the same yeast cells two alleles of *FAB1*, *FIG4*, and *VAC14* encoding two distinct epitopes (Figure 5D). We did not observe coIP between Fig4-Myc and Fig4-HA not between Fab1-Myc and Fab1-HA (Figure 5D). In contrast, Vac14-FLAG was retrieved with HA-Vac14 by IP (Figure 5D), which is consistent with yeast two-hybrid assays showing Vac14–Vac14 interaction (not illustrated; Dove *et al.*, 2002). Overall, these results indicate that Fab1, Vac14, and Fig4 can physically associate to form a membrane-bound protein assembly. Likely, there are multiple copies of Vac14 per complex, whereas the Fab1 kinase and the Fig4 phosphatase may be present at one subunit per complex.

Vac14 and Fig4 Exhibit Mutually Dependent Interaction with the Fab1 Kinase

To better understand how the Fab1 complex forms, we performed coIP experiments in different mutant backgrounds. We first determined that Vac14 and Fig4 bound to each other in the absence of the Fab1 kinase (Figure 6A). This is consistent with a putative Vac14–Fig4 subcomplex observed in the cytosolic fraction (Figure 5C). We also established that the Fab1 kinase and the Fig4 phosphatase were not retrieved together from *vac14Δ* cell lysates, which supports a role for Vac14 as a linker between the kinase and the phosphatase (Figure 6A). Remarkably though, Fab1 and Vac14 interacted poorly in the absence of Fig4 (Figure 6A), which suggests that Fig4 may play a regulatory/structural role in the assembly of the Fab1 complex.

Deletion of *VAC7* leads to undetectable levels of PtdIns(3,5)P₂ (Bonangelino *et al.*, 1997; Gary *et al.*, 2002). Because the mechanism for this regulation remains unknown, we postulated that Vac7 might be important for the association of Fab1 with Vac14-Fig4. However, deleting *VAC7* did not prevent Fab1 from interacting with Fig4, seemingly excluding this hypothesis (Figure 6A). Conversely, hyperosmotic shock of yeast cells causes a dramatic increase in PtdIns(3,5)P₂ levels after 5–10 min, followed by an abatement after 20–30 min (Dove *et al.*, 1997; Duex *et al.*,

Figure 6. Vac14 and Fig4 are both required for interaction with Fab1. (A) IPs were done with monoclonal anti-HA or anti-Myc from whole cell lysates from *fab1Δ* cells with or without HA-tagged Vac14 (top left), from *vac14Δ* cells with or without Myc-tagged Fab1 (top right), from *fig4Δ* cells with or without Myc-tagged Fab1 (bottom left) or from *vac7Δ* cells with or without Fab1-Myc (bottom right). IPs were separated by SDS-PAGE and probed accordingly and as described in *Materials and Methods*. Input lanes depict 10% of total protein used for each IP experiment. The asterisk (*) denotes degradation products of Fab1-myc. (B) Cellular levels of PtdIns(3,5)P₂ as a percentage of total PtdIns in *atg18Δ fig4Δ* cells transformed with an empty vector or vectors expressing *ATG18*, *FIG4*, or *fig4-1*. Bars represent SE, *n* = 3. (C) IPs using anti-HA antibodies against Vac14-HA from whole cell lysates derived from *atg18Δ fig4Δ* cells transformed with either empty vector, *FIG4* or *fig4-1*. Samples were handled and probed as described before.

2006b). Nonetheless, changes in PtdIns(3,5)P₂ did not seem to correlate with changes in the amount of Fab1 complex; we did not observe a significant change in the levels of Fig4 coIPed with Fab1-Myc upon exposure to salt-shock for 0, 8, or 25 min (Supplemental Figure S4). Together, these results indicate that Vac14 and Fig4 are mutually dependent for interaction with Fab1 and may explain the concomitant role of Vac14 and Fig4 in the synthesis and turnover of PtdIns(3,5)P₂ (Duex *et al.*, 2006a; Efe *et al.*, 2007).

Fig4, but Not Its Phosphatase Activity, Is Required for Atg18-dependent Hyperactivation of the Fab1 Kinase

Atg18 was identified as an effector of PtdIns(3,5)P₂ that regulates vacuolar morphology and membrane recycling. Atg18 also regulates PtdIns(3,5)P₂ levels—deletion of *Atg18* leads to an approximately ninefold increase in PtdIns(3,5)P₂ levels (Dove *et al.*, 2004). We showed previously that this increase is dependent on FIG4 (Efe *et al.*, 2007). Additionally, Fig4 is required to increase PtdIns(3,5)P₂ levels during hypertonic shock (Duex *et al.*, 2006a,b). This unexpected role for Fig4 in the synthesis of PtdIns(3,5)P₂ is likely explained by our observations—Fig4 is necessary for formation and/or stability of a productive Fab1-Vac14 interaction.

Conceivably, Fig4 may stimulate PtdIns(3,5)P₂ synthesis by mediating direct protein–protein interactions or through its phosphatase activity, possibly by acting as a protein phosphatase (Duex *et al.*, 2006b). To differentiate between these possibilities, we used a catalytic-inactive *FIG4* allele, *fig4-1*, which was initially isolated as a suppressor of the *vac7Δ* phenotype (Gary *et al.*, 2002; Rudge *et al.*, 2004).

The basal level of [³H]PtdIns(3,5)P₂ in *atg18Δ fig4Δ* cells was 0.13 ± 0.03% of total [³H]PtdIns, which is similar to wild-type [³H]PtdIns(3,5)P₂ levels (0.11%; Figure 6B). When *Atg18* was expressed epismally—recreating the *fig4Δ* condition—[³H]PtdIns(3,5)P₂ levels were slightly decreased (0.07 ± 0.01%). By contrast, expression of wild-type *FIG4* in *atg18Δ fig4Δ* cells recapitulated [³H]PtdIns(3,5)P₂ levels in *atg18Δ* cells (0.81 ± 0.05 vs. 0.9%; Figure 6B). Similarly, expression of *fig4-1* in *atg18Δfig4Δ* cells produced even higher levels of [³H]PtdIns(3,5)P₂ (1.37 ± 0.11% of total PtdIns; Figure 6B), likely because of abated turnover of the lipid. These results support the notion that Fig4 provides structural stability to the Fab1–Vac14 interaction independently of its phosphatase activity. To confirm this notion, we tested whether the Fab1 complex was reconstructed in *atg18Δ fig4Δ* cells expressing vector, *FIG4* or *fig4-1*. As shown in Figure 6A, the absence of Fig4 prevented coIP between Fab1-Myc and Vac14-HA (Figure 6C). In contrast, expression of *FIG4* or of the phosphatase-dead *fig4-1* allele permitted corecovery of Fab1-Myc and Vac14-HA (Figure 6C). Therefore, these observations support a structural role for Fig4 in stabilizing a productive Fab1 complex.

The GroL-like Domain of Fab1 Is Required for Interaction with Vac14 and Fig4 in Cells

Expression of Fab1^{CCT(T/I)} or of Fab1^{CCR(C/A)} crippled vacuole association of Vac14 and Fig4 (Figure 4 and Supplemental Figure S3). This suggests that the GroL-like region of Fab1 might be a binding site for Vac14 and/or Fig4. To test this hypothesis, we expressed a HA-tagged GroL-like fragment in *fab1Δ* cells and assessed the recovery of FLAG-tagged Vac14 and Fig4 by coIP. As shown, both Vac14-FLAG and Fig4 were recovered with the HA-GroL domain but not from control cells expressing the empty vector (Figure 7A). Importantly, mutating the TILLR motif in the CCT domain to ILLLA blocked interaction with Vac14 and Fig4, which is congruous with the release of Vac14 and Fig4 from the vacuole membrane in cells expressing Fab1^{CCT(T/I)} (Figure 3 and Supplemental Figure S3). Additionally, deletion of *FIG4* in *fab1Δ VAC14-FLAG* cells prevented coIP of Vac14-FLAG with the HA-tagged GroL-like fragment (Figure 7B), which recapitulated the requirement for Fig4 to stabilize the interaction between Vac14 and full-length Fab1 (Figure 6).

To further corroborate the interaction between Vac14 and the GroL-like region in vivo, we used fluorescence microscopy. To circumvent the cytosolic distribution of the GroL-

Figure 7. The GroL-like domain of Fab1 is sufficient to bind Vac14 and Fig4. (A and B) IP with anti-HA antibodies from whole cell lysates derived from *fab1Δ* VAC14-FLAG cells expressing empty vector, HA-GroL, or the mutated HA-GroL^{T/1} domain (A) or from *fab1Δ* fig4Δ Vac14-FLAG cells expressing empty vector or HA-GroL (B). IPs were separated by SDS-PAGE and probed for HA-GroL, Vac14-FLAG, and Fig4 as described previously. Inputs represent 10% of total material used during the IP. (C) *fab1Δ* VAC14-GFP cells expressing HA-ALP, HA-GroL-ALP, or HA-HA-GroL^{T/1}-ALP and labeled with FM4-64. FM4-64 and corresponding DIC images were merged. Line plot analysis was performed as described in Figure 2. Bar, 5 μm. (D) Quantification of V/C of Vac14-GFP signal as described in Figure 2. (E) Coomassie staining of purified bacterially expressed recombinant GST, GST-Vac14, GST-GroL, T7-Vac14-HIS₆ and T7-Fig4-HIS₆. Amount loaded represents ~20% of input samples for binding reactions. Small bars on the right indicate T7-Vac14-HIS₆ and T7-Fig4-HIS₆ and the asterisk (*) point to degradation products or contaminating proteins. (F) In vitro interaction between Vac14, Fig4, and the GroL-like region of Fab1. Left, GST and GST-Vac14 coupled to glutathione agarose beads were incubated with T7-Vac14-HIS₆ or T7-Fig4-HIS₆. Right, binding reactions between GST alone or GST-GroL fusion protein coupled to glutathione beads and incubated with T7-Vac14-HIS₆ or T7-Fig4-HIS₆. Blots were probed with monoclonal anti-T7 antibodies.

like region of Fab1 (Supplemental Figure S2B), we engineered a chimeric protein composed of the GroL-like domain and of the first 134 amino acids of ALP (ALP¹³⁴). ALP¹³⁴ contains the transmembrane domain and the AP3-pathway sorting motifs that target ALP to the vacuole (Klionsky and Emr, 1989; Bryant *et al.*, 1998). Indeed, GFP-GroL-ALP was clearly targeted to the limiting membrane of the vacuole (Supplemental Figure S2B).

Expression of HA-ALP in *fab1Δ* cells failed to mobilize Vac14-GFP to the vacuole (Figure 7, C and D). In sharp contrast, HA-GroL-ALP efficiently recruited Vac14-GFP to the vacuole. Introducing the ILLA mutation into HA-GroL-ALP (which generated HA-GroL^{T/L}-ALP) resulted in loss of Vac14-GFP localization to the vacuole (Figure 7C). Comparing the V/C ratio for Vac14-GFP in cells expressing HA-ALP, HA-GroL-ALP, or HA-HA-GroL^{T/L}-ALP substantiated our conclusions. The Vac14-GFP V/C ratio in cells expressing HA-ALP and the mutated HA-GroL-ALP were $1.04 \pm$

0.11 (n = 29) and 0.98 ± 0.12 (n = 35), respectively, and they were statistically indistinguishable ($p > 0.05$; Figure 7D). By comparison, the V/C ratio in HA-GroL-ALP-expressing cells was 1.72 ± 0.32 (n = 30) and was considerably greater than either HA-ALP or the mutated form ($p < 0.05$ for both comparisons; Figure 7D). In conclusion, we propose that the GroL-like region of Fab1 is an important docking site for Vac14 and Fig4, albeit it remains possible that Vac14/Fig4 may also interact with other regions on Fab1. The exact molecular events that translate into kinase activation upon Vac14-Fig4 binding will require further analysis.

In Vitro Binding between Vac14, Fig4, and a GroL-like Fragment of Fab1

To corroborate the above-mentioned observations and test for direct interaction, we used bacterially expressed recom-

binant proteins. GST-Vac14 and GST-GroL-like domain were purified from bacterial extracts by using glutathione-coupled agarose beads. We also expressed and purified Vac14 and Fig4 proteins tagged with an N-terminal T7 epitope and a C-terminal HIS₆ epitope (Figure 7E). We first tested and confirmed Vac14 interaction with itself *in vitro*; T7-Vac14-HIS₆ was recovered with GST-Vac14 but not with GST coupled to glutathione-agarose beads (Figure 7F). Similarly, T7-Fig4-HIS₆ bound to GST-Vac14 but not to GST alone (Figure 7F), showing that Vac14 and Fig4 interact directly. This is consistent with yeast two-hybrid analysis showing Vac14–Vac14 and Vac14–Fig4 interactions (not illustrated; Dove *et al.*, 2002).

We also tested whether recombinant GroL-like region of Fab1 was sufficient to interact directly with T7-Vac14-HIS₆ and/or T7-Fig4-HIS₆. We reproducibly observed enhanced recovery of T7-Vac14-HIS₆ with a GST-fusion of the GroL-like region compared with GST alone (Figure 7F). We could also detect an interaction between GST-GroL and T7-Fig4-HIS₆, although this interaction was weak and may reflect a nonspecific affinity by the GroL-related fragment. These results suggest that recombinant GroL-like region of Fab1 can directly bind to Vac14, and perhaps Fig4, *in vitro*.

In conclusion, these observations support a model where Vac14 and Fig4 bind to the GroL-like region of Fab1 to form a vacuole-bound complex in the cell to regulate PtdIns(3,5)P₂ levels. Our data suggest that Vac14 and Fig4 are mutually dependent for binding to Fab1 and for association with the vacuole. This can explain how Vac14 and Fig4 are concomitantly involved in synthesis and turnover of PtdIns(3,5)P₂.

DISCUSSION

The Functional Role for the Fab1 FYVE Domain

Disruption of the FYVE domain of Fab1 weakly affected PtdIns(3,5)P₂ levels and cellular function. This relatively weak phenotype is not likely to result from incomplete inactivation of the FYVE domain because cysteine²⁶² is at a vital position for folding of FYVE domains. Moreover, there was no apparent detriment to *fab1Δ* cells expressing a Fab1 mutant with a full deletion of its FYVE domain (Fab1^{ΔHindIII}) relative to cells expressing wild-type Fab1 or Fab1^{FYVE(C/S)} when grown at high temperature. There is precedent for these results as well; the *FAB1* allele that was originally cloned from a *fab1Δ* complementation screen lacked its FYVE domain (Yamamoto *et al.*, 1995). Consistent with this, several Fab1 orthologues lack a FYVE domain (Michell *et al.*, 2006).

In contrast, Fab1 was delocalized from the vacuole upon perturbation of its FYVE domain or ablation of PtdIns(3)P synthesis. Therefore, it is challenging to reconcile loss of membrane association while maintaining function (Figure 1). At least three plausible events could result in the appearance of a weak phenotype instead of extensive lipid loss that should accompany Fab1 membrane dissociation: 1) Fab1^{FYVE(C/S)}, possibly through its CCT domain, may transiently interact with the vacuolar membrane to synthesize a small amount of PtdIns(3,5)P₂, but 2) the extent of lipid loss is mitigated by the concomitant dissociation of the phosphatase from the vacuole and 3) such a transient association may favor the kinase over phosphatase activity, possibly because Fab1 is more proximal to the membrane.

Notably, the FYVE domain of Fab1 was sufficient to bind to the vacuole, whereas the FYVE domain of EEA1 distributed strictly to endosomes. Nevertheless, both FYVE do-

main required PtdIns(3)P for membrane association. Because vacuolar PtdIns(3)P is not thought to be abundant and is converted to PtdIns(3,5)P₂ by Fab1, this suggests an additional targeting mechanism for the FYVE domain of Fab1. Recently, we showed that Atg18 not only depends on PtdIns(3,5)P₂ for association with the vacuole but also that it requires an interaction with Vac7 (Efe *et al.*, 2007). Nevertheless, the FYVE domain of Fab1 maintained its vacuolar distribution in *vac7Δ* cells, indicating that Vac7 does not play a role in targeting the FYVE domain of Fab1 (Figure 3B). Further analysis is required to identify additional targeting mechanisms of Fab1 to the vacuole.

The GroL-like Domain of Fab1

The middle region of Fab1 is highly conserved across all of its orthologues. It consists of two distinct areas, the CCT and CCR domains. We refer to this combined region as GroL-related because recent comparisons with the conserved domain database retrieved the CCT and CCR regions over the GroL chaperonin. The GroL-related region is not critically important for vacuolar targeting, albeit it may stabilize Fab1 on the vacuole as suggested by the mild delocalization of Fab1^{CCT(T/I)} to the cytosol. Nevertheless, mutations in the CCT and CCR domains impeded Fab1 activity and function. Although it is likely that Fab1^{CCR(C/A)}, and especially Fab1^{CCT(T/I)}, are hypomorphs rather than representing full impairment of the respective domains, our data still support an important role for the GroL-related region. This is also consistent with inactivation of PIKfyve upon deletion of the CCT domain (Sbrissa *et al.*, 1999; Ikononov *et al.*, 2001).

The exact function of the GroL-related region remains poorly understood. Our data suggest that at least one function of the GroL-related region is to bind Vac14 and Fig4. Conceivably, the GroL-like region of Fab1 translates Vac14-Fig4 binding into Fab1 activity, which is consistent with reduced lipid levels in *vac14Δ* and in the CCT/CCR mutants (see below for Fig4 details). Possibly, docking of Vac14-Fig4 onto the GroL-like region of Fab1 might induce a conformational change that exposes the kinase domain to its substrate. Hyperactive mutants such as *fab1-5* and a number of *vac14Δ*-suppressing *fab1* mutants may bypass the need for Vac14 by constitutively stabilizing Fab1 in the open/active conformation (Gary *et al.*, 2002; Duex *et al.*, 2006b).

A Lipid Kinase-Lipid Phosphatase Complex That Modulates PtdIns(3,5)P₂

Vac7, Vac14, Atg18, the Fab1 lipid kinase and the Fig4 lipid phosphatase are the known components of an elaborate interaction network that modulates PtdIns(3,5)P₂ synthesis and turnover. Atg18, Fig4, and Vac14 buttress the complexity of this pathway by performing multiple tasks. In Atg18, it is a PtdIns(3,5)P₂ effector responsible for vacuole-to-endosome traffic and vacuole morphology. Remarkably, Atg18 is also a negative regulator of Fab1 whose elimination leads to a dramatic increase in PtdIns(3,5)P₂ levels (Dove *et al.*, 2004). Likewise, Fig4 and Vac14 play a dual role: regulating the turnover and synthesis of PtdIns(3,5)P₂ (Gary *et al.*, 2002; Rudge *et al.*, 2004; Duex *et al.*, 2006a,b; Efe *et al.*, 2007). The mechanism enabling this dual functionality was unknown and was particularly challenging to explain because previous attempts to show an interaction between Fab1 and Vac14/Fig4 were unsuccessful (Bonangelino *et al.*, 2002; Rudge *et al.*, 2004). Nonetheless, we identified a vacuolar signaling complex consisting of Vac14 and the antagonizing Fab1 lipid kinase and Fig4 lipid phosphatase, where Vac14 and Fig4 are mutually dependent for interaction with Fab1 in the cell (Figure 8). The discrepancy between our work and

Figure 8. The Fab1 complex model. Fab1 is recruited to the vacuolar membrane by binding to PtdIns(3)P through its FYVE domain. This localization is independent of Vac14 and Fig4 and is likely in dynamic exchange with the cytosol. Vac14 and Fig4 probably form a cytosolic subcomplex that subsequently binds to the CCT and CCR domains of Fab1 (the GroL-like region). Vac14 and Fig4 are mutually dependent for interaction with Fab1. Conceivably, the Fab1 complex is induced and/or stabilized by a vacuolar signal. Although Fab1 and Fig4 regulate PtdIns(3,5)P₂ lipid levels, the molecular mechanisms that coordinate the kinase and phosphatase activities are currently unknown.

that of others probably lies within technical difference; for example, we used 13-tandem copies of Myc to tag Fab1 for increased sensitivity and efficiency, a particularly important point because Fab1 is a very large, low-abundance protein. Regardless, our observations are consistent and provide a mechanistic insight into the observed complex interplay between Fab1, Fig4 and Vac14 as discussed below.

First, our observations indicate that Vac14 and Fig4 are tethered to the vacuolar membrane by binding to Fab1, not by an indirect mechanism as suggested previously (Rudge *et al.*, 2004). Additionally, the mutual dependency of Vac14 and Fig4 for binding to Fab1 explains their mutual dependency for vacuole association. Second, our data support a mechanism in which Vac14 binds to and activates Fab1 through its GroL-like domain, not through an intermediate as proposed previously (Rudge *et al.*, 2004). The exact molecular events leading to Vac14-mediated activation of Fab1 will require additional studies. Third, although Vac14 is clearly required for Fab1 activity, *vac14Δ* mutants are also impaired for down-regulation of PtdIns(3,5)P₂ (Duex *et al.*, 2006b). Likely, this is because in the absence of Vac14, Fig4 cannot bind Fab1, and therefore, it has reduced access to PtdIns(3,5)P₂. Fourth, the mechanism by which Fig4 sustains synthesis of PtdIns(3,5)P₂ in response to salt shock and in *atg18Δ* cells was not understood, although it was postulated to also function as a protein phosphatase that modulates Vac14 or Fab1 (Duex *et al.*, 2006a,b; Efe *et al.*, 2007). Instead, our data imply that Fig4 sustains PtdIns(3,5)P₂ synthesis by stabilizing the Fab1–Vac14 interaction independent of its catalytic activity—this is especially true in conditions that hyperactivate Fab1 such as *atg18Δ* and hyperosmotic shock.

Fig4 seems to contribute two opposing inputs to modulate PtdIns(3,5)P₂—the phosphatase activity comprises the negative input, whereas maintenance of the Fab1–Vac14 interaction constitutes the positive signal. This illuminates why elimination of the entire Fig4 protein has little effect on basal PtdIns(3,5)P₂, whereas elimination of its phosphatase activity alone (*fig4-1* allele) leads to an approximately threefold increase in this lipid (Gary *et al.*, 2002). In *fig4Δ* cells, there is a concomitant loss of the positive and the negative input, resulting (perhaps serendipitously) in unaffected PtdIns(3,5)P₂. In

contrast, *fig4-1* stabilizes the Fab1–Vac14 interaction (positive signal) in the absence of PtdIns(3,5)P₂ turnover, resulting in a significant increase in PtdIns(3,5)P₂ levels. In fact, the level of PtdIns(3,5)P₂ in the absence of Vac14 and Fig4 is only ~0.1 times that of wild-type cells but in the presence of Vac14 and Fig4-1 it is 3 times that of wild-type (thus, ~30-fold difference; Bonangelino *et al.*, 2002; Dove *et al.*, 2002; Gary *et al.*, 2002; Rudge *et al.*, 2004). Because elimination of phosphatase activity cannot solely explain this increase, it suggests that binding of Vac14–Fig4 to Fab1 stimulates its intrinsic kinase activity.

The assembly of the Fab1 complex is likely to be a controlled process. However, Vac7, Atg18, and hyperosmotic shock are not likely to regulate PtdIns(3,5)P₂ levels by modulating the assembly/disassembly of the Fab1 complex. The amount of the Fab1 complex did not seem to increase upon deletion of *ATG18* nor upon hyperosmotic shock. Similarly, Fab1 and Fig4 still interacted in *vac7Δ* cells, suggesting that Vac7 is not required for Fab1 complex assembly. In fact, this is consistent with the unperturbed vacuolar distribution of Fab1, Vac14, and Fig4 in *vac7Δ* cells (Rudge *et al.*, 2004).

It might be useful to compare the Fab1 and PIKfyve complexes (Sbrissa *et al.*, 2007). Notably, the union of the kinase and phosphatase activities into one complex is conserved in yeast and mammalian cells. However, in previous studies of the PIKfyve complex, the authors did not elaborate on the role of hVac14 and hSac3 (Fig4 orthologue) in the formation and stability of the PIKfyve complex nor did they explore whether hSac3 positively regulates PtdIns(3,5)P₂ synthesis, as is the case for Fig4. Strikingly, knockout mice for mFig4 (mouse orthologue of hSac3) exhibit reduced PtdIns(3,5)P₂ levels (Chow *et al.*, 2007), indicating that both mammalian and yeast Fig4 phosphatases may indeed have a role in PtdIns(3,5)P₂ synthesis.

It will be interesting to unravel the details of the Fab1 complex and study how Fab1 and Fig4 coordinate their kinase and phosphatase activities within the context of a common protein complex. The conserved nature of this complex indicates a fundamental requirement to tightly regulate PtdIns(3,5)P₂. Possibly, this may permit rapid and local adjustments in PtdIns(3,5)P₂ levels in specific subdomains of the vacuole membrane, which may assist in the generation of transport vesicles responsible for retrograde transport from the vacuole (Bryant *et al.*, 1998; Dove *et al.*, 2002). It is tempting to suggest that PtdIns kinase-phosphatase coupling may be a common mechanism to modulate PtdInsPs. Recently, the mammalian VPS34 PtdIns 3-kinase and the MTM1 PtdIns(3)P 3-phosphatase were shown to interact (Cao *et al.*, 2007).

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REFERENCES

- Balla, T. (2005). Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *J. Cell Sci.* 118, 2093–2104.
- Behnia, R., and Munro, S. (2005). Organelle identity and the signposts for membrane traffic. *Nature* 438, 597–604.

- Bonangelino, C. J., Catlett, N. L., and Weisman, L. S. (1997). Vac7p, a novel vacuolar protein, is required for normal vacuole inheritance and morphology. *Mol. Cell. Biol.* 17, 6847–6858.
- Bonangelino, C. J., Nau, J. J., Duex, J. E., Brinkman, M., Wurmser, A. E., Gary, J. D., Emr, S. D., and Weisman, L. S. (2002). Osmotic stress-induced increase of phosphatidylinositol 3,5-bisphosphate requires Vac14p, an activator of the lipid kinase Fab1p. *J. Cell Biol.* 156, 1015–1028.
- Bryant, N. J., Piper, R. C., Weisman, L. S., and Stevens, T. H. (1998). Retrograde traffic out of the yeast vacuole to the TGN occurs via the prevacuolar/endosomal compartment. *J. Cell Biol.* 142, 651–663.
- Burd, C. G., and Emr, S. D. (1998). Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol. Cell* 2, 157–162.
- Cao, C., Laporte, J., Backer, J. M., Wandinger-Ness, A., and Stein, M. P. (2007). Myotubularin lipid phosphatase binds the hVPS15/hVPS34 lipid kinase complex on endosomes. *Traffic* 8, 1052–1067.
- Chow, C. Y., Zhang, Y., Dowling, J. J., Jin, N., Adamska, M., Shiga, K., Szigeti, K., Shy, M. E., Li, J., Zhang, X., Lupski, J. R., Weisman, L. S., and Meisler, M. H. (2007). Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4j. *Nature* 448, 68–72.
- Cooke, F. T., Dove, S. K., McEwen, R. K., Painter, G., Holmes, A. B., Hall, M. N., Michell, R. H., and Parker, P. J. (1998). The stress-activated phosphatidylinositol 3-phosphate 5-kinase Fab1p is essential for vacuole function in *S. cerevisiae*. *Curr. Biol.* 8, 1219–1222.
- Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443, 651–657.
- Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J., and Michell, R. H. (1997). Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature* 390, 187–192.
- Dove, S. K., McEwen, R. K., Mayes, A., Hughes, D. C., Beggs, J. D., and Michell, R. H. (2002). Vac14 controls PtdIns(3,5)P₂ synthesis and Fab1-dependent protein trafficking to the multivesicular body. *Curr. Biol.* 12, 885–893.
- Dove, S. K. *et al.* (2004). Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. *EMBO J.* 23, 1922–1933.
- Duex, J. E., Nau, J. J., Kauffman, E. J., and Weisman, L. S. (2006a). Phosphoinositide 5-phosphatase Fig4p is required for both acute rise and subsequent fall in stress-induced phosphatidylinositol 3,5-bisphosphate levels. *Eukaryot. Cell* 5, 723–731.
- Duex, J. E., Tang, F., and Weisman, L. S. (2006b). The Vac14p-Fig4p complex acts independently of Vac7p and couples PI3,5P₂ synthesis and turnover. *J. Cell Biol.* 172, 693–704.
- Efe, J. A., Botelho, R. J., and Emr, S. D. (2005). The Fab1 phosphatidylinositol kinase pathway in the regulation of vacuole morphology. *Curr. Opin. Cell Biol.* 17, 402–408.
- Efe, J. A., Botelho, R. J., and Emr, S. D. (2007). Atg18 regulates organelle morphology and Fab1 kinase activity independent of its membrane recruitment by phosphatidylinositol 3,5-bisphosphate. *Mol. Biol. Cell* 18, 4232–4244.
- Gary, J. D., Sato, T. K., Stefan, C. J., Bonangelino, C. J., Weisman, L. S., and Emr, S. D. (2002). Regulation of Fab1 phosphatidylinositol 3-phosphate 5-kinase pathway by Vac7 protein and Fig4, a polyphosphoinositide phosphatase family member. *Mol. Biol. Cell* 13, 1238–1251.
- Gary, J. D., Wurmser, A. E., Bonangelino, C. J., Weisman, L. S., and Emr, S. D. (1998). Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. *J. Cell Biol.* 143, 65–79.
- Ikononov, O. C., Sbrissa, D., and Shisheva, A. (2001). Mammalian cell morphology and endocytic membrane homeostasis require enzymatically active phosphoinositide 5-kinase PIKfyve. *J. Biol. Chem.* 276, 26141–26147.
- Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153, 163–168.
- Klionsky, D. J., and Emr, S. D. (1989). Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J.* 8, 2241–2250.
- Lawe, D. C., Patki, V., Heller-Harrison, R., Lambright, D., and Corvera, S. (2000). The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization. *J. Biol. Chem.* 275, 3699–3705.
- Lemmon, M. A. (2003). Phosphoinositide recognition domains. *Traffic* 4, 201–213.
- Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Maniatis, T., Fritsch, E., and Sambrook, J. (1992). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Michell, R. H., Heath, V. L., Lemmon, M. A., and Dove, S. K. (2006). Phosphatidylinositol 3,5-bisphosphate: metabolism and cellular functions. *Trends Biochem. Sci.* 31, 52–63.
- Nicot, A. S., Fares, H., Payrastra, B., Chisholm, A. D., Labouesse, M., and Laporte, J. (2006). The phosphoinositide kinase PIKfyve/Fab1p regulates terminal lysosome maturation in *Caenorhabditis elegans*. *Mol. Biol. Cell* 17, 3062–3074.
- Rose, M., Winston, F., and Hieter, P. (1990). *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Rudge, S. A., Anderson, D. M., and Emr, S. D. (2004). Vacuole size control: regulation of PtdIns(3,5)P₂ levels by the vacuole-associated Vac14-Fig4 complex, a PtdIns(3,5)P₂-specific phosphatase. *Mol. Biol. Cell* 15, 24–36.
- Rusten, T. E., Rodahl, L. M., Pattni, K., Englund, C., Samakovlis, C., Dove, S., Brech, A., and Stenmark, H. (2006). Fab1 phosphatidylinositol 3-phosphate 5-kinase controls trafficking but not silencing of endocytosed receptors. *Mol. Biol. Cell* 17, 3989–4001.
- Sbrissa, D., Ikononov, O. C., Fu, Z., Ijuni, T., Gruenberg, J., Takenawa, T., and Shisheva, A. (2007). Core protein machinery for mammalian PtdIns(3,5)P₂ synthesis and turnover that regulates the progression of endosomal transport: novel Sac phosphatase joins the ArPIKfyve-PIKfyve complex. *J. Biol. Chem.* 282, 23878–23891.
- Sbrissa, D., Ikononov, O. C., and Shisheva, A. (1999). PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. *J. Biol. Chem.* 274, 21589–21597.
- Sbrissa, D., Ikononov, O. C., and Shisheva, A. (2000). PIKfyve lipid kinase is a protein kinase: downregulation of 5'-phosphoinositide product formation by autophosphorylation. *Biochemistry* 39, 15980–15989.
- Shisheva, A., Sbrissa, D., and Ikononov, O. (1999). Cloning, characterization, and expression of a novel Zn²⁺-binding FYVE finger-containing phosphoinositide kinase in insulin-sensitive cells. *Mol. Cell. Biol.* 19, 623–634.
- Stenmark, H., Aasland, R., Toh, B. H., and D'Arrigo, A. (1996). Endosomal localization of the autoantigen EEA1 is mediated by a zinc-binding FYVE finger. *J. Biol. Chem.* 271, 24048–24054.
- Takenawa, T., and Itoh, T. (2006). Membrane targeting and remodeling through phosphoinositide-binding domains. *IUBMB Life* 58, 296–303.
- Yamamoto, A., DeWald, D. B., Boronenkov, I. V., Anderson, R. A., Emr, S. D., and Koshland, D. (1995). Novel PI(4)P 5-kinase homologue, Fab1p, essential for normal vacuole function and morphology in yeast. *Mol. Biol. Cell* 6, 525–539.