Essential Role for the Myotubularin-related Phosphatase Ymr1p and the Synaptojanin-like Phosphatases Sjl2p and Sjl3p in Regulation of Phosphatidylinositol 3-Phosphate in Yeast

William R. Parrish, Christopher J. Stefan, and Scott D. Emr*

Department of Cellular and Molecular Medicine and the Howard Hughes Medical Institute, School of Medicine, University of California at San Diego, La Jolla, California 92093-0668

Submitted March 12, 2004; Revised May 6, 2004; Accepted May 18, 2004
Monitoring Editor: Suzanne Pfeffer

The requirement of Vps34p, the sole phosphatidylinositol (PI) 3-kinase in *Saccharomyces cerevisiae*, for protein sorting to the vacuole in yeast has exemplified the essential role for phosphoinositides, phosphorylated derivatives of PI, in membrane trafficking. To better understand mechanisms that regulate PI 3-phosphate [PI(3)P]-mediated signaling, the role of the yeast myotubularin-related PI(3)P phosphatase Ymr1p was investigated. We found that Ymr1p and the synaptojanin-like phosphatase Sjl3p function as key regulators of the localization and levels of PI(3)P. Our data indicated that the *ymr1Δ sjl3Δ* double mutant aberrantly accumulated PI(3)P and demonstrated a steady-state redistribution of this lipid that leads to enrichment on the vacuolar membrane. This resulted in vacuole protein sorting defects, vacuolar fragmentation, and the misregulation of PI(3)P-specific effectors. Triple deletion of *YMR1*, *SJL2*, and *SJL3* was lethal, suggesting an essential requirement for phosphatase-mediated PI(3)P regulation. Consistent with this, growth was restored to a *ymr1Δ sjl2Δ sjl3Δ* triple mutant by a PI(3)P-targeted Sac1p domain chimera (GFP-Sac1ΔC-FYVE_{EEA1}) that returned PI(3)P to levels comparable with wild-type cells. Together, this study demonstrated that Ymr1p, a myotubularin phosphatase family member, functions in the control of PI(3)P-dependent signaling and the maintenance of endosomal system integrity. In addition, this work defined an essential overlapping role for lipid phosphatases in the regulation of 3' phosphoinositides in yeast.

INTRODUCTION

Eukaryotic cells contain multiple membrane-bound organelles, each with a distinct composition and cellular function. To maintain the integrity and identity of each organelle, the cell selectively transports cargo molecules through an elaborate network of vesicle trafficking pathways to the appropriate target organelle (Bonifacino and Traub, 2003). Phosphoinositides, phosphorylated derivatives of phosphatidylinositol (PI), can be reversibly modified on the D-3, D-4, and D-5 positions of the inositol head-group (reviewed in Fruman *et al.*, 1998; Hughes *et al.*, 2000) and serve as key regulators of membrane trafficking events (Odorizzi *et al.*, 2000; Simonsen *et al.*, 2001). In particular, phosphoinositides generated by the action of PI 3-kinases have been the subject of much investigation and have been shown to play integral roles in various signal transduction and membrane trafficking pathways (Rameh and Cantley, 1999; Martin, 2001; Simonsen *et al.*, 2001; Pelham, 2002). In *Saccharo-*

myces cerevisiae, the demonstration that the sole PI 3-kinase Vps34 was required for efficient sorting of proteins from the late Golgi to the vacuole exemplified the importance of phosphatidylinositol 3-phosphate [PI(3)P] in vesicular trafficking within the endosomal system (Schu *et al.*, 1993; Stack *et al.*, 1993). Accordingly, a number of PI(3)P-specific effector proteins in yeast and higher eukaryotes have been found to contain FYVE (for Fab1, YGL023, Vps27, and EEA1) domains or PX (for phagocyte NADPH oxidase) domains, which can specifically recognize PI(3)P (Burd *et al.*, 1998; Corvera *et al.*, 1999; Sato *et al.*, 2001; Simonsen *et al.*, 2001).

The signaling events initiated by the binding of effectors to phosphoinositides are also regulated by PI phosphatases. Phosphatases that hydrolyze the D-3 or D-4 position phosphate share a signature CX₅R catalytic motif (where X is any amino acid), which is characteristic of Sac1p domain-containing phosphatases (reviewed in Hughes *et al.*, 2000), as well as DSP (dual specificity tyrosine and serine/threonine phosphatases) such as PTEN and myotubularin (reviewed in Nandurkar and Huysmans, 2002). Phosphoinositide 5-phosphatases such as synaptojanin or SHIP typically remove the D-5 phosphate from phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] or phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃] (Majerus *et al.*, 1999). *S. cerevisiae* encodes four Sac1p domain-containing proteins, Sac1p itself, Fig4p, and the synaptojanin-like phosphatases Sjl2p/Inp52p and Sjl3p/Inp53p (hitherto referred to as Sjl2p and Sjl3p) (Guo *et al.*, 1999). In vitro studies on the substrate specificity of the Sac1p domain-containing proteins in yeast have suggested that Fig4p is specific for the D-5 position of phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂] (Rudge *et al.*, 2004), whereas the Sac1p domains of Sac1p, Sjl2p, and Sjl3p are more promis-

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04-03-0209. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-03-0209.

* Corresponding author. E-mail address: semr@ucsd.edu.

Abbreviations used: Ape1, aminopeptidase 1; CPS, carboxypeptidase S; CPY, carboxypeptidase Y; CVT, cytoplasm-to-vacuole; 5-FOA, 5-fluoroorotic acid; GFP, green fluorescent protein; HPLC, high performance liquid chromatography; MVB, multivesicular body; PCR, polymerase chain reaction; PI, phosphatidylinositol; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(3,5)P₂, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; vps, vacuole protein sorting.

cuous and can convert PI(3)P, phosphatidylinositol 4-phosphate [PI(4)P], and phosphatidylinositol 5-phosphate [PI(5)P] as well as PI(3,5)P₂ to PI. In addition, Sjl2p and Sjl3p contain a separate PI(4,5)P₂ 5-phosphatase domain. This combination of phosphatase activities allows Sjl2p and Sjl3p to convert all major phosphoinositides in yeast to PI. Consistent with their *in vitro* activities, studies on Sjl2p and Sjl3p have indicated that they provide redundant functions with other PI phosphatases that are dedicated to the hydrolysis of specific phosphoinositides *in vivo*. For example, yeast mutants lacking the Sac1p domain-containing phosphatases Sac1p, Sjl2p, and Sjl3p demonstrated an essential overlapping role for these proteins in the control of PI(4)P and Golgi integrity (Foti *et al.*, 2001). In addition, Sjl1p, Sjl2p, and Sjl3p were shown to overlap in the regulation of PI(4,5)P₂ and actin cytoskeleton organization through their concerted PI 5-phosphatase activities (Stolz *et al.*, 1998; Stefan *et al.*, 2002).

Yeast also contains a single representative of the myotubularin family of phosphoinositide 3-phosphatases, Ymr1p (encoded by the YJR110W open reading frame) (Laporte *et al.*, 1998). Myotubularins are grouped together as a family based on a conserved core functional domain organization consisting of an N-terminal PH-like GRAM domain (for glucosyltransferase, Rab-like GTPase activator and Myotubularin), a RID domain (for Rac-induced recruitment domain), a DSP domain (dual specificity tyrosine and serine/threonine phosphatase), and a SID domain (SET-protein interaction domain) (Laporte *et al.*, 2003). The catalytically active myotubularins all exhibit specific activity against the D-3 position of PI(3)P and PI(3,5)P₂, leading to the generation of PI and PI(5)P, respectively (Schaletzky *et al.*, 2003). Among the 14 known human myotubularin-related phosphatases, three are mutated in severe genetic diseases: myotubular myopathy, and two distinct forms of Charcot-Marie-Tooth neuropathy (Nandurkar and Huysmans, 2002; Laporte *et al.*, 2003). Although it has been speculated that myotubularins may regulate of PI(3)P-mediated endosome functions, the precise cellular roles of these proteins remains unknown (Laporte *et al.*, 2003).

In this study, we sought to elucidate the cellular role of the yeast myotubularin-related phosphatase Ymr1p. Consistent with a previous study (Taylor *et al.*, 2000), we found that Ymr1p is a phosphoinositide 3-phosphatase *in vivo*. However, deletion of *YMR1* yielded no obvious phenotypes. Because previous work has indicated functional overlap between phosphoinositide phosphatases in yeast (Foti *et al.*, 2001; Gary *et al.*, 2002; Stefan *et al.*, 2002), we took a genetic approach to identify those that could compensate for the absence of Ymr1p activity. We identified a specific genetic interaction between *YMR1* and *SJL3*, because *ymr1Δ sjl3Δ* double mutant cells displayed an ~2.5-fold increase in cellular levels of PI(3)P, which aberrantly accumulated on the vacuolar membrane. Together, this study indicated that Ymr1p, a myotubularin family member, has a direct role in the regulation of PI(3)P-dependent signaling pathways that are important for the maintenance of endosome integrity. In addition, this work uncovered a novel requirement for phosphatase-mediated PI(3)P regulation in yeast; an essential function shared by Ymr1p, Sjl2p, and Sjl3p.

MATERIALS AND METHODS

Reagents and Media

Enzymes used for recombinant DNA techniques were purchased from commercial sources and used as per manufacturers' recommendations. Methods were performed essentially as described previously (Sambrook *et al.*, 1989). Sources for growth media for yeast and bacterial strains have been described

previously (Gaynor *et al.*, 1994). Standard yeast genetic methods were used throughout the study (Sherman *et al.*, 1979). All oligos used in this study are available upon request.

Strains, Plasmids, and Mutagenesis

All yeast strains used in this study are based on the parent strain SEY6210 (Table 1) (Robinson *et al.*, 1988). The *ymr1Δ* strain was generated using a *HIS3* deletion cassette strategy similar to that described previously (Audhya *et al.*, 2000). A strain expressing Atg24 (Cvt13, Snx4)-GFP was created using a polymerase chain reaction (PCR) approach that has been described previously (Longtine *et al.*, 1998). All gene deletions and epitope tag integrations were confirmed by genomic PCR. To generate all multiple mutant strains, the appropriate parent strains were mated and then sporulated. Tetrads were dissected, and germinated spores harboring the appropriate markers were selected. All disruptions were confirmed by genomic PCR analysis. To create the temperature-sensitive BPY13 strain, plasmid *pRS416sjl2-8^{ts}* was transformed into BPY03 diploid cells, which were subsequently sporulated, and Ura⁺ prototrophic *ymr1Δ sjl2Δ sjl3Δ* triple mutants were identified by PCR analysis. The same strategy was used to create BPY14. The BPY97 strain was generated by transforming BPY13 with a *LEU2*-marked *GFP-SAC1ΔC-FYVE_{EEA1}* plasmid and subsequently selecting for transformants that grew on plates containing 5-fluoroorotic acid (5-FOA). Generation of the *ymr1^{ts}* strain YTS1 is described below.

The *YMR1* gene (YJR110W) was amplified by PCR by using the *Pfu Turbo* DNA polymerase (Stratagene, La Jolla, CA) from yeast strain SEY6210 with oligos that added a *NotI* restriction site to the 5' end of the fragment, and a *SacI* site to the 3' end. The resulting ~2.75-kb *NotI-SacI* fragment was then ligated into the *LEU2*-marked plasmid pRS415 (Sikorski and Hieter, 1989) (plasmid pBP02), and the insert was confirmed by DNA sequence analysis. Subsequently, the *NotI-SacI* fragment of pBP02 was subcloned into the *URA3*-marked plasmid pRS416 (plasmid pBP03) and the high copy number *LEU2*-marked plasmid pRS425 (plasmid pBP29). Plasmids containing the lipid-targeted *GFP-SAC1ΔC* chimeric genes were constructed as follows. PCR was used to amplify the *SAC1* coding region from codons 1–522 lacking the transmembrane anchor of Sac1p (Foti *et al.*, 2001) by using oligos that added a unique *BglII* site at the 5' end and a unique *Sall* site at the 3' end. This fragment was then digested accordingly and ligated into plasmid pGO35 (Odorizzi *et al.*, 1998) to create plasmid *GFP-SAC1ΔC*. This plasmid was then used to create the lipid-targeted Sac1p domain constructs. For *GFP-SAC1ΔC-2 × PH_{PLCδ}*, the PI(4,5)P₂-binding plectrin homology (PH) domain of PLCδ (Kavran *et al.*, 1998) was amplified with oligos that added a *Sall* site (PH1), or a *BamHI* site (PH2) to the 5' end of the sequence, and a *BamHI* site (PH1), or a *KpnI* site (PH2) at the 3' end of the fragment. These PCR fragments were then digested with the appropriate enzymes and ligated into the *GFP-SAC1ΔC* plasmid cleaved with *Sall* and *KpnI*. For *GFP-SAC1ΔC-PH_{FAPP1}*, the PI(4)P-binding PH domain of FAPP1 (Dowler *et al.*, 2000) was amplified with oligos that added a 5' *Sall* site and a 3' *KpnI* site; the resulting PCR fragment was then digested and ligated into the *GFP-SAC1ΔC* plasmid as described above. The same strategy was used to generate the *GFP-SAC1ΔC-FYVE_{EEA1}* plasmid. *LEU2*-marked versions of the *GFP-SAC1ΔC-FYVE_{EEA1}* and *GFP-SAC1ΔC-2 × PH_{PLCδ}* were generated by subcloning the *NaeI-SpeI* fragment from these plasmids into plasmid pRS425.

The phosphatase inactive *GFP-Sac1ΔC-FYVE_{EEA1}* chimera was generated by QuikChange site-directed mutagenesis (Stratagene), substituting the active site cysteine residue for serine (*GFP-Sac1ΔC_(C392S)-FYVE_{EEA1}*) (Guo *et al.*, 1999). The subsequent plasmid (pBP33) was confirmed by DNA sequence analysis, and impairment of catalytic activity was confirmed by analysis of *in vivo* phosphoinositide metabolism of yeast cells harboring pBP33. A strain expressing a temperature conditional allele of *YMR1* was generated in the following manner. *YMR1* sequences from plasmid pBP03 were amplified by PCR under error-prone conditions as described previously (Muhlrad *et al.*, 1992). The mutagenized PCR fragment was then cotransformed with *SphI-PacI*-gapped pBP02 into BPY13 cells. Leu⁺ prototrophic transformants were then selected and screened for their ability to grow on 5-FOA at 26°C, but not at 38°C. From >6000 transformants, 10 putative *ymr1^{ts}* plasmids were recovered into *Escherichia coli* and retransformed into BPY13 to confirm the temperature conditional growth phenotype on 5-FOA. A strain displaying temperature conditional lethality at 34°C was selected for further studies (YTS1).

Metabolic Labeling and Immunoprecipitation

In vivo phosphoinositide analysis was done essentially as described previously (Rudge *et al.*, 2004), except that cells were labeled for 1 h after a 20-min preincubation at the appropriate temperature. Processed samples were resuspended in sterile water, and 10.5 × 10⁶ cpm of deacylated [³H]glycerophosphoinositols were analyzed.

Cell labeling and immunoprecipitations were performed as described previously (Gaynor *et al.*, 1994) with minor modifications. Briefly, mid-log phase cultures were concentrated to 1–2 OD₆₀₀ units/ml and labeled with Tran ³⁵S label (PerkinElmer Life and Analytical Sciences, Boston, MA) for 10 min in YNB containing 100 μg/ml bovine serum albumin. Cells were then chased with 5 mM methionine, 2 mM cysteine, and 0.2% yeast extract for the indicated times; proteins were precipitated with 9% trichloroacetic

Table 1. Strains used in this study

Strain	Genotype	Reference or source
SEY6210	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> , 1988
SEY6210.1	<i>MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> , 1988
JGY131	SEY6210; <i>sjl3Δ::TRP1</i>	Foti <i>et al.</i> , 2001
JGY132	SEY6210; <i>sjl2Δ::HIS3 sjl3Δ::TRP1</i>	Foti <i>et al.</i> , 2001
YCS62	SEY6210.1; <i>sjl1Δ::HIS3</i>	Stefan <i>et al.</i> , 2002
JGY130	SEY6210; <i>sjl2Δ::HIS3</i>	Foti <i>et al.</i> , 2001
MFY62	SEY6210.1; <i>sac1Δ::TRP1</i>	Foti <i>et al.</i> , 2001
YMR1Δ	SEY6210; <i>ymr1Δ::HIS3</i>	Sato and Emr, unpublished
BPY01	SEY6210.1; <i>ymr1Δ::HIS3 sjl3Δ::TRP1</i>	This study
BPY02	BPY01, except <i>sjl1Δ::HIS3</i>	This study
BPY03	SEY6210a/α; homozygotic <i>ymr1Δ::HIS3</i> heterozygotic <i>sjl2Δ::HIS3 sjl3Δ::TRP1</i>	This study
BPY04	SEY6210.1; <i>ymr1Δ::HIS3 sac1Δ::TRP1</i>	This study
BPY05	SEY6210.1; <i>ymr1Δ::HIS3 sjl1Δ::HIS3</i>	This study
BPY06	SEY6210; <i>ymr1Δ::HIS3 sjl2Δ::HIS3</i>	This study
YMR1Δ FIG4Δ	SEY6210.1; <i>ymr1Δ::HIS3 fig4Δ::HIS3</i>	Gary and Emr, unpublished
BPY13	SEY6210.1; <i>ymr1Δ::HIS3 sjl2Δ::HIS3 sjl3Δ::TRP1</i> carrying <i>pRS416sjl2-8^{ts}</i> (<i>URA3 CEN6</i>)	This study, <i>sjl2-8^{ts}</i> plasmid, Stefan <i>et al.</i> , 2002
BPY14	BPY13, except carrying <i>pRS416sjl2-D850S</i> (<i>URA3 CEN6</i>)	This study, <i>sjl2-D850S</i> plasmid, Stefan <i>et al.</i> , 2002
BPY16	BPY01, except <i>VPS10-GFP:TRP1</i>	This study
BPY97	BPY13, except carrying <i>pRS425GFP-SAC1ΔC-FYVE_{EEA1}</i> (2 μ <i>LEU2</i>)	This study
BPY115	BPY01, except <i>VPS17-GFP:TRP1</i>	This study
BPY122	BPY01, except <i>ATG24-GFP:HIS3-MX6</i>	This study
Snx4-GFP	SEY6210; <i>ATG24-GFP:HIS3-MX6</i>	This study
YTS1	BPY13, except carrying <i>pRS415ymr1^{ts}</i> (<i>LEU2 CEN6</i>) instead of <i>pRS416sjl2-8^{ts}</i>	This study

acid. Where required, cells were preincubated at the appropriate temperature for 20 min before the addition of label. To assay for secreted carboxypeptidase Y (CPY), cells were converted to spheroplasts after pulse-chase by adding an ice-cold 2× buffer containing 50 mM Tris (pH 7.5), 2 M sorbitol, 40 mM NaF, 40 mM NaN₃, and 10 mM dithiothreitol for 10 min. Zymolyase T100 (15 μg/OD₆₀₀ unit; Seikagaku Kogyo, Tokyo, Japan) was then added to the cell suspension and incubated at 30°C for 30 min. Cells were then centrifuged at 6000 rpm for 5 min, and the supernatants were harvested away from the cell pellets and both were precipitated with 9% trichloroacetic acid. In all cases, trichloroacetic acid-precipitated protein pellets were washed twice with ice cold acetone, dried in vacuo, and processed for immunoprecipitation as described previously (Gaynor *et al.*, 1994). Immunoprecipitated proteins were resuspended in sample buffer for resolution by SDS-PAGE, and gels were developed by autoradiography.

Fluorescence Microscopy

Labeling with *N*-[3-triethylammoniumpropyl]-4-[*p*-diethylaminophenyl-hexatrienyl] pyridinium dibromide (FM4-64); (Molecular Probes, Eugene, OR) was done essentially as described previously (Vida and Emr, 1995). All fluorescent images were obtained using an Axiovert S1002TV inverted fluorescent microscope (Carl Zeiss, Thornwood, NY) and processed using a Delta Vision deconvolution system (Applied Precision, Seattle, WA) and Photoshop 5.5 software (Adobe Systems, Mountain View, CA). All observations are based on the examination of at least 100 cells, and representative fields are shown. In all cases, the relative magnification is the same within a given figure.

RESULTS

Ymr1p Is a PI(3)P 3-Phosphatase In Vivo

The yeast *YMR1* gene product is an ortholog of the myotubularin family of PI(3)P phosphatases (Figure 1A) (Laporte *et al.*, 1998). As an *in vitro* PI(3)P-specific phosphatase (Taylor *et al.*, 2000), we reasoned that Ymr1p might function in the regulation of PI(3)P signaling *in vivo*. To test this, we first assayed PI(3)P levels in *ymr1Δ* cells and in cells overexpressing *YMR1* from a 2 μ *LEU2*-marked plasmid. We found that total cellular levels of PI(3)P were relatively unchanged in *ymr1Δ* cells compared with wild type; however, there was a subtle increase in PI(3,5)P₂ and PI(4,5)P₂ levels (Figure 1B). In contrast to the *ymr1Δ* mutant, overex-

pression of *YMR1* led to an ~25% decrease in PI(3)P levels compared with cells harboring an empty vector (Figure 1B), whereas other phosphoinositides remained relatively unaffected. These results were consistent with Ymr1p having specific activity against PI(3)P *in vivo*, as was reported for recombinant Ymr1p *in vitro* (Taylor *et al.*, 2000). Next, we examined vacuolar protein sorting by [³⁵S]methionine pulse-chase immunoprecipitation analysis in *ymr1Δ* cells and in *YMR1*-overexpressing cells by assaying the maturation of the vacuolar hydrolase CPY, which is delivered to the vacuole via a PI(3)P-dependent pathway (Schu *et al.*, 1993). Compared with wild-type cultures, the *ymr1Δ* mutant had no obvious defects in the maturation of CPY (Figure 1C). In contrast, overexpression of *YMR1* in wild-type cells caused a partial defect in CPY sorting in that roughly 15–20% was secreted in the Golgi-modified P2 precursor form (Figure 1C). These results suggested that *YMR1* overexpression depletes a pool of PI(3)P that is important for regulating CPY sorting from the Golgi to the vacuole and implied a role for Ymr1p in the regulation of PI(3)P-dependent protein sorting pathways *in vivo*.

Ymr1p and Sjl3p Control the Cellular Levels and Subcellular Distribution of PI(3)P

Previous studies on yeast phosphoinositide phosphatase mutants have indicated that Sac1p domain-containing proteins can provide redundant functions (Foti *et al.*, 2001; Gary *et al.*, 2002). Therefore, we tested whether Sac1p, Fig4p, Sjl1p, Sjl2p, or Sjl3p could compensate for Ymr1p function by generating a set of double mutants that were deleted of each corresponding open reading frame in combination with *ymr1Δ*. These double phosphatase mutant cells were labeled with *myo*-[2-³H]inositol, and the resulting deacylated glycerophosphoinositides were analyzed by high-performance liquid chromatography (HPLC). We found that only *ymr1Δ sjl3Δ* double mutant cells displayed a greater than twofold

Figure 1. Ymr1p metabolizes a pool of PI(3)P involved in vacuole protein sorting. (A) Schematic representation of the domain architecture of Ymr1p as predicted by the Prodom database. (B) Deletion and overexpression of *YMR1* alters PI(3)P levels in vivo. *YMR1* deletion cells, wild-type cells (SEY6210), and wild-type cells harboring a *2μYMR1* plasmid were grown under appropriate selection to early log phase and labeled with *myo*-[2-³H]inositol at 26°C for 60 min. Cellular lipids were recovered, deacylated, and separated by HPLC. Levels of deacylated products corresponding to the indicated phosphoinositides are shown as the % of total PI that was labeled. Data represent the mean $\pm$ SD of three independent experiments. (C) Overexpression of *YMR1* causes secretion of a pool of CPY; however, *YMR1* deletion has no effect. Cells were metabolically labeled at 26°C for 10 min with [³⁵S]methionine and then chased in the presence of excess unlabeled amino acids to the indicated time points. Intracellular pools (I) and secreted pools (E) of CPY were immunoprecipitated, and samples were resolved by SDS-PAGE and visualized by autoradiography. Positions of the endoplasmic reticulum-modified (P1), Golgi-modified (P2), and mature (M) CPY forms are shown.

elevation in PI(3)P levels over that of either single mutant (Figure 2A and Table 2). These findings indicated that Ymr1p and Sjl3p cooperate in the regulation of PI(3)P levels in yeast.

We next determined the intracellular distribution of PI(3)P in *ymr1Δ sjl3Δ* double mutant cells by using a PI(3)P-specific fluorescent reporter, GFP-FYVE_{EEA1} (Burd and Emr, 1998). Wild-type cells expressing this reporter exhibited a primarily punctate perivacuolar staining pattern representative of endosomal compartments (Figure 2B) (Burd and Emr, 1998). In contrast, the *ymr1Δ sjl3Δ* mutant displayed a dramatic enrichment of the green fluorescent protein (GFP) signal on vacuolar membranes, indicating that at steady state, PI(3)P accumulated on this organelle (Figure 2B). The vacuolar membrane staining by the GFP-FYVE_{EEA1} chimera in *ymr1Δ sjl3Δ* cells was accompanied by a reduction in perivacuolar punctate staining (Figure 2B). This could suggest that PI(3)P is depleted from endosomes in *ymr1Δ sjl3Δ* cells or that perhaps the integrity of these compartments is compromised in the double mutant. Interestingly, although PI(3)P

accumulated on the vacuole where the PI(3)P 5-kinase Fab1p is thought to be active (Odorizzi *et al.*, 1998; Rudge *et al.*, 2004), cellular PI(3,5)P₂ levels remained relatively unaffected in the *ymr1Δ sjl3Δ* mutant (Figure 2A and Table 2). This suggested that elevation in local PI(3)P concentration was not sufficient to drive Fab1p activity or perhaps that enhanced PI(3)P 5-kinase activity is met with elevated activity of a PI(3,5)P₂ phosphatase. Consistent with this, Fab1p has been found to be tightly regulated by several accessory factors such as Vac7p, Vac14p, and the Sac1p-domain-containing phosphatase Fig4p (Bonangelino *et al.*, 2002; Dove *et al.*, 2002; Gary *et al.*, 2002; Rudge *et al.*, 2004).

During the course of these PI(3)P localization studies, it became apparent that the misregulation of PI(3)P in *ymr1Δ sjl3Δ* mutant cells was associated with a fragmented vacuole phenotype not unlike that observed in class B *vps* mutants (Horazdovsky *et al.*, 1997). Therefore, *ymr1Δ sjl3Δ* mutant cells were labeled with FM4-64, a lipophilic dye that is endocytosed and trafficked to the vacuolar membrane (Vida and Emr, 1995), and observed by fluorescence microscopy. Compared with wild-type cultures labeled under the same conditions, the *ymr1Δ sjl3Δ* mutant displayed a multiple or fragmented vacuole phenotype (Figure 2B; see below), suggesting a role for Ymr1p and Sjl3p in the maintenance of vacuolar homeostasis.

Ymr1p and Sjl3p Regulate PI(3)P-specific Effectors that Direct Biosynthetic Cargo Transport

We next examined whether PI(3)P regulation by Ymr1p and Sjl3p was important for directing vesicle-mediated protein trafficking in the endosomal system. *ymr1Δ sjl3Δ* double mutant cells were pulse labeled with [³⁵S]methionine and assayed for their ability to sort and mature the vacuolar hydrolases CPY and carboxypeptidase S (CPS). Interestingly, the *ymr1Δ sjl3Δ* mutant displayed a significant defect in CPY sorting in that roughly 30–40% was secreted in the Golgi-modified P2 form (Figure 3A). In addition to missorting of CPY, there was a delay in the processing of CPS (Figure 3A) that correlated to a defect in multivesicular body (MVB) sorting (see below). Therefore, to gain insight into the mechanisms underlying these vacuole protein sorting defects in the *ymr1Δ sjl3Δ* double mutant, the localization and function of several PI(3)P-specific effectors that play important roles in directing endosomal sorting were examined.

The retromer complex functions in the recycling of the CPY receptor Vps10p from endosomes back to the Golgi (Cereghino *et al.*, 1995; Cooper and Stevens, 1996; Seaman *et al.*, 1998). Two retromer complex subunits, Vps5p and Vps17p, contain PX domains and are PI(3)P-specific effectors (Burda *et al.*, 2002). Therefore, to test whether the CPY sorting defect in the *ymr1Δ sjl3Δ* mutant could be due at least in part to the misregulation of these Vps10p sorting factors, the subcellular distribution of Vps5-GFP and Vps17-GFP was determined. Wild-type cells expressing Vps17-GFP displayed a perivacuolar punctate staining pattern consistent with previously published results (Burda *et al.*, 2002). In contrast, in *ymr1Δ sjl3Δ* double mutant cells, Vps17-GFP was diffuse in the cytoplasm with a partial enrichment on FM4-64-positive fragmented vacuoles (Figure 3B). Similar results also were obtained from cells expressing a Vps5-GFP fusion (our unpublished data). Thus, accumulation of PI(3)P in *ymr1Δ sjl3Δ* cells likely caused the mislocalization of these retromer complex components. Because mutants defective for Vps5p or Vps17p function also displayed a fragmented vacuole phenotype (Horazdovsky *et al.*, 1997), it remains tempting to speculate that defects in their regulation might

Figure 2. Ymr1p and Sjl3p coordinately regulate cellular levels and the subcellular distribution of PI(3)P. (A) Quantitative comparison of glycerophosphoinositols generated by wild-type cells (black bars) or *ymr1Δ sjl3Δ* mutant cells (gray bars). Cells were grown to early log phase in complete media and then labeled with *myo*-[2-³H]inositol as in Figure 1. Data represent the mean $\pm$ SD of three independent experiments. (B) Ymr1p and Sjl3p control the subcellular distribution of PI(3)P. Steady-state localization of the PI(3)P-specific GFP-FYVE^{EEA1} chimera in wild-type cells and in *ymr1Δ sjl3Δ* cells. Cells were maintained in early log phase under appropriate selection for 36–40 h and then harvested into YPD. Cells were stained for 10 min at room temperature with FM4-64, excess dye was washed away and cells were chased for 60 min at room temperature. Cells were then washed with YNB on ice, and aliquots were observed by fluorescence microscopy using a Delta Vision deconvolution system.

in part account for vacuolar morphology defects observed in the *ymr1Δ sjl3Δ* double mutant.

To investigate whether the mislocalization of Vps5p and Vps17p results in Vps10p trafficking defects, we expressed a chromosomally tagged *VPS10-GFP* chimera (Burda *et al.*, 2002) in *ymr1Δ sjl3Δ* mutant cells to visualize the subcellular localization of Vps10p. Consistent with previously published results (Burda *et al.*, 2002), wild-type cells displayed a punctate cytoplasmic staining pattern typical of Golgi and/or endosomal structures (Figure 3C). The *ymr1Δ sjl3Δ* mutant also displayed a cytoplasmic punctate staining pattern; however, these Vps10-GFP-containing structures were qualitatively different from the wild type in that they typically seemed larger, and

many colocalized with FM4-64 on vacuolar compartments (Figure 3C). Although these results indicated that Vps10-GFP was mislocalized in the *ymr1Δ sjl3Δ* mutant, this staining pattern does not phenocopy retromer loss of function mutants, in which case Vps10-GFP is found almost exclusively on the vacuole membrane (Seaman *et al.*, 1997; Burda *et al.*, 2002). Consistent with an incomplete defect in CPY sorting in the *ymr1Δ sjl3Δ* double mutant, this would suggest that retromer function was only partially compromised in these cells.

Proper sorting of Vps10p in the endosomal system is required to maintain its stability and function (reviewed in Pfeffer, 2001). Therefore, we next examined the stability of Vps10p by [³⁵S]methionine pulse-chase immunoprecipitation assays as an alternative means for examining endosomal sorting function in *ymr1Δ sjl3Δ* mutant cells. As expected from previous work (Cereghino *et al.*, 1995), in wild-type cells Vps10p remained stable throughout the 2-h time course (Figure 3D). In addition to the full-length protein observed in wild-type cells, after 30 min, the *ymr1Δ sjl3Δ* mutant also displayed a Vps10p breakdown product (Figure 3D). This roughly 20-kDa shift in mobility was consistent with Vps10p being clipped from its transmembrane anchor and cytoplasmic domain. In addition to clipping, there was nearly a complete loss of Vps10p by the end of the time course in *ymr1Δ sjl3Δ* cells, indicating a strong defect in endosome function (Figure 3D). This defect in Vps10p stability correlated with the CPY sorting defect, and was a specific property of the *ymr1Δ sjl3Δ* double mutant, because Vps10p stability was not altered in the *ymr1Δ* or the *sjl3Δ* single mutants (Figure 3D). Defects in Vps10p stability are typical of class E *vps* mutants that have altered endosome morphology and function (Cereghino *et al.*, 1995). Although *ymr1Δ sjl3Δ* cells do not exhibit an obvious class E compart-

Table 2. Phosphoinositide levels in phosphatase mutants

Strain	PI(3)P	PI(4)P	PI(3,5)P ₂	PI(4,5)P ₂
SEY6210	2.05 (.35)	1.63 (.31)	0.07 (.02)	1.60 (.21)
<i>ymr1Δsac1Δ</i>	2.40 (.02)	24.3 (.30)	0.06 (.01)	0.49 (.08)
<i>ymr1Δfig4Δ</i>	3.00 (.23)	1.72 (.11)	0.06 (.02)	1.64 (.21)
<i>ymr1Δsjl1Δ</i>	2.17 (.07)	1.11 (.14)	0.07 (.01)	2.05 (.08)
<i>ymr1Δsjl2Δ</i>	3.01 (.04)	1.62 (.06)	0.10 (.02)	1.62 (.12)
<i>ymr1Δsjl3Δ</i>	5.17 (.22)	1.66 (.19)	0.11 (.03)	1.22 (.10)
<i>ymr1Δ</i>	2.34 (.10)	1.78 (.13)	0.07 (.01)	1.59 (.03)
<i>sjl3Δ</i>	2.35 (.04)	2.14 (.07)	0.09 (.02)	1.57 (.07)

Strains were labeled with *myo*-[³H]inositol, and lipids were extracted and processed for HPLC analysis as described in MATERIALS AND METHODS. The mean peak area (cpm) is expressed as percentage of total ³H-labeled PIs. Results are the mean $\pm$ the standard deviation of at least two independent experiments done in duplicate.

Figure 3. Ymr1p and Sjl3p regulate vacuolar protein sorting pathways. (A) *ymr1Δ sjl3Δ* cells display defects in CPY sorting and CPS maturation. Wild-type and *ymr1Δ sjl3Δ* cells were analyzed by [³⁵S]methionine pulse-chase labeling followed by immunoprecipitation with antibodies directed against CPY (top) as in Figure 1 or CPS (bottom) as described in MATERIALS AND METHODS. The positions of precursor forms and mature forms are indicated. Results are representative of at least two independent experiments. (B) Vps17-GFP is mislocalized in the *ymr1Δ sjl3Δ* mutant. Wild-type and *ymr1Δ sjl3Δ* cells carrying a chromosomally integrated *VPS17-GFP* allele were grown to early log phase in complete media and stained with FM4-64 and subsequently processed for microscopy as in Figure 2. (C) Vps10-GFP is partially mislocalized in the *ymr1Δ sjl3Δ* mutant. Wild-type and *ymr1Δ sjl3Δ* cells carrying a chromosomally integrated *VPS10-GFP* allele were grown to early log phase in complete media and stained with FM4-64 and processed for microscopy as described above. (D) Vps10p is destabilized in the *ymr1Δ sjl3Δ* mutant. Cells were pulse labeled for 15 min with [³⁵S]methionine and chased with an excess of unlabeled amino acids for the indicated time. Lysates prepared from these cells were immunoprecipitated with antibodies specific to the luminal domain of Vps10p, and samples were resolved by SDS-PAGE and visualized by autoradiography. Results are representative of at least two independent experiments.

ment, it is possible that instability of Vps10p results from a combination of partial defects in retromer function and in the MVB sorting pathway, which is ablated in class E *vps*

mutants (reviewed in Katzmman *et al.*, 2002). Therefore, we next determined whether MVB sorting was affected by the accumulation of PI(3)P in the *ymr1Δ sjl3Δ* mutant.

Figure 4. Ymr1p and Sjl3p are required for proper MVB sorting. (A) GFP-Vps27 is redistributed to the vacuolar surface in the *ymr1Δ sjl3Δ* mutant. Wild-type and *ymr1Δ sjl3Δ* cells harboring a 2μ URA3-marked GFP-VPS27 plasmid were grown to early log phase in selective media and stained with FM4-64 and processed for microscopy as in Figure 2. (B) GFP-CPS mislocalization exemplifies a defect in MVB sorting in the *ymr1Δ sjl3Δ* mutant. Wild-type and *ymr1Δ sjl3Δ* cells harboring a 2μ URA3-marked GFP-CPS plasmid were grown to early log phase in selective media and stained with FM4-64 and processed for microscopy as described above.

YMR1 and SJL3 Regulate Vps27p Localization and MVB Sorting

The MVB sorting pathway depends on the proper localization and function of Vps27p. Vps27p is recruited to the endosome via its FYVE domain, which binds to PI(3)P. Subsequent to PI(3)P binding, Vps27p recruits/activates the ESCRT (for endosomal sorting complex required for transport) machinery required for the selection of MVB cargoes (Katzmann *et al.*, 2003). In wild-type cells, this MVB sorting process occurs at the endosome where PI(3)P is enriched. Mutants that are blocked in the MVB sorting pathway typically accumulate an aberrant endosome-like class E compartment and have severe defects in endosome function (Rieder *et al.*, 1996; Babst *et al.*, 1997). Therefore, we expressed GFP fusions of Vps27p (Katzmann *et al.*, 2003) or the MVB cargo protein CPS (Odorizzi *et al.*, 1998) in *ymr1Δ sjl3Δ* cells. Consistent with previously published results, wild-type cells localized GFP-Vps27 to punctate perivacuolar endosomal structures (Figure 4A) (Katzmann *et al.*, 2003). In *ymr1Δ sjl3Δ* cells, there was a redistribution of GFP-Vps27 to the surface of the vacuole (Figure 4A). The mislocalization of Vps27p correlated with a defect in MVB sorting of GFP-CPS in *ymr1Δ sjl3Δ* cells. Wild-type cells properly sorted GFP-CPS to the lumen of the vacuole; however, *ymr1Δ sjl3Δ* cells showed enrichment of GFP-CPS on the limiting membrane of the vacuole (Figure 4B). Thus, the delay in CPS maturation

that was observed in *ymr1Δ sjl3Δ* cells (Figure 3A) was most likely due to the mislocalization of Vps27p and subsequent defects in MVB sorting.

Cytoplasm-to-Vacuole Transport (CVT) Is Disrupted in the *ymr1Δ sjl3Δ* Double Mutant

The CVT pathway in yeast is also PI(3)P-dependent (Kametaka *et al.*, 1998) and uses the PX domain-containing sorting nexins Atg24p (Cvt13p, Snx4p) and Atg20p (Cvt20p, Snx42p) (Nice *et al.*, 2002). Therefore, we determined whether regulation of the CVT pathway was affected in the *ymr1Δ sjl3Δ* double mutant by assaying the maturation of the CVT cargo protein aminopeptidase 1 (Ape1p) by [35 S]methionine pulse-chase immunoprecipitation analysis. Compared with wild-type cells, the *ymr1Δ sjl3Δ* mutant displayed a strong defect in Ape1p processing, with the precursor form remaining stable throughout the course of the experiment (Figure 5A). We next generated a chromosomal Atg24-GFP fusion to test whether Atg24p was mislocalized in the *ymr1Δ sjl3Δ* double mutant. In wild-type cells, Atg24-GFP was found on punctate structures (Figure 5B) that are thought to represent preautophagosomal structures (PAS) (Nice *et al.*, 2002). In contrast to this, the *ymr1Δ sjl3Δ* double mutant redistributed Atg24-GFP to the cytoplasm (Figure 5B). Because this could result from a defect in PAS formation, the subcellular localization of two PAS markers,

Figure 5. CVT pathway sorting is defective in *ymr1Δ sjl3Δ* cells. (A) Processing of Ape1p through the CVT pathway, but not through macroautophagy, is severely defective in *ymr1Δ sjl3Δ* cells. Wild-type and *ymr1Δ sjl3Δ* cells were grown to early log phase and pulse labeled with [³⁵S]methionine for 15 min and chased with an excess of unlabeled amino acids to the indicated time points. Samples were resolved by SDS-PAGE and visualized by autoradiography. The positions of precursor and mature Ape1p are indicated. (B) Atg24p is mislocalized in *ymr1Δ sjl3Δ* cells. Wild-type and *ymr1Δ sjl3Δ* cells carrying a chromosomally integrated *Atg24-GFP* allele were grown to early log phase in complete media and stained with FM4-64 and processed for microscopy as described in Figure 2.

Cvt9 (Atg11)-GFP and Aut7 (Atg8)-GFP, also was analyzed (Kim *et al.*, 2001). The *ymr1Δ sjl3Δ* mutant displayed punctate staining for each PAS marker protein that was indistinguishable from the wild type (our unpublished data). Therefore, it is likely that Atg24p, in addition to binding of PI(3)P via its PX domain, requires other factors for stable membrane association that may not be present on the surface of vacuoles where PI(3)P accumulates in *ymr1Δ sjl3Δ* cells.

Sac1p Domain Activity of Sjl2p Is Required for Viability in the *ymr1Δ sjl3Δ* Mutant

The Sac1p domain-containing phosphatases, in particular the synaptojanin-like proteins Sjl2p and Sjl3p, were previously shown to significantly overlap in substrate specificity (Guo *et al.*, 1999) as well as cellular function (Stolz *et al.*, 1998; Foti *et al.*, 2001; Stefan *et al.*, 2002). Therefore, to address a possible role for Sjl2p in the regulation of PI(3)P, we sought to construct a *ymr1Δ sjl2Δ sjl3Δ* triple mutant. Interestingly, this mutant combination yielded a synthetic lethal phenotype (Table 3). Because Sjl2p and Sjl3p both contain a 5-phosphatase activity as well as Sac1p domain activity (Stefan *et al.*, 2002), we took advantage of this synthetic lethal phenotype to determine which activity of these phosphatases was required to support viability of the *ymr1Δ sjl2Δ sjl3Δ* triple mutant. To do this, a diploid strain that was homozygous for *ymr1Δ* and heterozygous for *sjl2Δ* and *sjl3Δ* was transformed with a *URA3*-marked plasmid carrying a temperature conditional *sjl2^{ts}-8* allele (Stefan *et al.*, 2002) and subsequently sporulated. A *ymr1Δ sjl2Δ sjl3Δ* triple mutant harboring the *sjl2^{ts}-8* plasmid was isolated (*ymr1Δ sjl2^{ts} sjl3Δ*) and transformed with *LEU2*-marked *sjl2-C446S* or *sjl2-D850S* plasmids, which impair the Sac1p domain activity or the 5-phosphatase activity of Sjl2p, respectively (Stefan *et al.*, 2002). The *sjl2-C446S* plasmid failed to rescue lethality of the *ymr1Δ sjl2^{ts} sjl3Δ* triple mutant at 38°C, whereas the *sjl2-D850S* plasmid fully restored growth to the strain (Figure 6), indicating that Sac1p domain activity was required to support viability. These results suggested that maintenance of PI(3)P regulation is an essential function that is disrupted in the *ymr1Δ sjl2Δ sjl3Δ* triple mutant.

To test this, we took advantage of the *ymr1Δ sjl2^{ts} sjl3Δ* triple mutant and determined the effects of inactivating Sjl2p on cellular phosphoinositide levels. In *ymr1Δ sjl2^{ts} sjl3Δ* cells at permissive temperature, there was a decrease in PI(4,5)P₂ levels, whereas PI(3)P, PI(4)P, and PI(3,5)P₂ levels remained nearly the same compared with the *ymr1Δ sjl3Δ* mutant [compare *ymr1Δ sjl3Δ* from Table 2 and *ymr1Δ sjl2Δ sjl3Δ* (*sjl2-8^{ts}*) from Table 3]. On shift to the restrictive temperature, the *ymr1Δ sjl2^{ts} sjl3Δ* mutant displayed further accumulation of 3' phosphoinositides, including a roughly 20% increase in PI(3)P, and an eightfold increase in PI(3,5)P₂ compared with the permissive temperature (Table 3). This

Table 3. Phosphoinositide levels in triple phosphatase deletion mutants and temperature sensitive mutants

Strain	PI(3)P	PI(4)P	PI(3,5)P ₂	PI(4,5)P ₂
<i>ymr1Δsjl3Δsjl1Δ</i>	3.88 (.07)	1.04 (.18)	0.08 (.01)	1.69 (.08)
<i>ymr1Δsjl3Δfig4Δ</i>	3.94 (.10)	1.44 (.02)	0.10 (.03)	0.93 (.04)
<i>ymr1Δsjl2Δsjl3Δ</i>	Lethal			
<i>ymr1Δsjl2Δsjl3Δ</i> (<i>sjl2-8^{ts}</i>) 26°C	5.56 (.38)	1.20 (.46)	0.12 (.02)	0.87 (.06)
<i>ymr1Δsjl2Δsjl3Δ</i> (<i>sjl2-8^{ts}</i>) 38°C ^a	6.90 (.38)	1.32 (.32)	0.97 (.07)	0.91 (.06)
<i>ymr1Δsjl3Δ</i> 38°C	4.89 (.40)	1.52 (.30)	0.37 (.08)	1.03 (.35)
SEY6210 38°C	1.84 (.23)	1.76 (.15)	0.24 (.03)	1.74 (.25)

Strains were labeled with *myo*-[³H]inositol, and lipids were extracted and processed for HPLC analysis as described in MATERIALS AND METHODS. The mean peak area (cpm) is expressed as percentage of total [³H]-labeled PIs. Results are the mean ± standard deviation of at least two independent experiments done in duplicate.

^a Greater than 90% of the cells in these cultures remained viable in the time course of these experiments as determined by incorporation of the vital dye CMAC (our unpublished data). Therefore, the levels of 3' phosphoinositides that result in loss of viability are likely to be underestimated in this analysis.

Figure 6. Sac1p domain activity of Sjl2p is required for viability in *ymr1Δ sjl3Δ* cells. *ymr1Δ sjl2^{ts} sjl3Δ* cells were transformed with either an empty vector or the indicated *sjl2* mutant allele impaired for either Sac1p domain function (C446S) or 5' phosphatase activity (D850S) carried on pRS415. Transformants were grown to log phase and 10-fold serial dilutions were spotted onto selective plates at either 26 or at 38°C, and growth was scored after 3 d.

indicated that Ymr1p, Sjl2p, and Sjl3p overlap in the regulation of PI(3)P. Together, these results strongly implicated the misregulation and/or accumulation of 3' phosphoinositides in the synthetic lethality of the *ymr1Δ sjl2Δ sjl3Δ* mutant.

3' Phosphoinositide Accumulation Is Toxic in Yeast

These results suggested that deficient phosphoinositide 3-phosphatase activity allows PI(3)P to become toxic in *ymr1Δ sjl2Δ sjl3Δ* triple mutant cells. To test this, we constructed several Sac1p domain chimeras in which the phosphatase domain of Sac1p was fused to various lipid targeting domains that have known phosphoinositide binding specificities (see below). To facilitate the detection of these

constructs in vivo, each was constructed as an N-terminal GFP fusion. By this approach cytoplasmic Sac1p domain activity could be targeted to PI(4,5)P₂ on the plasma membrane (GFP-Sac1ΔC-2 × PH_{PLCγ}), to PI(4)P on Golgi compartments (GFP-Sac1ΔC-PH_{FAPP1}), to PI(3)P on endosomes and the vacuole (GFP-Sac1ΔC-FYVE_{EEA1}), or left diffusely throughout the cytoplasm (GFP-Sac1ΔC) (Figure 7) (Foti *et al.*, 2001; Stefan *et al.*, 2002). Each of these chimeras localized to the intended target membrane by fluorescence microscopy, and Western blot analysis using GFP-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) showed no obvious differences in the expression levels or stability of these chimeras (Figure 7; our unpublished data). To facilitate the detection of subtle effects of these Sac1p domain chimeras on the growth properties of the *ymr1Δ sjl2Δ sjl3Δ* triple mutant, we generated a *ymr1^{ts}* allele that could not support viability above 34°C (see MATERIALS AND METHODS; our unpublished data). Next, each Sac1p domain chimera was expressed in *ymr1^{ts} sjl2Δ sjl3Δ* triple mutant cells, and its ability to promote viability at the restrictive temperature was determined. Interestingly, only the PI(3)P-targeted GFP-Sac1ΔC-FYVE_{EEA1} fusion was able to promote growth at restrictive temperature (Figure 8A). To confirm that rescue of the temperature conditional lethality was dependent on phosphatase activity, the conserved active site cysteine residue of the GFP-Sac1ΔC-FYVE_{EEA1} chimera was mutated to serine; a substitution that ablates the activity of Sac1p (Guo *et al.*, 1999). Although this mutant version, GFP-Sac1ΔC_{C392S}-FYVE_{EEA1} was expressed and localized comparably to the phosphatase-active chimera, it failed to rescue the *ymr1^{ts} sjl2Δ sjl3Δ* mutant, even at 34°C (Figure 8A; our unpublished data). This indicated that phosphatase activity

Figure 7. Subcellular localization of targeted GFP-Sac1ΔC chimeras. Wild-type cells were transformed with the indicated construct carried on pRS426, which target Sac1p phosphatase activity to the indicated subcellular compartment. Cells were grown to early log phase in selective media and GFP fluorescence was observed as described in Figure 2.

of the PI(3)P-targeted chimera was required to promote growth of the mutant at restrictive temperature. We next tested whether the GFP-Sac1ΔC-FYVE chimera could complement the synthetic lethality of the *ymr1Δ sjl2Δ sjl3Δ* triple mutant. To do this, a *LEU2*-marked version of the GFP-SAC1ΔC-FYVE_{EEA1} plasmid was constructed and transformed into *ymr1Δ sjl2^{ts} sjl3Δ* cells and their ability to grow on media containing 5-FOA, which selects against cells containing the *URA3*-marked *sjl2^{ts}* plasmid, was determined.

Interestingly, cells containing the GFP-Sac1ΔC-FYVE_{EEA1} chimera grew on 5-FOA, whereas control cells with an empty vector or the plasma membrane targeted GFP-Sac1ΔC-PH_{PLCδ} chimera did not (Figure 8B; our unpublished data). The ability of these cells to grow on 5-FOA indicated that the GFP-Sac1ΔC-FYVE_{EEA1} chimera was sufficient to support viability of the *ymr1Δ sjl2Δ sjl3Δ* mutant.

Analysis of *in vivo* phosphoinositides in *ymr1^{ts} sjl2Δ sjl3Δ* cells expressing the GFP-Sac1ΔC-FYVE_{EEA1} chimera at 38°C

Figure 8. Sac1p domain activity targeted to PI(3)P rescues lethality of the *ymr1Δ sjl2Δ sjl3Δ* mutant. (A) *ymr1^{ts} sjl2Δ sjl3Δ* cells were transformed with an empty vector or the indicated GFP-tagged Sac1p domain chimeras carried on pRS426. A phosphatase-inactive mutant (Sac1ΔC-C392S-FYVE_{EEA1}) was generated by site-directed mutagenesis. Transformants were grown to log phase and 10-fold serial dilutions were spotted as in Figure 6A. (B) *ymr1Δ sjl2^{ts} sjl3Δ* cells were transformed with either 2 μ LEU2-marked GFP-SAC1ΔC-FYVE_{EEA1} or GFP-SAC1ΔC-2 $\times$ PH_{PLC} plasmids and tested for their ability to grow on media containing 5-FOA, which selects against cells harboring the *URA3*-marked *sjl2^{ts}* plasmid. The PI(3)P-targeted GFP-SAC1ΔC-FYVE_{EEA1} plasmid supported viability of the resulting *ymr1Δ sjl2Δ sjl3Δ* triple mutant cells, whereas the PI(4,5)P₂-targeted GFP-SAC1ΔC-2 $\times$ PH_{PLC} plasmid did not. (C) GFP-Sac1ΔC-FYVE_{EEA1} chimera reduces PI(3)P and PI(3,5)P₂ in *ymr1^{ts} sjl2Δ sjl3Δ* cells to levels comparable with YMR1. *ymr1^{ts} sjl2Δ sjl3Δ* cells carrying an empty vector (black bars), the GFP-Sac1ΔC-FYVE_{EEA1} chimera (gray bars), or wild-type YMR1 (stippled bars) were grown to early log phase under appropriate selection, preincubated at 38°C for 20 min and labeled with *myo*-[2-³H]inositol for 60 min at 38°C. Samples were prepared for HPLC as in Figure 1. Levels of deacylated products corresponding to the indicated phosphoinositides are represented as percentage of total labeled PI. Data are representative of at least two independent experiments, and the SD was <15% for each data point.

revealed that the most striking differences, compared with cells harboring an empty vector, were a roughly 70% decrease in PI(3)P levels and a roughly 60% decrease in PI(3,5)P₂ levels, rendering the levels of both of these 3' phosphoinositides comparable with *YMR1*-rescued control cells (Figure 8C). Collectively, these results argued that phosphatase-mediated PI(3)P regulation was an essential function, most likely at post-Golgi compartments, and supported the idea that 3' phosphoinositide accumulation is toxic to cells, resulting in the synthetic lethal phenotype of the *ymr1Δ sjl2Δ sjl3Δ* mutant.

DISCUSSION

In this study, we have elucidated a role for the yeast myotubularin-related phosphatase Ymr1p in the regulation of PI(3)P-mediated vacuolar protein sorting. Our data argue that Ymr1p is a PI(3)P-specific phosphatase *in vivo*, and in conjunction with the synaptojanin-like phosphatase Sjl3p, controls the cellular levels and the subcellular distribution of PI(3)P. The misregulation of PI(3)P in *ymr1Δ sjl3Δ* cells impacts the localization and function of several PI(3)P effectors that play key roles in cargo sorting and vesicle trafficking in the endosomal system, which culminates in the dysfunction of endosomes as prevacuolar sorting compartments. Moreover, further deletion of *SJL2* in the *ymr1Δ sjl3Δ* double mutant yields a synthetic lethal phenotype that directly correlates with further defects in the regulation of 3' phosphoinositides. Together, this work provides the first experimental evidence that although PI(3)P is not an essential phosphoinositide (Schu *et al.*, 1993), the accumulation and loss of spatial control of PI(3)P lead to membrane trafficking defects and ultimately to a toxic effect that results in the loss of viability.

The Myotubularin Family of PI Phosphatases

The myotubularin family constitutes a large group of evolutionarily conserved phosphatases within the tyrosine/dual-specificity phosphatase superfamily (reviewed in Laporte *et al.*, 2003). The catalytically active human myotubularins that have been tested (Mtm1, Mtmr2, Mtmr3, Mtmr4, Mtmr6, and Mtmr7) are all specific 3-phosphatases for PI(3)P and PI(3,5)P₂ *in vitro*, generating PI and PI(5)P, respectively (Taylor *et al.*, 2000; Walker *et al.*, 2001; Tronchere *et al.*, 2003), of which PI(5)P seems to be an allosteric activator of phosphatase activity (Schaletzky *et al.*, 2003). In contrast to these mammalian myotubularins, our data suggest that Ymr1p, the sole myotubularin member in yeast, does not use PI(3,5)P₂ as a substrate *in vivo*. This is supported by the observations that overexpression of *YMR1* does not decrease the level of PI(3,5)P₂ in otherwise wild-type cells (Figure 1C), even upon hyperosmotic stress (Parrish and Emr, unpublished results), which stimulates PI(3,5)P₂ synthesis (Cooke *et al.*, 1998). In addition, PI(5)P, the product of PI(3,5)P₂ catalysis by myotubularins (Schaletzky *et al.*, 2003; Tronchere *et al.*, 2003), is not among the normal complement of phosphoinositides identified in yeast and was undetectable in all of our HPLC analyses.

Ymr1p and Sjl3p Coordinately Regulate Endosome Function by Controlling PI(3)P Signaling

The finding that deletion of *YMR1* did not affect vacuole protein sorting (Figure 1C; Taylor *et al.*, 2000) led us to predict that Sac1p domain-containing lipid phosphatases could compensate for the activity of Ymr1p in the context of the *ymr1Δ* mutant. This was indeed the case, because the deletion of *SJL3* in the *ymr1Δ* mutant caused a roughly

2.5-fold increase in cellular PI(3)P levels and the redistribution of this lipid onto the surface of vacuoles. Consistent with Ymr1p and Sjl3p functioning in a similar pathway, we found that like *YMR1*, overexpression of *SJL3* also significantly lowered PI(3)P levels and conferred defects in CPY sorting in otherwise wild-type cells (our unpublished data). Defects in PI(3)P regulation correlated with endosome dysfunction, fragmented vacuole morphology, and defects in PI(3)P-mediated vacuole protein sorting pathways in the *ymr1Δ sjl3Δ* double mutant. These phenotypes were apparently due to pleiotropic defects in the regulation of PI(3)P-specific effectors that carry out cargo sorting and vesicle trafficking. Together, these results indicate that PI(3)P-directed phosphatase activity of Ymr1p and Sjl3p maintains endosome function through the control of several key PI(3)P effectors. For Sjl3p, this function in PI(3)P metabolism and endosome maintenance may be in addition to its previously characterized role in the regulation of a clathrin/AP1 mediated "slow delivery" pathway of cargoes between the *trans*-Golgi and early endosomes, a function that requires both catalytic activities of Sjl3p (Ha *et al.*, 2003).

PI 3-Phosphatase Activity Is Required for Viability

Sjl2p and Sjl3p each possess two distinct phosphoinositide phosphatase activities, a Sac1p domain and a PI(4,5)P₂ 5-phosphatase domain, which renders them capable of converting all common phosphoinositides in yeast to PI (reviewed in Hughes *et al.*, 2000). Although each likely has a primary role in the regulation of distinct phosphoinositide signaling pathways, it is not surprising that Sjl2p and Sjl3p significantly overlap in function with each other, and with other lipid phosphatases that are dedicated to the hydrolysis of specific phosphoinositides. Consistent with this, *sjl2Δ sjl3Δ* double mutants are viable and demonstrate only modest phenotypes, including slowed growth rate, cell wall thickening, and defects in vacuole morphology and actin cytoskeleton organization (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998). Despite these phenotypes, *sjl2Δ sjl3Δ* double mutants do not display significant increases in cellular levels of PI(3)P, PI(4)P, or PI(4,5)P₂ (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998) and show only a small increase in PI(3,5)P₂ (Guo *et al.*, 1999), indicating that other phosphatases are sufficient to maintain the normal levels of these phosphoinositides. For example, in *sjl2Δ sjl3Δ* cells, further deletion of *SAC1* or *SJL1* results in lethality, which is attributable to defects in PI(4)P regulation (Foti *et al.*, 2001) or PI(4,5)P₂ regulation (Srinivasan *et al.*, 1997; Stolz *et al.*, 1998; Stefan *et al.*, 2002), respectively.

Our work presented here reveals a further essential requirement for phosphatase-mediated PI(3)P regulation through the elucidation of Ymr1p as a PI(3)P-specific phosphatase that supports viability in *sjl2Δ sjl3Δ* double mutant cells. Several observations are consistent with an essential overlapping role for Ymr1p, Sjl2p, and Sjl3p in PI(3)P regulation. First, Ymr1p is apparently sufficient to control PI(3)P signaling and promote proper endosomal sorting. Second, further elimination of the Sac1p domain activity of Sjl2p in *ymr1Δ sjl3Δ* cells results in lethality. Third, our studies on the *ymr1Δ sjl3Δ* double mutant combined with the analysis of phosphoinositide metabolism in *ymr1Δ sjl2^{ts} sjl3Δ* cells suggests that synthetic lethality is most likely due to exacerbated defects in 3' phosphoinositide regulation. Finally, soluble Sac1p domain activity was able to rescue *ymr1Δ sjl2Δ sjl3Δ* cells, but only if it was targeted to PI(3)P.

An intriguing question that arises from this study revolves around the essential process(es) that is disrupted upon the loss of phosphatase-mediated PI(3)P regulation.

Because PI(3)P is not an essential phosphoinositide (Schu *et al.*, 1993), it seems reasonable that 3' phosphoinositide accumulation might exert toxic effects by simultaneously interfering with the regulation of multiple phosphoinositide-dependent signaling pathways, potentially including those that regulate actin organization (reviewed in Yin and Janmey, 2003), cytokinesis (Casamayor and Snyder, 2003), or cell growth and survival (reviewed in Heinisch *et al.*, 1999), culminating in the inability of *ymr1Δ sjl2Δ sjl3Δ* cells to grow. Accordingly, further work will be required to determine the extent to which phosphatase-mediated regulation of 3' phosphoinositides and the integrity of the endosomal sorting system impacts the control of other phosphoinositide signaling systems.

In yeast, previous studies have indicated essential roles for the regulation of PI(4)P (Foti *et al.*, 2001) and PI(4,5)P₂ (Stolz *et al.*, 1998; Stefan *et al.*, 2002). In this study, we have elucidated an essential role for the regulation of cellular levels and the subcellular distribution of PI(3)P. To our knowledge, these findings represent the first clear demonstration that a myotubularin family member regulates PI(3)P-mediated endosomal sorting events. Thus, as has been speculated (Laporte *et al.*, 2003), it is likely that myotubularins antagonize type III PI 3-kinase (hVps34) signaling in higher eukaryotes as well. This may have important implications for the ontogeny of severe human genetic diseases such as myotubular myopathy and Marie-Charcot-Tooth neuropathy, which are caused by mutations in myotubularin family members.

ACKNOWLEDGMENTS

We are indebted to Steven Padilla, Perla Arcaira, Joshua Kaufman, Jane Atienza, and James Clegg for excellent technical support through the course of the study. We thank Drs. James Konopka, Jennifer Lin, Anne Savitt, and Greg Taylor for advice and critical comments on the work. We also thank members of the Emr laboratory, both past and present, especially Drs. Patrice Burda, Sriramonti Sarkar, Simon Rudge, Vicki Sciorra, Takeshi Noda, and Mitsuaki Tabuchi, for generosity with strains and reagents and invaluable discussions and suggestions regarding the manuscript. C.J.S. is a postdoctoral research associate of the Howard Hughes Medical Institute. W.R.P. is supported by National Research Service Award postdoctoral research training grant 5 T32 AI07036-25 from the National Institutes of Health/National Institute of Allergy and Infectious Diseases. S.D.E. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

- Audhya, A., Foti, M., and Emr, S.D. (2000). Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. *Mol. Biol. Cell* 11, 2673–2689.
- Babst, M., Sato, T.K., Banta, L.M., and Emr, S.D. (1997). Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *EMBO J.* 16, 1820–1831.
- Bonangelino, C.J., Nau, J.J., Duex, J.E., Brinkman, M., Wurmser, A.E., Gary, J.D., Emr, S.O., and Weisman, L.S. (2002). Osmotic stress-induced increase of Ptdins(3,5)-bisphosphate requires Vac 14p; an activator of the lipid kinase Fab1p. *J. Cell Biol.* 156, 1015–1028.
- Bonifacio, J., and Traub, L. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu. Rev. Biochem.* 72, 395–447.
- Burd, C., Babst, M., and Emr, S. (1998). Novel pathways, membrane coats and PI kinase regulation in yeast lysosomal trafficking. *Semin. Cell Dev. Biol.* 9, 527–533.
- 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.
- Burda, P., Padilla, S.M., Sarkar, S., and Emr, S.D. (2002). Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase. *J. Cell Sci.* 115, 3889–3900.
- Casamayor, A., and Snyder, M. (2003). Molecular dissection of a yeast septin: distinct domains are required for septin interaction, localization, and function. *Mol. Cell. Biol.* 23, 2762–2777.
- Cereghino, J.L., Marcusson, E.G., and Emr, S.D. (1995). The cytoplasmic tail domain of the vacuolar sorting receptor Vps10p and a subset of VPS gene products regulate receptor stability, function and localization. *Mol. Biol. Cell* 6, 1089–1102.
- Cooke, F., Dove, S., McEwen, R., Painter, G., Holmes, A., Hall, M., Michell, R., and Parker, P. (1998). The stress-activated phosphatidylinositol 3-phosphate 5-kinase Fab1p is essential for vacuole function in *S. cerevisiae*. *Curr. Biol.* 8, 1219–1222.
- Cooper, A.A., and Stevens, T.H. (1996). Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple yeast vacuolar hydrolases. *J. Cell Biol.* 133, 529–541.
- Corvera, S., D'Arrigo, A., and Stenmark, H. (1999). Phosphoinositides in membrane traffic. *Curr. Opin. Cell Biol.* 11, 460–465.
- Dove, S.K., McEwen, R.K., Mayes, A., Hughes, D.C., Beggs, J.D., and Michell, R.H. (2002). Vac14p controls Ptdins(3,5)-bisphosphate synthesis and Fab1p-dependent protein trafficking to the multivesicular body. *Curr. Biol.* 12, 885–893.
- Dowler, S., Currie, R., Campbell, D., Deak, M., Kular, G., Downes, C., and Alessi, D. (2000). Identification of plekstrin homology domain-containing proteins with novel phosphoinositide-binding specificities. *Biochem. J.* 351, 19–31.
- Foti, M., Audhya, A., and Emr, S.D. (2001). Sac1p lipid phosphatase and Stt4 phosphatidylinositol 4-kinase regulate a pool of phosphatidylinositol 4-phosphate that functions in the control of the actin cytoskeleton and vacuole morphology. *Mol. Biol. Cell* 12, 2396–2411.
- Fruman, D., Meyers, R., and Cantley, L. (1998). Phosphoinositide kinases. *Annu. Rev. Biochem.* 67, 481–507.
- 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.
- Gaynor, E.C., te Heesen, S., Graham, T.R., Aebi, M., and Emr, S.D. (1994). Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* 127, 653–665.
- Gillooly, D.J., Morrow, I.C., Lindsay, M., Gould, R., Bryant, N.J., Gaullier, J.M., Parton, R.G., and Stenmark, H. (2000). Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* 19, 4577–4588.
- Guo, S., Stolz, L.E., Lemrow, S.M., and York, J.D. (1999). SAC1-like domains of yeast SAC1, INP52, and INP53 and of human synaptojanin encode polyphosphoinositide phosphatases. *J. Biol. Chem.* 274, 12990–12995.
- Ha, S.-A., Torabinejad, J., DeWald, D.B., Wenk, M.R., Lucast, L., DeCamilli, P., Newitt, R.A., Aebersold, R., and Nothwehr, S.F. (2003). The synaptojanin-like protein Inp53/Sjl3 functions with clathrin in a yeast TGN-to-endosome pathway distinct from the GGA protein-dependent pathway. *Mol. Biol. Cell* 14, 1319–1333.
- Heinisch, J., Lorberg, A., Schmitz, H.-P., and Jacoby, J. (1999). The PKC-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 32, 671–680.
- Horazdovsky, B.F., Seaman, M.N.J., McLaughlin, S.A., Yoon, S.-H., and Emr, S.D. (1997). Vps5p, a sorting nexin homologue, forms a complex with Vps17p and is required for vacuolar protein sorting. *Mol. Biol. Cell* 8, 1529–1541.
- Hughes, W.E., Cooke, F.T., and Parker, P.J. (2000). Sac phosphatase domain proteins. *Biochem. J.* 350, 337–352.
- Kametaka, S., Okano, T., Ohsumi, M., and Ohsumi, Y. (1998). Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 22284–22291.
- Katzmann, D.J., Odorizzi, G., and Emr, S.D. (2002). Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell. Biol.* 3, 893–905.
- Katzmann, D.J., Stefan, C.J., Babst, M., and Emr, S.D. (2003). Vps27 recruits ESCRT machinery to endosomes during MVB sorting. *J. Cell Biol.* 162, 413–423.
- Kavran, J., Klein, D., Lee, A., Falaska, M., Isakoff, S., Skolnik, E., and Lemmon, M. (1998). Specificity and promiscuity in phosphoinositide binding by plekstrin homology domains. *J. Biol. Chem.* 273, 30497–30508.
- Kim, J., Huang, W.-P., Stromhaug, P.E., and Klionsky, D.J. (2001). Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to de novo vesicle formation. *J. Biol. Chem.* 277, 763–773.
- Laporte, J., Bedez, F., Bolino, A., and Mandel, J. (2003). Myotubularins, a large disease-associated family of cooperating catalytically active and inactive phosphoinositides phosphatases. *Hum. Mol. Genet.* 12, 285R–292R.

- Laporte, J., Blondeau, F., Buj-Bello, A., Tentler, D., Kretz, C., Dahl, N., and Mandel, J. (1998). Characterization of the myotubularin dual specificity phosphatase gene family from yeast to human. *Hum. Mol. Genet.* 7, 1703–1712.
- Longtine, M., McKenzie, A., Demarini, D., Shah, N., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Majerus, P., Kisseleva, M., and Norris, F. (1999). The role of phosphatases in inositol signaling reactions. *J. Biol. Chem.* 274, 10669–10672.
- Martin, T. (2001). PI(4,5)P₂ regulation of surface membrane traffic. *Curr. Opin. Cell Biol.* 13, 493–499.
- Muhlrad, D., Hunter, R., and Parker, R. (1992). A rapid method for localized mutagenesis of yeast genes. *Yeast* 8, 79–82.
- Nandurkar, H., and Huysmans, R. (2002). The myotubularin family: novel phosphoinositide regulators. *IUBMB Life* 53, 37–43.
- Nandurkar, H.H., Layton, M., Laporte, J., Selan, C., Corcoran, L., Caldwell, K.K., Mochizuki, Y., Majerus, P.W., and Mitchell, C.A. (2003). Identification of myotubularin as the lipid phosphatase catalytic subunit associated with the 3-phosphatase adapter protein, 3-PAP. *Proc. Natl. Acad. Sci. USA* 100, 8660–8665.
- Nice, D.C., Sato, T.K., Stromhaug, P.E., Emr, S.D., and Klionsky, D.J. (2002). Cooperative binding of the cytoplasm to vacuole targeting pathway proteins, Cvt13 and Cvt20, to phosphatidylinositol 3-phosphate at the pre-autophagosomal structure is required for selective autophagy. *J. Biol. Chem.* 277, 30198–30207.
- Odorizzi, G., Babst, M., and Emr, S.D. (1998). Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. *Cell* 95, 847–858.
- Odorizzi, G., Babst, M., and Emr, S. (2000). Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *Trends Biochem. Sci.* 25, 229–235.
- Pelham, H. (2002). Insights from yeast endosomes. *Curr. Opin. Cell Biol.* 14, 454–462.
- Pfeffer, S.R. (2001). Membrane transport: retromer to the rescue. *Curr. Biol.* 11, R109–R111.
- Rameh, L., and Cantley, L. (1999). The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* 274, 8347–8350.
- Rieder, S.E., Banta, L.M., Köhrer, K., McCaffery, J.M., and Emr, S.D. (1996). Multilamellar endosome-like compartment accumulates in the yeast vps28 vacuolar protein sorting mutant. *Mol. Biol. Cell* 7, 985–999.
- Robinson, J.S., Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8, 4936–4948.
- 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.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Sato, T., Overduin, M., and Emr, S. (2001). Location, location, location: membrane targeting directed by PX domains. *Science* 294, 1881–1885.
- Schaletzky, J., Dove, S., Short, B., Lorenzo, O., Clague, M., and Barr, F. (2003). Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases. *Curr. Biol.* 13, 504–509.
- Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D., and Emr, S.D. (1993). Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science* 260, 88–91.
- Seaman, M.N., Marcusson, E.G., Cereghino, J.L., and Emr, S.D. (1997). Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the VPS29, VPS30, and VPS35 gene product. *J. Cell Biol.* 137, 79–92.
- Seaman, M.N.J., Michael McCaffery, J., and Emr, S.D. (1998). A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J. Cell Biol.* 142, 665–681.
- Sherman, F., Fink, G.R., and Lawrence, L.W. (1979). *Methods in Yeast Genetics: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Sikorski, R., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- Simonsen, A., Wurmser, A., Emr, S., and Stenmark, H. (2001). The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* 13, 485–492.
- Srinivasan, S., Seaman, M., Nemoto, Y., Daniell, L., Suchy, S., Emr, S., De Camilli, P., and Nussbaum, R. (1997). Disruption of three phosphatidylinositol-polyphosphate 5-phosphatase genes from *Saccharomyces cerevisiae* results in pleiotropic abnormalities of vacuole morphology, cell shape, and osmohomeostasis. *Eur. J. Cell Biol.* 74, 350–360.
- Stack, J.H., Herman, P.K., Schu, P.V., and Emr, S.D. (1993). A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. *EMBO J.* 12, 2195–2204.
- Stefan, C.J., Audhya, A., and Emr, S.D. (2002). The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-biphosphate. *Mol. Biol. Cell* 13, 542–557.
- Stolz, L.E., Huynh, C.V., Thorner, J., and York, J.D. (1998). Identification and characterization of an essential family of inositol polyphosphate 5-phosphatases (INP51, INP52 and INP53 gene products) in the yeast *Saccharomyces cerevisiae*. *Genetics* 148, 1715–1729.
- Taylor, G.S., Maehama, T., and Dixon, J.E. (2000). Inaugural article: myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. *Proc. Natl. Acad. Sci. USA* 97, 8910–8915.
- Tronchere, H., Laporte, J., Pendaries, C., Chaussade, C., Liaubet, L., Pirola, L., Mandel, J., and Payrastra, B. (2003). Production of PtdIns(5)P by the phosphoinositide 3-phosphatase myotubularin in mammalian cells. *J. Biol. Chem.* 279, 7304–7312.
- Vida, T.A., and Emr, S.D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* 128, 779–792.
- Walker, D., Urbe, S., Dove, S., Tenza, D., Raposo, G., and Clague, M. (2001). Characterization of MTMR3, an inositol lipid 3-phosphatase with novel substrate specificity. *Curr. Biol.* 11, 1600–1605.
- Yin, H.L., and Janmey, P.A. (2003). Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* 65, 761–789.