Synthetic Genetic Array Analysis of the PtdIns 4-kinase Pik1p Identifies Components in a Golgi-specific Ypt31/rab-GTPase Signaling Pathway[D]

Vicki A. Sciorra,* Anjon Audhya,* Ainslie B. Parsons,[†] Nava Segev,[‡] Charles Boone,[†] 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, CA 92093-0668; [†]Banting and Best Department of Medical Research and Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada M5G 1L6; and [‡]Department of Biological Sciences, Laboratory for Molecular Biology, University of Illinois at Chicago, Chicago, IL 60607

Phosphorylated derivatives of phosphatidylinositol are essential regulators of both endocytic and exocytic trafficking in eukaryotic cells. In *Saccharomyces cerevisiae*, the phosphatidylinositol 4-kinase, Pik1p generates a distinct pool of PtdIns(4)P that is required for normal Golgi structure and secretory function. Here, we utilize a synthetic genetic array analysis of a conditional *pik1* mutant to identify candidate components of the Pik1p/PtdIns(4)P signaling pathway at the Golgi. Our data suggest a mechanistic involvement for Pik1p with a specific subset of Golgi-associated proteins, including the Ypt31p rab-GTPase and the TRAPPII protein complex, to regulate protein trafficking through the secretory pathway. We further demonstrate that TRAPPII specifically functions in a Ypt31p-dependent pathway and identify Gyp2p as the first biologically relevant GTPase activating protein for Ypt31p. We propose that multiple stage-specific signals, which may include Pik1p/PtdIns(4)P, TRAPPII and Gyp2p, impinge upon Ypt31 signaling to regulate Golgi secretory function.

INTRODUCTION

The Golgi apparatus plays an essential role in the intracellular trafficking and sorting of proteins and lipids within the secretory pathway. Although the Golgi complex in mammalian and yeast cells are morphologically different, they are both organized into functionally distinct compartments: the *cis*-, medial- and *trans*-Golgi (Franzusoff and Schekman, 1989; Graham and Emr, 1991). Each Golgi compartment has a unique repertoire of resident proteins that are responsible for the sequential posttranslational modification and maturation of cargo proteins, which are then secreted out of the Golgi and transported to their cellular destinations (i.e., to the plasma membrane, endosome, or lysosome/vacuole). Transport to and from the Golgi requires the concerted actions of specific rab-GTPases, SNAREs, phosphoinositides, and hetero-oligomeric protein complexes (Segev, 2001; Zerial and McBride, 2001; Pfeffer, 2003). The coordinated action of these diverse transport components provides spec-

ificity in each trafficking step, although the mechanism by which these components cooperate in Golgi trafficking is only partially understood.

Phosphorylated derivatives of phosphatidylinositol are particularly versatile regulators of protein transport and carry out essential modulatory functions within the secretory pathway by interacting with distinct lipid-binding effector molecules (Simonsen *et al.,* 2001). For instance, phosphatidylinositol 3-phosphate (PtdIns(3)P) is required for the anterograde traffic of cargo proteins from the Golgi to the vacuole by interacting with specific proteins that contain either a FYVE domain or a Phox homology (PX) domain, two motifs which specifically bind PtdIns(3)P (Simonsen *et al.,* 2001). Conversely, phosphatidylinositol 4-phosphate (PtdIns(4)P), regulates the traffic of cargo proteins from the Golgi to the plasma membrane (Hama *et al.,* 1999; Walch-Solimena and Novick, 1999; Audhya *et al.,* 2000) by a yet undefined mechanism.

Saccharomyces cerevisiae contains two essential phosphatidylinositol 4-kinases, Stt4p and Pik1p. Functional studies have demonstrated that Pik1p and Stt4p catalyze the production of the majority of PtdIns(4)P in yeast (Audhya *et al.,* 2000) and suggest unique roles for the two pools of PtdIns(4)P synthesized by each PtdIns 4-kinase. In part this is due to the differential subcellular targeting of Stt4p and Pik1p. Stt4p localizes to the cell periphery (Audhya and Emr, 2002) and regulates cell wall integrity and vacuole morphology but has no detected role in secretion (Audhya *et al.,* 2000). In contrast, Pik1p localizes to both the Golgi (Walch-Solimena and Novick, 1999) and the nucleus (Garcia-Bustos *et al.,* 1994; Walch-Solimena and Novick, 1999). Inactivation of Pik1p decreases PtdIns(4)P levels, impairs

secretory transport from the Golgi, and disrupts the structural integrity of the Golgi and vacuole (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). In addition, defects in cytokinesis and actin cytoskeletal organization have been observed after loss of Pik1p function (Garcia-Bustos *et al.*, 1994; Walch-Solimena and Novick, 1999). Consequently, the pleiotropic effects caused by the inactivation of Pik1p have obscured the precise function of PtdIns(4)P at the Golgi.

Mammalian cells express at least three Golgi-localized PtdIns 4-kinases, PI4KIIα (Wang *et al.*, 2003), PI4KIIIα (Nakagawa *et al.*, 1996), and PI4KIIIβ (Wong *et al.*, 1997). Subsequent studies have found that PI4KIIIβ is recruited to the Golgi by active, GTP-bound Arf (Godi *et al.*, 1999). Interfering with PI4KIIIβ by the overexpression of a kinase dead, dominant-negative mutant, disrupted the structural integrity of the Golgi (Godi *et al.*, 1999), providing additional evidence for PtdIns(4)P in the regulation of Golgi function. Arf also recruited an unidentified PtdIns(4)P 5-kinase to the Golgi, resulting in the conversion of PtdIns(4)P to PtdIns(4,5)P_2, making the respective roles of PtdIns(4)P and PtdIns(4,5)P_2 at the Golgi less clear (Godi *et al.*, 1999). Inactivation of temperature-sensitive alleles of *MSS4*, the sole phosphoinositide kinase responsible for the synthesis of PtdIns(4,5)P_2 in yeast, does not affect the morphology or secretory function of the Golgi complex (Audhya and Emr, 2002; Stefan *et al.*, 2002). Collectively, these data suggest that PtdIns(4)P serves multiple functions, as a direct mediator in Golgi function, and as a precursor to PtdIns(4,5)P_2.

To gain a better understanding of the essential role of Pik1p in yeast Golgi function, we have utilized a synthetic genetic array (SGA) analysis to identify genes that may participate in a Pik1p-dependent pathway. We report the identification of *PIK*-specific genetic interactions with genes known to have distinct roles in membrane trafficking and Golgi function. We demonstrate that Pik1p functionally interacts with Ypt31p, a Golgi-localized rab-GTPase, to regulate the trafficking of cargoes that require recycling through the early endosome, including the yeast SNARE, Snc1p, and chitin synthase, Chs3p. Using information obtained from the screen, we present evidence that transport protein particle (TRAPP) complex II, a large protein complex localized to the Golgi (Sacher *et al.*, 2001), interacts genetically with *PIK1* and functionally with Ypt31p. Further analysis of the interactions between TRAPPII and Ypt31p led to the identification of Gyp2p as a biologically relevant GTPase activating protein (GAP) for Ypt31p in vivo. Our comprehensive genetic analysis of the *pik1* mutation led to the dissection of relevant PtdIns(4)P-dependent pathways and to defining regulatory components that function in Ypt31p rab-GTPase signaling at the Golgi.

MATERIALS AND METHODS

Strains and Media

The genotypes of *S. cerevisiae* strains used in this study are listed in Table 1. All yeast strains were grown in YPD or SD minimal media containing the necessary amino acid supplements. Transformations into yeast were performed by the lithium acetate method (Ito *et al.*, 1983). All strains were constructed either by tetrad dissection of sporulated diploid strains or by integration of indicated disruption cassettes as previously described (Longtine *et al.*, 1998). Disruptions were confirmed by PCR. *pik1-139ts ypt31Δ*, *pik1-139ts kre11Δ*, and *pik1-139ts trs33Δ*, and *pik1–83ts ypt31Δ* were isolated by tetrad dissection and maintained on YPD containing 1 M sorbitol at 26°C. SEY6210 expressing *TAT2-GFP* or *CHS3-GFP* were engineered by transformation of PCR-amplified genomic integration construct using GFP-His3MX6 plasmid as template (Longtine *et al.*, 1998) and verified by PCR analysis generating CJSY298 and VSY184. SEY6210 expressing *GFP-SNC1* (VSY167) was engineered by integrating an expression plasmid linearized with *Stu*I at

URA3 (Lewis *et al.*, 2000) generating VSY167. *pik1-139ts*, *ypt31Δ* and *pik1-139ts ypt31Δ* double mutant cells expressing *TAT2-GFP*, *CHS3-GFP*, and *GFP-SNC1* were isolated by tetrad dissection. Expression of TAT2-GFP, CHS3-GFP, and GFP-SNC1 were screened by fluorescence microscopy and Western blotting.

Genetic and DNA Manipulations

SGA analysis was performed as described previously (Tong *et al.*, 2001). Standard molecular biology techniques were used for DNA manipulations (Maniatis *et al.*, 1992). Enzymes used for recombinant DNA techniques were purchased from Roche (Nutley, NJ) and New England Biolabs (Beverly, MA). PCR reactions were conducted as directed by manufacturer's instructions using PfuTurbo (Stratagene, La Jolla, CA) or AmpliTaq (Applied Biosystems, Foster City, CA) DNA polymerases for cloning and diagnostic reactions, respectively. Yeast genomic DNA was isolated by the method of Hoffman and Winston (1987). A 1.8-kb fragment containing the open reading frame (ORF) of *YPT31* was amplified from genomic DNA prepared from SEY6210 using oligonucleotide primers that incorporated unique *Bam*HI and *Sma*I restriction enzyme sites at the 5′ and 3′ ends, respectively. The PCR products were digested with *Bam*HI and *Sma*I and subcloned into *Bam*HI-*Sma*I–digested pRS416 (Sikorski and Hieter, 1989) creating VSB283. Haploid *ypt31Δ* and *ypt32Δ* single deletion mutants of opposite mating types, each expressing low-copy *YPT31* were mated and sporulated and tetrads were isolated. *ypt31Δ ypt32Δ* double mutants carrying *YPT31* were confirmed by PCR and demonstrated functionality of the *YPT31* clone. *ypt31 Q72L* (VSB333) and *N126I* (VSB334) were generated by site-directed mutagenesis of the wild-type plasmid (VSB327) using the Quik-change kit (Stratagene) and confirmed by DNA sequencing. To generate *GFP-YPT31*, the *YPT31* ORF (672 bp) was amplified from pRS416-*YPT31* engineered with *Bgl*II (5′) and *Pst*I (3′) and subcloned into pGO37 (Burd and Emr, 1998) creating VSB303. Expression of GFP-YPT31p was confirmed by Western blot analysis. *GFP-YPT31* was subsequently subcloned into *Not*I and *Sal*I sites of pRS415 (Sikorski and Hieter, 1989) creating VSB311. *ypt31Δ ypt32Δ* double mutants carrying low copies of *GFP-YPT31* were isolated and confirmed as described above.

To generate a construct for expression of *GFP-PIK1*, a *Xho*I-*Kpn*I fragment from pYcp50-*PIK1*, corresponding to the C-terminus of *PIK1*, was first ligated into *Xho*I-*Kpn*I–digested pGO36 (Burd and Emr, 1998), creating pJA519. The N-terminus of *PIK1* with an 8-alanine linker and *Xho*I sites was amplified by PCR and cloned into *Xho*I-digested pJA519, creating pJA520 (*GFP-PIK1*). Expression of *GFP-PIK1* in either *pik1Δ* or *pik1ts* mutant cells rescued lethality. To generate *GFP-GYP2*, the *GYP2* ORF was amplified from genomic DNA prepared from SEY6210 using oligonucleotide primers that incorporated unique *Eco*RI and *Cla*I restriction enzyme sites at the 5′ and 3′, respectively. The PCR product was digested with *Eco*RI and *Cla*I and ligated into *Eco*RI-*Cla*I–digested pGO35 (Burd and Emr, 1998) creating VSB363. In frame GFP fusions were confirmed by Western blot analysis. An *Not*I-*Cla*I fragment containing *GFP-GYP2* was subcloned into *Not*I-*Sma*I sites of pRS415 (Sikorski and Hieter, 1989) creating VSB367. *GFP-gyp2 G80E* (VSB368) and *GFP-gyp2 R295K* (VSB369) were generated by site-directed mutagenesis, as described above.

A strain expressing the *pik1-139ts* mutant plasmid was generated in several steps. First, pRS415-*PIK1* was linearized with *Nhe*I-*Nru*I. Together with a randomly mutagenized PCR product corresponding to the C-terminal kinase domain of PIK1, these DNA fragments were transformed into *pik1Δ::HIS3* cells carrying pRS416-*PIK1*. Cells were incubated at 26°C and then transferred to plates containing 5-FOA (to eliminate pRS416-*PIK1*) at both 26 and 37°C. Temperature-sensitive colonies were isolated, and individual plasmids were recovered. Temperature-sensitive alleles were retested, and *pik1-139* was chosen based on its ability to support robust growth of *pik1Δ::HIS3* cells at 26°C but not at temperatures above 35°C.

The *ypt32Δ ypt31-101ts* temperature-sensitive mutant was generated by a multistep process in which *YPT31* (i.e., the entire *YPT31* ORF including a 200-base pair fragment downstream of the *YPT31* stop codon) was randomly mutagenized using AmpliTaq DNA polymerase under error-prone PCR conditions. *URA3* was independently amplified under nonerror prone PCR conditions. The mutagenized *ypt31* and *URA3* PCR fragments were cotransformed, fused to each other, and integrated into the *YPT31* locus of *ypt32Δ* cells by homologous recombination. Ura+ prototrophic transformants were selected and screened for temperature-sensitive growth on YPD at 26 and 38°C. A *ypt32Δ* strain harboring *ypt31-101* (VSY468) displayed strong temperature-sensitive phenotypes was selected for further characterization.

pRC2240 *SEC7-DsRED* (Calero *et al.*, 2003) was a gift from R. Collins (Cornell University). pHXT1-GFP (Malinska *et al.*, 2003) was obtained from W. Tanner (Universitat Regensberg). pRS315-*YPT6* (Bensen *et al.*, 2001) was obtained from G. Payne (UCLA) and the 1.45-kb *Sac*I-*Bam*HI fragment from pRS315-*YPT6* was subsequently subcloned into pRS425 (Sikorski and Hieter, 1989) creating VSB372. Plasmids for the expression of *SEC4* and *sec4 Q72L* were gifts from P. Brenwald (UNC, Chapel Hill, NC). *GFP-SNC1* integration plasmid was from H. Pelham (MRC, United Kingdom).

Fluorescence and Electron Microscopy

Cells expressing *GFP-PIK1* or *GFP-YPT31* (or coexpressing *SEC7-DsRED*) were grown to early log phase at 26°C. After shifting to 38°C for the indicated

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

Strains	Genotype	Reference or source
SEY6210	*MAT a leu2-3112 ura3-52 his3-Δ200 trp1-Δ901 lys2–801 suc2-Δ9*	Robinson *et al.* (1988)
SEY6210.1	*MAT a leu2-3112 ura3-52 his3-Δ200 trp1-Δ901 lys2–801 suc2-Δ9*	Robinson *et al.* (1988)
SEY6210α/a	*MAT α/MAT a* homozygous diploid strain from SEY6210/6210.1 cross	Robinson *et al.* (1988)
AAY1139	SEY6210; *pik1Δ::HIS3* carrying pRS415 *pik1-139 (LEU2 CEN6 pik1-139)*	This study
AAY102	SEY6210; *stt4Δ::HIS3* carrying pRS415 *stt4-4 (LEU2 CEN6 stt4-4)*	Audhya *et al.* (2000)
AAY104	SEY6210; *pik1Δ::HIS3* carrying pRS314 *pik1-83 (TRP1 CEN6 pik1-83)*	Audhya *et al.* (2000)
AAY202	SEY6210; *mss4Δ::HIS3MX6* carrying pYCplac111 *mss4-102 (LEU2 CEN6 mss4-102)*	Stefan *et al.* (2002)
AAY1167	SEY6210; *drs2Δ::HIS3*	This study
AAY1169	SEY6210.1; *kre11Δ::HIS3*	This study
VSY378	SEY6210; *trs33Δ::HIS3*	This study
AAY1171	SEY6210.1; *trs33Δ::HIS3*	This study
VSY232	SEY6210.1; *ypt31Δ::TRP1*	This study
VSY322	SEY6210; *ypt31Δ::HIS3*	This study
AAY1182	AAY1139; *kre11Δ::HIS3*	This study
AAY1184	AAY1139; *trs33Δ::HIS3*	This study
VSY254	AAY1139; *ypt31Δ::TRP1*	This study
AAY1191	AAY102; *drs2Δ::HIS3*	This study
VSY475	AAY102; *kre11Δ::HIS3*	This study
VSY500	AAY102; *trs33Δ::HIS3*	This study
VSY312	AAY102; *ypt31Δ::TRP1*	This study
VSY479	AAY104; *ypt31Δ::TRP1*	This study
VSY342	AAY202; *drs2Δ::HIS3*	This study
VSY388	AAY202; *ypt31Δ::TRP1*	This study
VSY328	SEY6210; *drs2Δ::HIS3 ypt31Δ::TRP1*	This study
VSY409	SEY6210; *drs2Δ::HIS3 kre11Δ::HIS3*	This study
VSY167	SEY6120; *GFP-SNC1:URA3*	This study
VSY307	VSY232; *GFP-SNC1:URA3*	This study
VSY309	AAY1139; *GFP-SNC1:URA3*	This study
VSY314	VSY254: *GFP-SNC1:URA3*	This study
VSY184	SEY6210; *CHS3-GFP: HIS3MX6*	This study
VSY497	VSY184; *ypt31Δ::TRP1*	This study
VSY498	AAY1139; *CHS3-GFP:HIS3MX6*	This study
VSY499	VSY254; *CHS3-GFP: HIS3MX6*	This study
CJSY298	SEY6210; *TAT2-GFP:HIS3MX6*	This study
VSY492	CJS298; *ypt31D::TRP1*	This study
VSY491	AAY1139; *TAT2-GFP:HIS3MX6*	This study
VSY493	VSY254; *TAT2-GFP:HIS3MX6*	This study
VSY197	SEY6210.1; *ypt32D::TRP1*	This study
VSY468	SEY6210; *ypt32D::TRP1 ypt31-101^{ts}:URA3*	This study
VSY484	VSY468; *GFP-SNC1:URA3*	This study
AAY1151	SEY6210; *pik1D::HIS3* carrying pRS414 *GFP-PIK1*	This study
VSY531	VSY468; *pik1D::HIS3* carrying pRS414 *GFP-PIK1*	This study
VSY407	SEY6210; *ypt31D::HIS3 ypt32 D::TRP1* carrying pRS415 *GFP-YPT31*	This study
VSY380	SEY6210α/a; *trs130D::HIS3/TRS130*	This study
VSY379	SEY6210α/a; *trs120D::HIS3/TRS120*	This study
VSY446	SEY6210; *trs130-(33aa truncation)-HA^{ts} :HIS3MX6*	This study
VSY459	SEY6210; *TRS130-HA:HIS3MX6*	This study
VSY470	SEY6210.1; *gyp1D::HIS3*	This study
VSY471	SEY6210.1; *gyp2D::HIS3*	This study
VSY495	VSY470; *trs130-(33aa truncation)-HA^{ts} :HIS3MX6*	This study
VSY486	VSY446; *gyp2D::HIS3*	This study
VSY488	VSY468; *gyp1D::HIS3*	This study
VSY483	VSY468; *gyp2D::HIS3*	This study
VSY433	SEY6210; *trs33D::HIS3 kre11D::HIS3* carrying pRS425 *ypt31Q72L*	This study
VSY419	SEY6210; *trs130D::HIS3* carrying pRS425-*YPT31*	This study
VSY421	SEY6210; *trs130D::HIS3* carrying pRS425-*ypt31Q72L*	This study
VSY428	SEY6210; *trs120D::HIS3* carrying pRS425-*YPT31*	This study
VSY423	SEY6210; *trs120D::HIS3* carrying pRS425-*ypt31Q72L*	This study

duration, cells were concentrated and visualized by fluorescence microscopy. Cells expressing *GFP-SNC1*, *CHS3-GFP*, *TAT2-GFP*, or *HXT1-GFP* were grown to early log phase and visualized at 26°C. Fluorescent images were visualized using a Zeiss Axiovert S1002TV inverted fluorescent microscope (Thornwood, NY), acquired, and processed using a Delta Vision deconvolution system (Applied Precision Instruments, Issaquah, WA) and Adobe Photoshop 6.0 (San Jose, CA). Observations were based on examination of at least 200 cells. Vacuole morphology was examined by visualization of vacuole membranes by labeling with the fluorescent dye, FM4–64 (Molecular Probes, Eugene, OR) as previously described by Vida and Emr (1995). For ultrastructural analysis, 50 OD$_{600}$ of early log-phase cells incubated at the appropriate

temperature were harvested from YPD and fixed in 3% glutaraldehyde, 0.1 M Na cacodylate (pH 7.4), 5 mM CaCl$_2$, 5 mM MgCl$_2$, and 2.5% sucrose for 1 h. Cells were further processed for electron microscopy as described previously (Audhya *et al.*, 2000).

In Vivo Analysis of Phosphoinositides

Analysis of phosphoinositides levels was carried out as previously described (Audhya *et al.*, 2000; Rudge *et al.*, 2004). Briefly, cells were grown in synthetic medium with the appropriate amino acids. Cells from an early log phase culture were harvested, washed, and resuspended in inositol-free synthetic

medium (IFM). Cells were shifted to the appropriate temperature (26 or 38°C) for 10 min and labeled with 60 μCi of *myo*-[2-^{3}H]inositol (Amersham Biosciences, Piscataway, NJ) in IFM for an additional 45 min. Phosphoinositides were extracted by ice-cold precipitation with a final concentration of 4.5% perchloric acid and deacylated by treatment with methylamine. Extracts of deacylated lipids were further processed as described in Rudge *et al.* (2004). [^{3}H]glycerol-phosphoinositides were separated using an anion-exchange Partisphere SAX column (Whatman, Clifton, NJ) coupled to a GOLD HPLC system (Beckman Coulter, Fullerton, CA) and quantitated by liquid scintillation counting using an on-line radiomatic detector (Packard Instrument Co., Meriden, CT) utilizing Ultima Flo scintillation fluid (Packard).

Metabolic Labeling and Immunoprecipitation

Cell labeling and immunoprecipitations were performed as previously described (Audhya *et al.*, 2000). Log-phase cultures were concentrated to 5 OD600/ml and labeled with 2 μl/OD600 Trans-^{35}S label mix (DuPont New England Nuclear, Boston, MA) for 10 min in YNB, supplemented with 2% glucose, appropriate amino acids, and 100 μg/ml bovine serum albumin. Cells were chased with an excess of unlabeled methionine/cysteine for the indicated times, and proteins were precipitated with 9% trichloroacetic acid (TCA) on ice. For temperature shift experiments, cells were shifted to the appropriate temperature for 10–30 min before labeling. For analysis of media-secreted proteins, cells were separated from media by gentle centrifugation before precipitation. Extracts were immunoprecipitated with antisera against CPY (Cowles *et al.*, 1997) or Gas1p (R. Schekman, UC Berkeley). Immunoprecipitated proteins were resuspended in sample buffer, resolved by SDS-PAGE, and subjected to autofluorography.

Miscellaneous

Western blot analysis of SDS-PAGE–resolved proteins were performed as previously described (Rudge *et al.*, 2004). GFP and HA fusion proteins were detected using mouse monoclonal antibodies against GFP (Santa Cruz Biotechnology, Santa Cruz, CA) and HA (12CA5; Roche). Antibody against G6PDH was purchased from Sigma (St. Louis, MO). Immunoreactive proteins were detected using horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit (Zymed Laboratories, South San Francisco, CA) and SuperSignal chemiluminescent substrate (Pierce Chemical, Rockford, IL).

RESULTS

SGA Analysis Reveals a Network of PIK1 Genetic Interactions

To identify components of the Pik1p-dependent secretory apparatus required during transport out of the Golgi, we conducted a synthetic genetic interaction screen (Tong *et al.*, 2001) using a yeast strain expressing a functionally impaired temperature-sensitive allele of *pik1*. From such a sensitized genetic screen, we anticipated that we would identify gene products essential for Pik1p-dependent pathways. We used error-prone PCR mutagenesis targeted to the catalytic kinase domain of Pik1p, to engineer the *pik1-139^{ts}* mutant strain that could grow robustly up to temperatures of 35°C. Under permissive conditions (26°C), *pik1-139^{ts}* cells have a modest 20% reduction in PtdIns(4)P levels (SEY6210; 0.88 ± 0.06% compared with *pik1-139^{ts}*; 0.70 ± 0.06% of PtdIns labeled). Accordingly, at 26°C, *pik1-139^{ts}* cells lack any detectable growth defects associated with the loss of PtdIns(4)P signaling. Previously, it was shown that Pik1p and Stt4p each contribute ~50% of the total cellular PtdIns(4)P (Audhya *et al.*, 2000). As such, at the nonpermissive temperature (38°C, see Audhya *et al.*, 2000), *pik1-139^{ts}* cells have 43% reduced levels of PtdIns(4)P (SEY6210; 0.99 ± 0.08% compared with *pik1-139^{ts}*; 0.56 ± 0.08% of PtdIns labeled) and consequentially accumulate exaggerated Golgi as well as other membranous structures (unpublished data). Most of the remaining PtdIns(4)P observed in *pik1-139^{ts}* cells grown under nonpermissive conditions is due to the unaltered PtdIns 4-kinase activity of Stt4p (Audhya *et al.*, 2000 and unpublished data). These results are consistent with the proposed role for Pik1p-generated PtdIns(4)P in Golgi structure/function (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000).

The *pik1-139^{ts}* strain was then individually crossed with ~4700 single deletion strains that represent the complete set of viable haploid deletion mutants in *S. cerevisiae*. Double mutants exhibiting reduced growth at 26°C relative to the parental strains were scored three independent times. The results of the screen are listed in Supplementary Table 1. More than 30 genes, when deleted in the *pik1-139^{ts}* background, displayed a synthetic growth defect. The *pik1* interactions were highly enriched for genes with roles in membrane trafficking (Supplementary Table 1 and Figure 1A), indicative of an essential function for Pik1p in secretion. Consistent with previous studies suggesting roles for Pik1p/PtdIns(4)P in cytokinesis (Garcia-Bustos *et al.*, 1994), actin rearrangement (Walch-Solimena and Novick, 1999) and vacuole function (Audhya *et al.*, 2000), several genes involved in these processes were isolated. Surprisingly, gene products with known PtdIns(4)P-binding modules, such as a pleckstrin homology (PH) domain (Yu *et al.*, 2004), were not isolated. Further analysis of results obtained from this study and published studies revealed multiple interactions within subsets of genes involved in anterograde and retrograde Golgi traffic (Figure 1A).
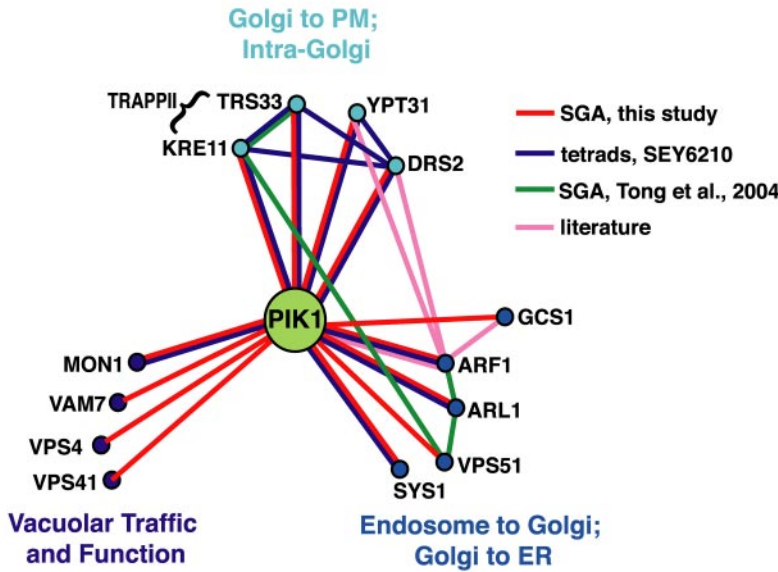
Specific Interactions with the Pik1p PtdIns 4-kinase

SGA analysis identified four genes: *DRS2*, *TRS33*, *KRE11*, and *YPT31*, with previous ascribed roles in Golgi anterograde trafficking to the plasma membrane. Drs2p, a Golgi-associated, integral membrane aminophospholipid translocase, is required for generating clathrin-coated exocytic vesicles (Chen *et al.*, 1999; Gall *et al.*, 2002). *TRS33* and *KRE11* encode nonessential subunits of the TRAPPII, a large Golgi-associated complex consisting of 10 proteins (Bet3p, Bet5p, Trs20p, Trs23p, Trs31p, Trs33p, Trs85p, Kre11p, Trs120p, and Trs130p; Sacher *et al.*, 2001). Temperature-sensitive mutants of the essential subunits display severe secretion defects, as well as an accumulation of aberrant Golgi structures (Sacher *et al.*, 2001). *YPT31* encodes a member of the Ypt/rab family of GTPases that is required for secretory transport, possibly by regulating vesicle formation at the *trans*-Golgi (Benli *et al.*, 1996; Jedd *et al.*, 1997).

Deletion of *DRS2*, *TRS33*, *KRE11*, or *YPT31* in either the *pik1-139^{ts}* or another temperature-sensitive allele of *PIK1*, *pik1–83^{ts}* strain (Hama *et al.*, 1999; Audhya *et al.*, 2000) resulted in either lethality or a severe growth defect (Figure 1B). Although Ypt31p shares 81% sequence identity with Ypt32p, only *YPT31* was uncovered in our SGA analysis using the *pik1^{ts}* mutation. Consistent with this observation, *pik1-139^{ts} ypt32Δ* double mutant cells did not exhibit a significant synthetic growth defect (lethality at 34°C) in contrast to *pik1-139^{ts} ypt31Δ* double mutant cells (Figure 1B). Because Ypt31p is more abundant than Ypt32p in the cell (Jedd *et al.*, 1997), these data suggest that Ypt31p may play a more dominant role in cells compared with Ypt32p.

We next compared the specificity of the genetic interactions obtained by SGA analysis by examining the effects of deleting these genes in strains harboring temperature-sensitive Stt4p, the second essential PtdIns 4-kinase in yeast, and Mss4p, the essential yeast PtdIns(4)P 5-kinase. As shown in Figure 1B, no significant genetic interactions with these PtdIns-kinases were identified. The PtdIns-kinase specificity of these genetic interactions suggests roles for Drs2p, Trs33p, Kre11p, and Ypt31p in a common or parallel pathway to Pik1p in Golgi function.

A

B

Figure 1. Phosphoinositide kinase specific genetic interactions. (A) Schematic of genes identified by SGA involved in membrane trafficking with known genetic interactions (synthetic sick or lethal). (B) Genetic interactions of four trafficking genes obtained from SGA (*drs2Δ*, *ypt31Δ*, *kre11Δ*, and *trs33Δ*) were analyzed in *pik1-139ts*, *pik1−83ts*, *stt4ts*, and *mss4ts*. Tetrads were isolated at 26°C and the growth of the double mutants was assessed at 26°C. +/- ; reduced growth, ++; similar to growth of single mutants. Temperature at which lethality is observed is given in parentheses.

Coordinated Regulation of the Secretory Pathway by Pik1p and Ypt31p

It has become increasingly apparent that cooperative protein-phosphoinositide and protein-protein interactions are required for the localization of specific signaling molecules to their site of action (Levine and Munro, 2002). For instance, localization of early endosome antigen 1 to the endosome requires its interaction with both active, GTP-bound Rab5 (Simonsen *et al.*, 1998), and PtdIns(3)P (Burd and Emr, 1998). In accordance with this model, we hypothesized that active, GTP-bound Ypt31p, and Pik1p-generated PtdIns(4)P may recruit effector molecules to function at the Golgi. We first investigated the phenotypes of *pik1-139ts ypt31Δ* double mutant cells to determine whether specific membrane trafficking pathways were attenuated in these cells. Growth and organelle morphology of *pik1-139ts* cells (grown at the permissive temperature) or *ypt31Δ* cells (Benli *et al.*, 1996; Jedd *et al.*, 1997) were similar to wild-type cells. However, electron microscopy of *pik1-139tsypt31Δ* double mutant cells grown under the same conditions (26°C) revealed an accumulation of abnormal Golgi structures, including Berkeley bodies, and fragmented vacuoles (unpublished data), indicating a synthetic defect in Golgi function.

We next investigated the localization of proteins that traffic from the Golgi to the plasma membrane. Snc1p is a yeast SNARE that mediates the fusion of exocytic vesicles with the plasma membrane (Protopopov *et al.*, 1993). Snc1p, which localizes to the plasma membrane at sites of bud formation, undergoes rapid endocytosis into early endocytic structures and is recycled to the Golgi where it is incorporated into another round of secretory vesicles (Lewis *et al.*, 2000). Consistent with previous studies (Lewis *et al.*, 2000), wild-type cells expressed GFP-Snc1p at the plasma membrane of newly formed daughter cells, and at the mother-daughter neck (Figure 2A). A few punctate structures were also visible, consistent with the dynamic recycling of this SNARE to and from the Golgi complex. Similar localization patterns of GFP-Snc1p fluorescence were also seen in *ypt31Δ* and *pik1-139ts* cells at the permissive temperature (Figure 2A). In *pik1-139tsypt31Δ* double mutant cells grown at the permissive temperature (26°C), the polarized plasma membrane localization of GFP-Snc1p was no longer observed, and instead GFP-Snc1p resided in punctate structures, some of which resembled "swollen Golgi," whereas others resembled tubular rings (Figure 2A). These data suggest that Pik1p and Ypt31p are required for GFP-Snc1p recycling to and/or secretion from the Golgi.

Chitin synthase III (Chs3p), a cell surface enzyme necessary for cell wall assembly, follows a recycling pattern similar to that of Snc1p. Chs3p localizes to the cell surface, to the mother-bud junction, and to intracellular punctate structures, referred to as chitosomes (Valdivia *et al.*, 2002).
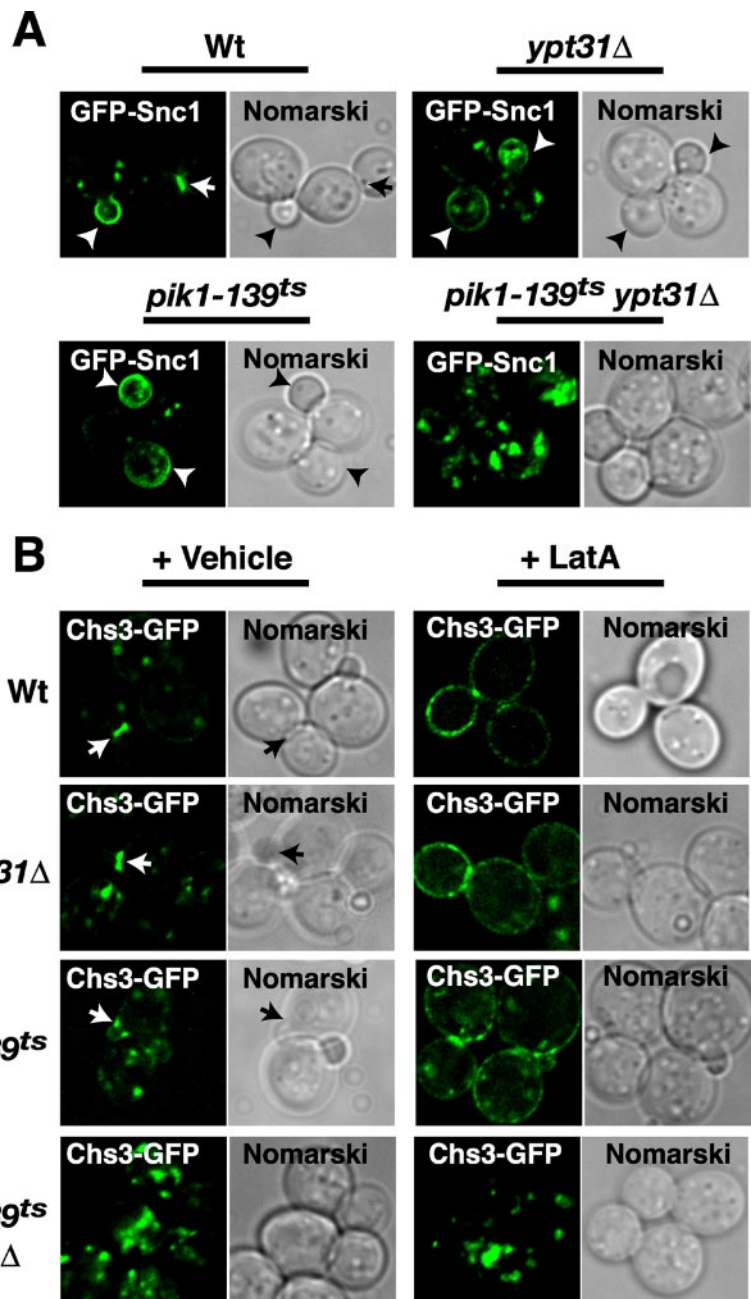
Figure 2. Trafficking of Snc1p and Chs3p requires Pik1p and Ypt31p. (A) GFP-Snc1p or (B) Chs3p-GFP distribution at 26°C in live cells, either wild-type (Wt) or carrying the indicated mutation, *ypt31Δ*, *pik1-139ts*, and *pik1-139ts ypt31Δ*. Arrows indicate bud necks and arrowheads indicate bud tips. (B) Chs3p-GFP transport to the cell surface was monitored by blocking endocytosis with LatA (200 μM, Molecular Probes) or treated with vehicle (dimethyl sulfoxide) as described in Valdivia *et al.* (2002).

Because chitosomes resemble early endosomes and Chs3p follows a trafficking pathway similar to Snc1p (Lewis *et al.*, 2000; Valdivia *et al.*, 2002), we next determined whether the transport of Chs3p to the bud neck required Pik1p and/or Ypt31p. Chs3p tagged with GFP at its C-terminus was previously found to be fully functional (Schorr *et al.*, 2001; Valdivia *et al.*, 2002). We examined the localization of Chs3p, chromosomally tagged with GFP (Figure 2B). In 48% of budded wild-type cells, Chs3p-GFP localized to the mother-bud neck. A modest decrease in the targeting of Chs3p-GFP to the bud neck was observed in *ypt31Δ* cells (39% of budded cells). Chs3p-GFP was localized to the bud neck in only 22% of budded *pik1-139ts* cells at the permissive temperature (26°C), suggesting that Pik1p/PtdIns(4)P is required for proper Chs3p targeting. Consistent with the defect in GFP-Snc1p localization (Fig-

ure 2A), <1% of budded *pik1-139ts ypt31Δ* double mutant cells (26°C) expressed Chs3p-GFP at the bud neck; Chs3p mostly accumulated intracellularly (Figure 2B). We also monitored the transport of Chs3p-GFP to the plasma membrane by blocking endocytosis with LatA (Figure 2B). Consistent with a defect in Golgi to plasma membrane traffic, Chs3p-GFP remained predominately in intracellular structures in *pik1-139ts ypt31Δ* double mutant cells treated with LatA (Figure 2B). These results provide evidence for a functional interaction between Pik1p and Ypt31p in Golgi to plasma membrane traffic.

We next examined the localization of other plasma membrane proteins that unlike Snc1p and Chs3p are trafficked to and degraded in the vacuole in response to environmental conditions. Consistent with previous reports (Malinska *et al.*, 2003), the plasma membrane hexose transporter, Hxt1p, lo-
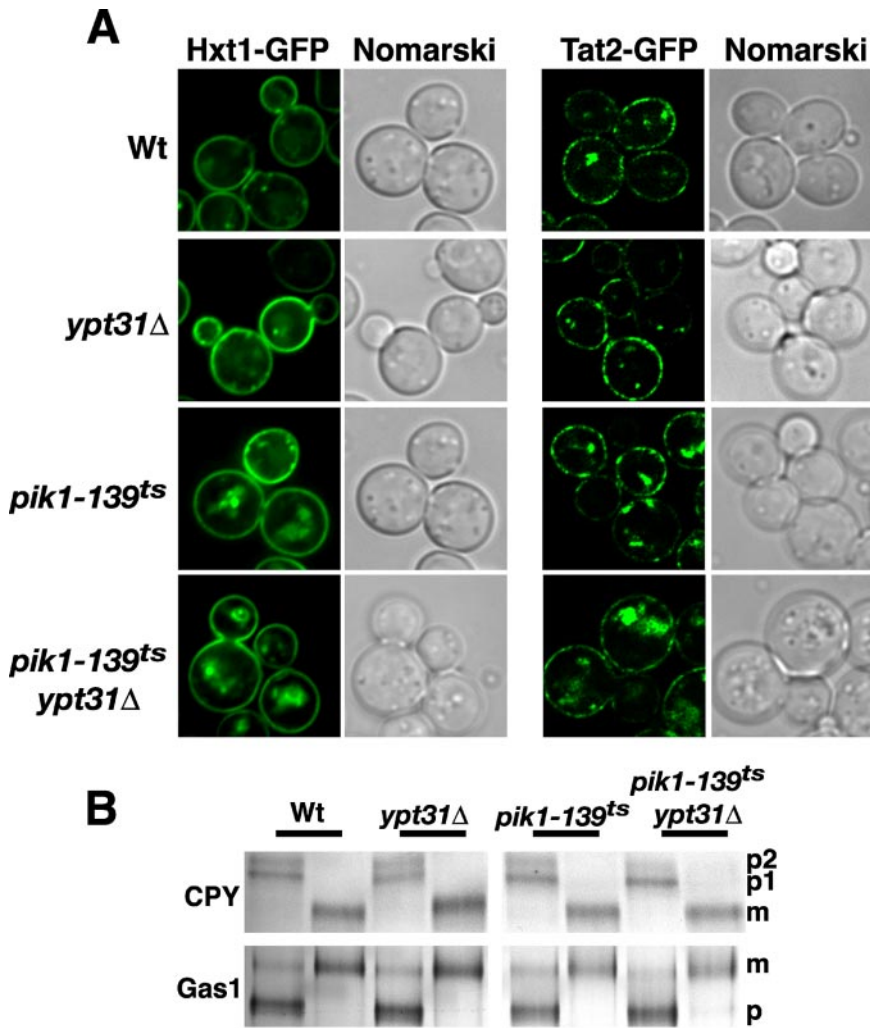
Figure 3. Trafficking competence of *pik1-139ts ypt31Δ* double mutant cells at permissive temperature. (A) Hxt1p-GFP and Tat2p-GFP distribution at 26°C in Wt, *ypt31Δ*, *pik1-139ts*, and *pik1-139ts ypt31Δ*. (B) Indicated yeast strains (Wt, *ypt31Δ*, *pik1-139ts*, and *pik1-139ts ypt31Δ*) were metabolically labeled with *Tran*-^{35}S for 10 min, and chased in the presence of excess methionine/cysteine for 30 min at 26°C. CPY and Gas1p were immunoprecipitated from cell lysates, resolved by SDS-PAGE, and visualized by fluorography. p, precursor form; m, mature form.

calized uniformly at the plasma membrane, with some fluorescence localized to punctate structures and/or the vacuole in wild-type cells (Figure 3A). This pattern of Hxt1p-GFP localization was also observed in *ypt31Δ* cells, *pik1-139ts* cells, and *pik1-139ts ypt31Δ* double mutant cells, demonstrating that transport of Hxt1p from the Golgi to the plasma membrane was unaffected (Figure 3A). We also examined the localization of another plasma membrane transporter, the tryptophan permease, Tat2p, chromosomally tagged with GFP to circumvent potential trafficking artifacts due to overexpression. Tat2p-GFP, like Hxt1p-GFP, is expressed uniformly at the plasma membrane with punctate and/or vacuole fluorescence observed in some cells (Umebayashi and Nakano, 2003 and Figure 3A). Under permissive growth conditions, the localization of Tat2p-GFP in *ypt31Δ* cells, *pik1-139ts* cells, and *pik1-139ts ypt31Δ* double mutant cells did not change relative to wild-type cells (Figure 3A). Additionally, the processing of several cargo proteins, the soluble vacuolar carboxypeptidase Y (CPY), and a glycophospholipid-anchored surface protein (Gas1p) were unaffected in *ypt31Δ* cells, *pik1-139ts* cells, and *pik1-139ts ypt31Δ* double mutant cells relative to wild-type cells (Figure 3B). Collectively, these results provide evidence for a functional interaction between Pik1p and Ypt31p in the specific targeting of proteins that continuously recycle during polarized growth.

Pik1p and Ypt31p Function in a Common Set of Trafficking Pathways

If Pik1p and Ypt31p function in similar trafficking pathways, then inactivation of Ypt31p should display similar defects in membrane traffic as the inactivation of Pik1p. To test our hypothesis, we generated a temperature-sensitive mutant of Ypt31p and then compared the phenotypes with those seen during the inactivation of Pik1p, in the same strain background, SEY6210. As previously mentioned, Ypt31p shares a high degree of sequence similarity with the Ypt32p GTPase, and deletion of both genes is lethal, indicating functional redundancy (Benli *et al.*, 1996; Jedd *et al.*, 1997). We therefore generated new temperature-sensitive alleles of *YPT31* by first mutating the ORF by random PCR mutagenesis and then integrating, via homologous recombination, the mutated PCR products into the genome of *ypt32Δ* mutant cells. We isolated two strains that exhibited temperature-sensitive growth. The strain, *ypt32Δypt31-101ts* that grew most robustly at 26°C was characterized further. *ypt32Δypt31-101ts* double mutant cells are temperature-sensitive for growth at elevated temperatures (>37°C), which can be fully rescued by the introduction of wild-type *YPT31* expressed from a low-copy plasmid (Figure 4A).

To further characterize *ypt32Δypt31-101ts* double mutant cells, we assessed the anterograde secretory transport of
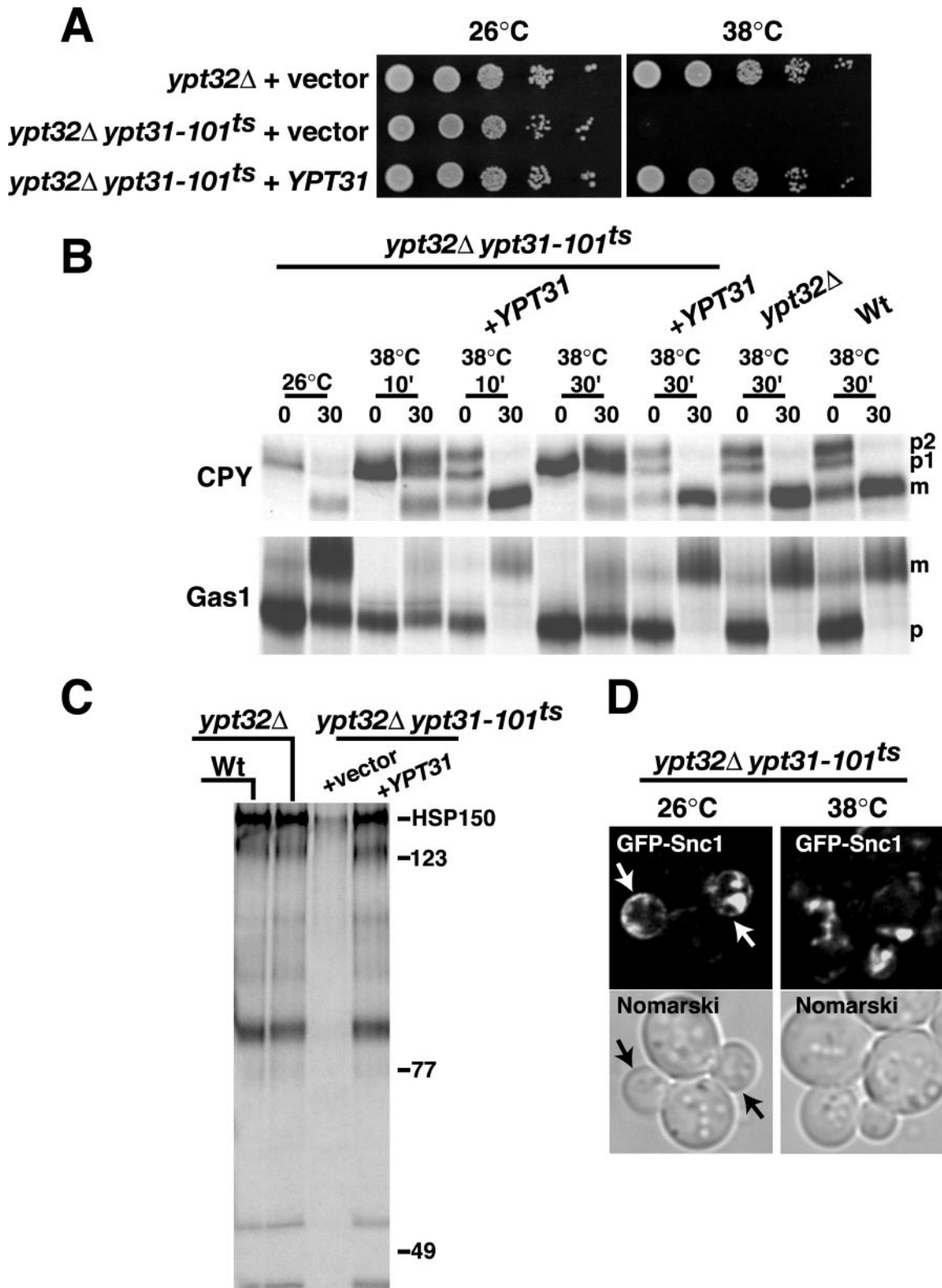
Figure 4. Pik1p and Ypt31p act in similar biological pathways. (A) Temperature-sensitive growth of yeast expressing *ypt31-101*[ts] in *ypt32Δ* background is rescued by expressing low-copy *YPT31*. (B and C) Wt, *ypt32Δ*, and *ypt32Δypt31-101*[ts] double mutant cells were incubated at the indicated temperature for 10 or 30 min, metabolically labeled with *Tran*-^{35}S for 10 min, and chased in the presence of excess methionine/cysteine for 30 min. CPY and Gas1p were analyzed as described in the legend to Figure 3. To assay for general secretion competence, cells and media were separated by centrifugation, and proteins secreted into the media during the pulse-chase were visualized by SDS-PAGE and fluorography. (D) *ypt32Δypt31-101*[ts] double mutant cells expressing GFP-Snc1p were grown to early/midlog phase, shifted to 26°C or 38°C for 30 min, and observed by fluorescence microscopy.

CPY and Gas1p. At the permissive temperature, CPY transport to the vacuole was unaffected (Figure 4B). However, after a 10- or 30-min shift to the restrictive temperature (38°C), CPY was predominately in the Golgi-modified form (p2) with minor amounts in the ER-modified (p1) and mature (m) forms. Gas1p acquires a glycophosphoinositol linkage in the ER (p) and is then glycosylated in the Golgi (m) before its transport to the plasma membrane. Although a modest defect is observed at the permissive temperature, shifting of *ypt32Δypt31-101ts* double mutant cells to the nonpermissive temperature (38°C, for 10 or 30 min) resulted in the accumulation of the ER-modified form (p) of Gas1p (Figure 4B). Expression of *YPT31* in the *ypt32Δypt31-101ts* double mutant cells resulted in full suppression of CPY and Gas1p trafficking defects (Figure 4B). We also examined general secretion competence of *ypt32Δypt31-101ts* double mutant cells, by taking advantage of the fact that only a few yeast proteins are efficiently secreted into the growth medium (Robinson *et al.,* 1988). As expected, at the nonpermissive temperature, *ypt32Δypt31-101ts* double mutant cells exhibited a near complete block in protein secretion into the medium, whereas double mutant cells expressing wild-type Ypt31p exhibited a normal pattern of protein secretion (Figure 4C). *ypt32Δypt31-101ts* double mutant cells at the nonpermissive temperature (38°C for 30 min) also accumulated GFP-Snc1p intracellularly, whereas *ypt32Δypt31-101ts* double mutant cells at the permissive temperature had only a partial defect in the polarized membrane localization of GFP-Snc1p (Figure 4D). These results reinforce the requirement of a functional Golgi for efficient Snc1p recycling. Collectively, the Golgi transport phenotypes observed upon Ypt31/32p inactivation resemble those observed when Pik1p is inactivated (Audhya *et al.,* 2000), suggesting that these proteins are involved in similar biological functions at the Golgi, consistent with the genetic interactions identified by our SGA analysis.

Ypt31/32 Does Not Regulate PtdIns(4)P Synthesis or Pik1p Localization

Because our genetic and phenotypic analysis suggests that Ypt31p and Pik1p functionally interact in specific biological pathways, it is possible that Ypt31p acts upstream of Pik1p by stimulating the kinase activity of Pik1p and/or by recruiting Pik1p to the Golgi. We next determined the levels of PtdIns(4)P under conditions where Ypt31/32p is transiently inactivated. *ypt32Δypt31-101ts* double mutant cells were grown at the permissive temperature (26°C) and then shifted to the nonpermissive temperature (38°C) for 10 min before labeling with *myo*-[³H]inositol for an additional 45 min at 38°C. Subsequent analysis of [³H]inositol–labeled lipids revealed no significant changes in the amounts of PtdIns(4)P at the nonpermissive temperature (Figure 5). This result suggests that the inactivation of Ypt31/32p does not alter production of PtdIns(4)P and therefore does not appear to regulate PtdIns 4-kinase activity in cells. Additionally, overexpression of *YPT31* or a GTPase-deficient mutant, *ypt31 Q72L*, does not restore the temperature-sensitive growth defect of *pik1ts* cells, nor does it affect PtdIns(4)P levels in wild-type or *ypt31Δ* cells (unpublished data). A 4.5-fold increase in PtdIns(3,5)P_2 was observed (SEY6210; 0.12 ± 0.02%; *ypt32Δ*; 0.16 ± 0.03%; *ypt32Δypt31-101ts*; 0.54 ± 0.11% of PtdIns labeled), and concurrently the vacuoles in *ypt32Δypt31-101ts* double mutant cells are highly fragmented (unpublished data), consistent with the role for PtdIns(3,5)P_2 in vacuolar membrane morphology (Rudge *et al.,* 2004). The levels of PtdIns(4,5)P_2 are significantly reduced by 2.8-fold (SEY6210; 2.13 ± 0.18%; *ypt32Δ*; 2.39 ± 0.04%; *ypt32Δypt31-*
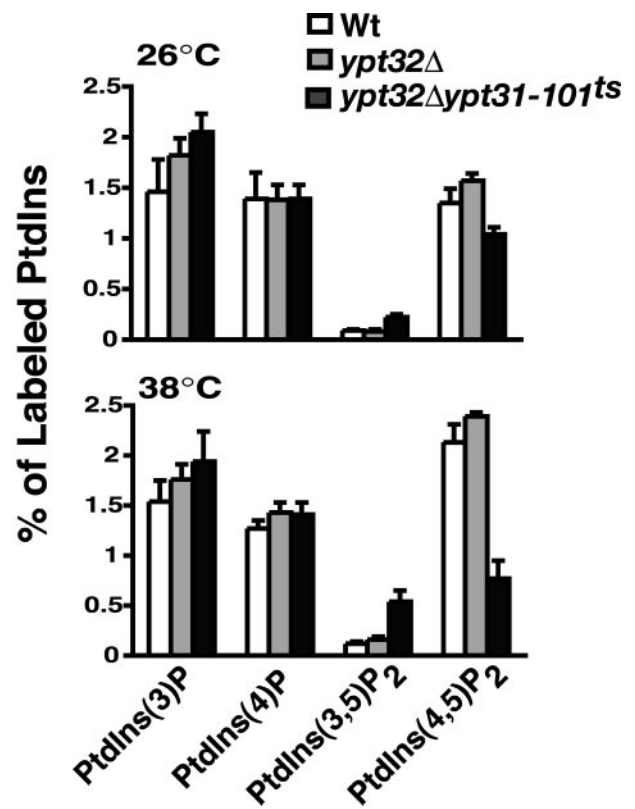

Figure 5. Phosphoinositide levels in Wt, *ypt32Δ*, and *ypt32Δypt31-101ts* double mutant cells. Cells were incubated at the temperature indicated for 10 min and labeled with myo-[2-³H]inositol for 45 min, and phosphoinositides were analyzed as previously described in Rudge *et al.* (2004). The levels of each indicated phosphoinositide are expressed as a percentage of the total ³H-labeled phosphoinositides analyzed by HPLC and represent the average of two independent experiments done in duplicate (n = 4).

101ts; 0.77 ± 0.18% of PtdIns labeled) at the nonpermissive temperature (38°C). The underlying basis of the elevated PtdIns(3,5)P_2 levels and the reduced PtdIns(4,5)P_2 levels is not clear. One possibility is that the observed accumulation of Golgi membranes and inappropriate sorting of several proteins may indirectly affect the distribution of the kinases and phosphatases involved in maintaining the levels of PtdIns(3,5)P_2 and PtdIns(4,5)P_2 in *ypt32Δypt31-101ts* double mutant cells.

We next epitope-tagged Pik1p with GFP to observe the cellular localization of GFP-Pik1p under conditions where Ypt31p is transiently inactivated. Haploid *pik1Δ* cells expressing GFP-Pik1p from a low-copy plasmid (as the only source of *PIK1*) were generated, indicating that GFP-Pik1p is functional. In wild-type backgrounds, GFP-Pik1p fluorescence localizes predominantly to concentrated puncta that colocalized with a Golgi marker protein, Sec7p (Figure 6A). Our observed localization of GFP-Pik1p is consistent with the primary role of Pik1p in Golgi structure and function. In *ypt32Δypt31-101ts* mutant background at both the permissive and nonpermissive temperatures, GFP-Pik1p remained associated with punctate structures (Figure 6A), demonstrating that a functional Ypt31/32p is not required for Pik1p to associate with intracellular membranes. Because overexpression of Sec7p in *ypt32Δypt31-101ts* double mutant cells has adverse effects on growth (Jones *et al.,* 1999), we were unable to determine whether the fluorescent puncta of GFP-Pik1p
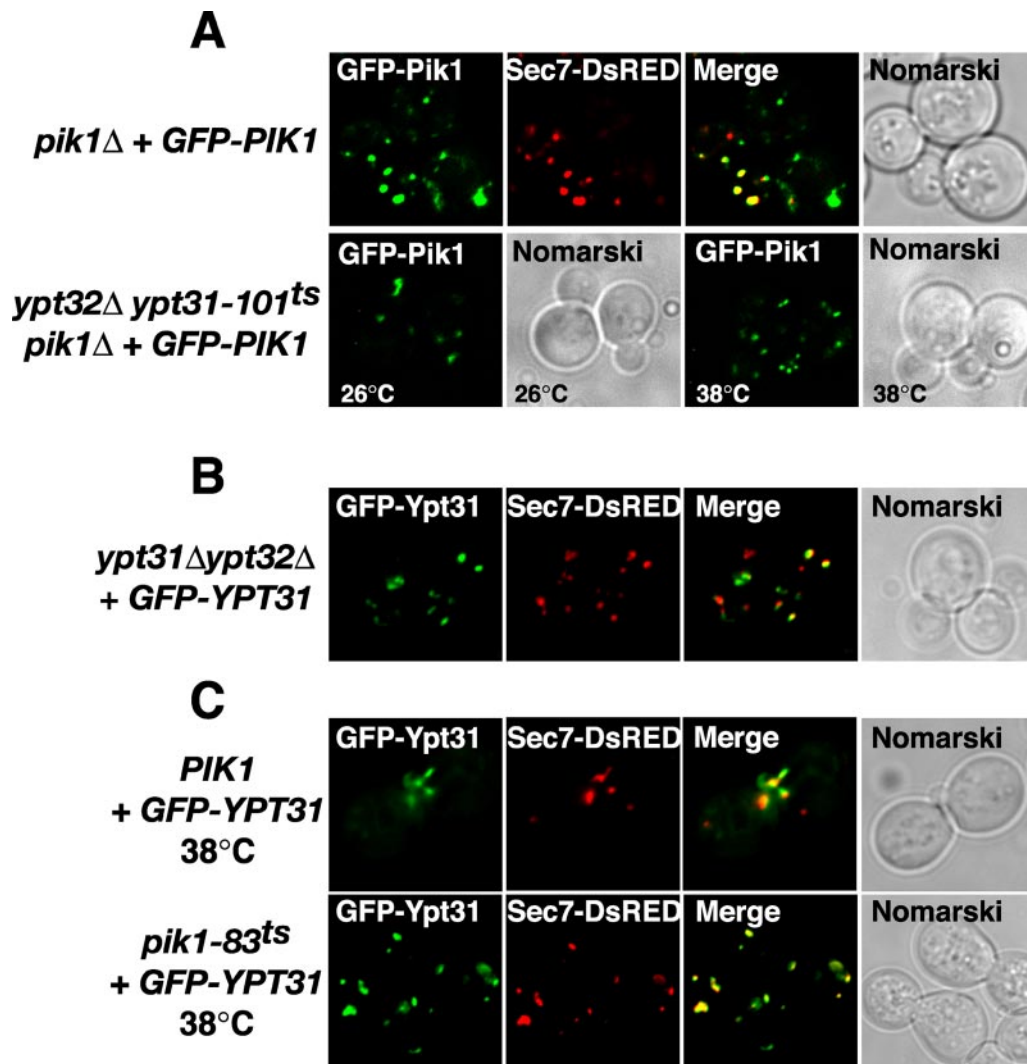
Figure 6. Independent localization of Pik1p and Ypt31p to the *trans*-Golgi network. (A) *pik1Δ* cells expressing GFP-Pik1p and Sec7p-DsRed were examined by fluorescence microscopy at 26°C. *ypt32Δypt31-101^{ts}pik1Δ* cells expressing GFP-Pik1p were grown at 26°C or shifted for 1 h at 38°C and examined by fluorescence microscopy. (B) *ypt31Δ ypt32Δ* cells expressing GFP-Ypt31p and Sec7p-DsRed were grown at 26°C and examined by fluorescence microscopy. (C) Wild-type (*PIK1*), *pik1–83^{ts}*, or *pik1-139^{ts}* (unpublished data) cells expressing *GFP-YPT31* from a low copy plasmid and Sec7p-DsRed were grown at 26°C (unpublished data) or shifted 1 h at 38°C and examined by fluorescence microscopy.

and Sec7p-DsRED colocalized. These data suggest that Ypt31p does not function as an upstream regulator of Pik1p kinase activity or localization.

Ypt31p Colocalizes with Sec7p at the trans-Golgi

Ypt31/32p has been reported to have a punctate distribution like that of the Golgi (Benli *et al.*, 1996; Jedd *et al.*, 1997), and we confirmed this using a functional GFP-tagged version of Ypt31p (Figure 6B). Furthermore, we extended this observation by demonstrating that GFP-Ypt31p localization was concentrated to Sec7p-containing compartments, indicative of *trans*-Golgi localization (Figure 6B). Similar to a previous report (Jedd *et al.,* 1997), GFP-Ypt31p fluorescence appeared to have a polarized distribution, being concentrated at the emerging bud or small bud tip in rapidly dividing cells (unpublished data). This localization pattern of Ypt31p is most consistent with the observations that late Golgi can preferentially localize to sites of polarized growth (Rossanese *et al.*, 2001).

We next investigated whether the localization of GFP-Ypt31p to the Golgi is mediated by Pik1p-generated PtdIns(4)P. When GFP-Ypt31p is expressed in yeast with a temperature-sensitive allele of *pik1*, incubation of cells at a nonpermissive temperature, which causes a substantial reduction in PtdIns(4)P (Audhya *et al.,* 2000 and this study) did not affect the localization of GFP-Ypt31p at Sec7p-enriched puncta compared with that of wild-type *PIK1* control cells (Figure 6C). These data indicate that Ypt31p does not require Pik1p kinase activity for normal membrane recruitment. We did note that the mobility of Sec7p/Ypt31p-positive compartments was reduced in *pik1–83^{ts}* cells when shifted to the nonpermissive temperature, and as a result of the reduced membrane dynamics, we were able to observe significant colocalization of GFP-Ypt31p fluorescence to Sec7p-labeled Golgi (Figure 6B). Taken together, the above results are consistent with a coordinated function of Ypt31p and Pik1p at a common membrane compartment, the *trans*-Golgi.
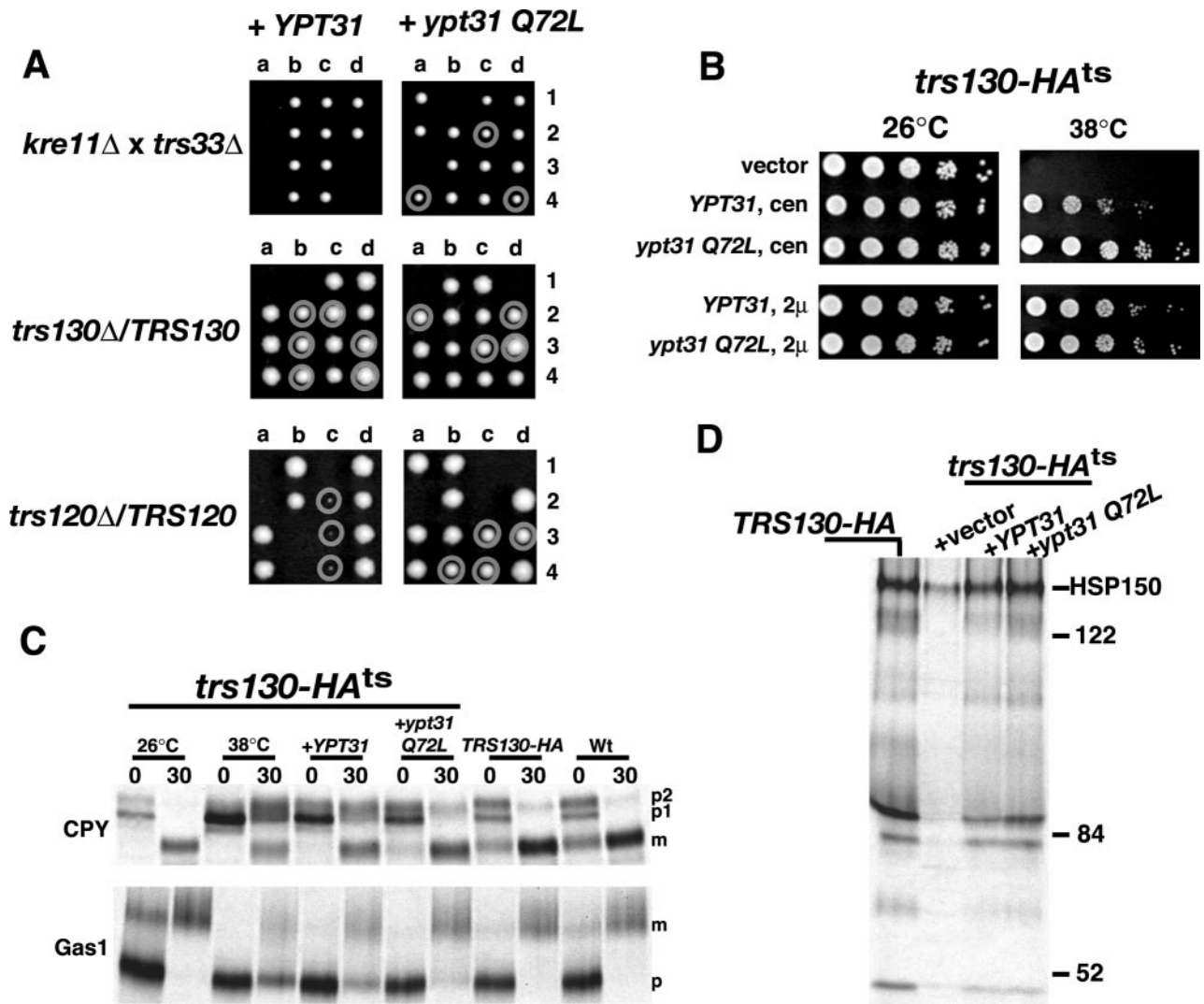
Figure 7. GTP-dependent suppression of TRAPPII lethality and trafficking defects by *YPT31*. (A) Tetrad analysis of diploid strains were transformed with high-copy wild-type *YPT31*, or *ypt31 Q72L*, sporulated, and dissected on YPD plates at 26°C. Haploid cells with deleted gene(s) carrying either *YPT31* or *ypt31 Q72L* are circled (confirmed by PCR and growth on selection media). (B) *trs130-HA*[ts] (33 aa deleted from C-terminus of TRS130, and tagged with HA) was examined for temperature-sensitive growth when expressing low (cen) or high (2 μ) copies of *YPT31* or *ypt31 Q72L*. (C) Wt, *TRS130-HA*, or *trs130-HA*[ts] cells expressing vector, *YPT31* or *ypt31 Q72L*, were incubated at 26 or 38°C for 30 min, metabolically labeled with *Tran*-^{35}S for 10 min, and chased in the presence of excess methionine/cysteine for 30 min. CPY and GAS1p were analyzed as described in the legend to Figure 3. (D) General secretion competence was analyzed as described in the legend to Figure 4.

TRAPPII Functions Upstream of Ypt31

Previous studies have demonstrated that overexpression of *YPT31* can suppress the lethality of *trs130*, which encodes an essential TRAPPII component (Yamamoto and Jigami, 2002; Zhang *et al.*, 2002), suggesting a functional relationship between TRAPPII and Ypt31p. *TRS33* and *KRE11* are nonessential subunits of the TRAPPII complex and were identified in our SGA analysis as having a functional interaction with *pik1*. To provide additional evidence that TRAPPII functions in Ypt31p signaling, we took advantage of our observation that deletion of *KRE11* in *trs33Δ* cells was lethal and tested whether the lethality of *kre11Δ trs33Δ* double mutants could be suppressed by the overproduction of *YPT31*. Haploid *trs33Δ* and *kre11Δ* single deletion mutants of opposite mating types, each expressing *YPT31* were mated and sporulated and tetrads were isolated. As shown in Figure 7A,

kre11Δ trs33Δ double mutants were never isolated. We next made a point mutation in *YPT31* to mimic the active, GTP-bound state (Walworth *et al.*, 1989) and expressed this mutant, *ypt31 Q72L*, in both haploid *trs33Δ* and *kre11Δ* single deletion mutants. Surprisingly, *kre11Δ trs33Δ* double mutants were now isolated only when *ypt31 Q72L* was overexpressed (Figure 7A). Consistent with previous findings (Zhang *et al.*, 2002), overexpression of *YPT31* in a *trs130Δ* (and to a lesser extent *trs120Δ)* deletion strains rescued the lethality in the SEY6210 background (Figure 7A). However, we found that *trs120Δ* deletion mutants obtained from trs120Δ heterozygotes grew better when *YPT31* was overexpressed in its GTP-locked form. Our data demonstrate that activated Ypt31p suppresses the mutant growth phenotypes exhibited by *trs130Δ* mutants, *trs120Δ* mutants, and *kre11Δ trs33Δ* double mutants.

To characterize the genetic interaction of *YPT31* and *TRS130* further, we constructed a temperature-sensitive mutant of *TRS130* by deleting the 33 amino acids from the C terminus as described by Sacher *et al.* (2001). Temperature-sensitive growth of *trs130-HA*[ts] mutant cells could be suppressed when cells expressed either wild-type or GTP-locked forms of *YPT31,* similar to the suppression observed in the *trs130Δ* deletion mutants (Figure 7B). We next examined whether the expression of *YPT31* could suppress the trafficking defects associated with inactivation of TRAPPII. In *trs130-HA*[ts] mutant cells shifted to the restrictive temperature, the majority of CPY was detected as a mixture of p1, p2, and mature forms, indicative of a defect in CPY trafficking out of the Golgi. Similarly, *trs130-HA*[ts] mutant cells exhibited a defect in the processing of Gas1p at the nonpermissive temperature. Expression of *YPT31* from a low-copy plasmid, which partially suppressed the growth phenotype (Figure 7B), resulted in partial suppression of the CPY and Gas1p trafficking defect (Figure 7C). However, expression of *ypt31 Q72L* from a low-copy plasmid, which suppresses the growth phenotype (Figure 7B), resulted in near complete suppression of the CPY and Gas1p trafficking defect (Figure 7C). Lastly, *trs130-HA*[ts] mutant cells at the nonpermissive temperature exhibit a strong block in protein secretion, whereas *trs130-HA*[ts] mutant cells expressing wild-type *YPT31* or *ypt31 Q72L* secreted proteins into the medium, similar to that of wild-type cells (Figure 7D). Moreover, expression of a nucleotide-free mutant of *YPT31*, *ypt31 N126I* was lethal when expressed in *trs130-HA*[ts] mutant cells (unpublished data). Collectively, these results support a role for TRAPPII functioning upstream of Ypt31p, potentially participating in the activation of Ypt31p.

Gyp2p Functions as a GAP for Ypt31p In Vivo

To provide additional evidence that TRAPPII functions upstream of Ypt31p, we reasoned that deletion of a specific rab-GAP would prolong the activated state of Ypt31p and thereby shift endogenous Ypt31p to an activated, GTP-bound state, analogous to the expression of *ypt31 Q72L*, which mimics the active, GTP-bound state (Figure 7). To date, a GAP for Ypt31p has not been identified. However, a similar approach was successful in identifying Gyp1p, a *cis*-Golgi localized protein, as the GAP for Ypt1p (Du and Novick, 2001), a rab-GTPase functioning in ER to Golgi transport. Deletion of either *GYP1* or a related homologue, *GYP2*, in wild-type cells did not have any effect on growth or trafficking (Figure 8A and unpublished data). Deletion of the *GYP1* in the *trs130-HA*[ts] mutant cells failed to suppress the growth defect at the nonpermissive temperature (Figure 8A), suggesting that Gyp1p does not exert significant GAP activity toward Ypt31p, which is consistent with in vitro studies (Albert *et al.,* 1999). It has been documented that Gyp2p has relatively low GAP activity toward Ypt31p in vitro (Albert and Gallwitz, 1999). Surprisingly, deletion of *GYP2* in *trs130-HA*[ts] mutant cells restored growth at the nonpermissive temperature of 37°C (Figure 8A) and partially at 38°C (unpublished data). Consistent with the restoration of growth at 37°C, deletion of *GYP2* also suppressed the trafficking defects (CPY, Gas1p, and HSP150 secretion) associated with the loss of Trs130p function (unpublished data).

It has been previously demonstrated that Gyp2p has high in vitro GAP activity toward Sec4p (which regulates the traffic and fusion of post-Golgi vesicles to the plasma membrane; Salminen and Novick, 1987) and Ypt6p (which regulates the fusion of late endocytic vesicles to the *trans*-Golgi; Siniossoglou and Pelham, 2001). Therefore, in order to elim-

inate the possibility that the suppression observed in *gyp2Δ trs130-HA*[ts] double mutant cells is due to the activity of Gyp2p toward Sec4p and/or Ypt6p, we determined whether the overexpression of either *SEC4* or *YPT6* could suppress the temperature-sensitive growth of *trs130-HA*[ts] mutant cells. However, no suppression of the *trs130-HA*[ts] mutant cells was observed when the wild-type or GTPase-deficient forms of *SEC4* or *YPT6* were expressed (Figure 8B and unpublished data). Our results suggest that Gyp2p functions as a physiologically relevant GAP for Ypt31p and that GAP activities measured in vitro may not reveal a true in vivo rab target.

Having demonstrated a role for Gyp2p as a potential GAP for Ypt31p in vivo, we rationalized that deletion of *GYP2* might also suppress the growth and trafficking defects of *ypt32Δypt31-101*[ts] double mutant cells. As anticipated, deletion of *GYP2*, but not *GYP1*, suppressed the growth defect of *ypt32Δypt31-101*[ts] double mutant cells fully at 37°C (Figure 8C) and partially at 38°C (unpublished data). More significantly, deletion of *GYP2* and not *GYP1*, suppressed the defects of CPY and Gas1p processing in *ypt32Δypt31-101*[ts] double mutant cells grown at 37°C (Figure 8D). This result demonstrates that the deletion of *GYP2* can alleviate the trafficking defects associated with loss of Ypt31p-dependent Golgi function.

GYP2, Conserved Member of GRAM-Domain Family of rab-GAPs

Most of the known rab GAPs share a region of homology, referred to as the TBC (Tre2, Bub2, Cdc16) domain (Neuwald, 1997), encoding the core catalytic machinery for GTPase activity (Albert *et al.,* 1999). The *S. cerevisiae* genome contains 11 genes encoding TBC/rab-GAP domain proteins. Compared with the other rab-GAP domain-containing proteins in yeast, Gyp2p is the only rab-GAP recognized to contain any additional protein motifs. In addition to a TBC/rab-GAP domain, protein motif search using SMART (Schultz *et al.,* 2000) revealed a GRAM (Glucosyltransferases, Rab-like GTPase activators, and Myotubularian) domain and an EfHand within Gyp2p (Figure 9A). Additional database searches revealed that the domain organization of Gyp2p is evolutionarily conserved, with putative orthologues in human (VRP and KIAA0676), *Drosophila* (CG7324) and *Caenorhabditis elegans* (Y45F10A.6; Figure 9A). This suggests that Gyp2p may represent a member of a unique set of conserved rab-GAPs.

To determine the in vivo functionality of the domains in Gyp2p, we developed an assay system based on our observation that deletion of *GYP2* restored the growth of the *trs130-HA*[ts] and *ypt32Δypt31-101*[ts] double mutant cells at 37°C (Figure 8). Suppression of the temperature-sensitive growth of the *trs130-HA*[ts] and *ypt32Δypt31-101*[ts] double mutant cells conferred by deleting *GYP2* is abrogated by the expression of wild-type *GYP2* from a low-copy plasmid (Figure 9B), demonstrating that *GYP2* expresses a functional protein and reinforces Gyp2p as a negative regulator of Ypt31p. To confirm that the GAP activity of Gyp2p is required for its in vivo function, we mutated the invariable arginine residue in the catalytic TBC/rabGAP domain (R295) that is predicted to be essential for GAP activity (Albert *et al.,* 1999). Expression of *gyp2 R295K* from a low copy plasmid in the *gyp2Δ trs130-HA*[ts] or *gyp2Δ ypt32Δypt31-101*[ts] mutant cells, failed to restore Gyp2p GAP function as shown by the ability of these mutants to grow at the elevated temperature (Figure 9B). Because the mutated protein, *gyp2 R295K* is stably expressed (Figure 9C), these results provide evidence that the GAP activity of Gyp2p is required to regulate Ypt31p function.
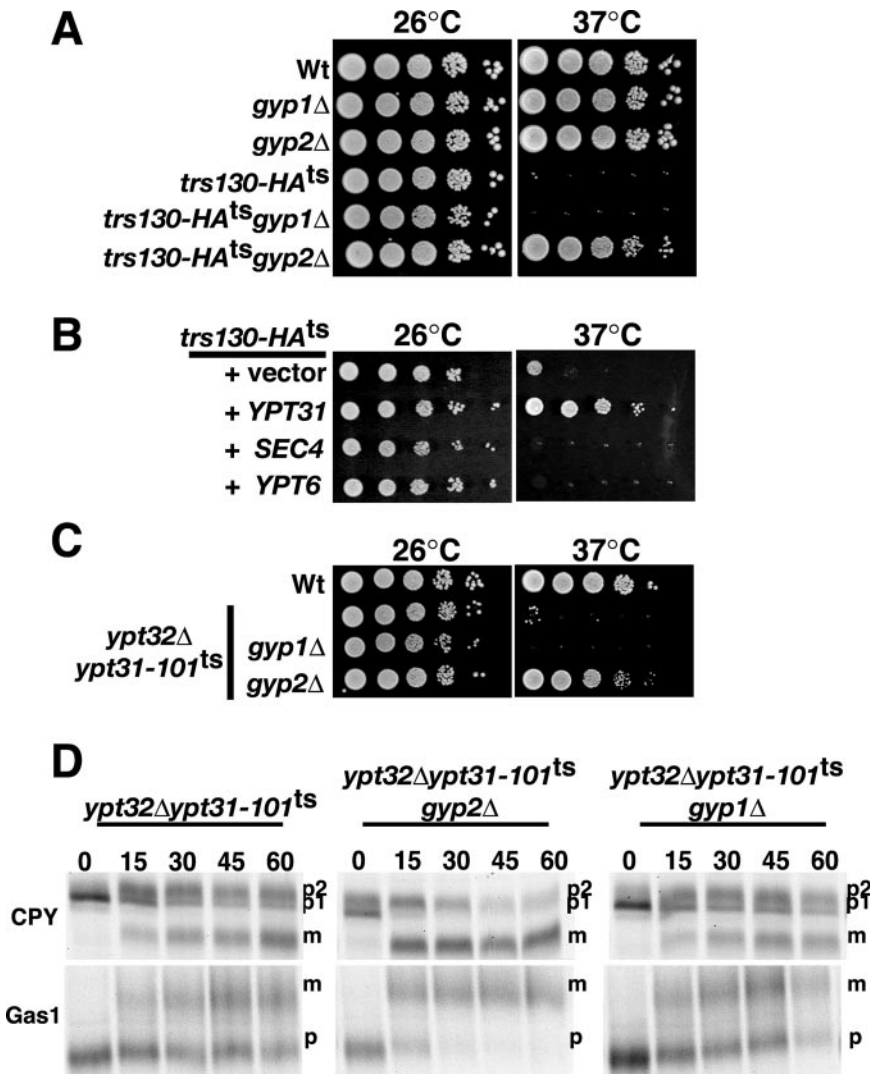
Figure 8. Gyp2p is a GAP for Ypt31p in vivo. (A) *gyp2* deletion suppresses the temperature-sensitive growth of *trs130-HA^ts*. (B) *SEC4* and *YPT6* (high-copy) do not suppress the temperature-sensitive growth defect of *trs130-HA^ts*. (C) *gyp2* deletion suppresses the temperature-sensitive growth of *ypt32Δypt31-101^ts* double mutant cells. (D) *gyp2* deletion specifically rescues trafficking defects of *ypt32Δypt31-101^ts* double mutant cells. *ypt32Δypt31-101^ts*, *ypt32Δypt31-101^ts gyp1Δ*, and *ypt32Δypt31-101^ts gyp2Δ* were incubated at 37°C for 30 min, metabolically labeled with *Tran*-³⁵S for 10 min, and chased in the presence of excess methionine/cysteine for the indicated minutes. CPY and Gas1p were analyzed as described in the legend to Figure 3.

The GRAM domain is a protein module of ∼70 amino acids that has been predicted to function as a protein- or lipid-binding motif (Doerks *et al.,* 2000). In the sequences identified so far, an invariable glycine residue in the GRAM domain (G80 in Gyp2p) has been proposed to be essential for function (Doerks *et al.,* 2000). When *gyp2 G80E* is expressed in *gyp2Δ trs130-HA^ts* or *gyp2Δ ypt32Δypt31-101^ts* mutant cells, they were still able to grow at the elevated temperature (Figure 9B), demonstrating that an intact GRAM domain is required for Gyp2p regulation of Ypt31p. Mutation of this residue (G80E), while not affecting the stability of Gyp2p (Figure 9C), impaired the Ypt31p-GTPase activating function of Gyp2p. The mechanism by which the GRAM motif participates in the GAP function of Gyp2p is presently unclear. Our data do not preclude the possibility that the GRAM motif transiently interacts with a protein or lipid to regulate Gyp2p function (see *Discussion*).

DISCUSSION

A Role for Pik1p in Vesicular Transport

Previous work (Hama *et al.,* 1999; Walch-Solimena and Novick, 1999; Audhya *et al.,* 2000) suggested that Pik1p-generated PtdIns(4)P plays a direct role in the regulation of Golgi traffic and function. To date, the only identified component of a Pik1p-dependent signaling pathway in yeast is Frq1p, a calcium-binding frequenin homologue that directly activates Pik1p catalytic activity in vitro (Hendricks *et al.,* 1999). It is currently unclear whether Frq1p regulates the pool of PtdIns(4)P that is relevant to vesicular trafficking (Hendricks *et al.,* 1999). To identify additional signaling pathway components of Pik1p, particularly those that function together with Pik1p in Golgi-mediated vesicle trafficking, an SGA analysis utilizing a temperature-conditional *pik1* mutant was undertaken.

In this study, we report the first genome-wide SGA analysis of *pik1*. This approach identified genetic interactions unique to *pik1*, which both confirm an essential role for Pik1p in Golgi-dependent trafficking and suggest a mechanistic involvement for Pik1p with a specific subset of Golgi-associated proteins, including the Ypt31p rab-GTPase, the multicomponent TRAPPII protein complex, and the aminophospholipid translocase, Drs2p. We further show that Pik1p and Ypt31p coordinately regulate traffic at the *trans*-Golgi, and that Ypt31p functions downstream of multiple TRAPPII components. On the basis of these genetic relationships, we were able to further elucidate the Ypt31p signaling pathway by identifying Gyp2p as a likely GAP for Ypt31p.
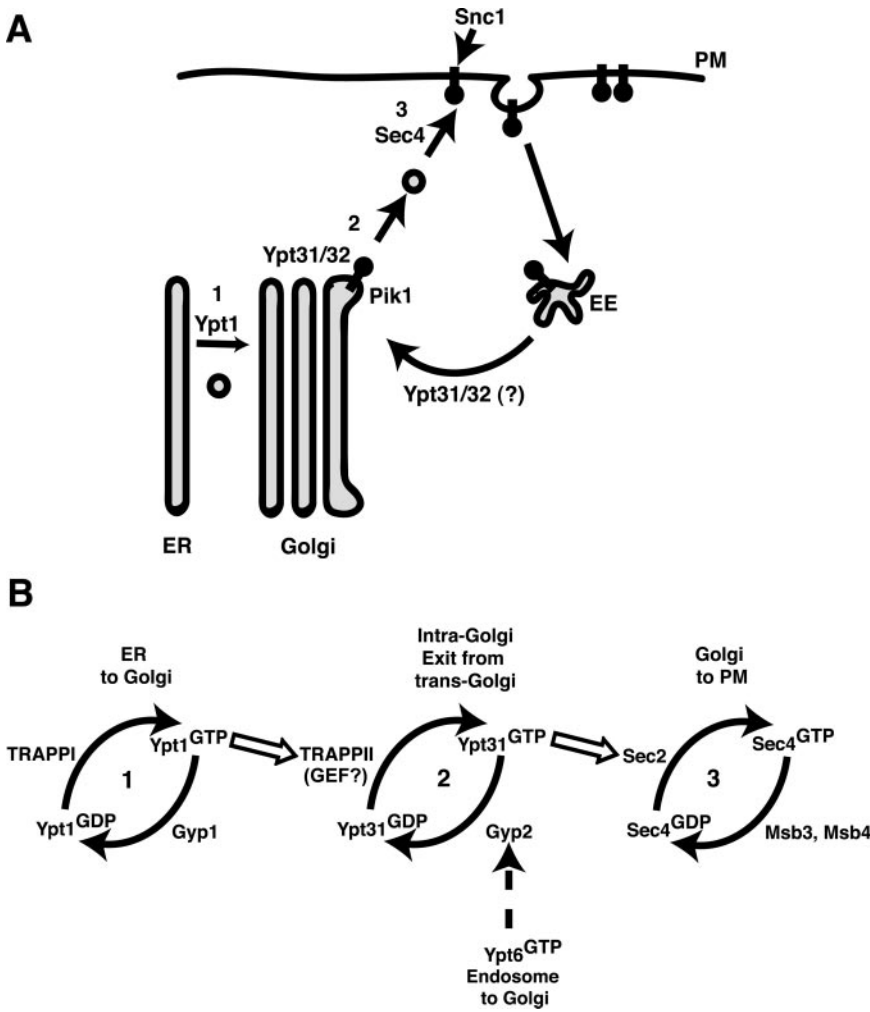
Figure 9. Gyp2p requires its GRAM domain and GAP activity for function in cells. (A) Schematic representation of the conserved domains in Gyp2 family in human, *Drosophila, C. elegans,* and yeast. EfH, EfHand; cc, coiled-coil. (B) Serial dilution of yeast cells expressing wild-type or mutants of *GYP2* from low-copy plasmids at 26 and 37°C. (C) *trs130-HA^ts gyp2Δ* cells expressing *GFP-GYP2, GFP-gyp2 G80E,* or *GFP-gyp2 R295K* from low-copy plasmids were grown at 26°C and shifted to 37°C for 1 h, and total cellular protein content were TCA-precipitated and resolved by Western blotting. Glucose 6-phosphate dehydrogenase (G6PDH) was examined as a loading control.

From our SGA and phenotypic analysis, we infer that a complex spatial and temporal interplay among Pik1p, Ypt31p, TRAPPII and possibly additional trafficking components converge to regulate Golgi secretory function.

Pik1p and Ypt31p Function Synergistically in Regulating Golgi Structure and Secretory Function

EM analysis demonstrated that *pik1-139^ts* (38°C), *ypt32Δypt31-101^ts* (38°C), and *pik1-139^ts ypt31Δ* double mutant cells (26°C) resulted in severe structural abnormalities of the Golgi (our unpublished results). These results suggest that the concerted action of Pik1p and Ypt31p was required to maintain the structural integrity and the unique membrane identity of the Golgi. Consistent with a primary role in Golgi function, we demonstrate that both Pik1p and Ypt31p colocalize to Sec7p-enriched puncta, indicative of *trans*-Golgi localization. Although the Golgi in *pik1-139^ts ypt31Δ* double mutant cells remains remarkably competent in the processing of several cargoes (CPY, Gas1p) and the traffic of several plasma membrane resident proteins (Tat2p, Hxt1p), we found that the collaborative function of Pik1p and Ypt31p was

required for the targeting of two proteins, the yeast SNARE Snc1p and the cell wall enzyme Chs3p to sites of polarized growth and membrane fusion (i.e., growing bud or septum). Snc1p and Chs3p are distinct from other transport cargoes in yeast, as they require continual recycling via early endosomes to maintain their proper subcellular localization (Lewis *et al.,* 2000; Valdivia *et al.,* 2002). Hence, the molecular components involved in the efficient recycling of Snc1p and Chs3p are starting to emerge, and our data suggest that Pik1p and Ypt31p activities are particularly important for this trafficking pathway (Figure 10A).

The observed genetic interaction between *pik1* and *ypt31* is indicative of an involvement of Pik1p and Ypt31p in a common signaling pathway that facilitates the transport function of the Golgi. The molecular basis of this genetic relationship is currently unclear. The simplest, putative explanation for the mis-localization of secretory cargoes in *pik1-139^ts ypt31Δ* double mutant cells is that the simultaneous perturbation of Pik1p and Ypt31p function resulted in severe defects in the generation and/or loading of cargoes into Golgi-derived vesicles (Figure 10A). It is conceivable that

Figure 10. (A) Snc1p trafficking to the plasma membrane (PM) in yeast requires Golgi and early endosome (EE) function. Inactivation of Pik1p or Ypt31p causes Snc1p to accumulate intracellularly. We propose that the primary role for Pik1p/PtdIns(4)P is in maintaining the structural integrity and molecular identity of the *trans*-Golgi and that unidentified multivalent adaptor proteins may be recruited by Pik1p/PtdIns(4)P and/or activated Ypt31/32p for the coordination of cargo-loading, budding, and/or fission at the *trans*-Golgi. Alternatively, Ypt31/32p may also participate in retrograde traffic of recycled cargoes by regulating early endosome-to-Golgi transport. (B) The orderly cascade of yeast exocytic rab-GTPases. The *cis*-Golgi rab-GT-Pase, Ypt1p and the multicomponent complex, TRAPPII act upstream to the *trans*-Golgi rab-GTPase, Ypt31p, possibly leading to its activation. Activated, GTP-bound Ypt31p interacts with Sec2p, the GEF for Sec4p (Ortiz *et al.*, 2002), whereas activated-Ypt6p may recruit Gyp2p (Siniossoglou and Pelham, 2001), which inactivates Ypt31p, allowing the rab-GTPase cycle of activation/inactivation at the Golgi to continue.

Pik1p and Ypt31p cooperatively recruit as yet unrecognized accessory proteins to the Golgi that efficiently package cargo into exocytic vesicles. It should be noted that no PH domain containing proteins (Yu *et al.*, 2004) and in addition no obvious candidate coat proteins that could drive cargo selection and secretory vesicle formation were identified in our SGA analysis. Another possible Pik1p/Ypt31p function stems from observations that some effectors of rab-GTPases interact with the SNARE machinery in ensuring the specificity and efficiency of vesicle fusion. For example, the Vps21p/Ypt51p rab-GTPase effector, Vac1p, associates with the t-SNARE, Pep12p and the Sec1 homolog, Vps45p (Peterson *et al.*, 1999). It is interesting to note that Vac1p also binds PtdIns(3)P, a phosphoinositide mediator of Golgi-to-vacuole transport (Burd and Emr, 1998). These studies suggest that through common effectors, rab-GTPases and phosphoinositides can coordinately function to ensure specificity of vesicular transport reactions

Recently, it was reported that FAPPs (glycolipid transfer proteins) interact with PtdIns(4)P and Arf through their PH domain necessary for their function at the Golgi in mammalian cells (Godi *et al.*, 2004). However, there are no true yeast homologues for the FAPPs, but a related family of proteins, the oxysterol-binding proteins (OSBPs), which also contain PtdIns(4)P-interacting PH domains, require both PtdIns(4)P and Arf1p for their Golgi localization (Levine and Munro, 2002). Genes encoding the yeast OSBPs were not isolated in

our SGA analysis and furthermore, deletion of all three yeast homologues did not affect viability nor Golgi morphology (Levine and Munro, 2001). At this time, it is not clear if our SGA analysis uncovered true effectors of Pik1p-generated PtdIns(4)P. It is likely that multiple effectors for PtdIns(4)P exist, possibly including effectors containing as yet unrecognized PtdIns(4)P-binding motifs or that are essential for viability (thereby absent from the array), contributing to the complexity of PtdIns(4)P signaling. These effectors may also be recognized by other Golgi-determinants, such as by Ypt31p or Arf1p that collectively contribute to the maintenance of Golgi structure and function. It is of interest to note that *ARF* was also identified in our SGA analysis to functionally interact with *PIK1* and it also interacts with *YPT31*, *DRS2*, and *TRS130* (see Figure 1A).

Another interpretation of our results invokes a model whereby Pik1p and Ypt31p physically interact. This hypothesis is supported by a recent report that the mammalian Pik1p homolog, PI4KIIIβ, associates with the GTP-bound form of the mammalian Ypt31p homolog, rab11, implying a role for PI4KIIIβ/Pik1p as an effector of rab11/Ypt31p (de Graaf *et al.*, 2004). However, our data do not support this interpretation because the inactivation of Ypt31p did not alter the localization of Pik1p or influence PtdIns(4)P levels in vivo (Figures 5 and 6). Additionally, in contrast to de Graaf *et al.* (2004), we did not detect an interaction between Pik1p and Ypt31p using two-hybrid analysis (Liang and

Segev, unpublished results). Collectively, our work and the findings of deGraff *et al.* (2004) favor a close relationship between Pik1p and Ypt31p. Further study will be necessary to mechanistically define the Pik1p/Ypt31p interaction.

TRAPPII in Ypt31 Signaling at the Golgi

Recent studies have suggested that phosphoinositides regulate the activity of small GTP-binding proteins by interacting with specific guanine-nucleotide exchange factors (GEFs) and/or GTPase activating proteins (GAPs); factors that control rab cycling between GTP-(active) and GDP-(inactive) bound forms. For instance, Mss4p-generated PtdIns(4,5)P_2 is required for the plasma membrane recruitment of Rom2p, a Rho1-GEF, and the activation of Rho1 (Audhya and Emr, 2002). Based on these models, we cannot overlook the possibility that Pik1-generated PtdIns(4)P regulates the GEF and/or GAP activities specific toward Ypt31/32p. It has been suggested that a TRAPPII complex component(s) functions as a GEF for Ypt31p (Jones *et al.,* 2000). GEF activity toward Ypt31p by an individual component of the TRAPPII complex has yet to be demonstrated (Wang and Ferro-Novick, 2002). Consistent with previous reports (Zhang *et al.,* 2002), we found that overexpression of the GTPase-deficient form of *YPT31*, but not the activated mutants of other rab-GTPases, can compensate for the loss of TRAPPII function (see Figure 7). This suggests that the TRAPPII components identified in our SGA analysis of *pik1* act upstream of Ypt31p. However, the precise biochemical function of TRAPPII (and its individual components) in Ypt31p signaling is unclear.

GYP2 Encodes a Biologically Relevant Ypt31p GAP

Before this study, the GAP or other downstream regulators for Ypt31p had not been identified. It has been suggested that Gyp2p interacts specifically with the GTP-bound form of Ypt6p (Siniossoglou and Pelham, 2001), a rab GTPase required for retrograde transport from the endosome to the Golgi, and that Gyp2p has GTPase activity toward Ypt6p in vitro (Albert *et al.,* 1999). It was thus inferred that Gyp2p functions as a GAP for Ypt6p in vivo. However, overexpression of *YPT6* (Bensen *et al.,* 2001) but not the deletion of *GYP2* (Lafourcade *et al.,* 2003) suppressed the recycling defects associated with *ric1* and *rgp1* mutants, components of the heterodimeric GEF for Ypt6p. These results suggest that deletion of *GYP2* did not significantly shift Ypt6p to its active, GTP-bound form in vivo. Our observations complement these data because we demonstrate that the deletion of *GYP2* can suppress the lethality and trafficking defects associated with temperature-sensitive alleles of *YPT31* and *TRS130*, suggesting that Gyp2p functions as a GAP for Ypt31p in vivo.

Gyp2p is the only known rab-GAP protein in yeast that contains the GRAM domain, a newly recognized protein motif (Doerks *et al.,* 2000). Based on the crystal structure of the GRAM domain-containing phosphoinositide phosphatase, MTMR2, the GRAM domain contains five beta strands that are part of a larger structural motif that resembles a PH domain (Begley *et al.,* 2003). In common with the phosphoinositide-binding activity of the PH domain (Yu *et al.,* 2004), recent reports have suggested that the GRAM domain also binds phosphoinositides (Berger *et al.,* 2003). The GRAM domain of MTMR2 bound PtdIns(4)P, PtdIns(5)P, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 and was required for MTMR2 correct subcellular targeting (Berger *et al.,* 2003). The phosphoinositide binding potential of the GRAM domain has become controversial, however, because another report did not detect significant binding using similar assay systems (Begley *et al.,* 2003). We find that the GRAM domain of Gyp2p bound mono-phosphoinositides (PtdIns(3)P, PtdIns(4)P, and PtdIns(5)P) using a similar protein-lipid overlay assay; however, mutations in the GRAM domain, which renders Gyp2p dysfunctional (Figure 9), did not significantly alter the cytoplasmic localization of the full-length protein (our unpublished results). Gyp2p, via its GRAM domain, therefore has the potential to couple phosphoinositides to the regulation of Ypt31p rab-GTPase activity.

Novick and colleagues recently proposed a rab-GTPase protein cascade in the regulation of yeast exocytosis, by which individual transport steps are functionally linked with each rab-GTPase and the corresponding regulators of each rab-GTPase in the cascade (Ortiz *et al.,* 2002). As illustrated in Figure 10, genetic and phenotypic analysis previously demonstrated that 1) the *cis*-Golgi associated Ypt1p rab-GTPase (Segev *et al.,* 1988) acts upstream of 2) the *trans*-Golgi-associated Ypt31/32p rab-GTPase (Figure 6B), both of which act upstream of 3) Sec4p, a rab-GTPase associated with post-Golgi–derived secretory vesicles (Salminen and Novick, 1987). The orderly regulation of rab-GTPase activity would then ensure the sequential completion of transport steps necessary for proper transport through the Golgi (Figure 10B). Likewise, recycling back to the *trans*-Golgi must be coupled with exit from the *trans*-Golgi in order to maintain its structure and function. Consistent with a rab-GTPase cascade of sequential activation events (Ortiz *et al.,* 2002), we propose that multiple, stage-specific signals impinge upon each rab-GTPase to regulate the rab activation/inactivation cycle. In the case of Ypt31p, our data suggest that these signaling interactions are likely to include the activities of the Pik1p PtdIns 4-kinase, the TRAPPII complex and the inactivating function of the Gyp2p GAP.

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