

Phospholipid Transfer Protein Sec14 Is Required for Trafficking from Endosomes and Regulates Distinct *trans*-Golgi Export Pathways*

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A protein known to regulate both lipid metabolism and vesicular transport is the phosphatidylcholine/phosphatidylinositol transfer protein Sec14 of *Saccharomyces cerevisiae*. Sec14 is thought to globally affect secretion from the *trans*-Golgi. The results from a synthetic genetic array screen for genes whose inactivation impaired growth of cells with a temperature-sensitive *SEC14* allele implied Sec14 regulates transport into and out of the Golgi. This prompted us to examine the role of Sec14 in various vesicular transport pathways. We determined that Sec14 function was required for the route followed by Bgl2, whereas trafficking of other secreted proteins, including Hsp150, Cts1, Scw4, Scw10, Exg1, Cis3, and Ygp1, still occurred, indicating Sec14 regulates specific *trans*-Golgi export pathways. Upon diminution of Sec14 function, the v-SNARE Snc1 accumulated in endosomes and the *trans*-Golgi. Its accumulation in endosomes is consistent with Sec14 being required for transport from endosomes to the *trans*-Golgi. Sec14 was also required for trafficking of Ste3 and the lipophilic dye FM4-64 from the plasma membrane to the vacuole at the level of the endosome. The combined genetic and cell biology data are consistent with regulation of endosome trafficking being a major role for Sec14. We further determined that lipid ligand occupancy differentially regulates Sec14 functions.

The composition of lipids within a membrane affects vesicle fission, transport, and fusion. Information on proteins that integrate lipid metabolism with vesicular transport is sparse. One protein that does so is Sec14 from *Saccharomyces cerevisiae*. Sec14 is an essential protein that extracts phosphatidylcholine (PC)² and phosphatidylinositol (PI) from membranes *in vitro* and regulates PC and phosphoinositide metabolism in cells (1). Decreasing Sec14 function results in a reduction in Golgi PI 4-phosphate levels, an increase in the rate of PC syn-

thesis, increased turnover of PC via the PC-phospholipase D Spo14, and decreased PC turnover by the PC-phospholipase B Nte1 (Fig. 1A) (2–5). The mechanisms by which Sec14 regulates PI 4-phosphate and PC metabolism are not known; however, this appears to be a major function in its regulation of cell biology as inactivation of numerous genes that mediate PC and phosphoinositide metabolism enhances or impairs growth of cells with defective Sec14 function (2, 4–6, 8–11).

Sec14 is known to be required for the transport of vesicles from the *trans*-Golgi to the plasma membrane and vacuole (equivalent to the lysosome in mammalian cells) as reduced Sec14 function results in a rapid decrease in Golgi-derived transport of invertase and acid phosphatase out of the cell and carboxypeptidase Y to the vacuole (2–6, 8–10, 12–15). Regulation of PC and phosphoinositide metabolism by Sec14 is hypothesized to provide the appropriate membrane environment for fission of vesicles from the *trans*-Golgi, although the precise step(s) where Sec14 regulates vesicular transport has yet to be determined.

The human genome contains at least 29 Sec14 domain-containing genes that code for more than 45 proteins (16). Mutations in several human Sec14 domain-containing proteins result in the onset of human diseases, including neurodegeneration, blindness, and cancer (17–22). Sec14 domains from higher eukaryotes are often embedded as part of a larger protein, many of which are guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs), indicating that regulation of small G protein activity is a unifying theme for several members of the Sec14 superfamily. Human Sec14 domains bind hydrophobic ligands, including phospholipids (16).

In this study, we used synthetic genetic array (SGA) analysis to determine more precisely how *S. cerevisiae* Sec14 regulates vesicular transport. Genetic interactions were observed between a *SEC14* allele with reduced function and GTPases that regulate vesicular transport in and out of the Golgi via endosomes. This is the first instance of Sec14 regulating import into the Golgi.

A main route for fusion of endosome-derived vesicles with the *trans*-Golgi is facilitated by the t-SNARE Tlg2 (23). Tlg2, along with Vps45 (a protein of the Sec1/Munc18 family), tethers endosome-derived vesicles to the *trans*-Golgi to facilitate membrane fusion (23) (Fig. 1B). The tethering complex that directly interacts with Tlg2 is the Golgi-associated retrograde protein (GARP) complex. The GARP complex is composed of four subunits Vps51–54 (23). The GARP complex directly

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² The abbreviations used are: PC, phosphatidylcholine; PI, phosphatidylinositol; GEF, guanine exchange factor; GAP, GTPase-activating protein; TRAPP, transport protein particle; GARP, Golgi-associated retrograde protein; DIC, differential interference contrast; MS, mass spectrometry; Lat-A, latrunculin-A; SNARE, soluble NSF attachment protein receptors; PI-4P, phosphatidylinositol 4-phosphate; SGA, synthetic genetic array.

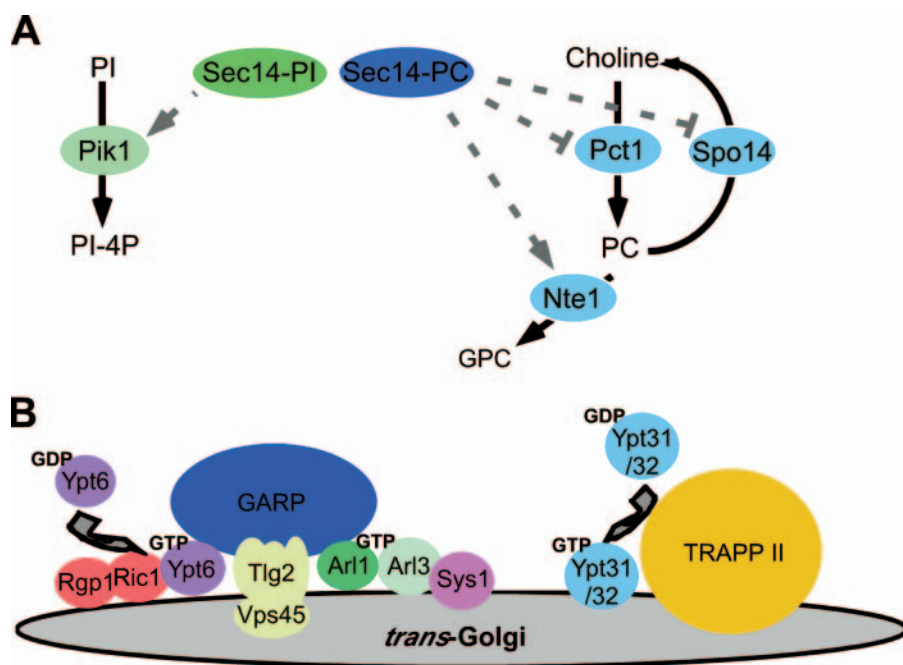


FIGURE 1. Sec14 regulation of phospholipid metabolism. *A*, regulation of phospholipid metabolism by Sec14 is thought to be lipid ligand-dependent. Sec14 bound to PC regulates PC metabolism, whereas Sec14 bound to PI controls phosphoinositide metabolism. PC-bound Sec14 is thought to inhibit PC synthesis at the rate-limiting step of Pct1 while at the same time inhibiting PC turnover by the phospholipase D Spo14 (to decrease the production of choline to prevent supply of PC precursor). At the same time Sec14-PC shunts PC turnover toward the phospholipase B Nte1, away from supplying choline for PC synthesis. In this way, Sec14 acts as a homeostat for regulation of PC levels. Sec14 bound to PI maintains PI-4P levels, although whether this is by activating the PI 4-kinase Pik1 is not known. The *solid arrows* indicate metabolic pathways; the *dotted arrows* activation, and the *dotted lines* inhibition. GPC, glycerophosphocholine. *B*, pathways in and out of the *trans*-Golgi. A subset of vesicles destined to fuse with the *trans*-Golgi do so via the GARP complex (contains Vps51, Vps52, Vps53, and Vps54). The GARP complex binds Ypt6, a Rab regulated by the GEF pair Ric1/Rgp1. The GARP complex also directly interacts with the Arf-like GTPase Arl1 that is anchored to the *trans*-Golgi via Arl3 and Sys1. Fusion to the *trans*-Golgi is mediated in part by the t-SNARE Tlg2 and its accessory protein Vps45. A second Golgi-associated complex, the transport protein particle II (TRAPP II) complex (contains the essential subunits Bet3, Bet5, Trs20, Trs23, Trs31, Trs120, and Trs130 and the nonessential subunits Trs33, Trs65, and Trs85), acts as a GEF for the Rab pair Ypt31/32 and mediates transport both in and out of the *trans*-Golgi.

interacts with Ypt6 (a Rab GTPase) and Arl1 (an Arf-like GTPase) that together comprise the two main routes for endosome fusion with the *trans*-Golgi. Ypt6 is activated by a heterodimeric GEF comprised of Ric1 and Rgp1. The GEF for Arl1 is unclear; however, the localization of Arl1 to the Golgi is dependent on Arl3 and Sys1 (24, 25). Arl3 is also an Arf-like GTPase, whereas Sys1 is an integral membrane protein that targets Arl3 to the Golgi.

A second Golgi-associated tethering complex is the transport protein particle (TRAPP). TRAPP I regulates early Golgi events in part by acting as a GEF for the Rab Ypt1. TRAPP II is specific for transport in and out of the *trans*-Golgi and is a GEF for the Rab pair Ypt31/32 (Fig. 1*B*) (26). TRAPP I and II share seven subunits (Bet3, Bet5, Trs20, Trs23, Trs31, Trs33, and Trs35) with three subunits (Trs65, Trs120, and Trs130) added to form TRAPP II.

Sec14 has previously been implied to globally regulate vesicular transport out of the Golgi. Our genetic screen dramatically expanded the network of genes required for vesicular transport when Sec14 function is impaired, including those comprising GARP and TRAPP complexes. The results from the genetic screen prompted us to examine the role of Sec14 in multiple vesicular transport routes. We report that Sec14 regulates a

subset of vesicular transport pathways out of the *trans*-Golgi. The genetic analysis, coupled with assessments of vesicular transport pathways, go on to indicate that a primary function of Sec14 is regulation of vesicular transport through endosomes. We also demonstrate that ligand binding by Sec14 differentially regulates function.

MATERIALS AND METHODS

Synthetic Genetic Array Screen—SGA analysis was performed essentially as described (27, 28) with the below modifications. CMY503 (contains a temperature-sensitive *SEC14* allele, *sec14^{ts}*) was mated with 4,800 *S. cerevisiae* single gene deletion strains at 25 °C; diploids were selected, and cells were sporulated for 5 days at 25 °C. To ensure that the haploid cells obtained were from mated diploids, cells were selected for histidine prototrophy followed by growth on medium containing G418 and nourseothricin. The resulting haploids were incubated at 25 or 35 °C. Three independent screens were performed. Mutants whose inactivation resulted in decreased growth when in combination with the *sec14^{ts}* allele in at least two of three screens were subjected to random

spore analysis to determine whether the genetic interactions observed were true.

Yeast Strain Construction—The *sec14^{ts}* allele linked to the nourseothricin drug resistance cassette was constructed as follows (mutated sites in primer sequences are underlined). Plasmid-borne *SEC14* was subjected to site-directed mutagenesis to convert Gly-266 to Asp using primers 5'-CTTACCA-GTCAAATTTGGCGATAAGTCTGAAGTTGATGATCA-3' and 5'-GATTCATCAACTTCAGACTTATCGCCAAATTT-GACTGGTAAG-3'. This mutation confers temperature sensitivity to Sec14. To insert the natMX4 cassette next to the *sec14^{ts}* gene, an SphI site was generated 300 bp downstream of the *SEC14* stop codon by site-directed mutagenesis with the primers 5'-GGCAGTATATACATTTATCTTATAGAATACGCATGCACGTATTAACAATAAAAAGAACATAAG-3' and 5'-CTTATGTTCTTTTTATTGTTTAATACGTGCAT-GCGTATTCTATAAGATAAATGTATATACTGCC-3'. The natMX4 cassette was amplified with SphI sites at each end using primers 5'-CTGAGAGCATGCACATGGAGGCCAG-AATACCC-3' and 5'-CTGAGAGCATGCCAGTATAGCGACCAGCATTAC-3'. An SphI site in the natMX4 DNA sequence was removed by site-directed mutagenesis (without changing the amino acid sequence) using primers 5'-CGCTC-

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TACATGAGTATGCCCTGCCCTA-3' and 5'-TAGGGGC-AGGGCATACTCATGTAGAGCG-3', and this DNA fragment was subcloned into the SphI site downstream of the *sec14^{ts}* open reading frame. The *sec14^{ts}:natMX4* disruption cassette was PCR-amplified from the resulting vector using primers 5'-CCTTTCTTGGATCCAGTCACTG-3' and 5'-GGCATGGACTTGATATCCTAG-3' to give a fragment containing both the *sec14^{ts}* mutation and the natMX4 cassette along with 5' and 3' *SEC14*-flanking DNA. This DNA fragment was transformed into the Y2454 yeast strain to replace the *SEC14* gene with the *sec14^{ts}* allele linked to the natMX4 cassette to generate strain CMY503 (*MAT α mfa1 Δ ::MFA1pr-HIS3 can1 Δ 0 ura3 Δ 0 leu2 Δ 0 his3 Δ 1 lys2 Δ 0 sec14^{ts}:natMX4*). The *sec14^{ts}:natMX4* cassette was transformed into the indicated strains for the construction of isogenic sets of *sec14^{ts}* containing yeast (Table 1). Strain genotypes were confirmed by genomic PCR and/or genomic Southern blot using the digoxigenin High Prime DNA Labeling and Detection Kit II (Roche Applied Science).

Monitoring Bulk Protein Secretion and Mass Spectrometry Identification of Secreted Proteins—Cells were grown to mid-log phase at 25 °C, and an equal cell number was shifted to 37 °C for 10 min and incubated with [³⁵S]methionine/cysteine for 10 min and then with 10 mM unlabeled methionine/cysteine for 15 or 30 min. Proteins in the medium were precipitated with 10% trichloroacetic acid and separated by SDS-PAGE, and the gel was exposed to x-ray film. Similarly, equal numbers of log phase cells were shifted to 37 °C for 1 h in fresh YPD medium and proteins precipitated with 10% trichloroacetic acid, separated by SDS-PAGE, and stained with Gelcode (Thermo Scientific). Proteins contained in gel slices were reduced with dithiothreitol, carboxamidomethylated with iodoacetamide, and digested with trypsin. Peptides were extracted with 70% acetonitrile, 1% formic acid. The extraction solvent was removed under vacuum, and the tryptic peptides were resuspended in 5% methanol, 0.5% formic acid.

Liquid chromatography MS/MS was performed using an Ultimate pump and Famos auto-sampler (LC Packings, Amsterdam, Netherlands) interfaced to the nanoflow electrospray ionization source of a hybrid triple quadrupole linear ion trap mass spectrometer (QTrap, Applied Biosystems). Samples were injected onto a capillary column (0.10 × 150 mm Chromolith C18, monolithic, Merck) at a flow rate of 1.2 μ l per min. Solvent A consisted of 98% water, 2% acetonitrile, 0.1% formic acid, and Solvent B consisted of 2% water, 98% acetonitrile, 0.1% formic acid, and the linear gradient was as follows: 5% solvent B to 35% solvent B over 35 min then 90% B for 6 min before re-equilibration at 5% solvent B. The sample was sprayed through distal coated fused silica emitter tips, 75- μ m inner diameter with 15- μ m inner diameter tip (New Objectives Pico Tip). The capillary voltage was 2.10 kV with a declustering potential of 60 V, and the curtain gas was set to 15 (arbitrary units). Spectra were acquired using the information-dependent acquisition mode. The two most intense ions from the survey scan (375–1200 *m/z*) were selected for tandem MS, and the collision energy was set based on the mass of the precursor as determined by the *m/z* and charge. The raw MS/MS data were searched against NCBI yeast

entries and against all SwissProt entries using the MASCOT algorithm (Matrix Science). Search parameters were peptide mass and fragment mass tolerance 0.8 and 0.5 Da, respectively, with one missed cleavage allowed. Oxidized methionines and carboxamidomethylated cysteines were chosen as variable modifications.

Invertase and Bgl2 Secretion Assays—The invertase secretion assay was performed as described (29) with modifications (13). Bgl2 secretion was determined as described (30). Mid-log phase cells were incubated at 25 °C or shifted to 37 °C for 15–60 min. Cells were then harvested by centrifugation at 1000 × *g* for 5 min. Cell pellets were resuspended in 10 mM NaN₃, 10 mM KF solution, incubated on ice for 10 min, and then transferred to microcentrifuge tubes. Cells were centrifuged at 10,000 × *g* for 1 min, and pellets were resuspended in fresh pre-spheroplasting buffer (100 mM Tris-H₂SO₄, pH 9.4; 50 mM β -mercaptoethanol; 10 mM NaN₃; 10 mM KF), incubated on ice for 15 min, centrifuged as before, washed with 0.5 ml of spheroplast buffer (50 mM KH₂PO₄-KOH, pH 7; 1.4 M sorbitol; 10 mM NaN₃), and pelleted. Cells were resuspended in spheroplast buffer containing 167 μ g/ml Zymolyase 100T and incubated for 30 min at 25 °C. Spheroplasts were then pelleted at 5,000 × *g* for 10 min and resuspended in 2× SDS-PAGE sample buffer. Proteins were separated using a 10% SDS-polyacrylamide gel, and Bgl2 was detected by Western blot using a rabbit polyclonal antibody against Bgl2. Bgl2 antibodies were the kind gifts of Randy Schekman (University of California, Berkeley) and Wei Guo (University of Pennsylvania).

Ste3 Internalization—MAT α cells were grown to mid-log phase at 25 °C and shifted to 37 °C for 2 h. Proteins were extracted, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies for Ste3 and Pgk1 (Molecular Probes). Ste3 antibodies were the kind gift of George Sprague (University of Oregon).

Microscopy—Live cells were observed using a Zeiss Axiovert 200 M microscope fitted with a plan-neofluor 100× oil immersion lens. Images were captured using a Zeiss Axio Cam HR using Axiovision 4.5 software. Cells were visualized using differential interference contrast (DIC), green fluorescent protein, or rhodamine filters as required. GFP-Snc1 subcloned into the plasmid pRS416 (pMjL1) was the kind gift of Hugh Pelham (MRC Laboratory of Molecular Biology, Cambridge, UK). The plasmid expressing the Vps27-GFP fusion was from Christopher Stefan (Cornell University, Ithaca, NY). The yeast strains expressing red fluorescent protein-tagged organelle marker proteins were from Erin O'Shea (University of California, San Francisco).

RESULTS

SGA Analysis of *sec14^{ts}* Cells Uncovers Novel Genetic Interactions—*S. cerevisiae* contains 6607 genes of which ~5000 are not essential. A haploid query strain containing a temperature-sensitive *sec14^{ts}* allele was mated to ~4800 viable single gene deletion haploid yeast strains of the opposite mating type using SGA technology (28). The resulting diploids were isolated and forced to undergo meiosis, and haploid cells were selected that contained the *sec14^{ts}* allele in combination with each single gene deletion. The resulting strains, obtained from three sepa-

TABLE 1

Yeast strains used in this study

UCSF is University of California, San Francisco.

Strain	Genotype	Source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	EUROSCARF
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	EUROSCARF
Y2454	<i>MATa mfa1Δ::MFA1pr-HIS3 can1Δ0 his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0</i>	49
CMY503	<i>Y2454 sec14^{ts}::natMX4</i>	This study
CMY505	<i>BY4741 sec14^{ts}::natMX4</i>	This study
CMY506	<i>BY4742 sec14^{ts}::natMX4</i>	This study
Y01846	<i>BY4741 gyp1Δ::kanMX4</i>	EUROSCARF
CMY514	<i>BY4741 gyp1Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y06583	<i>BY4741 ypt31Δ::kanMX4</i>	EUROSCARF
CMY515	<i>BY4741 ypt31Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y02371	<i>BY4741 trs33Δ::kanMX4</i>	EUROSCARF
CMY516	<i>BY4741 trs33Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y01709	<i>BY4741 tlg2Δ::kanMX4</i>	EUROSCARF
CMY517	<i>BY4741 tlg2Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y05102	<i>BY4741 spo14Δ::kanMX4</i>	EUROSCARF
CMY518	<i>BY4741 spo14Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y06437	<i>BY4741 ric1Δ::kanMX4</i>	EUROSCARF
CMY526	<i>BY4741 ric1Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
CMY553	<i>BY4741 rgp1Δ::LEU2</i>	This study
CMY554	<i>BY4741 rgp1Δ::LEU2 sec14^{ts}::natMX4</i>	This study
Y04462	<i>BY4741 vps45Δ::kanMX4</i>	EUROSCARF
CMY527	<i>BY4741 vps45Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y05171	<i>BY4741 ypt6Δ::kanMX4</i>	EUROSCARF
CMY528	<i>BY4741 ypt6Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y05091	<i>BY4741 vps51Δ::kanMX4</i>	EUROSCARF
CMY530	<i>BY4741 vps51Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y04318	<i>BY4741 vps52Δ::kanMX4</i>	EUROSCARF
CMY531	<i>BY4741 vps52Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
FRY107	<i>BY4742 vps53Δ::kanMX4</i>	50
CMY532	<i>BY4742 vps53Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y03966	<i>BY4742 vps54Δ::kanMX4</i>	EUROSCARF
CMY533	<i>BY4741 vps54Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y06772	<i>BY4741 sys1Δ::kanMX4</i>	EUROSCARF
CMY547	<i>BY4741 sys1Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y03304	<i>BY4741 arl1Δ::kanMX4</i>	EUROSCARF
CMY548	<i>BY4741 arl1Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y02777	<i>BY4741 arl3Δ::kanMX4</i>	EUROSCARF
CMY549	<i>BY4741 arl3Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y04576	<i>BY4741 ypt32Δ::kanMX4</i>	EUROSCARF
CMY550	<i>BY4741 ypt32Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y04796	<i>BY4741 trs65Δ::kanMX4</i>	EUROSCARF
CMY551	<i>BY4741 trs65Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
Y04738	<i>BY4741 trs85Δ::kanMX4</i>	EUROSCARF
CMY552	<i>BY4741 trs85Δ::kanMX4 sec14^{ts}::natMX4</i>	This study
CMY566	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-SNF7</i>	UCSF
CMY557	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-CHC1</i>	UCSF
CMY559	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-ANP1</i>	UCSF
CMY563	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-SEC13</i>	UCSF
CMY562	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-COP1</i>	UCSF
CMY564	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-ERG6</i>	UCSF
CMY537	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-SNF7 sec14ts::natMX4</i>	This study
CMY535	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-CHC1 sec14ts::natMX4</i>	This study
CMY534	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-ANP1 sec14ts::natMX4</i>	This study
CMY536	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-SEC13 sec14ts::natMX4</i>	This study
CMY538	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-COP1 sec14ts::natMX4</i>	This study
CMY539	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (S288C) RFP-ERG6 sec14ts::natMX4</i>	This study
X2180-1A	<i>MATa mal mel gal2 CLIP1 SLUC2</i>	Randy Sheckman
SF292-1A	<i>MATa sec14-3 (sec14^{ts}) isogenic to X2180-1A</i>	Randy Sheckman
SF282-1D	<i>MATa sec18-1 (sec18^{ts}) isogenic to X2180-1A</i>	Randy Sheckman
CBY926	<i>MATα ura3-52 his3Δ200 lys2-801 leu2-3,112 trp1Δ901 suc2Δ9 osh1Δ::kanMX4 osh2Δ::kanMX4 osh3Δ::LYS2 osh4Δ::HIS3 osh5Δ::LEU2 osh6Δ::LEU2 osh7Δ::HIS3 [pCB255 (osh4-1 TRP1)]</i>	30

rate screens, were incubated at permissive (25 °C) and semi-permissive (35 °C) temperatures for growth of cells containing the *sec14^{ts}* allele alone. We considered only strains with the *sec14^{ts}* gene in combination with each single gene deletion able to grow at 25 °C, but not 35 °C, to eliminate genes unable to grow because of defective mating, sporulation, or restoration of vegetative growth from spores.

Forty genes were isolated from the SGA screen whose inactivation aggravated growth of *sec14^{ts}*-containing cells (Fig. 2A and Table 2). Inactivation of *SPO14* (PC phospholipase D) had been previously determined to diminish growth of *sec14^{ts}* cells

(1, 5, 9), and it was isolated from our screen. Of the 40 genes isolated, the largest group contained genes known to regulate Golgi-dependent vesicular transport processes consistent with Sec14 acting as an essential regulator of this process (Fig. 2A). As genetic interactions can be representative of direct regulation of a protein/process, or may indicate processes whose functions are linked but by several degrees of separation, we focused on the genes isolated from the SGA screen with known roles in vesicular transport.

To independently confirm the results of the SGA screen, a set of isogenic strains was constructed whereby the wild type

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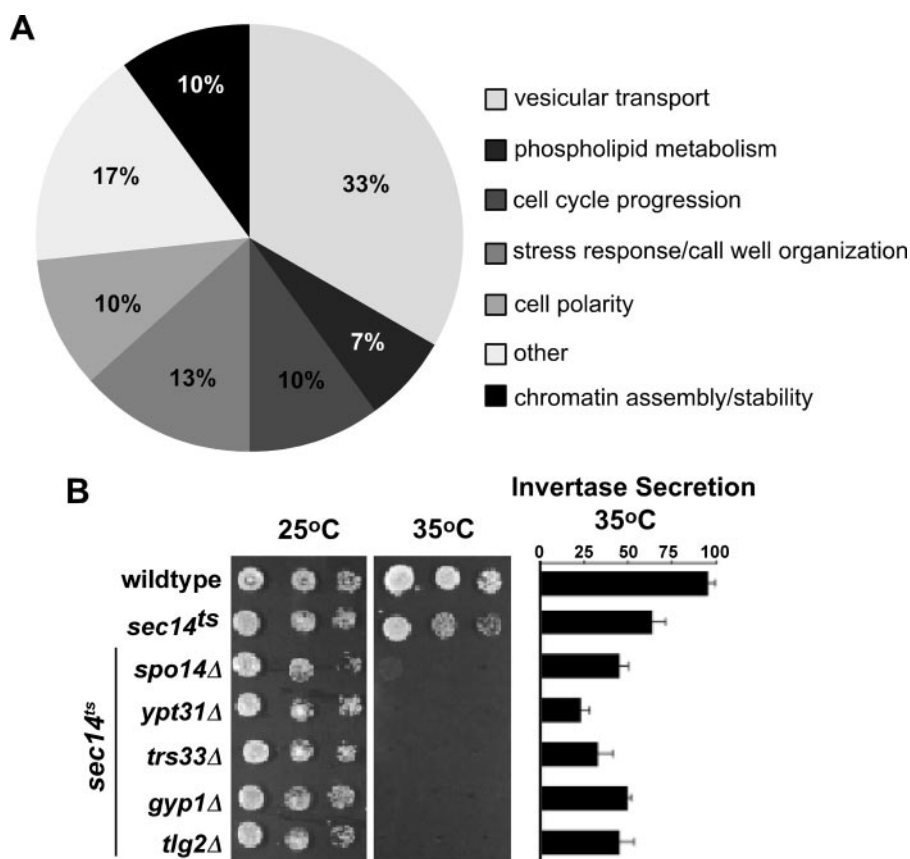


FIGURE 2. Genes whose inactivation aggravates growth of *sec14^{ts}* cells. *A*, pie chart of the functions of the 40 genes identified by the SGA screen whose inactivation decreased growth of *sec14^{ts}* cells. A subset of these genes (*SPO14*, *YPT31*, *TRS33*, *TLG2*, *GYP1*, and *VPS1*) had been identified previously as genetic interactors in cells with decreased Sec14 function (2). *B*, isogenic mutant strains with the indicated genotypes were grown to mid-log phase in synthetic complete medium at 25 °C; identical numbers of cells were serially diluted, spotted onto agar plates, and grown for 3 days at the indicated temperatures. Invertase secretion is the ratio of external invertase activity to total (internal + external) invertase activity. Invertase data are the mean of three individual experiments performed in triplicate. Standard errors of the mean are indicated in the figure.

SEC14 gene was replaced with the *sec14^{ts}* allele in strains with inactivated genes for *SPO14* (PC phospholipase D) (1, 5, 9), *YPT31* (Rab that regulates Golgi vesicle export and import) (31), *TRS33* (a component of TRAPP complex that acts as a GEF for Ypt31 and its homologue Ypt32) (32, 33), *GYP1* (GAP for the essential Rab Ypt1) (34), and *TLG2* (syntaxin-like t-SNARE that mediates fusion of endosome-derived vesicles with the *trans*-Golgi) (34). Growth of cells containing the *sec14^{ts}* allele alone was slightly slower than wild type at the semi-permissive temperature for this allele (35 °C) (Fig. 2*B*). This growth defect was exacerbated when a cell containing the *sec14^{ts}* allele had in combination an inactivated gene for *SPO14*, *YPT31*, *TRS33*, *GYP1*, or *TLG2*. Inactivation of any of these genes alone did not affect cell growth. The SGA analysis successfully revealed previously unknown genetic interactions with *SEC14*.

Secretion Pathways Are Differentially Affected in Cells Defective in Sec14 Function—Sec14 regulates fission of vesicles from the *trans*-Golgi. A hallmark of Sec14 deficiency is reduced invertase secretion from the cell. Invertase follows a pathway from the *trans*-Golgi via endosomes on its route to the plasma membrane (35, 36). We monitored invertase secretion in *sec14^{ts}* cells and compared it with *sec14^{ts}* cells containing inac-

tivated genes isolated from our SGA screen. In *sec14^{ts}* cells invertase secretion was reduced to 66% at 35 °C (Fig. 2*B*) (compared with ~30% at 37 °C, which is the non-permissive temperature for the *sec14^{ts}* allele; data not shown). In *sec14^{ts}* cells that also contained inactivated *SPO14*, *YPT31*, *TRS33*, *GYP1*, or *TLG2* invertase secretion was reduced to 22–48% of wild type at 35 °C (Fig. 2*B*). All strains secreted invertase similar to wild type at 25 °C and in addition all single gene deletions secreted invertase at wild type levels at 35 and 37 °C (data not shown). Therefore, inactivation of *SPO14*, *YPT31*, *TRS33*, *GYP1*, or *TLG2* decreases Sec14-dependent vesicular transport of invertase from the *trans*-Golgi.

Two types of secretory vesicles have been isolated from cells based on differences in density (35, 36). The endosome-dependent route fractionates with higher density vesicles that contain invertase and acid phosphatase. These are the only two proteins that have been shown to be inefficiently secreted in *sec14^{ts}* cells.

A second route to the plasma membrane is found in lower density vesicles containing Bgl2 and Pma1 and represent an endosome-independent route from the *trans*-Golgi to the plasma membrane. To assess if a deficiency in Sec14 function also affects secretion through the endosome-independent route, we monitored Bgl2 secretion. Bgl2 accumulated in *sec14^{ts}* cells compared with wild type after incubation at the non-permissive temperature for the *sec14^{ts}* allele. We observed a build up of Bgl2 in cells with defective Sec14 function as early as 15 min after temperature shift, and this was obvious within 1 h (Fig. 3*A*). The *oshΔ osh4^{ts}* strain is known to accumulate Bgl2 and served as a positive control (30). Bgl2 observed outside the cell in all strains is standard for this assay as Bgl2 is present in the periplasmic space prior to onset of the secretory defect.

The high and low density vesicles that contain invertase/acid phosphatase and Bgl2/Pma1, respectively, were isolated from cells with defects in fusion of Golgi-derived vesicles with the plasma membrane (36). These experiments did not rule out other routes to the plasma membrane from the *trans*-Golgi. To determine the extent and specificity of the protein secretion defect in *sec14^{ts}* cells, wild type and *sec14^{ts}* cells (from two different genetic backgrounds) were grown at 37 °C for 10 min, incubated with [³⁵S]methionine/cysteine for 10 min to radiolabel cellular protein, and chased with 10 mM unlabeled methionine/cysteine for various times, and radiolabeled proteins secreted into the medium were separated by SDS-PAGE. Wild

TABLE 2

Yeast genes isolated from the synthetic genetic array screen whose inactivation aggravated growth of *sec14^{ts}* cells

Gene	Open reading frame	Description	Cellular process
<i>SPO14</i>	YKR031C	Phospholipase D, catalyzes hydrolysis of phosphatidylcholine to phosphatidic acid and choline	Vesicular transport/phospholipid metabolism
<i>YPT31</i>	YER031C	GTPase of Rab family involved in the endocytic and exocytic pathways	Vesicular transport
<i>TRS33</i>	YOR115C	Protein of the TRAPP2 complex involved in endocytic and exocytic pathways; GEF activity toward Ypt31	Vesicular transport
<i>GYP1</i>	YOR070C	Golgi GTPase-activating protein for Rab family members	Vesicular transport
<i>TLG2</i>	YOL018C	t-SNARE involved in endocytosis and maintenance of resident proteins in the <i>trans</i> -Golgi	Vesicular transport
<i>UBP3</i>	YER151C	Ubiquitin-specific protease, interacts with Bre5	Vesicular transport
<i>BRE5</i>	YNR051C	Required by Ubp3 to form an active de-ubiquitination complex which protects Sec23, a COPII subunit, from degradation	Vesicular transport
<i>KEX2</i>	YNL238W	Calcium-dependent serine protease involved in the activation of proproteins of the secretory pathway	Vesicular transport/Golgi function
<i>VPS1</i>	YKR001C	Dynamamin-like GTPase for vacuolar protein sorting and high density vesicle production for secretion of invertase	Vesicular transport
<i>TCB2</i>	YNL087W	Synaptotagmin like protein; contains 3 C2 domains	Golgi localized
<i>INP51</i>	YIL002C	PI-4,5-bisphosphate 5-phosphatase	Phospholipid metabolism
<i>YBR030W</i>	YBR030W	Protein of unknown function. Likely involved in phospholipid metabolism as it has Ino2/4-binding sites in promoter	Phospholipid metabolism
<i>CLN2</i>	YPL256C	G ₁ /S-specific cyclin, interacts with Cdc28 protein kinase to control events at START	Cell cycle progression
<i>CDH1</i>	YGL003C	Cell cycle-regulated activator of the anaphase-promoting complex required for exit from mitosis	Cell cycle progression
<i>WHI2</i>	YOR043W	Protein involved in stress response and growth regulation; negative regulator of G ₁ cyclin expression	Cell cycle progression/stress response
<i>RIM9</i>	YMR063W	Protein of unknown function; involved in proteolytic activation of Rim101 in response to pH	Stress response
<i>TUS1</i>	YLR425W	GDP-GTP exchange factor that functions to modulate Rho1 activity as part of the cell integrity pathway	Stress response/cell wall organization
<i>SLG1</i>	YOR008C	Plasma membrane protein required for maintenance of cell wall integrity and for the stress response	Cell wall organization
<i>ECM33</i>	YBR078W	Glycosylphosphatidylinositol anchor protein of unknown function; possible role cell wall organization	Cell wall organization
<i>NCS2</i>	YNL119W	Role in urmylation	Cell polarity
<i>NCS6</i>	YGL211W	Protein of unknown function; role in urmylation and invasive/pseudohyphal growth	Cell polarity
<i>PEA2</i>	YER149C	Coiled-coil polarisome protein	Cell polarity
<i>CRN1</i>	YLR429W	Coronin, actin and microtubules associated protein	Actin patch assembly
<i>ICE2</i>	YIL090W	Protein of unknown function; integral endoplasmic reticulum membrane protein	Endoplasmic reticulum organization
<i>PER1</i>	YCR044C	Vacuolar membrane protein; mutant is dependent on activation of the unfolded protein response	Protein Processing
<i>DSD1</i>	YGL196W	D-Serine dehydratase	Amino acid metabolism
<i>HXT8</i>	YJL214W	PM protein with strong similarity to hexose transporters; expression induced by low glucose	Hexose transport
<i>LSM1</i>	YJL124C	Protein involved in mRNA degradation in cytoplasm	mRNA catabolism
<i>CBF1</i>	YJR060W	Helix-loop-helix protein that binds to promoters in <i>CDE1</i> and <i>MET</i> genes; required for chromosome stability	DNA/chromosome organization
<i>RAD27</i>	YKL113C	Single-stranded DNA endonuclease and 5'-3'-exonuclease, 5'-flap endonuclease	DNA/chromosome organization
<i>EAF7</i>	YNL136W	Subunit of the NuA4 histone acetyltransferase complex	DNA/chromosome organization
<i>RAD34</i>	YDR314C	Nucleotide excision repair	DNA repair
<i>YSA1</i>	YBR111C	Nucleosidediphosphate sugar hydrolase	Unknown
<i>CUE3</i>	YGL110C	Protein of unknown function; has CUE domain that may facilitate intramolecular monoubiquitination	Unknown
<i>YFR045W</i>	YFR045W	Protein with similarity to mitochondrial transporter family	Unknown
<i>YMR003W</i>	YMR003W	Protein of unknown function	Unknown
<i>YGR064W</i>	YGR064W	Protein of unknown function	Unknown
<i>YPL261C</i>	YPL261C	Protein of unknown function	Unknown
<i>YJL007C</i>	YJL007C	Protein of unknown function	Unknown
<i>YMR052C-a</i>	YMR052C-a	Protein of unknown function	Unknown

type and *sec14^{ts}* cells secreted a similar amount of protein. The pattern of proteins secreted was similar but not completely identical (Fig. 3B). As a control we monitored the amount of radiolabeled protein secreted from *sec18^{ts}* cells that possess a well characterized defect in secretion (Sec18 encodes the ATPase-*N*-ethylmaleimide-sensitive fusion protein that primarily regulates endoplasmic reticulum to Golgi transport). The *sec18^{ts}* cells secreted significantly less protein.

To determine whether the radiolabeled bands could be due to protein breakdown, or whether they represented a series of proteins, proteins secreted into the medium were acid-precip-

itated and separated by SDS-PAGE, and gels were stained with Coomassie Blue and silver nitrate. Like the secretion of [³⁵S]methionine/cysteine-labeled proteins, the banding pattern and amount were similar between wild type and *sec14^{ts}* cells (data not shown). Identity was determined by mass spectrometry and included Hsp150, Cts1, Scw4, Scw10, Exg1, Cis3, and Ygp1, proteins known to be secreted by *S. cerevisiae*.

Decreasing Sec14 function does not appear to diminish all vesicular transport routes from the *trans*-Golgi, but it affects specific vesicular transport pathways emanating from this organelle.

Sec14-mediated Vesicular Transport

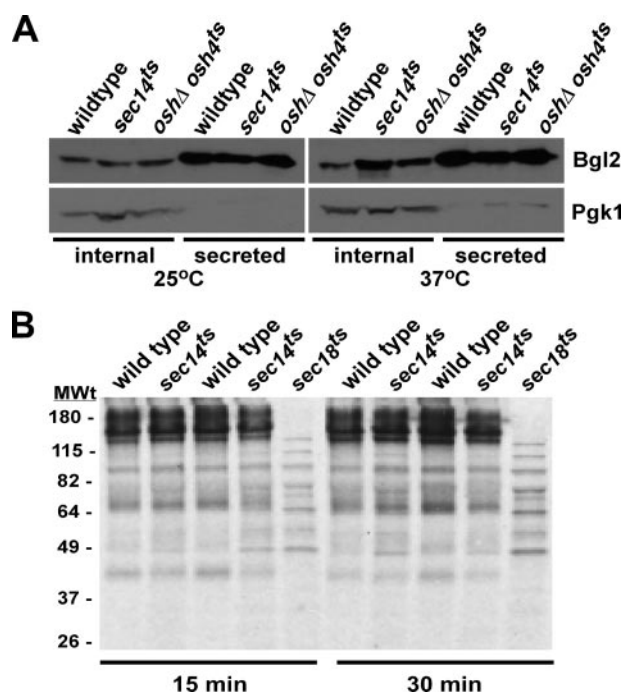


FIGURE 3. Cells with reduced Sec14 function do not have a major defect in bulk protein secretion. *A*, mid-log phase cells were incubated at 25 °C or shifted to 37 °C for 1 h. Cells were harvested and resuspended in 10 mM Na₃, 10 mM KF and incubated on ice. Cells were washed and resuspended in buffer containing 1.4 M sorbitol and zymolyase. Resulting spheroplasts were pelleted and proteins separated by SDS-PAGE. Bgl2 was detected by Western blot. *B*, wild type, *sec14^{ts}*, and *sec18^{ts}* cells (strains used were BY4741 (wild type), CMY505 (*sec14^{ts}*), X2180 (wild type), SF292-1A (*sec14^{ts}*), and SF282-1D (*sec18^{ts}*)) were grown to mid-log phase at 25 °C, shifted to 37 °C for 10 min, incubated with [³⁵S]methionine/cysteine for 10 min, and then with 10 mM unlabeled methionine/cysteine for 15 and 30 min at 37 °C. Proteins in the medium were separated by SDS-PAGE, and the gel was exposed to x-ray film.

GFP-Snc1 Trafficking Is Defective in Cells with Diminished Sec14 Function—Invertase transits through early endosomes on its way to the cell surface (35, 36). A possible explanation for the defect in delivery of invertase to the cell surface is that Sec14 is required for trafficking via endosomes. Consistent with this notion, genes isolated from the SGA screen (*YPT31*, *TRS33*, *TLG2*, and *VPS1*) regulate vesicular transport pathways through endosomes (Table 2). The endosome is a central organelle required for transit of a subset of proteins from the *trans*-Golgi to the plasma membrane, as well as trafficking of proteins from the plasma membrane back to the Golgi as well as the vacuole. To further assess if Sec14 regulated endosome requiring pathways we first monitored trafficking of the v-SNARE Snc1.

Snc1, the *S. cerevisiae* homologue of synaptobrevin, continually cycles between the plasma membrane and *trans*-Golgi. Snc1 is found in both low and high density Golgi-derived vesicles during transit to the plasma membrane (37), and thus the SNARE machinery is thought to be shared among vesicular transport pathways emanating from the *trans*-Golgi. Subsequently, Snc1 is transported from the plasma membrane back to the *trans*-Golgi via endosomes (36, 38). At steady state Snc1 is primarily found at the plasma membrane (34, 38), and consistent with prior studies GFP-Snc1 was primarily localized at the plasma membrane as well as a few punctate structures in the cytoplasm (endosomes and *trans*-Golgi) in wild type cells (Fig.

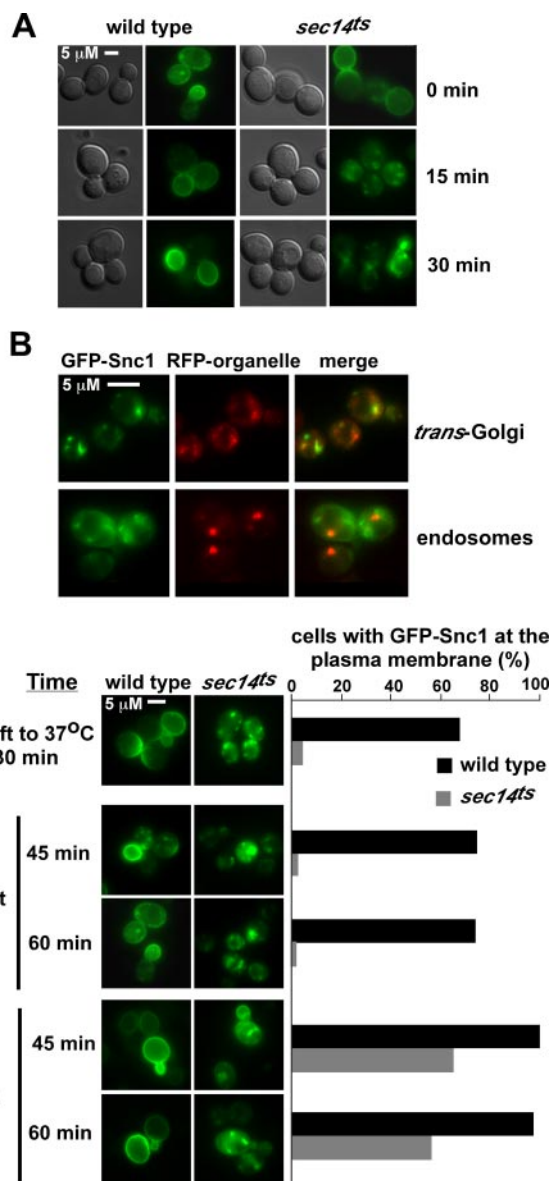


FIGURE 4. Sec14 regulates Snc1 cycling. *A*, wild type and *sec14^{ts}* cells expressing GFP-Snc1 were grown to early log phase at 25 °C; a portion was shifted to 37 °C, and live cells were imaged using DIC and fluorescence microscopy at the indicated time points. *B*, wild type *SEC14* gene was replaced with the *sec14^{ts}* allele in yeast strains where RFP was fused to the organellar markers *Chc1* (*trans*-Golgi) and *Snf7* (endosomes). These strains were transformed with the plasmid expressing GFP-Snc1, and colocalization of GFP-Snc1 was determined in *sec14^{ts}* cells after cells were shifted to 37 °C for 30 min. *C*, wild type and *sec14^{ts}* cells (strains BY4741 and CMY505) were shifted to 37 °C for 30 min resulting in GFP-Snc1 localization to the *trans*-Golgi in *sec14^{ts}* cells. Lat-A was added for 10 min to prevent GFP-Snc1 cycling back into the cell from the plasma membrane, and cells were grown for a further 15–30 min at 37 °C. Live cells were visualized using DIC and fluorescence microscopy. The number of cells from random fields (four fields from two separate experiments totaling at least 100 cells) was visually assessed for GFP-Snc1 at the plasma membrane using a double-blinded protocol.

4A). A similar localization was observed in *sec14^{ts}* cells grown at the permissive temperature for function of this allele (25 °C); however, when *sec14^{ts}* cells were shifted to the non-permissive temperature (37 °C), GFP-Snc1 rapidly localized to large intracellular punctate spots. Localization of GFP-Snc1 with organellar marker proteins revealed that in the *sec14^{ts}* cells shifted to 37 °C for 30 min GFP-Snc1 largely colocalized with the *trans*-

Golgi with a smaller fraction colocalizing with endosomes (Fig. 4B). GFP-Snc1 accumulation in endosomes indicates reduced endosome trafficking to the *trans*-Golgi.

Accumulation of GFP-Snc1 at the *trans*-Golgi could be due to either reduced fusion to or reduced transit from this organelle. To assess whether the GFP-Snc1 accumulating in the *trans*-Golgi upon reduction of function of Sec14 can exit this organelle, we treated cells with latrunculin-A (Lat-A). Lat-A blocks actin polymerization and blocks endocytosis from the plasma membrane to endosomes in *S. cerevisiae*. These conditions allowed us to monitor *trans*-Golgi to plasma membrane transport in the absence of recycling of GFP-Snc1 out of the plasma membrane. Cells were grown at 37 °C for 30 min to localize GFP-Snc1 to the *trans*-Golgi in *sec14^{ts}* cells, and Lat-A was added to inhibit endocytosis, and cells were grown for a further 15–30 min at 37 °C to determine whether GFP-Snc1 could exit the *trans*-Golgi and traffic to the plasma membrane. As expected, wild type cells exhibit only plasma membrane GFP-Snc1 after treatment with Lat-A. A significant number of the Lat-A-treated *sec14^{ts}* cells (58–67%) were able to transport GFP-Snc1 from the *trans*-Golgi to the plasma membrane (Fig. 4C). This is consistent with a Sec14-independent vesicular transport route from the *trans*-Golgi to the plasma membrane.

Endosome-dependent Routes from the Plasma Membrane Are Defective in Cells with Reduced Sec14 Function—Endosomes are also required for transport of vesicles from the plasma membrane to the vacuole. Ste3 is a mating factor receptor that, in the absence of mating factor, is transported from the plasma membrane via endosomes to the vacuole for degradation (39). Ste3 was observed in wild type cells at 25 °C with reduced levels at 37 °C (Fig. 5A). At increased temperature the rate of endocytosis is increased (40) resulting in the lower steady-state levels of Ste3 observed in wild type cells at 37 °C. In cells with defective Sec14, Ste3 was stabilized at 37 °C. Sec14 functions in the transport of Ste3 from the plasma membrane to the vacuole.

We also monitored internalization of the lipophilic dye FM4-64 as a separate assay to determine whether there were defects in endosome to vacuole trafficking upon reduction of function of Sec14. FM4-64 incorporates into the plasma membrane and traffics to the vacuole via early and late endosomes (40). In wild type cells FM4-64 was transported to the vacuole in a matter of minutes at 37 °C, whereas in *sec14^{ts}* cells shifted to 37 °C, FM4-64 accumulated in punctate spots in the cytoplasm and was unable to transit out of these punctate regions (Fig. 5B). These punctate structures colocalized with the endosomal marker Vps27-GFP (Fig. 5C). The inhibition of FM4-64 trafficking and stabilization of Ste3 upon inactivation of Sec14 is consistent with Sec14 regulating plasma membrane-derived endosome trafficking pathways.

Genetic Interactions of sec14^{ts} with Genes That Regulate Endosome Trafficking—Our data are consistent with Sec14 regulating endosome function, and genes isolated from our SGA screen participate in this process. A main strength of the SGA strategy is comprehensiveness; however, SGA screens can miss genetic interactions depending on several factors, including the allele used in the screen, the nature of the screen, the fitness of each single gene deletion strain, and the effect of the gene(s) on

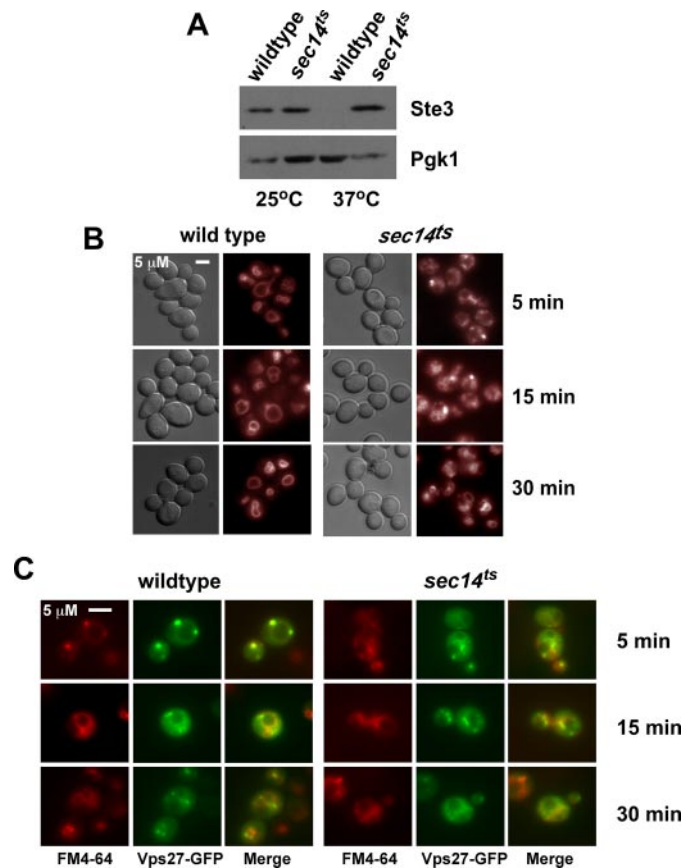


FIGURE 5. Sec14 is required for trafficking of endosomes to the vacuole. A, wild type and *sec14^{ts}* MAT α cells (BY4742 and CMY506) were grown to mid-log phase at 25 °C and shifted to 37 °C for 2 h. Proteins were extracted, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies for Ste3 and Pgk1. B, wild type (BY4741) and *sec14^{ts}* (CMY505) cells were grown to mid-logarithmic phase at 25 °C, shifted to 37 °C for 15 min, labeled for 2 min with 40 μ M FM4-64, and incubated post-label in fresh pre-warmed 37 °C medium for the indicated times. Live cells were visualized using DIC and fluorescence microscopy. C, FM4-64 was colocalized with the endosome marker Vps27-GFP at various time points after the shift to 37 °C.

mating, sporulation, and the transition from spore to growing cell (41). Therefore, we tested all nonessential genes involved in regulation of tethering and fusion of endosome-derived vesicles at the *trans*-Golgi for aggravating effects on growth of *sec14^{ts}* cells (see Fig. 1B).

The wild type *SEC14* gene was replaced with the *sec14^{ts}* allele in an isogenic set of strains containing inactivated genes that regulate GARP-mediated fusion from endosome to the *trans*-Golgi, including *VPS51*, *VPS52*, *VPS53*, *VPS54*, *VPS45*, *YPT6*, *RIC1*, *RGPI*, *ARL1*, *ARL3*, or *SYS1*. Inactivation of any of these genes in *sec14^{ts}* cells decreased growth at 35 and 33 °C compared with cells containing the *sec14^{ts}* allele alone, except for cells containing an inactivated *RGPI* gene that was inviable at any temperature (Fig. 6).

Inactivation of *YPT31* was found by our SGA screen to aggravate growth of *sec14^{ts}* cells. Ypt31/Ypt32 and the TRAPP II complex regulate both export from the *trans*-Golgi to the plasma membrane and endosome trafficking to the *trans*-Golgi (26, 31, 32). Inactivation of any of the non-essential subunits of the TRAPP complexes, including

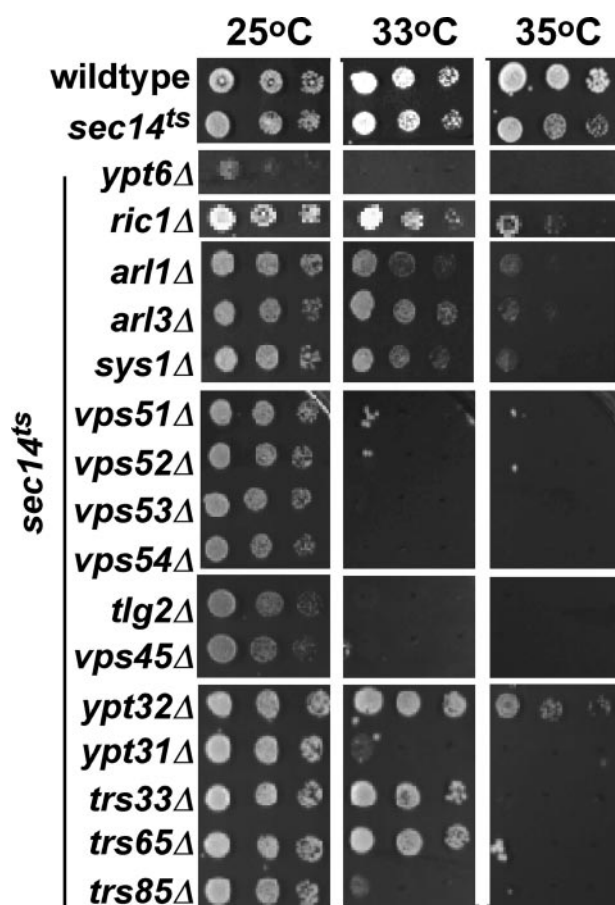


FIGURE 6. **Tethering complex defects aggravate Sec14 function.** Isogenic mutant strains with the indicated genotypes were grown to mid-log phase in synthetic complete medium at 25 °C, and identical numbers of cells were serially diluted, spotted onto agar plates, and grown for 3 days at the indicated temperatures.

TRs65 which is specific to the TRAPP II complex, aggravated growth of *sec14^{ts}* cells (Fig. 6).

Inactivation of *YPT32* did not aggravate growth of *sec14^{ts}* cells. Ypt32 is found in cells at lower levels than Ypt31 (42), and thus inactivation of the *YPT32* gene may have its phenotype masked by the Ypt31 still present in the cell. The converse would not be true as the low level of Ypt32 could not buffer the effect of inactivation of the *YPT31* gene.

For all of the genes tested, growth of isogenic strains containing inactivating mutations for each gene in the presence of the wild type *SEC14* gene was determined at 35, 33, and 25 °C. All grew at rates similar to wild type except strains with inactivated *TRS85*, *YPT6*, or *RIC1* genes that displayed moderately reduced growth at 35 °C. However, the combination of the *sec14^{ts}* allele along with inactivation of *TRS85*, *YPT6*, or *RIC1* resulted in much more severe growth defects than inactivation of any one gene alone (data not shown). Combined, the cell biology and genetic evidence clearly shows that Sec14 regulates endosome trafficking from the plasma membrane to both the *trans*-Golgi and the vacuole.

Specific Lipid Ligands Regulate Discrete Vesicular Transport Processes—Sec14 extracts PC and PI from membranes, and this activity is essential for its function in cells (4, 10, 43). A mutant version of Sec14 (Sec14^{K66A,K239A} known as Sec14-PC) that

transfers PC with much higher affinity than PI *in vitro* was found to restore growth and secretion to cells lacking Sec14 function (7, 43). Yeast contain five other Sec14 homologues (Sfh1–5) capable of PI (but not PC) transfer, and overexpression of two of these, Sfh2 and Sfh4, restored growth and secretion to cells lacking Sec14 function.

To facilitate analysis of roles for PC *versus* PI binding by Sec14, we transformed plasmids expressing wild type Sec14, the Sec14-PC allele, or Sfh4 into the strains containing the *sec14^{ts}* allele in combination with every gene deletion determined in this study to diminish Sec14-dependent growth. If there are separate functions for PC and PI binding by Sec14, then a subset may be rescued by only one or more of wild type Sec14, Sec14-PC and Sfh4.

Growth of *sec14^{ts}* cells lacking *VPS51–53*, three of the four nonessential components of the endosome to Golgi tethering complex GARP, required Sec14 or Sfh4, whereas Sec14-PC was insufficient (Fig. 7). Growth of cells inactivated for the fourth member of the GARP complex, *VPS54*, was only restored by wild type Sec14. Inactivation of the GARP complex as a whole requires Sec14 that can exchange PC and PI *in cis* within the same Sec14 molecule.

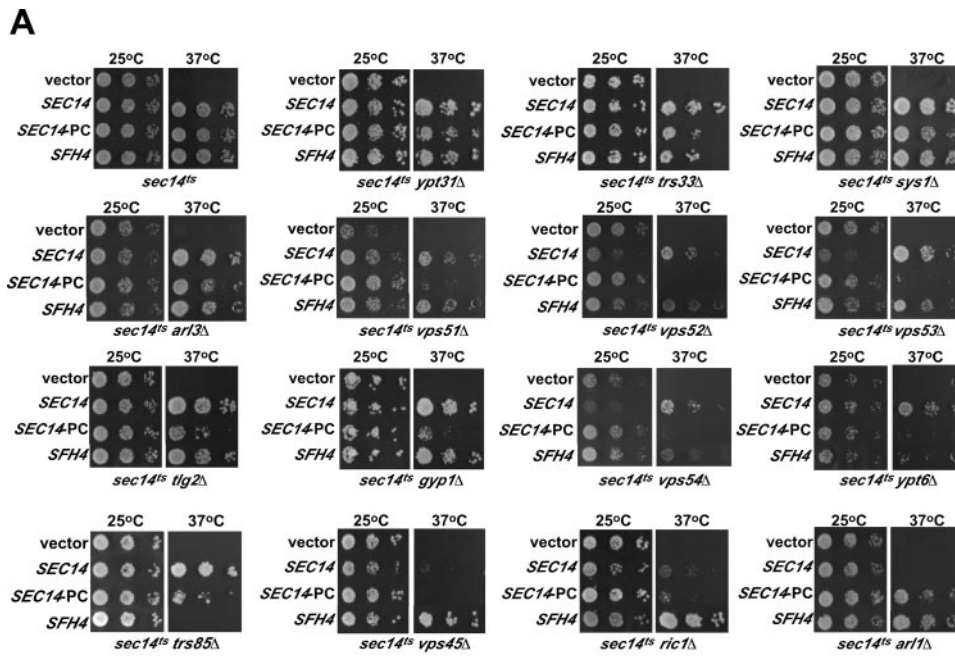
Endosome to Golgi fusion by the GARP complex is facilitated by the Rab Ypt6, and *sec14^{ts}* cells lacking *YPT6* were similar to those lacking *VPS54* in that growth was supported only by wild type Sec14 but not by Sec14-PC or Sfh4 (Fig. 7). Ypt6 is activated by the GEF Ric1, and growth of *sec14^{ts}* cells with inactivated *RIC1* was supported by Sfh4 but not by wild type Sec14 or Sec14-PC. A similar *sec14^{ts}* rescue phenotype associated with loss of *RIC1* was observed for loss of function of *VPS45*. Vps45 is required for the t-SNARE Tlg2 to tether the GARP complex to the Golgi. The *sec14^{ts}* cells with inactivated *TLG2* could grow normally when expressing wild type Sec14 or Sfh4, with some growth observed if Sec14-PC was expressed.

The second complex capable of activating GARP for fusion of endosomes with the Golgi is mediated by Arl1, Arl3, and Sys1. Growth was restored upon inactivation of *ARL3* or *SYS1* in *sec14^{ts}* cells by Sec14, Sec14-PC, and Sfh4 (Fig. 7). *ARL1* was unique in that its inactivation in *sec14^{ts}* cells was not restored by wild type Sec14, but it was restored by either Sec14-PC or Sfh4. The two pathways that mediate GARP complex fusion of endosomes with the Golgi have specific lipid ligand occupancy requirements by Sec14. Growth of *sec14^{ts}* cells lacking *YPT31* or *TRS33* was restored by Sec14, Sec14-PC, or Sfh4, whereas growth of *sec14^{ts}* cells with inactivated *TRS85* required wild type Sec14 or Sec14-PC (although growth was restored to a lesser extent).

Growth of *sec14^{ts}* cells lacking the GAP Gyp1 (has specificity for several small G proteins *in vitro*, and the essential Rab Ypt1 *in vivo*; with Ypt1 activity activated by the TRAPP complex) was restored by wild type Sec14, Sfh4, and to a lesser extent Sec14-PC. Processes influenced by Sec14 function are differentially affected depending on Sec14 lipid ligand occupancy.

DISCUSSION

Sec14 Regulates Specific *trans*-Golgi Export Pathways—Sec14 was known to be required for secretion through the endosome-



B Restoration of Growth at 37°C

Plasmid	Inactivated Gene
SEC14, SEC14-PC, SFH4	YPT31, TRS33, SYS1, ARL3, GYP1, TLG2
SEC14, SFH4	VPS51, VPS52, VPS53,
SEC14	VPS54, YPT6, TRS85
SEC14-PC, SFH4	ARL1
SFH4	VPS45, RIC1

FIGURE 7. Regulation of specific vesicular transport processes based on Sec14 lipid ligand occupancy. A, isogenic mutant strains with the indicated genotypes transformed with low copy plasmids expressing Sec14, Sec14-PC, or a high copy plasmid expressing Sfh4 were grown to mid-log phase in synthetic complete medium at 25 °C, and identical numbers of cells were serially diluted, spotted onto agar plates, and grown for 3 days at the indicated temperatures. B, tabulation of the ability of Sec14, Sec14-PC, and Sfh4 to rescue cell growth of *sec14^{ts}* cells with the indicated inactivated genes.

dependent route followed by invertase and acid phosphatase. In this study we demonstrate that Sec14 also regulates the endosome-independent route from the *trans*-Golgi to the plasma membrane used by Bgl2. Moreover, not all secretion was inhibited upon diminution of Sec14 function as many proteins were still secreted at a similar level to wild type, including Hsp150, Cts1, Scw4, Scw10, Exg1, Cis3, and Ygp1. This implies that there is at least one more route from the *trans*-Golgi to the plasma membrane, and it is independent of Sec14 (Fig. 8).

The v-SNARE Snc1 traffics from the *trans*-Golgi to the plasma membrane via both the endosome-dependent and -independent routes (35, 36). Decreasing Sec14 function resulted in ~50% of Snc1 still being able to traffic from the *trans*-Golgi to the plasma membrane, implying Snc1 may also transit to the plasma membrane by the route used by Hsp150, Cts1, Scw4, Scw10, Exg1, Cis3, and Ygp1. This is consistent with the idea

that the SNARE machinery required for secretion is shared by the *trans*-Golgi to plasma membrane routes.

Regulation of Endosome Trafficking by Sec14—We also determined in this study that Sec14 was required for trafficking from the plasma membrane to the vacuole via endosomes. FM4-64 internalization was inhibited at the endosome stage of transport, and consistent with this Ste3 was stabilized in cells with diminished Sec14 function. Indeed, trafficking of FM4-64 was dramatically reduced within 5 min of reduction of Sec14 function. These findings imply a role for Sec14 in regulation of endosome trafficking from the plasma membrane to the vacuole.

We observed that inactivation of Sec14 resulted in the accumulation of a portion of GFP-Snc1 in endosomes implying a defect in endosome to *trans*-Golgi transport. We also present substantive genetic evidence pointing to an important role for Sec14 in this pathway. Cells with defective Sec14 function were further impaired for growth by inactivation of nonessential components of the GARP or TRAPP II complexes and their regulators, including Rabs, Arf-like proteins, and their GEFs. In total, this study has revealed that endosome trafficking is likely a major role for Sec14 (Fig. 8).

Sec14 Affects Phospholipid-mediated Regulation of Vesicular

Trafficking—Sec14 binds PC and PI and affects the metabolism of both these ligands, although precisely how is not known (1–6, 8, 9, 11, 44, 45). It is believed that occupancy of Sec14 by PC regulates PC synthesis and turnover, whereas occupancy by PI regulates PI-4P levels (2, 4, 16, 45, 46). Two separate SGA screens using different temperature-sensitive alleles of the Golgi PI 4-kinase *PIK1* determined that inactivation of many of the genes in the current study (as aggravating growth of *sec14^{ts}* cells) exacerbated *Pik1*-dependent vesicular trafficking from the Golgi, including *YPT31* (but not *YPT32*), *TRS33*, *TRS65*, *ARL1*, *SYS1*, *VPS51*, *VPS54*, *YPT6*, *RIC1*, and *VPS1* (47, 48). As inactivation of a similar set of genes was found to inhibit growth of *pik1^{ts}* and *sec14^{ts}* cells (this study), and the fact that Golgi PI-4P levels are reduced in cells with diminished *Pik1* and Sec14 function (2, 11, 44), it is likely that inactivation of these genes inhibits growth when Golgi PI-4P levels are reduced. Indeed,

Sec14-mediated Vesicular Transport

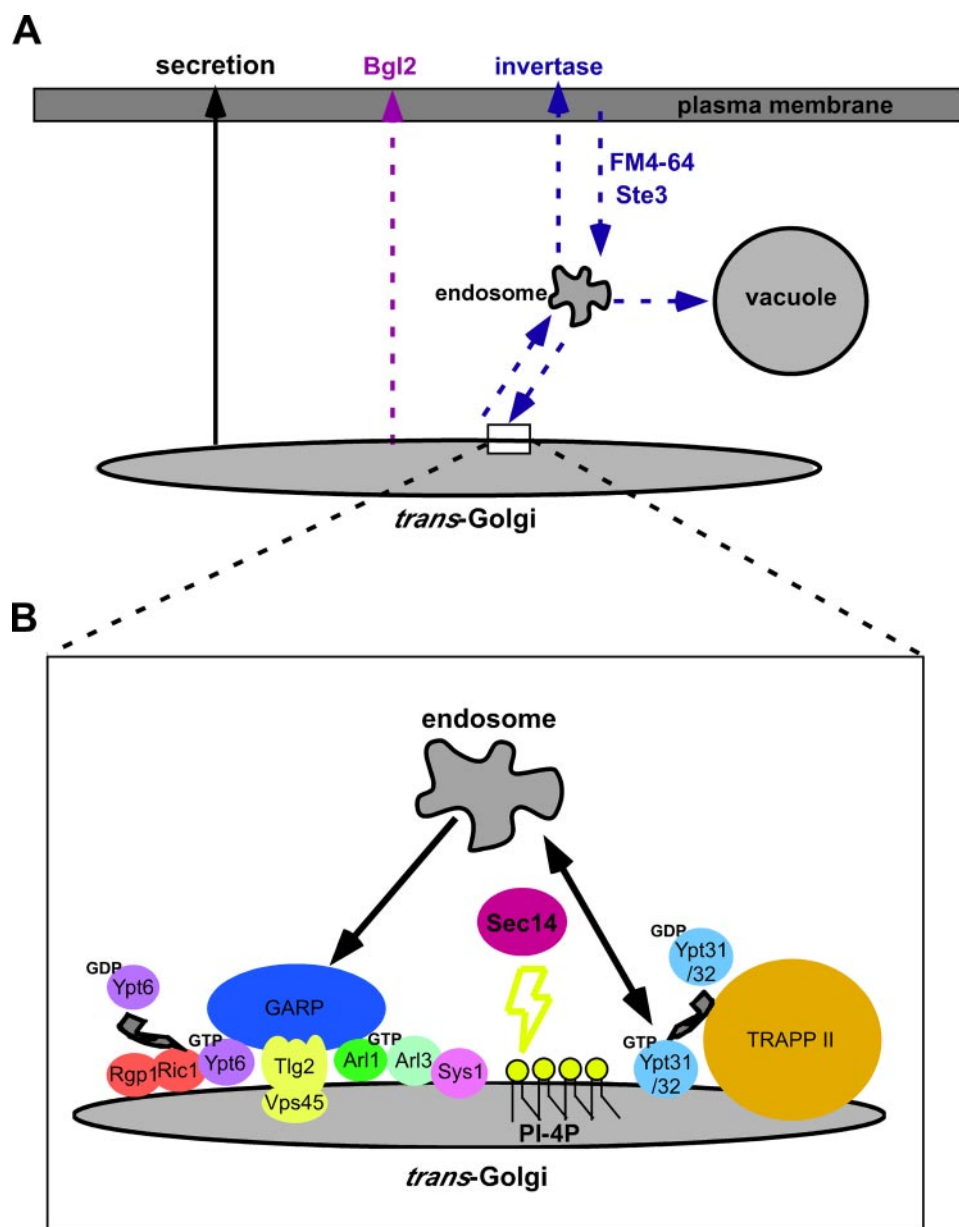


FIGURE 8. Regulation of vesicular transport by Sec14. *A*, Sec14 facilitates nonendosomal and endosomal transport pathways from the *trans*-Golgi to the plasma membrane (pink and blue dotted lines). At least one other route from the *trans*-Golgi to the plasma membrane is not facilitated by Sec14. Transport from the plasma membrane to both the *trans*-Golgi and vacuole via endosomes is also regulated by Sec14. *B*, genetic evidence suggest Sec14 regulates endosome trafficking to the *trans*-Golgi via all major routes into this organelle by regulating Golgi PI-4P levels. Many of the gene deletions that aggravate growth of *sec14^{ts}* cells also aggravate growth of cells containing a temperature-sensitive allele of the Golgi PI-4-kinase, *PIK1*; Golgi PI-4P levels are decreased in *sec14^{ts}* and *pik1^{ts}* cells (2). This implies that regulation of Golgi PI-4P levels by Sec14 is necessary for regulation of import and export of vesicles from the *trans*-Golgi. The processes affected could include GARP complex (Vps51–54) binding to the t-SNARE Tlg2 that, together with Vps45, aid in targeting and fusion of endosome-derived vesicles with the *trans*-Golgi. Fusion is promoted by interaction of the GARP complex with the Rab GTPase Ypt6 that is in turn activated by the heterodimeric GEF Ric1/Rgp1. GARP also binds the Arf-like GTPase Arl1 that is localized to the Golgi by a second Arf-like GTPase Arl3 that interacts with the Golgi resident protein Sys1. The function of the Rab GTPase Ypt31 appears to be to regulate trafficking both in and out of the Golgi. Ypt31 is activated by the 10-subunit TRAPP II complex of which Trs33, Trs65, and Trs85 are nonessential. Reduction of Sec14 function results in aberrant Golgi PI-4P levels directly confounding vesicle import and export. The defects in endosome trafficking from the plasma membrane to the vacuole could be due to improper endosome trafficking because of miscommunication between Rab cascades into and out of the *trans*-Golgi.

the majority of the genes we identified in the *sec14^{ts}* SGA screen could be rescued by wild type Sec14 or Sfh4, suggesting that they are dependent on the function of PI-bound Sec14.

Specifically, in the absence of Vps51–53, wild type Sec14 and Sfh4 restored life but Sec14-PC did not. This implies that nor-

mal phosphoinositide metabolism is required in the absence of these components of the GARP complex. Strains lacking Vps54, Ypt6, or Trs85 only grew when transformed with a plasmid expressing wild type Sec14 indicating that both PC and phosphoinositide metabolism have to be intact and/or Sec14 has to exchange PI and PC in *cis* within the same protein when these functions are absent. Remarkably, in the absence of some proteins (Arl1, Vps45, and Ric1) wild type Sec14 did not restore growth, with growth of cells lacking Arl1 restored by Sec14-PC or Sfh4, and growth of cells lacking Vps45 or Ric1 restored only by Sfh4. Although Sec14 and its analogues were expressed from their own promoters, they were plasmid-borne, with Sec14 and Sec14-PC on low copy plasmids (2–3 copies per cell) and Sfh4 on a high copy 2- μ m plasmid (~20 copies per cell). The inability of plasmid-borne Sec14 to restore growth to cells lacking Arl1, Vps45, or Ric1 could be due to inhibition of growth upon increased dose of a Sec14 that exchanges PI and PC in *cis* and regulates both PC and phosphoinositide metabolism, whereas either one of these functions alone is not growth-inhibitory. Regulation of components of these pathways by specific lipids or membrane properties needs to be investigated to allow for a more precise determination of how lipid metabolism interfaces with and regulates vesicular trafficking.

Sec14 Domain and Regulation of GTPases—The Sec14 domain of human p50RhoGAP is required for its association with endosomes and for its interaction with the small GTPase Rab11 (20). Rab11 associates with endosomes and the *trans*-Golgi and regulates trafficking through these organelles. Recruitment of p50RhoGAP to activated Rab11 does not result in GAP activity

toward Rab11 but instead is thought to temporally coordinate Rab and Rho signaling events. Human Rab11 is the homologue of *S. cerevisiae* Ypt31, and interestingly Rab11 directly interacts with mammalian PI 4-kinase β (the homologue of *S. cerevisiae* Pik1). Abolishing the Rab11-PI kinase β interaction

resulted in mislocalization of Rab11 implying a conserved role for PI-4P in regulation of Rab signaling as inactivation of *YPT31* affected growth and vesicular transport in *S. cerevisiae* cells with reduced Pik1 or Sec14 function (7).

Indeed, a similar coordination in Rab signaling appears to operate in *S. cerevisiae*. Inactivation of the *S. cerevisiae* Rab *YPT6* was also aggravating for growth of *sec14^{ts}* cells. GTP-bound Ypt6 physically interacts with the GAP for Ypt31, Gyp2, while exhibiting no GAP activity toward Ypt6 (47). Regulation of timing of the Ypt31 and Ypt6 Rab cascades has been proposed to facilitate transport between endosomes and the *trans*-Golgi, similar to coordination of mammalian Rab and Rho pathways. There may be a conserved role for the Sec14 domain in regulation of Rab/Rho signaling cascades.

REFERENCES

- Patton-Vogt, J. L., Griac, P., Sreenivas, A., Bruno, V., Dowd, S., Swede, M. J., and Henry, S. A. (1997) *J. Biol. Chem.* **272**, 20873–20883
- Fairn, G. D., Curwin, A. J., Stefan, C. J., and McMaster, C. R. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104**, 15352–15357
- Henneberry, A. L., Lagace, T. A., Ridgway, N. D., and McMaster, C. R. (2001) *Mol. Biol. Cell* **12**, 511–520
- Fernandez-Murray, J. P., and McMaster, C. R. (2005) *J. Biol. Chem.* **280**, 8544–8552
- Sreenivas, A., Patton-Vogt, J. L., Bruno, V., Griac, P., and Henry, S. A. (1998) *J. Biol. Chem.* **273**, 16635–16638
- Cleves, A. E., McGee, T. P., Whitters, E. A., Champion, K. M., Aitken, J. R., Dowhan, W., Goebel, M., and Bankaitis, V. A. (1991) *Cell* **64**, 789–800
- de Graaf, P., Zwart, W. T., van Dijken, R. A., Deneka, M., Schulz, T. K., Geijsen, N., Coffey, P. J., Gadella, B. M., Verkleij, A. J., van der Sluijs, P., and van Bergen en Henegouwen, P. M. (2004) *Mol. Biol. Cell* **15**, 2038–2047
- Xie, Z., Fang, M., and Bankaitis, V. A. (2001) *Mol. Biol. Cell* **12**, 1117–1129
- Xie, Z., Fang, M., Rivas, M. P., Faulkner, A. J., Sternweis, P. C., Engebrecht, J. A., and Bankaitis, V. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12346–12351
- Schaaf, G., Ortlund, E. A., Tyeryar, K. R., Mousley, C. J., Ile, K. E., Garrett, T. A., Ren, J., Woolls, M. J., Raetz, C. R., Redinbo, M. R., and Bankaitis, V. A. (2008) *Mol. Cell* **29**, 191–206
- Li, X., Rivas, M. P., Fang, M., Marchena, J., Mehrotra, B., Chaudhary, A., Feng, L., Prestwich, G. D., and Bankaitis, V. A. (2002) *J. Cell Biol.* **157**, 63–77
- Bankaitis, V. A., Aitken, J. R., Cleves, A. E., and Dowhan, W. (1990) *Nature* **347**, 561–562
- Bankaitis, V. A., Malehorn, D. E., Emr, S. D., and Greene, R. (1989) *J. Cell Biol.* **108**, 1271–1281
- Sha, B., Phillips, S. E., Bankaitis, V. A., and Luo, M. (1998) *Nature* **391**, 506–510
- Chang, H. J., Jones, E. W., and Henry, S. A. (2002) *Genetics* **162**, 29–43
- Curwin, A. J., and McMaster, C. R. (2008) *Future Lipidol.* **3**, 399–410
- D'Angelo, I., Welte, S., Bonneau, F., and Scheffzek, K. (2006) *EMBO Rep.* **7**, 174–179
- Kostenko, E. V., Mahon, G. M., Cheng, L., and Whitehead, I. P. (2005) *J. Biol. Chem.* **280**, 2807–2817
- Phillips, S. E., Vincent, P., Rizzieri, K. E., Schaaf, G., Bankaitis, V. A., and Gaucher, E. A. (2006) *Crit. Rev. Biochem. Mol. Biol.* **41**, 21–49
- Sirokmany, G., Szidonya, L., Kaldi, K., Gaborik, Z., Ligeti, E., and Geiszt, M. (2006) *J. Biol. Chem.* **281**, 6096–6105
- Ueda, S., Kataoka, T., and Satoh, T. (2004) *Cell. Signal.* **16**, 899–906
- Welte, S., Fraterman, S., D'Angelo, I., Wilm, M., and Scheffzek, K. (2007) *J. Mol. Biol.* **366**, 551–562
- Panic, B., Whyte, J. R., and Munro, S. (2003) *Curr. Biol.* **13**, 405–410
- Behnia, R., Panic, B., Whyte, J. R., and Munro, S. (2004) *Nat. Cell Biol.* **6**, 405–413
- Setty, S. R., Strohlic, T. I., Tong, A. H., Boone, C., and Burd, C. G. (2004) *Nat. Cell Biol.* **6**, 414–419
- Cai, H., Zhang, Y., Pypaert, M., Walker, L., and Ferro-Novick, S. (2005) *J. Cell Biol.* **171**, 823–833
- Fairn, G. D., and McMaster, C. R. (2005) *Methods (Orlando)* **36**, 102–108
- Tong, A. H., Lesage, G., Bader, G. D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G. F., Brost, R. L., Chang, M., Chen, Y., Cheng, X., Chua, G., Friesen, H., Goldberg, D. S., Haynes, J., Humphries, C., He, G., Hussein, S., Ke, L., Krogan, N., Li, Z., Levinson, J. N., Lu, H., Menard, P., Munyana, C., Parsons, A. B., Ryan, O., Tonikian, R., Roberts, T., Sdicu, A. M., Shapiro, J., Sheikh, B., Suter, B., Wong, S. L., Zhang, L. V., Zhu, H., Burd, C. G., Munro, S., Sander, C., Rine, J., Greenblatt, J., Peter, M., Bretscher, A., Bell, G., Roth, F. P., Brown, G. W., Andrews, B., Bussey, H., and Boone, C. (2004) *Science* **303**, 808–813
- Goldstein, A., and Lampen, J. O. (1975) *Methods Enzymol.* **42**, 504–511
- Kozminski, K. G., Alfaro, G., Dighe, S., and Beh, C. T. (2006) *Traffic* **7**, 1224–1242
- Chen, S. H., Chen, S., Tokarev, A. A., Liu, F., Jedd, G., and Segev, N. (2005) *Mol. Biol. Cell* **16**, 178–192
- Morozova, N., Liang, Y., Tokarev, A. A., Chen, S. H., Cox, R., Andrejic, J., Lipatova, Z., Sciorra, V. A., Emr, S. D., and Segev, N. (2006) *Nat. Cell Biol.* **8**, 1263–1269
- Liang, Y., Morozova, N., Tokarev, A. A., Mulholland, J. W., and Segev, N. (2007) *Mol. Biol. Cell* **18**, 2533–2541
- Lafourcade, C., Galan, J. M., Gloor, Y., Haguenaer-Tsapis, R., and Peter, M. (2004) *Mol. Cell Biol.* **24**, 3815–3826
- Harsay, E., and Schekman, R. (2002) *J. Cell Biol.* **156**, 271–285
- Gurunathan, S., David, D., and Gerst, J. E. (2002) *EMBO J.* **21**, 602–614
- Lustgarten, V., and Gerst, J. E. (1999) *Mol. Cell Biol.* **19**, 4480–4494
- Robinson, M., Poon, P. P., Schindler, C., Murray, L. E., Kama, R., Gabriely, G., Singer, R. A., Spang, A., Johnston, G. C., and Gerst, J. E. (2006) *Mol. Biol. Cell* **17**, 1845–1858
- Chen, L., and Davis, N. G. (2000) *J. Cell Biol.* **151**, 731–738
- Vida, T. A., and Emr, S. D. (1995) *J. Cell Biol.* **128**, 779–792
- Tong, A. H., and Boone, C. (2006) *Methods Mol. Biol.* **313**, 171–192
- Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) *Nature* **425**, 686–691
- Phillips, S. E., Sha, B., Topalof, L., Xie, Z., Alb, J. G., Klenchin, V. A., Swigart, P., Cockcroft, S., Martin, T. F., Luo, M., and Bankaitis, V. A. (1999) *Mol. Cell* **4**, 187–197
- Hama, H., Schnieders, E. A., Thorner, J., Takemoto, J. Y., and DeWald, D. B. (1999) *J. Biol. Chem.* **274**, 34294–34300
- Skinner, H. B., McGee, T. P., McMaster, C. R., Fry, M. R., Bell, R. M., and Bankaitis, V. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 112–116
- Mousley, C. J., Tyeryar, K. R., Vincent-Pope, P., and Bankaitis, V. A. (2007) *Biochim. Biophys. Acta* **1771**, 727–736
- Sciorra, V. A., Audhya, A., Parsons, A. B., Segev, N., Boone, C., and Emr, S. D. (2005) *Mol. Biol. Cell* **16**, 776–793
- Demmel, L., Gravert, M., Ercan, E., Habermann, B., Muller-Reichert, T., Kukhtina, V., Haucke, V., Baust, T., Sohrmann, M., Kalaidzidis, Y., Klöse, C., Beck, M., Peter, M., and Walch-Solimena, C. (2008) *Mol. Biol. Cell* **19**, 1991–2002
- Tong, A. H., Evangelista, M., Parsons, A. B., Xu, H., Bader, G., Pagé, N., Robinson, M., Raghibzadeh, S., Hogue, C. W., Bussey, H., Andrews, B., Tyers, M., and Boone, C. (2001) *Science* **294**, 2364–2368
- Reggiori, F., Wang, C. W., Stromhaug, P. E., Shintani, T., and Klionsky, D. J. (2003) *J. Biol. Chem.* **278**, 5009–5020