Journal of Cell Science 113, 4545-4555 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS1891

Identification of Rgp1p, a novel Golgi recycling factor, as a protein required for efficient localization of yeast casein kinase 1 to the plasma membrane

Heather R. Panek[1], Elizabeth Conibear[2], Joshua D. Bryan[1], Richard T. Colvin[1], Chan D. Goshorn[1] and Lucy C. Robinson[1,*]

[1]Department of Biochemistry, Louisiana State University Health Science Center, Shreveport, LA 71130, USA
[2]Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA
*Author for correspondence: (e-mail: lrobin@lsumc.edu)

Accepted 18 October; published on WWW 16 November 2000

SUMMARY

The Yck1p and Yck2p casein kinase 1 isoforms in yeast are essential peripheral plasma membrane-associated protein kinases with roles in endocytosis, cellular morphogenesis and cytokinesis. The membrane targeting of these cytoplasmically oriented protein kinases requires normal secretory pathway function, but specific targeting factors have not been identified. To learn more about Yckp targeting, we characterized mutations that cause synthetic lethality with impairment of Yck function. We report here that these include mutations in two gene products that function in protein trafficking. One of these is the previously described t-SNARE Tlg2p, which participates in recycling of proteins to the Golgi. The other is a previously uncharacterized protein, Rgp1p, which appears to have a similar function. Loss of either Tlg2p or Rgp1p causes inefficient localization of Yck2p, suggesting that its transport may be directed, in part, by a targeting factor that must be recycled back to the Golgi.

Key words: Protein trafficking, Casein kinase 1, Synthetic interaction, t-SNARE, GFP

INTRODUCTION

Characterization of casein kinase I (CKI) in the budding yeast *Saccharomyces cerevisiae* has demonstrated that this activity is required for normal cell growth (Hoekstra et al., 1991; Robinson et al., 1992; Wang et al., 1992; Wang et al., 1996). *YCK1* and *YCK2* encode functionally redundant, plasma membrane-localized CKI proteins (Robinson et al., 1992; Wang et al., 1992; Vancura et al., 1994). Deletion of both *YCK1* and *YCK2* results in aberrant cellular morphology and loss of viability (Robinson et al., 1992; Wang et al., 1992). A *yck*^{ts} strain, which has a temperature-sensitive allele of *YCK2* (*yck2-2*^{ts}) and a deletion of *YCK1*, retains 60% of wild-type kinase activity and displays relatively normal morphology at permissive temperature (Robinson et al., 1993). However, at the restrictive temperature, *yck*^{ts} cells show less than 25% of wild-type levels of kinase activity and undergo aberrant cell cycles with hyperpolarized growth and without cell division, resulting in dramatically elongated buds (Robinson et al., 1993). The elongated cell phenotype suggests that Yck could be required for regulating the switch from polarized to isotropic growth at mitosis. Yck2p is highly enriched in membranes of emerging and small buds, and at the mother-bud junction at the time of cytokinesis and cell separation (Robinson et al., 1999). Thus, Yck2p is correctly located to have a role in regulation of growth polarity. Moreover, the Yck protein kinases are required for internalization of plasma membrane proteins (Panek et al., 1997; Hicke et al., 1998). This effect is observed for a *yck*^{ts} strain even at permissive temperature, suggesting that wild-type kinase levels are necessary for efficient endocytosis. Together the phenotypic and endocytic defects point to a possible role for Yck activity in regulated cell surface remodeling.

Localization of Yck2p to the plasma membrane requires a functioning secretory pathway (our unpublished results). The secretory pathway is a highly regulated process involving proteins that are conserved among eukaryotes (Ferro-Novick and Jahn, 1994; Rothman, 1994). In yeast, vesicle-mediated protein transport to the plasma membrane has been extensively studied (reviewed in Kaiser et al., 1997). However, much of what is known about trafficking is based on proteins that enter the secretory pathway after translocation into the endoplasmic reticulum (ER). Yck2p has no signal sequence for ER translocation and is cytoplasmically oriented, arguing against an alternative method of entry into this pathway. Yck2p may interact with the cytoplasmic surface of secretory membranes by virtue of its presumed C-terminal isoprenyl modification, since removal of the consensus sequence for geranylgeranylation results in the inability both to associate with the plasma membrane (Vancura et al., 1994) and to interact with secretory membranes (our unpublished results). Yck2p interaction with membranes does not appear to be random, since association appears to proceed temporally from ER to Golgi to the plasma membrane. Therefore, specific trafficking factors linking Yck2p

to internal membranes may direct transport of the kinase to the cell surface. Prenylated proteins are found in diverse membranes, but factors required for trafficking to those membranes and for specificity of localization have not been identified (Zhang and Casey, 1996; Sinensky, 2000). A previous genetic screen using the *yck^{ts}* mutant identified an AP-3 adaptor complex that acts in vesicular trafficking (Panek et al., 1997). However, loss of this complex has no effect on Yck2p localization (our unpublished results). To identify proteins acting in pathways that affect Yck activity or localization, we have isolated mutations that exacerbate the growth defect of a *yck^{ts}* strain. Using this synthetic enhancement screen, we identified two proteins that function in protein trafficking and indirectly in Yck2p trafficking. One of these, Tlg2p, is a member of the t-SNARE family of vesicle trafficking proteins (Holthuis et al., 1998a); the other is a novel protein encoded by the *RGP1* gene (Aguilera et al., 1990).

MATERIALS AND METHODS

Yeast culture and manipulation

Yeast strains (Table 1) were grown on standard rich (YEPD) or synthetic media (Sherman et al., 1986) at temperatures as indicated. Formamide, which destabilizes noncovalent bonds, was added to 3% in YEPD (Aguilera, 1994). Standard techniques were used for phenotypic and genetic analyses (Sherman et al., 1986).

Yeast transformation was by the lithium acetate procedure (Ito et al., 1983), modified by buffering all solutions with Tris-EDTA at pH 8.0. All gene disruptions were constructed in vitro and introduced into diploid strains by one-step gene replacement (Rothstein, 1983). Strains confirmed by genomic Southern analysis to carry a disrupted allele were sporulated and haploid progeny were analyzed for cosegregation of the disruption marker and associated growth traits.

DNA manipulation and analysis

Standard techniques were used for DNA manipulation (Maniatis et al., 1982; Sambrook et al., 1989). Restriction and modification enzymes were used as recommended by the manufacturer (Promega, Madison, WI, USA). The Sequenase enzyme (US Biochemical, Cleveland, OH, USA) and dideoxy chain termination method (Sanger et al., 1977) were used with synthetic oligonucleotide primers (Integrated DNA Technologies, Coralville, IA, USA) to obtain DNA sequence from double-stranded templates. For cloning, the low-copy YCp50-based libraries of D. S. Conklin (unpublished) were used. DNA sequences were analyzed using the Genetics Computer Group (GCG; Madison, WI, USA) software package. Database searches with predicted protein sequences were carried out from the *Saccharomyces* Genome Database, using the Blast algorithm (Altschul et al., 1990).

Isolation of *yck^{ts}* synthetic enhancement mutations

To isolate *yck^{ts}* synthetic enhancement mutations, an *ade2 ade3*

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

Strain	Genotype	Source
HPY15	*MAT***a** *his leu2 ura3-52 ade2 ade3*	This study
HPY16	*MAT***a** *his leu2 ura3-52 ade2 ade3 yck1::ura3$^-$ yck2-2^{ts}*	This study
HPY17	*MAT*α *his leu2 ura3-52 ade2 ade3 yck1::ura3$^-$ yck2-2^{ts}*	This study
HPY18	*MAT***a** *his leu2 ura3-52 yck1::ura3$^-$*	This study
HPY19	*MAT*α *his leu2 ura3-52 yck1::ura3$^-$*	This study
HPY29	*MAT***a**/*Mat*α *his3/his3 leu2/leu2 ura3-52/ura3-52 yck1::ura3$^-$/yck1::ura3$^-$*	this study
HPY90	*MAT***a** *his leu2 ura3-52 yck1::LEU2 GFP:yck2-2^{ts}*	This study
HPY119	*MAT***a** *his leu2 ura3-52 yck1::LEU2 tlg2::LEU2*	This study
HPY122	*MAT***a** *his3 leu2 ura3-52 yck1::ura3$^-$ rgp1::HIS3-2*	This study
HPY133	*MAT***a** *his leu2 ura3-52 tlg2::LEU2*	This study
HPY134	*MAT*α *his leu2 ura3-52 tlg2::LEU2*	This study
HPY142	*MAT***a** *his3 leu2 ura3-52 rgp1::HIS3-2*	This study
HPY150	*MAT*α *his leu2 ura3-52 SEC7:GFP*	This study
HPY178	*MAT*α *his3 leu2 ura3-52 rgp1::HIS3-2 pep4Δ-1*	This study
HPY185	*MAT*α *his3 leu2 ura3-52 rgp1::HIS3-2*	This study
HPY189	*MAT*α *his leu2 ura3-52 GFP:YCK2 tlg2::LEU2*	This study
HPY191	*MAT***a** *his leu2 ura3-52 GFP:yck2-2^{ts}*	This study
HPY192	*MAT***a** *his leu2 ura3-52 GFP:yck2-2^{ts} tlg2::LEU2*	This study
HPY193	*MAT***a** *his3 leu2 ura3-52 GFP:yck2-2^{ts} rgp1::HIS3-2*	This study
HPY195	*MAT***a** *his3 leu2 ura3-52 rgp1::HIS3-2*	This study
HPY204	*MAT*α *his leu2 ura3-52 pep12::LEU2*	This study
HPY208	*MAT*α *his3 leu2 ura3-52 vps27::LEU2 pep4Δ-1*	This study
HPY210	*MAT*α *his leu2 ura3-52 tlg2::LEU2 pep4Δ-1*	This study
HPY217	*MAT*α *his3 leu2 ura3-52 GFP:YCK2 rgp1::HIS3-2*	This study
LRB503	*MAT*α *his3 leu2 ura3-52 yck1::ura3$^-$ yck2-2^{ts}*	Robinson collection
LRB687	*MAT***a** *his3 leu2 ura3-52 pep4Δ-1*	Robinson collection
LRB756	*MAT***a** *his3 leu2 ura3-52 yck1::ura3$^-$ yck2-2^{ts}*	(Panek et al., 1997)
LRB757	*MAT*α *his3 leu2 ura3-52 yck1::ura3$^-$ yck2-2^{ts}*	(Panek et al., 1997)
LRB758	*MAT***a** *his3 leu2 ura3-52*	(Panek et al., 1997)
LRB759	*MAT*α *his3 leu2 ura3-52*	(Panek et al., 1997)
LRB824	*MAT*α *his3 leu2 ura3-52 pep4Δ-1*	Robinson collection
LRB854	*MAT***a** *his3 leu2 ura3-52 yck1::ura3$^-$ GFP:YCK2*	(Robinson et al., 1999)
LRB856	*MAT***a** *his3 leu2 ura3-52 yck1::LEU2*	Robinson collection
LRB861	*MAT***a** */MAT*α *his3/his3 leu2/leu2 ura3-52/ura3-52*	(Robinson et al., 1999)
LRB939	*MAT***a** *his3 leu2 ura3-52*	Robinson collection
AACY5	*MAT*α *his3 leu2 ura3-52 vps27::LEU2*	T. Stevens collection
BFY106-4D	*MAT*α *can1 ade2 his3 leu2 ura3-1 trp1 kex2Δ2::HIS3*	(Wilcox et al., 1992)
JM162	*MAT*α *his4 leu2 ura3-52 jnm1::LEU2 ade2 ade3*	K. Tatchell collection
RH240	*MAT*α *his3 leu2 ura3-52 prc1*	H. Riezman collection

colony color sectoring assay was used. HPY15 (*ade2 ade3*), derived from JM162, was crossed to a *yck1::ura3*$^{-}$ *yck2-2*^{ts} strain (LRB503) to generate the *yck1::ura3*$^{-}$ *yck2-2*^{ts} *ade2 ade3* strains HPY16 (*MAT***a**) and HPY17 (*MAT*α). HPY16 and HPY17 cells, transformed with pHRP5 (p*YCK2 ADE3 URA3*), were mutagenized to approx. 10% viability with ethylmethane sulfonate (EMS; Sherman et al., 1986). Mutagenized colonies were grown at semipermissive temperature for *yck*^{ts}, 30°C, on rich medium and 45 remained Sect^{-} after several platings to nonselective medium. Each mutant was backcrossed to the parental *yck*^{ts} strain to determine dominant or recessive and single gene character of mutations by segregation in meiotic progeny. After three serial backcrosses, 12 strains displayed consistent 2:2 segregation of synthetic defects. These were designated *dwy* (defective with *yck*^{ts}) mutants. Mutants were then crossed by one another and the 12 strains were assigned to 10 complementation groups. Additional crosses were carried out to *YCK2*$^{+}$ strains to assay for conditional growth phenotypes in haploid progeny.

Cloning and gene disruptions

The *DWY1* and *DWY2* genes were cloned by complementation of the *dwy1* and *dwy2* conditional growth phenotypes. To test for identity of isolated clones with mutant loci, genomic sequences from library plasmids were subcloned into a *URA3* integrating vector, linearized within the genomic fragment, and introduced into wild-type strains by transformation. Genomic Southern hybridization analysis confirmed integration at the appropriate loci. Genetic linkage of the Ura^{+} phenotype with the original *dwy* mutation was assayed by tetrad analysis. The *dwy1* complementing region was narrowed down to a 2.1 kb *Hin*dIII-*Bam*HI (the *Bam*HI site is from the library vector YCp50) fragment from one of the library clones (pHP101) and was subcloned into pRS316, creating pHP102. This construct contains the entire *TLG2* coding sequence with approx. 500 base pairs (bp) flanking each side. The *dwy2* complementing region is contained on a *Bgl*II-*Sph*I fragment from the original library plasmid (pHP203) and was cloned into YCp50, creating pHP209. This plasmid contains the entire *RGP1* open reading frame as well as approx. 500 bp flanking each side.

The *DWY1/TLG2* gene (*Hin*dIII-*Bam*HI fragment in pUC18) was disrupted by substitution of a 4.2 kb *Pst*I fragment from YEp13 containing *LEU2* for a 336 bp *Pst*I fragment (codons 126-242 of 397 total) within the gene (pHP117). The *DWY2/RGP1* gene (*Bgl*II-*Sph*I fragment in pUC18) was disrupted by replacing a 1350 bp *Eco*RI fragment within the coding sequence (codons 23-475 of 663 total) with a 2.5 kb *Eco*RI fragment from pJA50 (Elledge and Davis, 1988) containing *HIS3*/*Kan*^{r}, creating pHP212.

The *pep12::LEU2* disruption strain was made by digesting pBJ4351 (kindly provided by the E. Jones laboratory, Carnegie Mellon University, Pittsburgh, PA, USA) with *Bam*HI and *Sph*I and transformation of the fragment into LRB861. The resulting diploid strain was sporulated and HPY204 was among the *pep12::LEU2* meiotic progeny.

Immunoblot analysis

To assay carboxypeptidase Y (CPY) secretion, strains were overlaid with prewetted nitrocellulose filters and plates were incubated at 30°C. Cells were rinsed off the filter and the filter was immunoblotted with monoclonal CPY antisera (Molecular Probes, Eugene, OR, USA) and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP, CalBiochem, San Diego, CA, USA), with subsequent detection using the ECL system (Amersham, Piscataway, NJ, USA). To examine steady state forms of CPY or Kex2p by immunoblot analysis, strains were grown in YEPD to approx. 3.5×10^{7} cells/ml. Total protein was harvested as described previously (Davis et al., 1993) and electrophoresed in SDS/10% polyacrylamide gels. Immunoblotting was carried out either with anti-CPY as above or using polyclonal anti-Kex2p (a gift from R. Fuller, University of Michigan, Ann Arbor, MI, USA) and goat anti-rabbit IgG conjugated to HRP (CalBiochem), with detection using the ECL system.

Vacuole visualization

Yeast vacuoles were observed using the vacuole lumen-specific fluorescent probe CMAC (7-amino-4-chloromethylcoumarin; Cell Tracker Blue; Molecular Probes). Cells were grown in rich medium at 24°C to 1×10^{7} cells/ml, pelleted gently, and resuspended in 10 mM Hepes buffer pH 7.5 containing 5% glucose. CMAC was added to a final concentration of 100 μM, cells were incubated at 24°C for 20 minutes and observed by fluorescence microscopy using DAPI (UV) optics.

GFP and YFP fusion construction and analysis

Primers were designed to add *Cla*I sites (underlined; Cla-GFP-1: 5′ GCTGAATTCATCGATATGAGTAAAGGAGAAG 3′ and Cla-GFP-2: 5′ TTCGAATTCATCGATTTTGTATAGTTCATC 3′) by PCR to both the 5′ and 3′ ends of the coding sequence of the F64L, S65T bright GFP variant (Cormack et al., 1996; Robinson et al., 1999). The PCR product was cloned into pRSETB (creating pHRP29), transformed into BL21(DE3) cells for expression of GFP from the T7 promoter in pRSETB, and checked for function by monitoring colony fluorescence under UV light. Yellow fluorescent protein (YFP) is a variant of GFP (GFP-10c) with red-shifted excitation and emission wavelengths (Ormö et al., 1996), and is encoded on a plasmid kindly provided by R. Tsien (University of California, San Diego, USA). An *Nco*I-*Pvu*II fragment that contained all of the *YFP* mutations was swapped with the corresponding *GFP* fragment using the construct described above, resulting in a *Cla*I-ended *YFP* fragment in pHRP30.

For addition of *YFP* to *TLG2* (in pUC18), a *Cla*I site was added immediately following the initiating ATG of *TLG2* by PCR, creating pHP118. Primers were DWY1CLA-1: 5′ CGGATCGATTTTAGAGATAGAACT 3′ and DWY1CLA-2: 5′ CGGATCGATCATGTTTGTAACGAC 3′. *YFP* was then inserted into *TLG2* at the *Cla*I site, creating pHP124. *YFP:TLG2* was then cloned into pRS316 (*Hin*dIII-*Bam*HI fragment), resulting in pHP125. The *Cla*I-ended *YFP* gene was added in-frame to the *RGP1* coding sequence by insertion at a unique *Cla*I site corresponding to codons 6 and 7, creating pHP223.

A carboxy-terminal fusion of *GFP* coding sequence to *SEC7* was kindly provided by O. Rossanese and B. Glick (University of Chicago, Chicago, IL, USA). The plasmid pUSE3+URA (Seron et al., 1998) was linearized by digesting at an unique *Spe*1 site within the *SEC7* gene. This fragment was transformed into wild-type strain LRB759 for pop-in/pop-out gene replacement (Guthrie and Fink, 1991).

The *GFP:yck2-2*^{ts} fusion gene was created by swapping an *Xba*I-*Bcl*I fragment carried in pL2.992 (Robinson et al., 1999), which contains the *GFP* coding sequence and 280 bp of 5′ *YCK2* sequence, for the corresponding fragment contained in a plasmid-borne *yck2-2*^{ts} gene (pL2.330; Robinson et al., 1993), creating the plasmid pRC2. The fusion gene was integrated at the wild-type *YCK2* locus by excising an *Xba*I-*Sac*I fragment that contained the entire coding sequence plus flanking regions and transforming into LRB856 (*yck1Δ*). Temperature-sensitive transformants were screened for GFP fluorescence and positive clones included HPY90. HPY90 was crossed to wild-type LRB759 and a Leu^{-} (*YCK1*$^{+}$) fluorescent spore clone (HPY191) was isolated. HPY191 was crossed to HPY134 (*tlg2Δ*) or HPY185 (*rgp1Δ*) to create the strains HPY192 (*GFP:yck2-2*^{ts} *tlg2Δ*) and HPY193 (*GFP:yck2-2*^{ts} *rgp1Δ*), respectively.

Expression of the GFP and YFP fusion proteins (except for GFP-Snc1p, see below) was monitored by fluorescence microscopy using an Olympus (Melville, NY, USA) AX-70 microscope equipped for DIC optics and epifluorescence. For observation of both GFP and YFP in the same cells, filter sets selective for S^{65}T GFP (JP1) and YFP (JP2) excitation and emission spectra (Chroma Tech. Corp., Brattleboro, VT, USA) were used. For observation of GFP only, we used the filter set 41001 (Chroma Tech.), which is selective for S^{65}T GFP, but has broader bandwidth than JP1. Images were captured with a Photometrics (Tucson, AZ, USA) cooled CCD (*CE200A*) camera using IPLab *Spectrum* software run by a Macintosh G3 computer. GFP-Snc1p was visualized by FITC optics and photographed with an

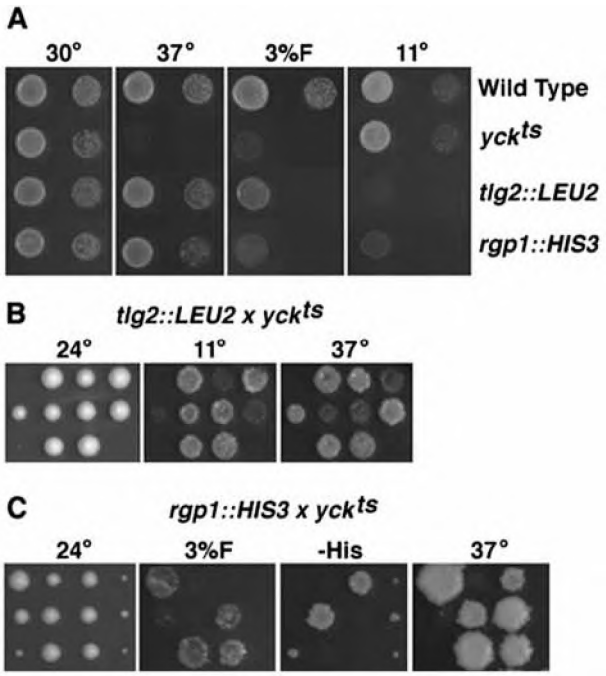

Fig. 1. Mutation of *TLG2* or *RGP1* results in synthetic growth defects when combined with a *yck*^{ts} mutation and in growth defects in a *YCK*$^{+}$ genetic background. (A) Comparison of wild type (LRB758), *yck*^{ts} (LRB756), *tlg2* (HPY133) and *rgp1* (HPY142) strains. Cultures were grown to 10^7 cells/ml and 3 μl of undiluted (left) and fiftyfold diluted (right) cultures were spotted on rich medium containing the indicated additional components (3%F=3% formamide). Plates were incubated at 30°C or the indicated temperature for 24-48 hours (except for 11°C, 5 days) before photographing. (B,C) Tetrad analysis of progeny of crosses of (B) HPY119 (*yck1::LEU2 tlg2::LEU2*) to LRB757 (*yck1::LEU2 yck2-2*^{ts}) and (C) HPY122 (*yck1::ura3- rgp1::HIS3-2*) to LRB757 (*yck1::LEU2 yck2-2*^{ts}) reveal synthetic growth defects.

Orca digital camera (Hamamatsu Photonics). All images for a given experiment were captured with the same exposure time and image composites were constructed using Adobe *Photoshop*.

To capture images of GFP and YFP fluorescence within the same cells, we used a custom IPLab *Spectrum* script written by K. Tatchell that automates switching between filter sets. This minimized the time between capture of the image pair, necessary due to rapid movement of the fluorescent structures within live cells. IPLab *Spectrum* software was also utilized to compare intracellular staining to membrane staining of GFP-Yck2p in mutants. Fluorescence levels were determined along linear regions across cells. The fluorescence level at membranes was set as the average of the peak levels generated at each end of the cell. The internal level was determined by calculating the average level within the cell section. Internal fluorescence level is expressed as a percentage of the membrane level.

RESULTS

Isolation of synthetic enhancement mutations

To identify proteins that participate in pathway(s) that impinge on, or are regulated by, Yck activity, we used a colony color sectoring assay (Bender and Pringle, 1991). *yck*^{ts} synthetic enhancement mutations were isolated at the semi-permissive temperature of 30°C (Robinson et al., 1993). Upon introduction of a *YCK2 ADE3* plasmid, *yck*^{ts} *ade2 ade3* strains were Sect^{+} under nonselective conditions (retained viability upon plasmid loss, not shown). After mutagenesis, cells containing mutations that synthetically enhance the *yck*^{ts} growth defect should require wild-type Yckp activity for continued viability and thus display the Sect^{-} phenotype. Strains HPY16 and HPY17 were mutagenized with EMS (Materials and Methods) and 12 Sect^{-}, *dwy* (defective with *yck*^{ts}) strains were isolated. All mutations were recessive, and were assigned to ten complementation groups. The *dwy* mutants were crossed to *YCK*$^{+}$ and *yck1*$^{-}$ *YCK2*$^{+}$ strains to determine if they displayed any growth defects in these genetic backgrounds. Mutations in *DWY1* confer cold sensitivity (11°C) and mutations in *DWY2* impart sensitivity to 3% formamide (not shown). Since mutations in *DWY1* and *DWY2* conferred both conditional growth phenotypes and the strongest synthetic growth defects, these were selected for further characterization.

Cloning and disruption of *DWY1* and *DWY2*

The *DWY1* and *DWY2* genes were cloned from low copy genomic libraries by complementation of the *dwy1* and *dwy2* conditional growth defects in a *YCK2*$^{+}$ background. Two overlapping library plasmids complemented the cold sensitivity of the *dwy1* strain. After delimiting the complementing region and demonstrating linkage between the library sequences and *dwy1-1* (not shown; Materials and Methods), we found that *DWY1* is the previously described gene *TLG2* (t-SNARE affecting a late Golgi compartment; Holthuis et al., 1998a). *TLG2* encodes a member of the syntaxin (t-SNARE) family of vesicle trafficking proteins, which interact with cognate v-SNARES located on transport vesicles and mediate vesicle fusion (Sollner et al., 1993; Weber et al., 1998). Tlg2p function has been implicated in endocytic processes, in retention of late Golgi proteins (Abeliovich et al., 1998; Seron et al., 1998; Holthuis et al., 1998a), and in delivery of internalized Chs3p and Snc1p to the Golgi for redirection to the plasma membrane (Holthuis et al., 1998b; Lewis et al., 2000). Disruption of *TLG2* in our genetic background confirmed that this gene is not essential for growth on rich medium at 30°C. However, it is essential for growth in the cold and in the *yck*^{ts} background, as double mutant spore clones were not recovered (Fig. 1A,B).

Eight overlapping library clones from chromosome IV complemented the *dwy2* conditional phenotype and contain the *RGP1* (reduced growth phenotype; Aguilera et al., 1990) gene. *RGP1* is predicted to encode a 663-amino-acid protein that has no significant sequence similarity to any previously described protein and contains no distinctive signal sequences or any other described sequence motifs. Analysis of the *rgp1::HIS3* disruption allele revealed that *RGP1* is not essential for growth. However, it is required for growth in the presence of 3% formamide, for wild-type growth rates at temperature extremes in *YCK*$^{+}$ strains (Fig. 1A), and for normal growth in the *yck*^{ts} genetic background (Fig. 1C). No function has been assigned to Rgp1p, but because it was isolated in the same genetic screen as Tlg2p, we explored whether it also has a role in protein trafficking. Hereafter, we will refer to *DWY1* as *TLG2* and *DWY2* as *RGP1*, and unless stated otherwise, all further studies were done with strains disrupted for *TLG2* or *RGP1*.

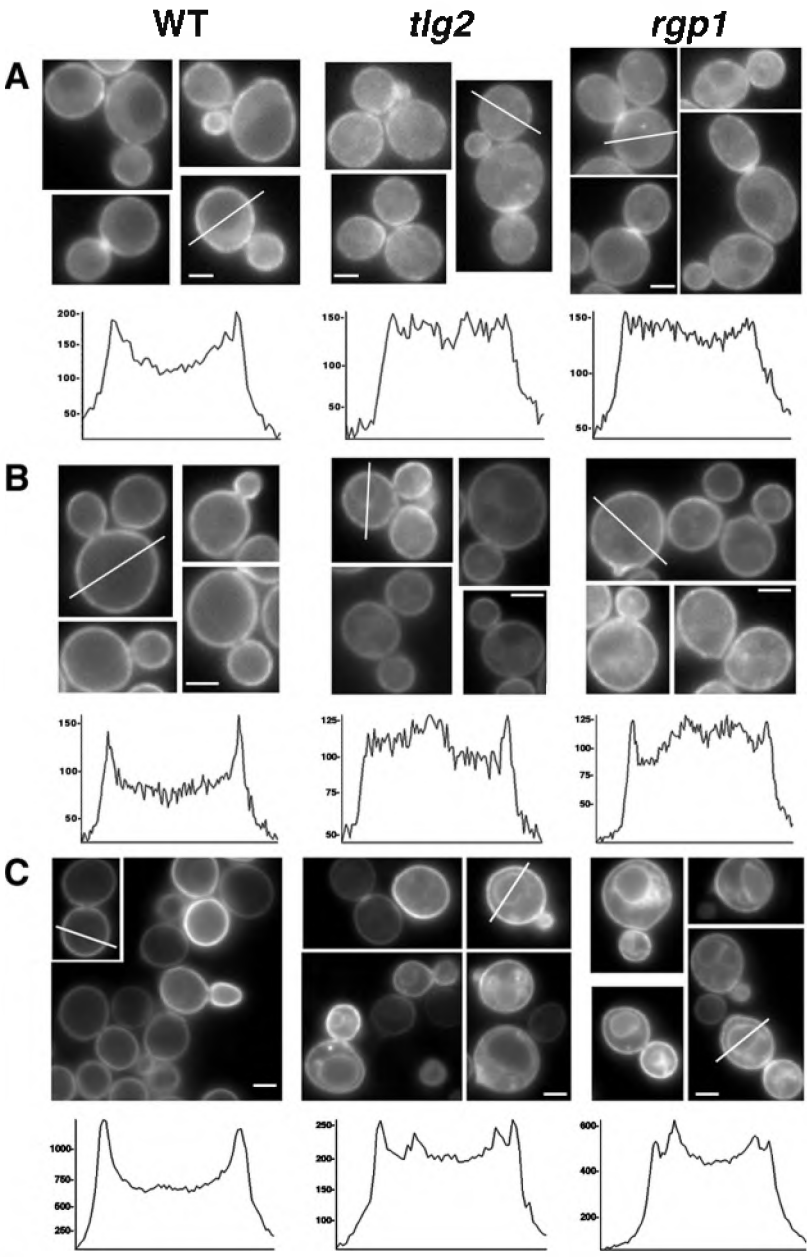

Fig. 2. GFP-yck2^{ts}p and GFP-Yck2p fluorescence patterns are altered in *rgp1* and *tlg2* strains. (A) Fluorescence of GFP-yck^{ts}p in wild type (HPY191), *tlg2* (HPY192) and *rgp1* (HPY193) cells. (B) Fluorescence of GFP-Yck2p in wild-type (LRB854), *tlg2* (HPY189) and *rgp1* (HPY217) strains. All strains in A and B carried the fusion gene integrated at the *YCK2* locus. Cells were grown at 30°C in synthetic medium to OD_{600} approx. 0.7, harvested, and resuspended in synthetic complete medium for viewing. Representative graphs with fluorescence levels on the *y*-axis were generated for the indicated cells. (C) Localization of overexpressed GFP-Yck2p. Wild type (LRB758), *tlg2* (HPY133) and *rgp1* (HPY142) cells expressing GFP-Yck2p from a galactose-inducible promoter carried in pJB1 (Robinson et al., 1999) were grown for 10 hours at 30°C in selective synthetic medium containing galactose before viewing. Bars, 2 μm.

Tlg2p and Rgp1p are required for efficient targeting of Yck2p to the plasma membrane

The simplest explanation for a mutation in a trafficking protein causing a synthetic growth defect with *yck*^{ts} is that this mutation affects targeting of Yckp to the plasma membrane. The *yck2-2*^{ts} gene product has reduced kinase activity even at permissive temperatures, so perturbing its localization to the plasma membrane would further lower Yckp activity at the cell surface. To test this hypothesis, the localization of a GFP-yck2^{ts} fusion protein was monitored in strains disrupted for *rgp1* or *tlg2* and carrying a chromosomal copy of *GFP:yck2-2*^{ts}. These strains contain the wild-type *YCK1* gene to maintain viability. As shown in Fig. 2A, the GFP-yck2^{ts} fusion protein in otherwise wild-type cells is primarily plasma membrane-localized at 24°C. This localization is similar to the wild-type fusion protein (see below); however, internal fluorescence relative to plasma membrane fluorescence is increased by at least 10% over the wild-type ratio, suggesting that targeting of GFP-yck2^{ts}p to the cell surface is less efficient than that of wild type.

Consistent with the notion that these proteins affect Yck2p targeting, cells lacking Tlg2p or Rgp1p function show altered patterns of GFP:yck2^{ts}p fluorescence. Internal fluorescence relative to plasma membrane staining was quantitated using graphical representation of the fluorescence levels across single cells. A representative graph is shown below each set of cells and the region selected to generate the graph is depicted as a line through the cell being analyzed. For the wild-type strain expressing GFP-yck2^{ts}p, the internal fluorescence level averaged 84% (n=16) of that of the plasma membrane level. Internal fluorescence appears to be mostly excluded from vacuolar structures. *tlg2* cells showed a higher level of internal fluorescence (Fig. 2A, middle panels), averaging 95% (n=25) that of the plasma membrane level. The small vacuoles in these cells (see below) are somewhat masked by internal fluorescence. The average internal level for *rgp1* cells was similarly increased to 93% that of the plasma membrane (n=24, right panels). Prominent vacuoles are present by Nomarski optics in most of these cells (not shown) and fluorescence appears excluded from these structures as in wild-type cells.

To determine if loss of Tlg2p or Rgp1p activity also affects localization of wild-type Yck2p, mutant strains containing an integrated *GFP:YCK2* fusion gene were generated. At 30°C wild-type cells carrying *GFP:YCK2* show typical plasma membrane Yck2p association (Fig. 2B). However, in both mutant backgrounds the GFP-Yck2p fluorescence is more diffuse, with an increase in cytoplasmic staining. In wild-type cells, the average internal fluorescence level of GFP:Yck2p is 76% (n=21) of the fluorescence level at the plasma membrane. For both *tlg2* (n=24) and *rgp1* (n=20) cells, the internal fluorescence level is increased to approx. 90% of that at the cell periphery.

If Yck2p interacts with a sorting factor(s) during its transport, Yck2p overexpression could saturate its localization

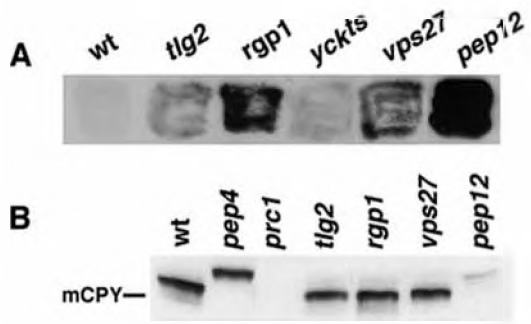

Fig. 3. CPY sorting is affected by loss of Rgp1p, but internal CPY is processed accurately. (A) CPY secretion assay. To detect external CPY, strains LRB758 (WT), HPY133 (*tlg2*), HPY142 (*rgp1*), LRB756 (*yck2*^{ts}), AACY5 (*vps27*) and HPY204 (*pep12*), were grown overnight on nitrocellulose filters, cells were rinsed from the filters, and secreted CPY was detected by immunoblot analysis. (B) Internal CPY is processed in *rgp1* and *tlg2* strains. To examine the steady state processing of CPY, total intracellular protein was isolated from strains and CPY was detected by immunoblot analysis. Cells lacking PrA (*pep4*) and CPY (*prc1*), which contain the p2 form of CPY and no CPY, respectively, were included for comparison purposes. Strains used: LRB758 (WT), LRB687 (*pep4*), RH240 (*prc1*), HPY133 (*tlg2*), HPY142 (*rgp1*), AACY5 (*vps27*) and HPY204 (*pep12*).

machinery. Consistent with this idea, when GFP-Yck2p is greatly overexpressed from a galactose-inducible promoter in wild-type cells, fluorescence is sometimes observed on vacuolar membranes, a localization rarely observed with lower kinase levels (Fig. 2C, left panels). In contrast to the faint vacuolar staining in wild-type cells, the fluorescence in *rgp1* and *tlg2* mutant cells appears equivalent on peripheral and vacuolar membranes (Fig. 2C, middle and right panels). We approximated vacuolar membrane versus plasma membrane fluorescence levels for cells overexpressing GFP-Yck2p from the *GAL1* promoter (n=10 for each strain). In the 70% of wild-type cells that exhibit vacuolar fluorescence, the vacuolar membrane level is 72% that of the plasma membrane level. Strikingly, the vacuolar membrane level of fluorescence is as high or higher than that at the cell periphery in both *tlg2* (101%) and *rgp1* (114%) cells. These results are consistent with the missorting of a significantly larger fraction of the kinase in the mutant strains.

Rgp1p is required for efficient CPY sorting

Since deletion of *TLG2* or *RGP1* results in similar effects on Yck2p localization, Rgp1p, like Tlg2p, could function in protein trafficking. Mutations in *TLG2* confer a mild carboxypeptidase Y (CPY) sorting defect, causing missorting of approx. 20% of this vacuolar enzyme to the cell surface (Abeliovich et al., 1998), so we examined *rgp1* mutant cells for this phenotype. Whereas wild-type and *yck*^{ts} strains do not secrete significant amounts of CPY (Fig. 3A), mutants defective in trafficking of this vacuolar protease, such as *vps27* and *pep12*, secrete approx. 50% and greater than 75% of their CPY, respectively (Piper et al., 1995; Becherer et al., 1996) and were used for comparative purposes. We used a filter overlay assay to detect CPY secretion by the *rgp1* strain and compared it to *tlg2*, *vps27* and *pep12* mutant strains. As shown in Fig. 3A, *rgp1* cells secrete more of this vacuolar hydrolase than do *tlg2* cells, and an amount intermediate between that of *vps27* and *pep12* mutant cells.

Immunoblot analysis was performed on total protein extracts to examine the intracellular steady state forms of CPY in these mutants. CPY is present in the ER as a 67 kDa precursor form (p1) and is modified in the Golgi to a larger p2 form of 69 kDa. Upon delivery to the vacuole, CPY is processed to its mature, active 61 kDa form, in part by Proteinase A (PrA), which is encoded by the *PEP4* gene (reviewed in Jones et al., 1997). As illustrated in Fig. 3B, CPY in *tlg2* and *rgp1* strains is in the mature form, indicating that these mutations do not significantly affect processing of intracellular enzyme. Further, significant amounts of CPY are observed in *rgp1* cells, suggesting that the missorting is not as severe as that observed in cells lacking Pep12p, in which internal, mature CPY is virtually undetectable in our hands.

rgp1 cells contain numerous small vacuoles

Since vacuole morphology is often perturbed in mutants that missort vacuolar hydrolases (Raymond et al., 1992), and *tlg2* mutant cells contain fragmented vacuoles (Holthuis et al., 1998a), vacuolar structure was examined in *rgp1* cells. As Fig. 4 shows, at 24°C, most *rgp1* mutant cells (>80%) contain highly fragmented vacuoles when visualized by staining with CMAC (staining was carried out in buffered 5% glucose to prevent coalescence of vacuole structures (Roberts et al., 1991; Materials and Methods). In these conditions, *rgp1* vacuoles appear to be smaller and even more numerous than those in *tlg2* mutant cells, whereas wild-type and *yck*^{ts} cells show one to several larger vacuoles. Staining of both *tlg2* and *rgp1* mutants with quinacrine, a weakly basic dye that accumulates in acidic compartments (Guthrie and Fink, 1991), demonstrated that these structures are acidified (not shown), indicating that the vacuolar H^+-ATPase is targeted to and functional in these vacuoles.

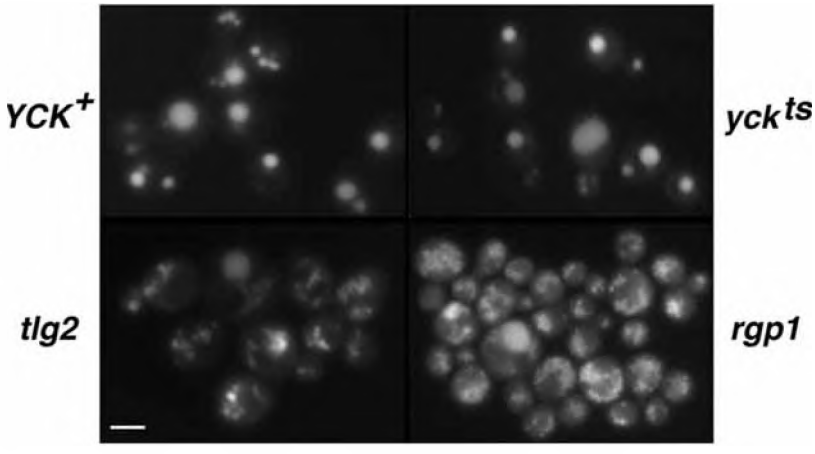

Fig. 4. *rgp1* cells have fragmented vacuoles. Wild-type (LRB758), *yck*^{ts} (LRB756), *tlg2* (HPY133), and *rgp1* (HPY142) cells were stained with the vacuole lumen-specific marker CMAC (Materials and Methods). CMAC accumulation was visualized by fluorescence microscopy using a DAPI (UV) filter set. Bar, 2 μm.

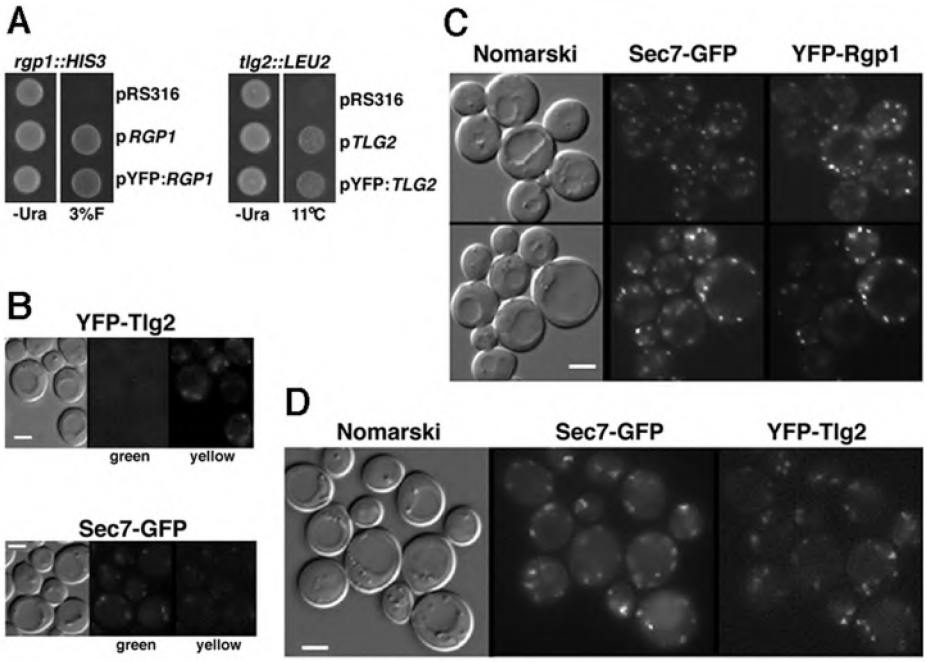

Fig. 5. The YFP-Rgp1 and YFP-Tlg2 fusion proteins are functional and YFP-Rgp1p fluorescence is associated with punctate Golgi-like structures. (A) Growth tests of cells disrupted for either *rgp1* (HPY142, left panel) or *tlg2* (HPY133, right panel). Cells were transformed with empty vector or low-copy plasmids containing wild-type (pHP209 and pHP102) or YFP fusion (pHP223 and pHP125) *RGP1* and *TLG2* genes, respectively. 3×10^4 cells from log-phase cultures were spotted on solid medium selective for the plasmid at either permissive conditions or conditions/medium restrictive for the *rgp1* and *tlg2* mutants. (B) Photographs of cells expressing only YFP-Tlg2p (upper panels) or GFP-Sec7p (lower panels) demonstrate that the filter set selective for GFP does not pass yellow fluorescence but the YFP selective filter does pass low levels of green fluorescent signal. (C,D) Comparison of YFP and GFP fluorescent fusion protein localization. The *SEC7:GFP* fusion gene was integrated into a wild-type strain (LRB759), creating HPY150. Low-copy plasmids containing the YFP fusions were introduced into this strain. YFP-Rgp1p (C) and YFP-Tlg2p fluorescence (D) overlap that of Sec7-GFP. Bars, 2 μm.

Rgp1p colocalizes with the Golgi marker Sec7p

Based on the CPY sorting defect and abnormal vacuolar morphology seen in cells lacking Rgp1p, this protein could participate in protein trafficking. To ascertain where Rgp1p functions, a red-shifted green fluorescent protein variant, yellow fluorescent protein (YFP, Materials and Methods), was fused in-frame to the 5′ end of the *RGP1* coding sequence. Introduction of this fusion on a low-copy vector complemented the formamide sensitivity of cells lacking *RGP1* (Fig. 5A, left panel). By fluorescence microscopy, cells expressing the fusion protein showed staining of small punctate structures dispersed throughout the cell (Fig. 5C). The YFP variant was also fused in-frame to the *TLG2* coding sequence following the initiating ATG of *TLG2*. Introduction of this fusion protein on a low-copy vector complemented the cold sensitivity of the *tlg2* mutant (Fig. 5A, right panel) and the pattern of YFP-Tlg2p fluorescence (Fig. 5D) was similar to that of YFP-Rgp1p.

Golgi cisternae in yeast are observed in punctate staining patterns (Redding et al., 1991) and the structures labeled by the YFP-Rgp1 fusion protein are reminiscent of that organelle. Sec7p is required for secretory traffic through the Golgi (Franzusoff and Schekman, 1989) and the fluorescence pattern of a functional Sec7-GFP fusion protein reflects Golgi localization (Seron et al., 1998). To determine if YFP-Rgp1 is also Golgi-localized, we transformed low-copy vectors carrying the *YFP* fusion genes into cells that contain the *SEC7:GFP* fusion integrated at the wild-type *SEC7* locus and imaged the fusion proteins with filter sets designed to selectively observe YFP or GFP. Fig. 5B demonstrates that the filter set selective for GFP does not pass significant amounts of yellow fluorescence, but the filter set selective for YFP passes a low level of green fluorescent signal. However, the YFP fluorescence is always significantly brighter than the GFP signal in the images using the yellow filter set. To assess colocalization of the fusion proteins, we compared spots in corresponding GFP and YFP images of individual cells. Percentage colocalization was determined by the ratio of overlapping spots to the total number of spots counted. Fig. 5C shows that the fluorescence of YFP-Rgp1p overlaps with that of Sec7-GFP. The calculated 85% colocalization (n=76 cells) suggests that Rgp1p functions at the Golgi. Only 60% colocalization (n=100 cells) was observed for the Tlg2 and Sec7 fusion proteins (Fig. 5D), which is in agreement with previous observations that Tlg2p distribution overlaps with both Golgi and endosomal markers (Abeliovich et al., 1998; Seron et al., 1998; Holthuis et al., 1998a; Conibear and Stevens, 2000).

Rgp1p is required for Kex2p stability

Tlg2p has a role in retrograde protein trafficking at the Golgi

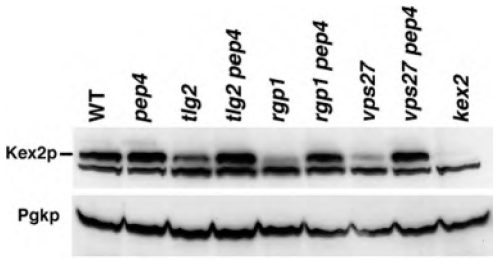

Fig. 6. Steady state levels of Kex2p are reduced in *rgp1* cells. Immunoblot of Kex2p (upper panel) in cells of indicated genotype. The strains used were: LRB759 (WT), LRB824 (*pep4*), HPY134 (*tlg2*), HPY210 (*tlg2 pep4*), HPY185 (*rgp1*), HPY178 (*rgp1 pep4*), AACY5 (*vps27*), HPY208 (*vps27 pep4*) and BFY106-4D (*kex2*), respectively. The faster migrating (lower) band is a crossreacting protein consistently seen in all strains tested. The blot was reprobed with anti-PGK to provide a loading control (lower panel).

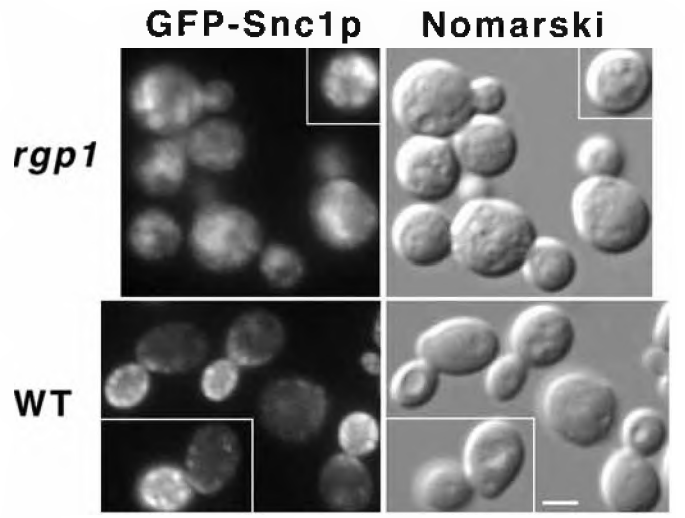

Fig. 7. GFP-Snc1p is mislocalized in *rgp1* mutant cells. *GFP:SNC1* (Lewis et al., 2000) was linearized with *Stu*I and integrated at *URA3* in LRB939 (WT) and HRP195 (*rgp1*) backgrounds. Cells were grown in rich medium to log phase and placed on concanavalin-A treated slides for viewing. Bar, 2 μm.

(Holthuis et al., 1998a). The localization data and the common phenotypes of *rgp1* and *tlg2* cells together suggest that Rgp1p may also function in this process. One function of this retrograde pathway is to retrieve Golgi membrane proteins that have inadvertently been delivered to the late endosome (Roberts et al., 1992; Cooper and Stevens, 1996). Kex2p is one such Golgi enzyme that must be recycled from the endosome to avoid delivery to the vacuole, where it is degraded (Wilcox et al., 1992; Nothwehr et al., 1993). Thus, we assessed steady state levels of Kex2p in *rgp1* mutant cells. If Kex2p is not recycled, the steady state Kex2p levels should be lower in *rgp1* cells than in wild-type cells. We also assessed Kex2p levels in a *vps27* strain, which, like the *tlg2* strain, is defective in retrograde Golgi trafficking (Piper et al., 1995; Nothwehr et al., 1996), and in strains lacking *pep4*, since mislocalized Kex2p is not efficiently degraded in PrA-deficient vacuoles (Wilcox et al., 1992). As shown in Fig. 6, Kex2p levels are reduced by 60% in *rgp1* strains as compared to wild type. This reduction is similar to that seen for *tlg2* (50%) and *vps27* (65%) cells. Kex2p protein levels are restored to wild type in the *rgp1 pep4* double mutant strain, consistent with the idea that the reduced amount in the *rgp1* strain reflects inaccurate Kex2p trafficking and subsequent vacuolar degradation rather than decreased Kex2p synthesis.

Rgp1p is required for Snc1p targeting

The SNARE protein Snc1p mediates fusion of exocytic vesicles with the plasma membrane (Protopopov et al., 1993). After exocytosis, Snc1p is endocytosed and delivered to the Golgi via an early endosomal compartment to participate in another round of transport to the cell surface (Lewis et al., 2000). This recycling is dependent on Tlg2p function, as cells lacking *tlg2* accumulate Snc1p in internal punctate structures with very little cell surface localization (Lewis et al., 2000). To determine if Rgp1 is also required for recycling of Snc1p, we utilized a functional GFP-tagged Snc1p (Lewis et al., 2000). GFP-Snc1p has a polarized distribution at the cell surface of wild-type cells, where it is concentrated at the plasma membrane of small and emerging buds (Fig. 7, lower panels). In contrast, GFP-Snc1p is localized intracellularly in *rgp1* mutant cells (Fig. 7, upper panels), which show a hazy cytosolic fluorescence that could correspond to transport vesicles as well as fluorescence at internal membranes. A defect in forward transport of Snc1p to the cell surface is most likely not the cause of its mislocalization in *rgp1* mutant cells as secretion of invertase and transport of a GFP fusion to the hexose transporter Hxt2p to the cell surface (Kruckeberg et al., 1999) are indistinguishable from wild-type cells (not shown).

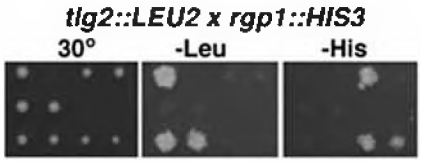

Fig. 8. The *rgp1* and *tlg2* mutations show synthetic lethality. Tetrads shown resulted from a cross between HPY133 and HPY185, and are representative of 40 that were scored. In every case, double mutants failed to form macrocolonies.

Rgp1p function is essential in *tlg2* mutant cells

Elimination of *TLG2* or *RGP1* causes very similar trafficking phenotypes. If these gene products act only at the same trafficking step, combination of the two null mutations should have no additive detrimental effect (Guarente, 1993). To test this, we generated double *rgp1 tlg2* mutants by crossing single mutant strains. As shown in Fig. 8, meiotic progeny of this cross do not include viable double mutants, indicating that while Rgp1p and Tlg2p may both play a role in retrograde Golgi trafficking, they may also act in different pathways.

The t-SNARE Tlg1p has overlapping functions with Tlg2p (Holthuis et al., 1998a; Holthuis et al., 1998b; Lewis et al., 2000). We attempted to examine the requirement for Rgp1p in cells lacking *tlg1*, but deletion of this t-SNARE in our genetic background (derived from S288c) results in lethality, as it does in the W303 background (Coe et al., 1999).

DISCUSSION

The yeast CK1 protein kinases Yck1p and Yck2p are together essential for viability, and although they rely on the secretory pathway for localization, how they are targeted to the plasma membrane is unknown. To identify proteins that act in pathways that affect or are affected by Yck2p, a synthetic enhancement screen was carried out using a thermosensitive *yck* mutant. This screen identified mutations in *TLG2* and *RGP1*, which cause a requirement for wild-type levels of Yck activity for normal cell growth.

Tlg2p is distributed on both Golgi and endosomal membranes, perhaps localized to structures at the junction between endocytic and exocytic pathways (Abeliovich et al., 1998; Holthuis et al., 1998a; Lewis et al., 2000). This t-SNARE is required for efficient endocytic turnover and for retrieval of Golgi resident proteins (Abeliovich et al., 1998; Seron et al., 1998; Holthuis et al., 1998a). Recently it has been demonstrated that Tlg2p also has a role in delivery of Chs3p

(Holthuis et al., 1998b) and Snc1p (Lewis et al., 2000) to the Golgi from the cell surface.

RGP1 encodes a novel protein (Aguilera et al., 1990). Although Rgp1p shares no sequence similarity with any known trafficking protein, mutations in the *RGP1* gene confer several traits in common with mutations in *TLG2*. Neither gene is essential for viability under optimal growth conditions, nor are they required directly for secretion as invertase secretion is unaffected in either mutant strain (not shown, see also Holthuis et al., 1998a). Further, cells with mutations in either display a fragmented vacuolar phenotype and affect the accurate trafficking of CPY, Kex2p and Snc1p.

Like Tlg2p, Rgp1p appears to be Golgi-localized. Although the overlap between Rgp1p and Sec7p localization is extensive, it is not complete. The lack of complete colocalization could reflect the fact that Sec7p is present on all Golgi compartments (Franzusoff et al., 1991) whereas Rgp1p functions at a subset of them. However, we observed Rgp1p not only in a subset of Sec7p-containing structures, but also in Sec7p-negative structures. Alternatively, as the localization study was done in live cells and the fluorescent structures were in motion, the lack of colocalization may be simply due to this movement. Indeed, when we compared the localization of YFP-Rgp1 to that of GFP-Rgp1, colocalization never exceeded 90% (not shown), suggesting that the colocalization observed between YFP-Rgp1 and Sec7-GFP is at the limit of overlap detectable in live cells using our microscope system. Thus, Rgp1 may act exclusively at the Golgi and not also at endosomal structures as has been proposed for Tlg2p.

Based upon its localization, the *rgp1* phenotypes, and the similarities of these phenotypes to Golgi recycling mutants, we propose that Rgp1 participates in retrograde trafficking at the Golgi. A defect in this recycling pathway would explain some phenotypes exhibited by *rgp1* cells. Failure to recycle the CPY sorting receptor Vps10p would result in default transit of some CPY to the cell surface (Cooper and Stevens, 1996). The decreased levels of Kex2p in *rgp1* cells would stem from the inability to recycle this Golgi enzyme from an endosomal compartment, causing its degradation in the vacuole. Finally, the inability to deliver GFP-Snc1p to the Golgi for redirection into exocytic vesicles would result in its mislocalization.

The *rgp1* and *tlg2* mutants were isolated in the same genetic screen, and display phenotypes that suggest similar roles in recycling. Thus, Rgp1p could work in conjunction with Tlg2p in delivery of retrograde vesicles at the Golgi. However, several observations indicate that they do not function only at the same trafficking step. The mutant strains do not have identical phenotypes. Tlg2p is necessary for growth in the cold, as well as for efficient turnover of endocytosed proteins. In contrast, mutations in *RGP1* confer sensitivity to formamide and a slow-growth phenotype especially at temperature extremes, but do not affect Ste3p endocytosis (not shown). If these proteins function only in Golgi recycling, no synthetic defects would be expected for cells containing both null mutations, yet this combination is lethal. Therefore, they may also act in different trafficking steps/pathways, one of which must be operational for viability. Tlg2p is present on endosomal membranes and a unique role for the t-SNARE at this compartment may be required when Rgp1p function is lacking.

Recently, a complex composed of Vps52p, Vps53p and Vps54p was isolated that localizes to the Golgi and is required for retrograde trafficking (Conibear and Stevens, 2000). This complex was identified in a screen for mutants that secrete CPY and are synthetically lethal with *end4*. Although Rgp1p appears to function at a similar step as the Vps52/53/54 complex, we have not observed synthetic effects upon combining *rgp1* and *end4*. Thus, if Rgp1p works in conjunction with the Vps52/53/54 complex, it is not required for the Vps52/53/54p function which, when lacking, results in the requirement for End4p. One possibility is that Rgp1p function increases efficiency of Golgi recycling. In this case, loss of Rgp1p function would slow delivery, but not completely block it. Only upon loss of both efficiency and specificity (such as with *tlg2* or *vps52/53/54* mutations) does the essential nature of retrograde trafficking become apparent. A role for Rgp1p as a guanine nucleotide exchange factor for Ypt6, in conjunction with Ric1p, was recently determined (Siniossoglou et al., 2000). This role fits well with our genetic data.

The most probable reason for the isolation of *tlg2* and *rgp1* in the *yck*^{ts} synthetic lethal screen is their effects on localization of the Yck kinases. Yck2p requires the secretory pathway for plasma membrane delivery and most likely travels on the cytoplasmic surface of secretory vesicles (our unpublished results). As this delivery appears to be proceed in a regulated fashion (our unpublished results), we speculate that Yck interacts with a trafficking factor(s) for accurate delivery. Recently, it was demonstrated that targeting of farnesylated Ras to the plasma membrane also requires the secretory pathway; however, no specific factor(s) linking it to the cytoplasmic face of vesicles has been identified (Choy et al., 1999). Mutations in either *tlg2* and *rgp1* affect efficient delivery of GFP-Yck2 and GFP-yck2^{ts} proteins to the cell surface. Thus, Tlg2p and Rgp1p may be required for recycling of a membrane-bound factor that interacts with Yck2p during its transit to the plasma membrane. If the *rgp1* and *tlg2* mutations perturb Yck2p transport indirectly due to a defect in recycling of its sorting factor to the Golgi, overexpression of the kinase in these mutants should result in pronounced missorting, as efficient recycling would become crucial under these conditions. This is what was observed upon overexpression of Yck2p in *tlg2* and *rgp1* mutants. Based on these results, it is possible that factors directing transport of prenylated proteins on the cytoplasmic surface of vesicles utilize classical trafficking pathways for their recycling. De novo synthesis of the Yck2p trafficking factor could allow delivery of sufficient wild-type Yck kinase to the membrane for it to perform its essential function in *rgp1* and *tlg2* mutant cells. However, when the yck2^{ts} kinase is the sole source of Yck activity in the *rgp1* or *tlg2* mutant cell, inefficient delivery coupled with reduced activity would be lethal, even at permissive temperature. Alternatively, the Yck kinases, which regulate internalization of several plasma membrane proteins (Panek et al., 1997; Hicke et al., 1998; Marchal et al., 2000), could enter the cell on endocytic vesicles and be recycled back to the cell surface. An endosome-plasma membrane recycling pathway appears to deliver a mutant form of the major plasma membrane ATPase, Pma1-7p, to the plasma membrane, supporting the idea that recycling to this membrane can occur in yeast (Luo and Chang, 2000). Either explanation leads to the prediction that other mutations that affect recycling of proteins to the Golgi from the cell surface could result in mislocalization of Yck2p. This is in fact what we observed

upon loss of the Vps52/53/54 complex (our unpublished results). We also predict that synthetic effects would be observed upon removal of this complex in a *yck*^{ts} background. Interestingly, mutants that affect trafficking at the late endosome do not result in synthetic lethality with the *yck*^{ts} mutation (our unpublished results). The proposed Yck2p targeting factor may therefore be sorted away from other endocytosed proteins in the early endosome and may not travel through the late endosome before reaching the Golgi. A similar scenario was proposed for the trafficking of Snc1p (Lewis et al., 2000). Experiments to identify the Yck trafficking factor(s) are currently underway.

We thank K. Tatchell for help with microscopy and N. Mathias and K. Tatchell for critical evaluation of this manuscript. We also thank S. Emr, R. Fuller, B. Glick, E. Jones, G. Payne, T. Stevens, K. Tatchell, R Tsien and the members of their laboratories for generously providing strains, plasmids and reagents. We are grateful to Kim Marks, Allison Jerome and Jennifer Morgan for superb technical assistance. This work was supported by NSF grant MCB-9601294 to L.C.R. H.R.P. was supported by Louisiana Educational Quality Support Fund doctoral student training award GF11 to D. J. O'Callaghan and R. E. Rhoads.

REFERENCES

Abeliovich, H., Grote, E., Novick, P. and Ferro-Novick, S. (1998). Tlg2p, A yeast syntaxin homolog that resides on the Golgi and endocytic structures. *J. Biol. Chem.* **273**, 11719-11727.

Aguilera, A. (1994). Formamide sensitivity: a novel conditional phenotype in yeast. *Genetics* **136**, 87-91.

Aguilera, A., Moskowitz, P. and Klein, H. L. (1990). Molecular analysis of *RGP1*, a new yeast gene required for proper mitotic growth. *Nucleic Acids Res.* **18**, 1064.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Becherer, K. A., Rieder, S. E., Emr, S. D. and Jones, E. W. (1996). Novel syntaxin homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. *Mol. Biol. Cell* **7**, 579-594.

Bender, A. and Pringle, J. R. (1991). Use of a screen for synthetic lethal and multicopy suppressee mutants to identify two new genes involved in morphogenesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**, 1295-1305.

Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I. E. and Philips, M. R. (1999). Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* **98**, 69-80.

Coe, J. G. S., Lim, A. C. B., Xu, J. and Hong, W. (1999). A role for Tlg1p in the transport of proteins within the Golgi apparatus of *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **10**, 2407-2423.

Conibear, E. and Stevens, T. H. (2000). Vps52p, Vps53p and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. *Mol. Biol. Cell* **11**, 305-323.

Cooper, A. A. and Stevens, T. H. (1996). Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple yeast vacuolar hydrolases. *J. Cell Biol.* **133**, 529-541.

Cormack, B. P., Valdivia, R. H. and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33-38.

Davis, N. G., Horecka, J. L. and Sprague, G. F., Jr. (1993). *Cis-* and *trans*-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell Biol.* **122**, 53-65.

Elledge, S. J. and Davis, R. W. (1988). A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. *Gene* **70**, 303-312.

Ferro-Novick, S. and Jahn, R. (1994). Vesicle fusion from yeast to man. *Nature* **370**, 191-193.

Franzusoff, A., Redding, K., Crosby, J., Fuller, R. S. and Schekman, R. (1991). Localization of components involved in protein transport and processing through the yeast Golgi apparatus. *J. Cell Biol.* **112**, 27-37.

Franzusoff, A. and Schekman, R. (1989). Functional compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO J.* **8**, 2695-2702.

Guarente, L. (1993). Synthetic enhancement in gene interaction: a genetic tool come of age. *Trends Genet.* **9**, 362-366.

Guthrie, C. and Fink, G. R. (1991). Guide to Yeast Genetics and Molecular Biology. *Methods Enzymol.* vol. 194 (ed. J. Abelson and M. I. Simon). San Diego: Academic Press Ltd.

Hicke, L., Zanolari, B. and Riezman, H. (1998). Cytoplasmic tail phosphorylation of the α-factor receptor is required for its ubiquitination and internalization. *J. Cell Biol.* **141**, 349-358.

Hoekstra, M. F., Liskay, R. M., Ou, A. C., DeMaggio, A. J., Burbee, D. J. and Heffron, F. (1991). HRR25, a putative protein kinase from budding yeast: association with repair of damaged DNA. *Science* **253**, 1031-1034.

Holthuis, J. C. M., Nichols, B. J., Dhruvakumar, S. and Pelham, H. R. B. (1998a). Two syntaxin homologues in the TGN/endosomal system of yeast. *EMBO J.* **17**, 113-126.

Holthuis, J. C. M., Nichols, B. J. and Pelham, H. R. B. (1998b). The syntaxin Tlg1p mediates trafficking of chitin synthase III to polarized growth sites in yeast. *Mol. Biol. Cell* **9**, 3383-3397.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163-168.

Jones, E. W., Webb, G. C. and Hiller, M. A. (1997). Biogenesis and function of the yeast vacuole. In: *The Molecular and Cellular Biology of the Yeast Saccharomyces: Cell Cycle and Cell Biology*, vol. 3 (ed. J. R. Pringle, J. R. Broach and E. W. Jones), pp. 363-470. Plainview, NY: Cold Spring Harbor Laboratory Press.

Kaiser, C. A., Gimeno, R. E. and Shaywitz, D. A. (1997). Protein secretion, membrane biogenesis, and endocytosis. In: *The Molecular and Cellular Biology of the Yeast Saccharomyces, Cell Cycle and Cell Biology*, vol. 3 (ed. J. R. Pringle, J. R. Broach and E. W. Jones), pp. 91-227. Plainview, NY: Cold Spring Harbor Laboratory Press.

Kruckeberg, A., Ye, L., Berden, J. A. and vanDam, K. (1999). Functional expression, quantification and cellular localization of the Hxt2 hexose transporter of *Saccharomyces cerevisiae*. *Biochem. J.* **339**, 299-307.

Lewis, M. J., Nichols, B. J., Prescianotto-Baschong, C., Riezman, H. and Pelham, H. R. B. (2000). Specific retrieval of the exocytic SNARE Snc1p from early endosomes. *Mol. Biol. Cell* **11**, 23-38.

Luo, W.-J. and Chang, A. (2000). An endosome-to-plasma membrane pathway involved in trafficking of a mutant plasma membrane ATPase in yeast. *Mol. Biol. Cell* **11**, 579-592.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor NY: Cold Spring Harbor Laboratory Press.

Marchal, C., Haguenauer-Tsapis, R. and Urban-Grimal, D. (2000). Casein kinase I-dependent phosphorylation within a PEST sequence and ubiquitination at nearby lysines signal endocytosis of yeast uracil permease. *J. Biol. Chem.* **275**, 23608-23614.

Nothwehr, S. F., Bryant, N. J. and Stevens, T. H. (1996). The newly identified yeast *GRD* genes are required for retention of late-Golgi membrane proteins. *Mol. Cell. Biol.* **16**, 2700-2707.

Nothwehr, S. F., Roberts, C. J. and Stevens, T. H. (1993). Membrane protein retention in the yeast Golgi apparatus: dipeptidyl aminopeptidase A is retained by a cytoplasmic signal containing aromatic residues. *J. Cell Biol.* **121**, 1197-1209.

Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y. and Remington, S. J. (1996). Crystal structure of the *Aequorea victoria* Green Fluorescent Protein. *Science* **273**, 1392-1395.

Panek, H. R., Stepp, J. D., Engle, H. M., Marks, K. M., Tan, P. K., Lemmon, S. K. and Robinson, L. C. (1997). Suppressors of *YCK*-encoded yeast casein kinase 1 deficiency define the four subunits of a novel clathrin AP-like complex. *EMBO J.* **16**, 4194-4204.

Piper, R. C., Cooper, A. A., Yang, H. and Stevens, T. H. (1995). *VPS27* controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae*. *J. Cell Biol.* **131**, 603-617.

Protopopov, V., Govindan, B., Novick, P. and Gerst, J. E. (1993). Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell* **74**, 855-861.

Raymond, C. K., Howald-Stevenson, I., Vater, C. A. and Stevens, T. H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: Evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell* **3**, 1389-1402.

Redding, K., Holcomb, C. and Fuller, R. S. (1991). Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **113**, 527-538.

Roberts, C. J., Nothwehr, S. F. and Stevens, T. H. (1992). Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. *J. Cell Biol.* **119**, 69-83.

Roberts, C. J., Raymond, C. K., Yamashiro, C. T. and Stevens, T. H. (1991). Methods for studying the yeast vacuole. *Meth. Enzymol.* **194**, 644-661.

Robinson, L. C., Bradley, C., Bryan, J. D., Jerome, A., Kweon, Y. and Panek, H. R. (1999). The Yck2 yeast casein kinase 1 isoform shows cell cycle-specific localization to sites of polarized growth and is required for proper septin organization. *Mol. Biol. Cell* **10**, 1077-1092.

Robinson, L. C., Hubbard, E. J. A., Graves, P., DePaoli-Roach, A. A., Roach, P. J., Kung, C., Haas, D. W., Hagedorn, C. H., Goebl, M., Culbertson, M. R. and Carlson, M. (1992). Yeast casein kinase I homologues: an essential gene pair. *Proc. Natl. Acad. Sci. USA* **89**, 28-32.

Robinson, L. C., Menold, M. M., Garrett, S. and Culbertson, M. R. (1993). Casein kinase I-like protein kinases encoded by *YCK1* and *YCK2* are required for yeast morphogenesis. *Mol. Cell. Biol.* **13**, 2870-2881.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature* **372**, 55-63.

Rothstein, R. J. (1983). One-step gene disruption in yeast. *Methods Enzymol.* **101**, 202-210.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Seron, K., Tieaho, V., Prescianotto-Baschong, C., Aust, T., Blondel, M.-O., Guillaud, P., Devilliers, G., Rossanese, O. W., Glick, B. S., Riezman, H., Keranen, S. and Haguenauer-Tsapis, R. (1998). A yeast t-SNARE involved in endocytosis. *Mol. Biol. Cell* **9**, 2873-2889.

Sherman, F., Fink, G. R. and Hicks, J. B. (1986). *Methods in Yeast Genetics*. Cold Spring Harbor NY: Cold Spring Harbor Laboratory Press.

Sinensky, M. (2000). Recent advances in the study of prenylated proteins. *Biochim. Biophys. Acta* **1484**, 93-106.

Siniossoglou, S., Peak-Chew, S. Y. and Pelham, H. R. B. (2000). Ric1p and Rgp1p form a complex that catalyses nucleotide exchange on Ypt6p. *EMBO J.* **19**, 4885-4894.

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318-323.

Vancura, A., Sessler, A., Leichus, B. and Kuret, J. (1994). A prenylation motif is required for plasma membrane localization and biochemical function of casein kinase I in budding yeast. *J. Biol. Chem.* **269**, 19271-19278.

Wang, P.-C., Vancura, A., Mitcheson, T. G. and Kuret, J. (1992). Two genes in *Saccharomyces cerevisiae* encode a membrane bound form of casein kinase-1. *Mol. Biol. Cell* **3**, 275-286.

Wang, X., Hoekstra, M. F., DeMaggio, A. J., Dhillon, N., Vancura, A., Kuret, J., Johnston, G. C. and Singer, R. A. (1996). Prenylated isoforms of yeast casein kinase I, including the novel Yck3p, suppress the *gcs1* blockage of cell proliferation from stationary phase. *Mol. Cell. Biol.* **16**, 5375-5385.

Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H. and Rothman, J. E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* **92**, 759-772.

Wilcox, C. A., Redding, K., Wright, R. and Fuller, R. S. (1992). Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. *Mol. Biol. Cell* **3**, 1353-1371.

Zhang, F. L. and Casey, P. J. (1996). Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* **65**, 241-269.