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Genomic Screen for Vacuolar Protein Sorting Genes in *Saccharomyces cerevisiae*[D]

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The biosynthetic sorting of hydrolases to the yeast vacuole involves transport along two distinct routes referred to as the carboxypeptidase Y and alkaline phosphatase pathways. To identify genes involved in sorting to the vacuole, we conducted a genome-wide screen of 4653 homozygous diploid gene deletion strains of *Saccharomyces cerevisiae* for missorting of carboxypeptidase Y. We identified 146 mutant strains that secreted strong-to-moderate levels of carboxypeptidase Y. Of these, only 53 of the corresponding genes had been previously implicated in vacuolar protein sorting, whereas the remaining 93 had either been identified in screens for other cellular processes or were only known as hypothetical open reading frames. Among these 93 were genes encoding: 1) the Ras-like GTP-binding proteins Arl1p and Arl3p, 2) actin-related proteins such as Arp5p and Arp6p, 3) the monensin and brefeldin A hypersensitivity proteins Mon1p and Mon2p, and 4) 15 novel proteins designated Vps61p-Vps75p. Most of the novel gene products were involved only in the carboxypeptidase Y pathway, whereas a few, including Mon1p, Mon2p, Vps61p, and Vps67p, appeared to be involved in both the carboxypeptidase Y and alkaline phosphatase pathways. Mutants lacking some of the novel gene products, including Arp5p, Arp6p, Vps64p, and Vps67p, were severely defective in secretion of mature α-factor. Others, such as Vps61p, Vps64p, and Vps67p, displayed defects in the actin cytoskeleton at 30°C. The identification and phenotypic characterization of these novel mutants provide new insights into the mechanisms of vacuolar protein sorting, most notably the probable involvement of the actin cytoskeleton in this process.

INTRODUCTION

Lysosomes have long been recognized as the major site for the degradation of both exogenous and endogenous macromolecules within mammalian cells (De Duve and Wattiaux, 1966; Kornfeld and Mellman, 1989). The biogenesis of lysosomes, however, remains poorly understood. Helpful insights have been gained from the study of the yeast vacuole, which is functionally analogous to the mammalian lysosome. To the extent that they have been compared, the biosynthetic pathways and molecular machineries involved in the formation of mammalian lysosomes and the yeast vacuole appear remarkably conserved (reviewed by Lemmon and Traub, 2000; Mullins and Bonifacino, 2001a). Thus, further studies of the yeast vacuole are likely to provide new insights into the mechanisms of lysosome biogenesis.

The main pathway for the delivery of newly synthesized proteins to the vacuole is referred to as the carboxypeptidase Y (CPY) pathway based on the utilization of this pathway by CPY (reviewed by Burd *et al.*, 1998; Conibear and Stevens, 1998; Mullins and Bonifacino, 2001a). CPY is a soluble hydrolase that normally resides within the vacuole lumen. Another soluble hydrolase, proteinase A (PrA), as well as the membrane-bound hydrolase carboxypeptidase S are also transported to the vacuole via the CPY pathway. This pathway involves transport from the late-Golgi complex (equivalent to the mammalian *trans*-Golgi network) to a prevacuolar compartment (PVC). From the PVC, some proteins are subsequently transferred to the vacuole, whereas others return to the late-Golgi complex for further rounds of transport. Membrane-bound enzymes involved in the processing of pro-α-factor, such as the Kex2p dibasic endopeptidase, are also transported along parts of the CPY pathway as they cycle between the late-Golgi complex and the PVC. A second pathway, referred to as the alkaline phosphatase (ALP) pathway, is followed by the membrane-bound hydrolase ALP and the t-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) Vam3p and Nyv1p (Burd *et al.*, 1998; Conibear and Stevens, 1998; Reggiori *et al.*, 2000; Mullins and Bonifacino, 2001a). The ALP pathway differs from the CPY pathway in that it uses a different type

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[D] Online versions of this article contain complete data sets.

of Golgi-derived carrier vesicle (Rehling *et al.*, 1999) and bypasses the PVC (Cowles *et al.*, 1997b; Piper *et al.*, 1997) en route to the vacuole.

Genetic screens based on the detection of reduced proteolytic activity of the vacuole (Jones, 1977) or missorting of vacuolar hydrolases to the periplasmic space (Robinson *et al.*, 1988; Rothman *et al.*, 1989) have identified >40 vacuolar protein sorting (*VPS*) genes. Other screens devised for different purposes uncovered additional genes that, although not termed *VPS*, also participate in vacuolar protein sorting. The products of most vacuolar protein sorting genes identified to date control transport exclusively along the CPY pathway (reviewed by Burd *et al.*, 1998; Conibear and Stevens, 1998; Mullins and Bonifacino, 2001a). These include proteins involved in transport from the late-Golgi complex to the PVC (e.g., Vps1p, Vps8p, Vps9p, Vps10p, Vps15p, Vps19p, Vps21p, Vps34p, Vps45p, clathrin, the AP-1 complex, Gga1p/Gga2p, Drs2p, and Mvp1p); PVC maturation, including multivesicular body formation (e.g., Vps2p, Vps4p, Vps20p, Vps22p, Vps23p, Vps24p, Vps27p, Vps28p, Vps32p, and Vps36p); and retrieval from the PVC to the Golgi complex (e.g., Vps5p, Vps17p, Vps26p, Vps29p, Vps30p, Vps35p, Vps51p, Vps52p, Vps53p, Vps54p, and Grd19p). The ALP pathway is controlled by a surprisingly simpler machinery, of which the heterotetrameric adaptor protein complex AP-3 is the only specific component known to date (reviewed by Burd *et al.*, 1998; Conibear and Stevens, 1998; Mullins and Bonifacino, 2001a). AP-3 participates, together with Vps39p and Vps41p, in the formation of ALP carriers at the late-Golgi complex (Rehling *et al.*, 1999). The CPY and ALP pathways converge at the step of fusion with the vacuole, for which reason they share a number of gene products involved in vacuole targeting and fusion (e.g., Vam3p, Vam7p, Vps11p, Vps16p, Vps18p, Vps33p, Vps39p, Vps41p, and Ypt7p).

As numerous as the vacuolar protein sorting genes may seem at present, the total number of genes involved in this process may be even higher. Indeed, studies over the past 2 yr alone have shown that mutations in several novel genes, including Mrl1p (Whyte and Munro, 2001b), Ric1p (Siniossoglou *et al.*, 2000; Bensen *et al.*, 2001), Rgp1p (Siniossoglou *et al.*, 2000; Bensen *et al.*, 2001), Did2p (Amerik *et al.*, 2000), Did4p (Amerik *et al.*, 2000), Ccz1p (Kucharczyk *et al.*, 2000), Mos10p (Kranz *et al.*, 2001), and Swa2p (Gall *et al.*, 2000) impair CPY sorting to the vacuole. The steady pace of discovery of new vacuolar protein sorting genes suggests that previous screens were not saturating and that additional genes involved in this process might still remain to be identified.

To obtain a global view of all the genes involved in vacuolar protein sorting, we decided to perform a genome-wide screen for *VPS* genes in *Saccharomyces cerevisiae*. The approach used in our study consisted of screening a collection of 4653 homozygous diploid gene deletion strains (Winzeler *et al.*, 1999) for secretion of CPY into the medium. This approach benefited from the fact that most vacuolar protein sorting genes are nonessential in yeast. We identified 146 mutant strains that secreted strong-to-moderate levels of CPY. Of these, only 53 of the corresponding genes had been previously implicated in vacuolar protein sorting. The identity of the remaining 93 genes and the phenotypic characterization of the mutant strains reported herein provide surprising new insights into vacuole biogenesis.

MATERIALS AND METHODS

Media, Strains, and Molecular Biology Procedures

S. cerevisiae cells were grown in yeast extract-peptone-dextrose (YEPD) medium. DNA manipulations and transformation into *Escherichia coli* DH5α cells were performed by standard protocols. Homozygous diploid deletion strains and Mat α haploid deletion strains were obtained from Research Genetics (Invitrogen, Huntsville, AL) and compared with the corresponding parental strains, BY4743 (diploid), BY4739 (haploid), and BY4742 (haploid). For genotypes see the Research Genetics Web site at http://www.resgen.com/products/YEASTD.php3.

CPY Colony Blots

The CPY colony blot assay was adapted from Roberts *et al.* (1991). Briefly, yeast strains were transferred from the 96-well plates to 15-cm^2 YEPD plates with a pinning tool and placed at 30°C for 2 to 3 d. Once strains had grown in the round patches, they were replica-plated to a fresh YEPD plate and overlaid with a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), taking care to remove any trapped bubbles. The plates were then incubated at 30°C for 18–22 h. The nitrocellulose membranes were washed several times with double-distilled H_2O and then with standard phosphate-buffered saline. The membranes were then subjected to immunoblotting with mouse anti-CPY (Molecular Probes, Eugene, OR) performed as described previously (Mullins and Bonifacino, 2001b) with slight modifications. Briefly, the mouse anti-CPY was used at 1:1000 dilution. Donkey anti-mouse IgG-horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) at 1:5000 dilution was used as the secondary antibody, followed by visualization with enhanced chemiluminescence reagents (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's protocols.

Metabolic Labeling and Immunoprecipitation

Metabolic labeling of yeast cells with ^{35}S Express label (PerkinElmer Life Sciences), pulse-chase analyses, and immunoprecipitations were performed at 30°C as described by Bonifacino and Dell'Angelica (1998). A 10-min pulse, followed by a 10-min chase were used in all the pulse-chase assays. Immunoprecipitations were performed overnight at 4°C and the immunoprecipitates analyzed by SDS-PAGE and autoradiography. Mouse anti-CPY antibodies were purchased from Molecular Probes. Anti-PrA and rabbit anti-ALP antibodies were the generous gifts of Carol Woolford (Carnegie Mellon University, Pittsburgh, PA) and Tom Stevens (University of Oregon, Eugene, OR), respectively. For α-factor experiments, cells were pulse-labeled at 25°C for 7.5 min to visualize α-factor–processing intermediates (Graham and Emr, 1991) and immunoprecipitated with anti-α-factor (generously provided by Todd Graham, Vanderbilt University, Nashville, TN).

Labeling Yeast Vacuoles with FM4-64

Yeast vacuoles were visualized in vivo by labeling log-phase cells with 80 μM *N*-(3-triethylammoniumpropyl)-4-(*p*-diethyl-aminophenylhexatrienyl) pyridinium dibromide (FM4-64) (Vida and Emr, 1995; Bonangelino *et al.*, 1997). Cells were viewed with a 100× objective lens on an Olympus IX-70 fluorescence microscope (excitation, 560 nm; dichroic mirror at 595 nm; emission, 630 nm) combined with a low level of transmitted light to reveal cell outlines. Images were captured digitally with an IMAGO charge-coupled device camera controlled by TILLvisION software (TILL Photonics, Eugene, OR). Images were processed using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA).

Labeling Yeast Actin with Oregon-Green Phalloidin

Cells were grown in 5 ml of YEPD at 30°C to midlog phase (OD_{600} ~0.5) and subsequently fixed by the addition of 1/9 volumes of

37.5% formaldehyde (final concentration ~3.7%). This fixation was carried out at room temperature for 1 h with gentle agitation. The fixed cells were harvested and washed twice with phosphate-buffered saline. The cells were then resuspended in 100 μl of phosphate-buffered saline, added Oregon green-phalloidin (Molecular Probes) to 1.1 μM (20 μl of 6.6 μM stock), and incubated at 30°C with agitation in the dark overnight (~16–20 h.). Labeled cells were viewed with a 100× objective lens on an IX-70 fluorescence microscope (excitation, 485 nm; dichroic mirror at 520 nm; emission, 540 nm; Olympus, Tokyo, Japan). Images were captured and processed as described above.

Analysis of α-Factor Secretion

Halo assays for α-factor secretion were performed essentially as described previously (Mullins and Bonifacino, 2001b). Briefly, the *sst1-3* mutant strain RC634 (Mat a) was grown at 30°C to stationary phase in YEPD. Cells were harvested, washed with YEPD and resuspended in fresh medium to a final concentration of ~9.0 OD_{600}/ml. Then, a 120-μl aliquot of the *sst1-3* mutant strain was spread evenly on a YEPD agar plate and used immediately in the assay. Strains to be tested for α-factor secretion were grown to 1–1.5 OD_{600}/ml at 30°C, concentrated to 1 OD_{600}/ml, and serially diluted in YEPD to the concentrations indicated in the figure. Three microliters of each dilution was spotted on plates containing RC634 cells and incubated at 30°C for ~72 h to visualize the α-factor–induced growth inhibition (i.e., growth halo). Strains that produced no visible halos when spotted on the *sst1-3* lawn were retested in the same way by using Xbhb-2C, a Mat α *sst2-1* mutant strain, to ensure that these strains were not secreting a-factor.

RESULTS AND DISCUSSION

Screening of a Collection of Gene Deletion Strains for CPY Secretion

To identify vacuolar protein sorting mutants, we screened a collection of 4653 homozygous diploid deletion strains developed by the Saccharomyces Genome Deletion Project (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html) (Winzeler *et al.*, 1999) and available through Research Genetics (http://www.resgen.com/products/YEASTD.php3). The collection was made by polymerase chain reaction-based disruption of all open reading frames (ORFs) larger than 100 codons in the BY4743 wild-type strain. Because the diploid strains are homozygous for the deletions, only nonessential genes (~82% of the total) are represented in the collection. The primary screening consisted of analyzing each deletion strain for secretion of CPY by using a colony blotting assay (Roberts *et al.*, 1991). In wild-type cells, CPY is efficiently sorted to the vacuole and therefore is not secreted. In vps mutants, on the other hand, CPY sorting to the vacuole is impaired and different levels of the protein are secreted. Figure 1a, top, shows examples of the CPY colony blotting assay for wild-type (negative control) and *vps39*, a *vps* mutant known to secrete high levels of CPY (positive control).

Screening of the complete collection resulted in the identification of 362 mutant strains (7.8% of the total) that secreted various amounts of CPY. The strains were categorized as strong (46 strains), moderate (100 strains), and weak (216 strains) based on a visual evaluation of the signal intensity, as exemplified by the colony blots shown in Figure 1, a and b. The majority of known *VPS* genes and other genes previously shown to be involved in vacuolar protein sorting were found distributed among the strong and moderate categories. For this reason, genes whose deletion gave strong and moderate phenotypes were treated as a single class in all subsequent analyses. Tables 1 and 2 list the genes with strong-moderate and weak phenotypes, respectively, that were identified in the screen. Supplemental Tables 1 and 2 are available online and contain additional information on these genes, their products and the phenotypes of the corresponding mutant strains. Genes in most tables are grouped according to the known or presumed functions of their products.

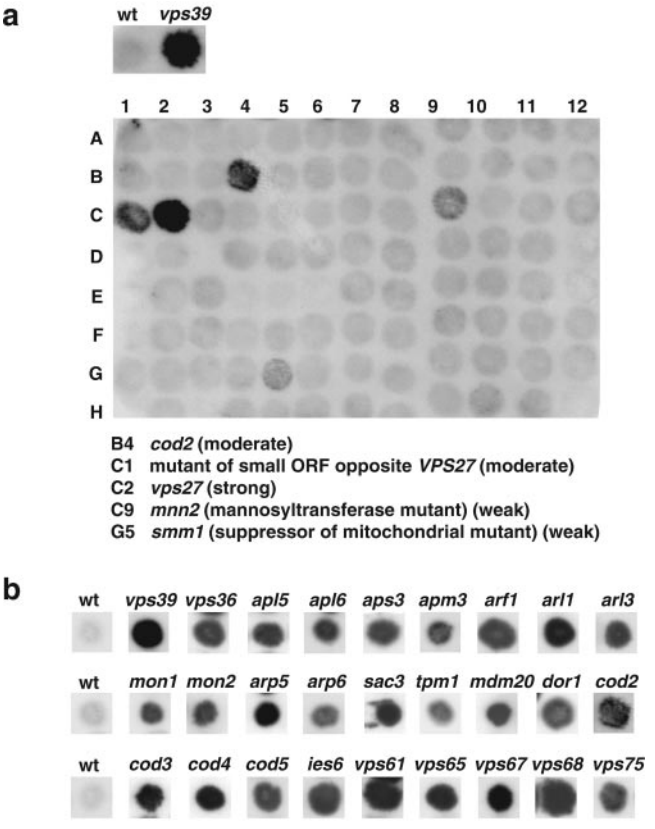

Figure 1. Identification of CPY-secreting strains. The collection of 4653 homozygous diploid deletion strains developed by the Saccharomyces Genome Deletion Project was screened using a CPY colony blot assay (Roberts *et al.*, 1991). The membranes were subjected to immunoblotting with mouse anti-CPY at 1:1000 dilution. Results for either one 96-well plate (a) or examples of several mutants taken from different blots (b) are shown. Wild-type (wt) and *vps39* strains are included for comparison.

Genes whose deletions gave strong-moderate phenotypes encoded: most known Vps proteins, other proteins previously implicated in vacuolar protein sorting, vacuolar ATPase subunits, components of the glycosylation machinery, AP-3 subunits, Arf1 and Arf-related proteins, actin-related proteins, monensin and brefeldin A hypersensitivity proteins, ribosomal proteins, miscellaneous proteins, and the products of a number of hypothetical ORFs (Table 1 and Supplemental Table 1). Genes whose deletions gave weak phenotypes included members of some of the same groups discussed above, plus genes involved in protein trafficking to compartments other than the vacuole, and a large number

Table 1. Genes identified in CPY secretion screen (strong or moderate phenotypes)

Known *VPS* Genes (40)
VPS1, VPS3, VPS4, VPS5, VPS6, VPS8, VPS9, VPS10, VPS13, VPS16, VPS17, VPS18, VPS19, VPS20, VPS21, VPS22, VPS23, VPS24, VPS25, VPS26, VPS27, VPS28, VPS29, VPS30, VPS32, VPS33, VPS34, VPS35, VPS36, VPS37, VPS38, VPS39, VPS41, VPS44, VPS45, VPS52, VPS53, VPS54, VPS55, VPS60
Other Genes Implicated in Vacuole Biogenesis or Function (13)
CCZ1, DID4, GOS1, MRL1, MVP1, PTC1, RGP1, RIC1, SYS1, TLG2, VAM7, YPT6, YPT7
Vacuolar ATPase (12)
VMA1, VMA2, VMA4, VMA6, VMA8, VMA10, VMA11, VMA12, VMA13, VMA21, VMA22, VPH1
Glycosylation (9)
FKS1, KRE6, MNN2, MNN9, MNN11, OCH1, OST3, PMT1, VAN1
AP-3 Subunits (4)
APL5, APL6, APM3, APS3
ARF1 and ARF-related (3)
ARF1, ARL1, ARL3
Monensin and Brefeldin A Hypersensitive (2)
MON1, MON2
Actin-related (10)
ANP1, AOR1, ARP5, ARP6, CAX4, HOF1, MDM20, PSL10, SAC3, TPM1
Ribosomal Proteins (5)
RPL21A, RPL27A, RPL35A, RPP1A, RPS6A
Miscellaneous (35)
ADH1, ARC1, BDF1, BRO1, BUD14, BUD32, CKB1, COD2, COD3, COD4, COD5, DHH1, DOR1, GSG1, GUP1, GYP1, IES6, IRS4, KRE25, MAK3, MAK10, NPL6, OAR1, PER1, PKR1, PMR1, RAI1, SNA2, RSC2, SNF3, SPF1, STP4, THP1, TOM37, YAF9
Hypothetical ORFs (15)
VPS61 (YDR136C), *VPS62* (YGR141W), *VPS63* (YLR261C), *VPS64* (YDR200C), *VPS65* (YLR322W), *VPS66* (YPR139C), *VPS67* (YKR020W), *VPS68* (YOL129W), *VPS69* (YPR087W), *VPS70* (YJR126C), *VPS71* (YML041C), *VPS72* (YDR485C), *VPS73* (YGL104C), *VPS74* (YDR372C), *VPS75* (YNL246W)

This table lists a total of 148 genes whose deletion results in CPY secretion. The number of genes in each group is indicated in parentheses. The majority of the corresponding deletion strains display strong or moderate CPY secretion phenotypes. Genes corresponding to a few weak CPY secreting strains (VPS60 and TPl1) are also included because they belong to the same structural or functional class as some of the other genes. For the sake of simplicity, we use the *VPS* or *VMA* classifications whenever possible, even though a different designation may have been originally used for some genes. Some gene designations have not yet been published in the literature but are either registered in the Saccharomyces Genome Database or reported in abstract form. Additional information on the genes listed in this table, their products and/or the phenotypes of the corresponding mutant strains can be found in Supplemental Table 1 (online).

Table 2. Genes identified in CPY secretion screen (weak phenotype)

Vacuole Biogenesis or Function (11)
APG17, AUT4, BPH1, GRD19, IMH1, SNX4, SWA2, VAC14, VAM3, VID21, VID22
Vacuolar ATPase (5)
RAV1, RAV2, VMA3, VMA7, VMA5
Glycosylation or Cell Wall Assembly (11)
ALG5, CWH36, HOC1, KRE1, KRE11, KRE6, PMT2, RHK1, SMI1, YER083C, *YUR1*
ARF GAPs (3)
AGE2, GCS1, GLO3
Actin-related (3)
BNI1, SAC1, SAC7
Brefeldin A Sensitive (3)
BRE5, BFR1, ERG4
Protein Trafficking (13)
AKL1, AKRI, BST1, CNE1, ERD1, ERV41, LHS1, RUD3, SEC13, SEC22, SEC66, SSH1, YPT31
Ribosomal Proteins (16)
RPL11B, RPL12A, RPL13B, RPL16B, RPL17B, RPL2A, RPL36A, RPL37A, RPL39, RPL40A, RPL6A, RPL7A, RPP1B, RPS21B, RPS29A, RPS9B
Miscellaneous (102)
ADE3, ADOI, APN1, ARO2, ATP11, BAS1, BIM1, BSD2, BTS1, BUD31, CDC10, CHL1, CTF19, CTF4, CTK1, CTK3, CTP1, CYK3, DAL1, DBP7, DOT5, DPP1, EFD1, ELM1, ERG24, ERG28, ESC2, GAS1, GCN5, GDA1, GEF1, GIF1, HCM1, HMT1, HNT3, HPR1, HTA1, HTL1, HTZI, IOC2, ISC1, ISWI, KIM3, KRE20, KRE28, LAS21, LOC1, LPP1, LSM6, LYP1, MAE1, MAL11, MIHI, MND2, MRT4, MSC1, MSL1, MSS18, MUD2, NEM1, NGL2, NIP100, NUP120, NUP84, OSM1, PFD1, PIN2, PLC1, PMC1, POS5, PRO1, RAD61, RAM1, REF2, RLR1, RSC1, SBP1, SGS1, SHP1, SICI, SKN7, SMM1, SNF1, SNF5, SPO7, STO1, SUM1, SWR1, SXM1, TCI1, TDH1, TIF3, UME6, UPF3, URM1, YAK1, YDL033C, YNL080C, YNL099C, *YTA7, YVH1, ZMS1*
Hypothetical ORFs (47)
YAL014C, YAL011W, YAL004W, YLR111W, YML013C-A, YMR299C, YOR292C, YPL184C, YBR266C, YBR246W, YBR267W, YDR105C, YDR426C, YDR425W, YEL043W, YGR206W, YHL029C, YHR111W, YHR199C, YLR181C, YLR225C, YOR135C, YLR374C, YLR381W, YDR186C, YDR203W, YGL198W, YGL072C, YKR035C, YHR009C, YLR435W, YLR426W, YJR079W, YJR100C, YCR050C, YDL096C, YJR018W, YGR064W, YIL067C, YGL020C, YIL110W, YNL136W, YNL127W, YCR033W, YJL075C, YLR350W, YDL119C

This table lists a total of 214 genes whose deletion results in a weak CPY secretion phenotype. The number of genes in each group is indicated in parentheses. Some genes have not been published in the literature but are either registered in the Saccharomyces Genome Database or reported in abstract form.

of miscellaneous genes and hypothetical ORFs (Table 2 and Supplemental Table 2). Because of the greater likelihood that mutations in genes whose deletions have a weak phenotype could affect vacuolar protein sorting indirectly, we focused our subsequent analyses on deletion strains with strong-moderate CPY secretion.

The CPY-secretion phenotypes of 146 of the strong-moderate strains were confirmed at least twice by colony blotting of the homozygous diploid mutants and the corresponding haploid mutants. All diploid, as well as all but two haploid strains (*adh1* and *bud14*), retested positive in these assays. Seventy-four mutant strains were selected for further analyses. Additional properties analyzed included growth at 30° or 37°C (Table 3); biosynthetic processing of CPY, PrA, and ALP (Figure 2); vacuole morphology (Figure 3); integrity of the actin cytoskeleton (Figure 4); and secretion of processed α-factor (Figure 5). Representative results for some of the mutants are presented in Figures 2–5, and a summary of these results is presented in Tables 3 and 4. The characteristics of selected genes and their products are discussed below.

Known VPS Genes and Other Genes Previously Implicated in Vacuolar Protein Sorting

Forty genes identified in our screen had been previously reported in the literature or listed in the Saccharomyces Genome Database as *VPS* genes (Table 1 and Supplemental Table 1). Of the *VPS* genes characterized to date (reviewed by Burd *et al.*, 1998; Conibear and Stevens, 1998; Mullins and Bonifacino, 2001a), only *VPS11* and *VPS15* were not identified in our screen, but this was because these genes were not represented in the deletion collection. The recovery of virtually all known *VPS* genes attests to the accuracy of the collection and the reliability of the CPY secretion assay. From this, we infer that the majority of the other genes have been accurately identified. Thirteen other genes (*CCZ1*, *DID4*, *GOS1*, *MRL1*, *MVP1*, *PTC1*, *RGP1*, *RIC1*, *SYS1*, *TLG2*, *VAM7*, *YPT6*, *YPT7*) had also been previously implicated directly or indirectly in vacuolar protein sorting (Table 1 and Supplemental Table 1). These known genes will not be further discussed herein, except for their relationship to other genes of interest.

Vacuolar ATPase

Twelve genes whose deletions resulted in strong-moderate phenotypes (Table 1 and Supplemental Table 1) and six genes whose deletions gave weak phenotypes (Table 2 and Supplemental Table 2) encoded either subunits of the vacuolar proton-translocating ATPase or proteins involved in its assembly or regulation (reviewed by Graham *et al.*, 2000). The subunits belonged to both the catalytic V_1 subcomplex (Vma1p, Vma2p, Vma4p, Vma5p, Vma7p, Vma8p, Vma10p, and Vma13p) and the proton-translocating V_0 subcomplex (Vma3p, Vma6p, Vma11p, and Vph1p). The only remaining subunit of the V_0 subcomplex, Vma16p, was not represented in the deletion collection used for the screen. The assembly factors included Vma12p, Vma21p, and Vma22p, which facilitate assembly but are not stable components of either subcomplex. Thus, the full integrity of the vacuolar ATPase appears to be required for efficient CPY sorting to the vacuole. The identification of vacuolar ATPase genes in our screen is in line with previous observations that mutations in the *VMA2* gene (Yamashiro *et al.*, 1990) or treatment with the vacuolar ATPase inhibitor, bafilomycin A1 (Klionsky and Emr, 1989), cause partial missorting and secretion of CPY. This points to a possible requirement for acidification of the vacuole and perhaps the PVC for efficient CPY sorting. However, the V_0 subcomplex has also been shown to participate directly in vacuole fusion, in a role independent of acidification (Peters *et al.*, 2001); thus, it is possible that this subcomplex may also play a more direct role in CPY sorting.

Glycosylation

Nine genes whose deletions resulted in strong-moderate phenotypes (Table 1 and Supplemental Table 1) encoded proteins involved in glycosylation or modification of carbohydrate chains. Some of these proteins (e.g., Van1p, Mnn9p, Mnn11p, and Anp1, the latter of which is listed under actin-related proteins) are known to form multiprotein complexes with mannosyl-transferase activity in the Golgi complex (Jungmann and Munro, 1998; Kojima *et al.*, 1999). At present it is unclear how defects in glycosylation could cause missorting of CPY, although it has been reported that unglycosylated CPY is transported more slowly through the secretory pathway (Winther *et al.*, 1991). Thus, it is possible that abnormal glycosylation of CPY or transmembrane components of the vacuolar protein sorting machinery (e.g., Vps10p, Mrl1p) could decrease the efficiency of sorting to the vacuole. Given that glycosylating enzymes are also involved in the biosynthesis of cell wall materials, it is also possible that their deficiency renders the cell wall fragile and causes lytic release of vacuolar CPY.

AP-3

We were surprised to find that strains with deletions of the genes encoding the four subunits of the AP-3 complex (Apl5p, Apl6p, Apm3p, and Aps3p) (Cowles *et al.*, 1997a; Panek *et al.*, 1997; Stepp *et al.*, 1997) secreted moderate levels of CPY (Figure 1b and Table 1 and Supplemental Table 1). AP-3 had been shown to be involved in the ALP pathway but not the CPY pathway (Cowles *et al.*, 1997a; Stepp *et al.*, 1997). Pulse-chase analyses revealed delayed processing of the Golgi precursor forms of pro-CPY and pro-PrA in the AP-3 mutant strains, although this was much less severe than that of the strongest *vps* mutants (Figure 2). Processing of ALP was completely blocked in the AP-3 mutants (Figure 2), as reported previously (Cowles *et al.*, 1997a; Stepp *et al.*, 1997). The detection of a slight CPY sorting defect in our study could be due to the greater sensitivity of the assays or to the different genetic background of the yeast strains used herein. We suspect that this CPY sorting defect could be an indirect consequence of the drastic inhibition of the ALP pathway. The vacuolar t-SNAREs Vam3p and Nyv1p, for example, normally traffic via the ALP pathway, but function in vesicle fusion in the CPY pathway (Darsow *et al.*, 1997; Reggiori *et al.*, 2000). Although, Vam3p and Nyv1p are rerouted through the CPY pathway in AP-3 mutants (Darsow *et al.*, 1998; Reggiori *et al.*, 2000), it is possible that this rerouting does not completely compensate for the loss of the AP-3 pathway.

The *apl5*, *apl6*, *apm3*, and *aps3* mutant strains have similar vacuole morphology, with slightly fewer lobes than the wild-type strain and lobes that seem somewhat unfolded (Figure 3). This phenotype, although subtle, may indicate a decrease in vacuole fission, or an increase in vacuole fusion. This may also be an indirect consequence of reduced Vam3p at the vacuole, as factors required for normal vacuole morphology may have difficulty reaching the vacuole under these conditions.

ARF1 and ARF-related Proteins

Three strong-moderate genes encoded the small GTP-binding proteins of the Ras superfamily, Arf1p (ADP-ribosylation factor 1 product), Arl1p (Arf-like 1 product), and Arl3p (Arf-like 3 product) (Table 1 and Supplemental Table 1). In addition, three weak genes encoded the Arf GTPase-activating proteins Age2p, Gcs1p, and Glo3p (Table 2 and Supplemental Table 2). Arf proteins had been previously implicated in the regulation of protein trafficking along the secretory pathway by virtue of their interactions with multiple effectors, including several coat proteins (Donaldson and Jackson, 2000). *S. cerevisiae* expresses two Arf proteins, Arf1p and Arf2p, of which Arf1p is the most abundant (Stearns *et al.*, 1990). Disruption of both genes is lethal,

whereas disruption of only *ARF1* is not (Stearns *et al.*, 1990). Although viable, *arf1* mutant strains display various phenotypes, including slower kinetics of CPY transport through the secretory pathway (Chen and Graham, 1998; Huang *et al.*, 1999; Yahara *et al.*, 2001).

In agreement with these reports, we found that deletion of the *ARF1* gene results in delayed processing of CPY and PrA (Figure 2). Some of this delay could be due to a reduced rate of transport from the endoplasmic reticulum (ER) to the Golgi complex. However, our finding that the *ARF1*-deletion strain secretes CPY (Figure 1) indicates that Golgi-to-vacuole sorting is also compromised in this strain. This interpretation agrees with the observation that *ARF1* displays genetic interactions with clathrin and auxilin (Swa2p, also identified in our screen; Table 2 and Supplemental Table 2), two proteins involved in late-Golgi transport events (Chen and Graham, 1998). Moreover, Arfs are involved in the recruitment of the GGA adaptor proteins to the late-Golgi complex (Zhdankina *et al.*, 2001). We also observed delayed processing of ALP in the *ARF1*-deletion strain (Figure 2), which could be due to inhibition of either ER-to-Golgi or Golgi-to-vacuole transport. In mammalian cells, Arf1 and Arf3 regulate the association of AP-3 with membranes (Ooi *et al.*, 1998; Drake *et al.*, 2000), and it is possible that Arf1p could play a similar role in *S. cerevisiae*. In addition to defects in protein trafficking, the *arf1* strain exhibited defects in vacuole morphology, actin cytosketelon, and α-factor secretion. The vacuole morphology of *arf1* was fragmented, exhibiting a larger number of vacuole lobes than the wild-type (Figure 3). However, the degree of fragmentation in *arf1* was less severe than that observed for *vps39*, in which protein traffic is more impaired. This suggests that inefficient trafficking of factors required for vacuole fusion or biogenesis may account for the vacuole morphology observed in *arf1*. We also observed a decrease in the number and thickness of polarized actin filaments in *arf1* mutants (Figure 4). Finally, we found that the *arf1* mutant strain secreted less mature α-factor, as determined by a halo assay for growth arrest (Figure 5A). This reduced secretion of mature α-factor is likely due to a block in Kex2p recycling to the Golgi complex as glycosylated pro-α-factor accumulates in *arf1* (Figure 5B), suggesting a deficiency in α-factor processing.

The three Arf GAPs, Age2p, Gcs1p, and Glo3p, that were identified among the weak secretors might play overlapping roles in the inactivation of yeast Arfs at the late-Golgi complex. In support of this notion, a recent study has shown that yeast mutants lacking both Age2p and Gcs1p function exhibit substantial defects in CPY and ALP sorting to the vacuole (Poon *et al.*, 2001).

S. cerevisiae Arl1p and Arl3p are structurally related to Arf1p (55 and 37% amino acid sequence identity, respectively), but their functions are less well understood. An *ARL1*-deletion strain was previously reported to display normal biosynthetic processing of CPY (Lee *et al.*, 1997). Another study showed that deletion of *ARL3* also resulted in normal processing of CPY, but processing of ALP was delayed at the nonpermissive temperature of 15°C (Huang *et al.*, 1999). We observed that *ARL1*- or *ARL3*-deletion strains exhibited secretion of CPY into the medium (Figure 1), delayed processing of both CPY and PrA (Figure 2), and normal processing of ALP (Figure 2) at 30°C. Both *arl1*Δ and *arl3*Δ have vacuole morphology defects similar to those of *arf1*Δ (Figure 3). Unlike Arf1p, however, Arl1p and Arl3p do not seem to be required for a normal polarized actin cytoskeleton (Figure 4) or in α-factor secretion (Figure 5).

These observations place Arl1p and Arl3p in the CPY pathway of biosynthetic sorting to the vacuole. Like Arf1p, Arl1p and Arl3p would be expected to exert their functions through GTP-dependent binding to effector proteins. Human Arl1 has been shown to bind to various putative effector proteins, including one termed SCOCO (for short coiled-coil) (Van Valkenburgh *et al.*, 2001). This protein has two *S. cerevisiae* homologs, Vps30p and Imh1p, both of which were also identified in our screen (Tables 1 and 2 and Supplemental Tables 1 and 2).

Monensin and Brefeldin A Hypersensitivity Genes

Two genes whose deletions gave strong-moderate phenotypes, *MON1* and *MON2*, and two genes whose deletions gave weak phenotypes, *BRE5* and *ERG4*, identified in our screen had been previously found in a screen for mutant strains hypersensitive to the drugs monensin and/or brefeldin A (Muren *et al.*, 2001). We observed that both *MON1*- and *MON2*-deletion strains secreted CPY (Figure 1). Proteolytic processing of CPY and PrA was completely blocked in *mon1*, as in the strongest of the *vps* mutants (Figure 2). Processing of ALP was partially blocked in *mon1* (Figure 2). Processing of all three vacuolar enzymes was also impaired in the *mon2* strain, but the pattern differed from that of the *mon1* strain in that processing of CPY and PrA was less affected than that of ALP (Figure 2). Interestingly, the block in ALP processing in *mon2* was similar to that observed in *vps39* and the AP-3 mutants (Figure 2).

Consistent with defects in Golgi-to-vacuole traffic, both *mon1* and *mon2* have fragmented vacuoles (Figure 3). However, the vacuoles in *mon1* are smaller and tend to remain clustered together (Figure 3), whereas those of *mon2* are slightly larger but more dispersed (Figure 3). The distinct vacuolar phenotypes of these mutants may reflect their preferential function in the CPY and ALP pathways, respectively. Neither Mon1p nor Mon2p is required for secretion of α-factor (Figure 5).

A noteworthy feature of Mon2p is a 115-amino acid segment of homology (26% amino acid sequence identity) to Sec7p, a guanine nucleotide exchange factor (GEF) for Arf (Peyroche *et al.*, 1996). The homologous segment, however, does not overlap with the so-called Sec7 domain, which has intrinsic Arf GEF activity (Chardin *et al.*, 1996).

Actin-related Proteins

Another intriguing finding was the identification in our screen of 10 genes (*ANP1*, *AOR1*, *ARP5*, *ARP6*, *CAX4*, *HOF1*, *MDM20*, *PSL10*, *SAC3*, and *TPM1*) encoding proteins that are structurally or functionally related to actin (Table 1 and Supplementary Table 1). Arp5p and Arp6p are two of 10 actin-related proteins identified in *S. cerevisiae* (Schafer and Schroer, 1999). In addition to secreting CPY (Figure 1), *arp5* and *arp6* mutant strains displayed delayed processing of CPY and PrA (*arp5* more than *arp6*), but not ALP (Figure 2), placing them in the CPY pathway. Both *arp5* and *arp6* have similar defects in vacuolar morphology, often displaying a large vacuole lobe surrounded by much smaller lobes (Figure 3). Furthermore, both mutant strains were defective in secretion of mature α-factor (Figure 5), defects that seem to

Table 3. Phenotypic characteristics of selected CPY-secreting strains

Strain	CPY secretion	CPY processing	PrA processing	ALP processing	α-Factor halo	Growth at 30°C	Growth at 37°C
adh1	+++	+	++	+++	mat a	+++	+++
aor1	++	+	++	+++	+++	+++	+++
apl5	++	++	++	−	+++	+++	+++
apl6	++	++	++	−	+++	+++	+++
apm3	++	++	++	−	+++	+++	+++
aps3	++	++	++	−	+++	+++	+++
arf1	++	+	+	+	+	+++	+++
arl1	+++	++	+++	+++	+++	+++	+++
arl3	++	++	+++	+++	+++	+++	+++
arp5	+++	+	++	+++	−	++	+/−
arp6	++	++	++	+++	+	+++	+++
bro1	++	+/−	++	+++	++	++	+
bud14	++	+++	++	+++	+	+++	+++
bud32	++	+	+	++	+/−	+	−
cax4	+++	++	++	++	−	+	+
ckb1	++	++	++	+++	+++	++	+
cod2	+++	+	++	+++	++	+++	+++
cod3	+++	+/−	+	+	+/−	+	+
cod4	+++	+	++	+++	−	+++	+++
cod5	++	++	++	+++	++	+	+
dhh1	++	+	++	+++	++	+++	+++
did4	++	+	++	+++	+++	++	++
dor1	++	+	++	+++	+	+++	+++
gos1	+++	+	+	++	+++	+++	+++
gup1	++	ND	ND	++	++	+++	+++
gyp1	++	+	++	+++	+++	+++	+++
hex3	+	++	++	+++	+++	++	++
hof1	++	++	+++	+++	++	+++	++
ies6	++	+	++	+++	−	+	+/−
irs4	+++	+	++	+++	++	+++	+++
kre25	++	+	+	+++	+++	++	−
mak3	++	++	+++	+++	+++	+++	+++
mak10	++	++	++	+++	+++	+++	+++
mdm20	++	++	+	++	+/−	+	−
mon1	++	−	+	+	+++	+++	+++
mon2	++	+	++	−	+++	+++	+
mrl1	++	++	++	+++	+++	+++	+++
mvp1	++	+	++	+++	+	+	+
pkr1	++	+	+	+++	+	+++	+++
psl10	++	+	++	+++	++	+++	+++
ptc1	++	++	++	+++	++	++	+/−
rai1	++	++	++	+++	+	++	++
ric1	++	+	++	++	++	+++	+/−
rgp1	++	+	++	+++	++	+++	+
rpl27a	++	++	++	+++	no haploid	++	++
sac3	++	++	++	+++	+	+++	+++
sna2	++	++	++	+++	+++	+++	+++
snf3	++	+++	++	+++	+++	+++	+++
sys1	++	+	++	+++	+++	+++	+++
thp1	++	++	++	+++	+/−	+++	+++
tpm1	+	++	++	+++	+	++	++
vps22	++	+	+	+++	+	+++	+++
vps36	++	+	+	+++	++	+++	+++
vps37	+++	+/−	+	+++	++	+++	+++
vps39	+++	−	−	−	+++	+++	+++
vps44	+++	+	+	+++	+	+++	+++
vps55	+++	+	++	+++	++	+++	+++
vma4	+++	+/−	+	+++	+	+	+
vps61	+++	+	+	+	++	+++	+/−
vps62	++	++	++	+++	+++	+++	+++
vps64	++	++	++	++	−	+++	+++
vps65	++	+	++	+++	++	+	+/−
vps66	++	++	++	+++	++	+++	+++

Table 3 (Continued).

Strain	CPY secretion	CPY processing	PrA processing	ALP processing	α-Factor halo	Growth at 30°C	Growth at 37°C
vps67	+++	+/−	++	+	−	+	+/−
vps68	++	+	++	+++	++	+++	+++
vps69	++	++	++	++	++	++	+
vps70	++	+++	+++	+++	+++	+++	+++
vps71	++	+	++	+++	+	+++	+++
vps72	++	++	++	+++	+++	+++	+++
vps73	++	++	+++	+++	++	+++	+++
vps74	++	+++	++	+++	+++	+++	+++
vps75	++	++	+	++	+++	++	++
yaf9	++	++	++	+++	+++	+++	++
WT	−	+++	+++	+++	+++	+++	+++

This table summarizes results of various assays done for 74 strains. CPY secretion was estimated visually from CPY colony blots performed. Range employed: −, no detectable CPY secreted above background (i.e. similar to wt); +, very low levels of CPY detected; ++, moderate levels of CPY secreted; and +++, high levels of CPY secreted. For pulse-chase experiments, the scale indicates the amount of each enzyme processed to the mature form after a 10-min chase. The range used is as follows: +++, 90–100% enzyme found as mature form (similar to wt); ++, 60%–90% enzyme converted to mature form; + 30–60% of enzyme converted to the mature form; +/−, 10–30% of enzyme converted to the mature form; −, 0–10% of the enzyme was converted to the mature form (i.e. similar to *vps39*); ND, not determined. For halo assays the scale indicates the size of halo (or the amount of α-factor secreted). The range used was determined by visual inspection. +++, halos were similar to wt at each concentration of cells spotted; ++, halos were smaller than wt at the lowest concentration of cells plated; +, halos were much smaller than wt independent of the concentration of cells spotted; +/−, halos were very small at any concentration of cells spotted; −, no halos were detected at any concentration of cells spotted. Two of the strains selected could not be tested for α-factor secretion by the halo assay because either the haploid strain was not present in the Mat α knockout collection purchased (*rpl27a*) or the strain in the Mat α knockout collection purchased was actually a Mat a strain (gave a halo on Mat α *sst2-1* cells) (*adh1*). Growth at 30 and 37°C were determined by streaking cells onto YPD plates and incubating at the indicated temperatures for 2–3 d until robust growth could be seen for the wt strains. The size of the colonies were then inspected visually. The scale used indicates the growth and size of colonies observed. +++, good growth/large colonies (similar to wt); ++, moderate growth/medium colonies; +, poor growth/small colonies; +/− very poor growth/indistinct colonies (growth only on streak); −, no growth.

stem from a delay in α-factor processing indicated by the accumulation of pro-α-factor and α-factor–processing intermediates in these strains (Figure 5B; our unpublished data). Neither Arp5 nor Arp6 has severe defects in the actin cytoskeleton structure at 30°C, although polarized actin cables in the mother cells may be slightly more disorganized than those observed in wild type (Figure 4). Other ARPs are components of stable complexes with other proteins (Schafer and Schroer, 1999). Arp2p and Arp3p, for example, are part of a complex that regulates the assembly of actin networks and also participates in endocytosis in *S. cerevisiae* (Moreau *et al.*, 1997). In mammalian cells, the Arp2/3 complex mediates propulsion of endosomes in the cytoplasm (Taunton *et al.*, 2000). It is thus tempting to speculate that Arp5p and Arp6p could play a role in transport to the vacuole analogous to that of Arp2p and Arp3p in endocytosis.

anp1, *psl10*, *cax4*, *aor1*, and *hof1* displayed severe defects in the actin cytoskeleton with few or no polarized actin cables visible in the mother cells [Novick *et al.*, 1989; Kamei *et al.*, 1998; Sekiya-Kawasaki *et al.*, 1998; our unpublished data), similar to those observed for *tpm1* and *mdm20* (Liu and Bretscher, 1989; Hermann *et al.*, 1997; Figure 4). All of these mutants also exhibited defects in secretion of mature α-factor (Figure 5). *cax4*, which, like *arp5*, secretes very little mature α-factor, also accumulates a large amount of α-factor–processing intermediates (Figure 5B). In fact, all the actin mutants analyzed accumulate some of these intermediates, suggesting that the integrity of the actin cytoskeleton may be important for recycling of Kex2 from the prevacuolar compartment back to the Golgi.

The actin cytoskeleton is known to mediate vacuole movements in the process of mitotic vacuolar inheritance (Catlett and Weisman, 2000). Thus, it is possible that some of the actin-related proteins identified in our screen could participate in the translocation of vesicular intermediates on their way to the vacuole. This role could be analogous to the well-established function of the actin cytoskeleton in endocytosis in yeast (Munn, 2001). It is also possible that some of the genes in this group could be more directly involved in vesicle tethering or fusion events and only indirectly involved in association with the actin cytoskeleton. A case in point is the product of the *VPS52* gene, a component of the Vps52p-Vps53p-Vps54p complex involved in tethering endosome-derived vesicles to the Golgi apparatus (Conibear and Stevens, 2000; Siniossoglou and Pelham, 2001), which was first identified as a suppressor of actin mutations, Sac2p (Novick *et al.*, 1989). Interestingly, *sac2* also displays similar defects in the actin cytoskeleton as the other mutants, exhibiting a decrease in the number of polarized actin cables visible in the mother cells (Novick *et al.*, 1989; our unpublished data).

Ribosomal Proteins

Five strong-moderate genes (Table 1 and Supplemental Table 1) and 16 weak genes (Table 2 and Supplemental Table 2) encoded components of both the large and small ribosome subunits. One intriguing explanation for the secretion of CPY by these mutants would be the existence of a regulatory network that reduces vacuolar protein sorting when translation is impaired. In this regard, *RIC1* was initially identi-

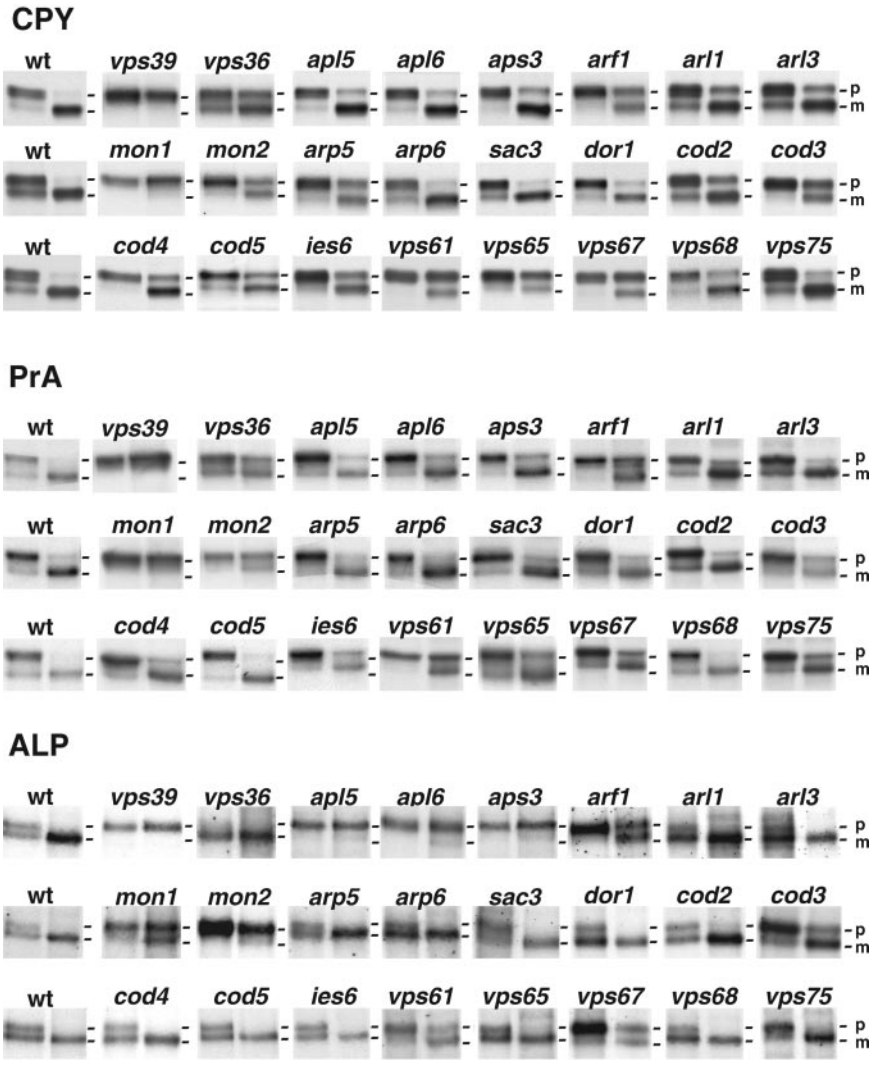

Figure 2. Analysis of CPY, PrA, and ALP processing. Wild-type (wt) and deletion strains were pulse-labeled with [^{35}S]methionine for 10 min (first lane of each pair) and chased for 10 min (second lane of each pair). Sequential immunoprecipitations were performed from the lysates by using antibodies against CPY (a), PrA (b), or ALP (c) as described in MATERIALS AND METHODS. Results are shown for wild-type (wt) and 24 mutant strains. The positions of precursor (p) and mature (m) forms of the hydrolases examined are indicated. The p1 and p2 precursor forms of CPY were not well resolved for all strains, in some cases due to aberrant Golgi glycosylation that resulted in comigration of both precursor species. A summary of results for these and other strains not shown in this figure is presented in Table 3.

fied in a screen for mutations that decrease ribosome synthesis (Mizuta *et al*., 1997), but was later found to encode a protein that, together with the product of *RGP1*, functions as a GEF for Ypt6p (Siniossoglou *et al*., 2000; Bensen *et al*., 2001) (both *RIC1* and *RGP1* were identified in our screen; Table 1 and Supplemental Table 1). We cannot rule out, however, that decreased translation of one or more key components of the vacuolar protein sorting machinery might underlie the CPY sorting defects observed in ribosomal proteins mutants.

Miscellaneous Proteins

This group includes the products of 35 genes whose deletions resulted in strong-moderate phenotypes but that were previously identified in screens other than for vacuolar protein sorting (Table 1 and Supplemental Table 1). A subgroup of these genes has been implicated in Golgi function. These include *GYP1*, *PMR1*, *BRO1*, *DOR1*, *COD2*, *COD3*, *COD4*, and *COD5.* The products of five of these genes (*DOR1*, *COD2*, *COD3*, *COD4*, and *COD5*) have recently been shown to be components of an eight-subunit complex with Sec34p and Sec35p (Whyte and Munro, 2001a) referred to as the Sec34/35 complex (Kim *et al*., 1999; VanRheenen *et al*., 1999). This complex has been proposed to mediate vesicle tethering to the Golgi complex based on the weak homology of some of its subunits to subunits of the Vps52p-Vps53p-Vps54p (Conibear and Stevens, 2000; Siniossoglou and Pelham, 2001) and exocyst (TerBush *et al*., 1996) complexes (Whyte and Munro, 2001a). Deletion of genes encoding some of the subunits of the Sec34/35 complex has been shown to result in abnormal accumulation of intracellular membranes and glycosylation defects (Whyte and Munro, 2001a). Other observations suggest that the role of this complex may not be restricted to the Golgi complex. For example, the Golgi-plasma-membrane v-SNARE Snc1p became trapped in internal membranes in *dor1* mutants (Whyte and Munro, 2001a). Moreover, a *sec34* (also known as *grd20*) mutant strain exhibited mislocalization of Kex2p and secretion of CPY (Spelbrink and Nothwehr, 1999).

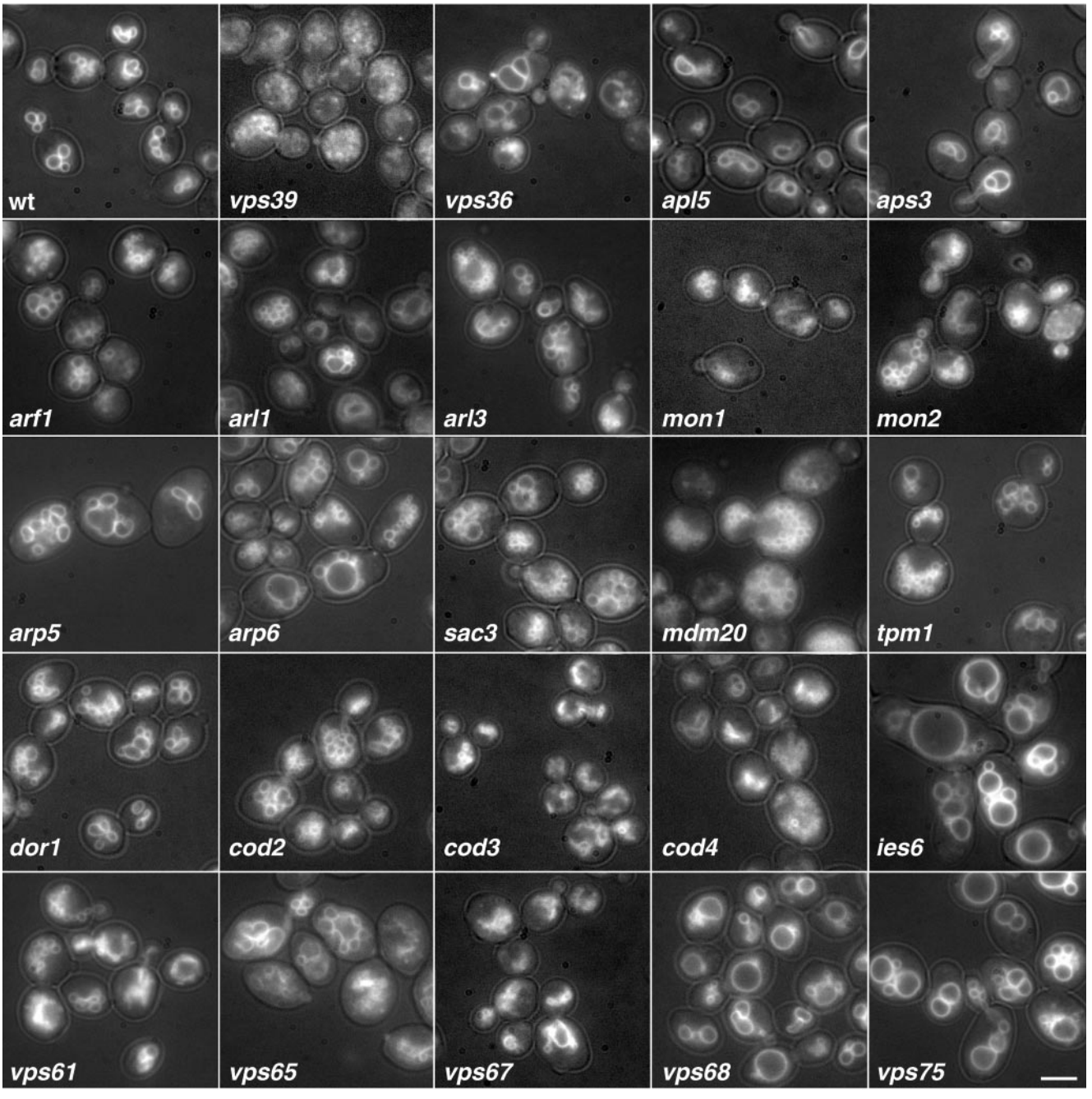

Figure 3. Visualization of vacuole morphology in wild-type and mutant strains by light microscopy. Vacuole morphology was examined by labeling with FM4-64 as described in MATERIALS AND METHODS. The photographs were taken with both fluorescence and a low level of transmitted light. Results are shown for wild-type (wt), *vps39* (as a control for aberrant vacuole morphology), and 23 selected vacuolar sorting mutants identified in our screen. Many of the deletions resulted in fragmented vacuole morphology, which was intermediate, and not as severe as the class B *vps* mutants (*vps39*). These include *arf1*, *arl1*, *arl3*, *mon1*, *mon2*, *mdm20*, *tpm1*, *dor1*, *cod2*, *cod3*, *vps61*, and *vps67*. Bar, 9 μm.

We found that the *DOR1-*, *COD2-*, *COD3-*, *COD4-*, and *COD5*-deletion strains displayed secretion of CPY (Figure 1) and delayed processing of CPY and PrA (Figure 2). These observations are consistent with the notion that the Sec34/35 complex could play a role in late-Golgi or post-Golgi sorting events. However, only *cod3* had a significant delay in ALP processing (Figure 2). In addition, *dor1*, *cod2*, *cod4*, and *cod5* had similar vacuolar morphologies, exhib-

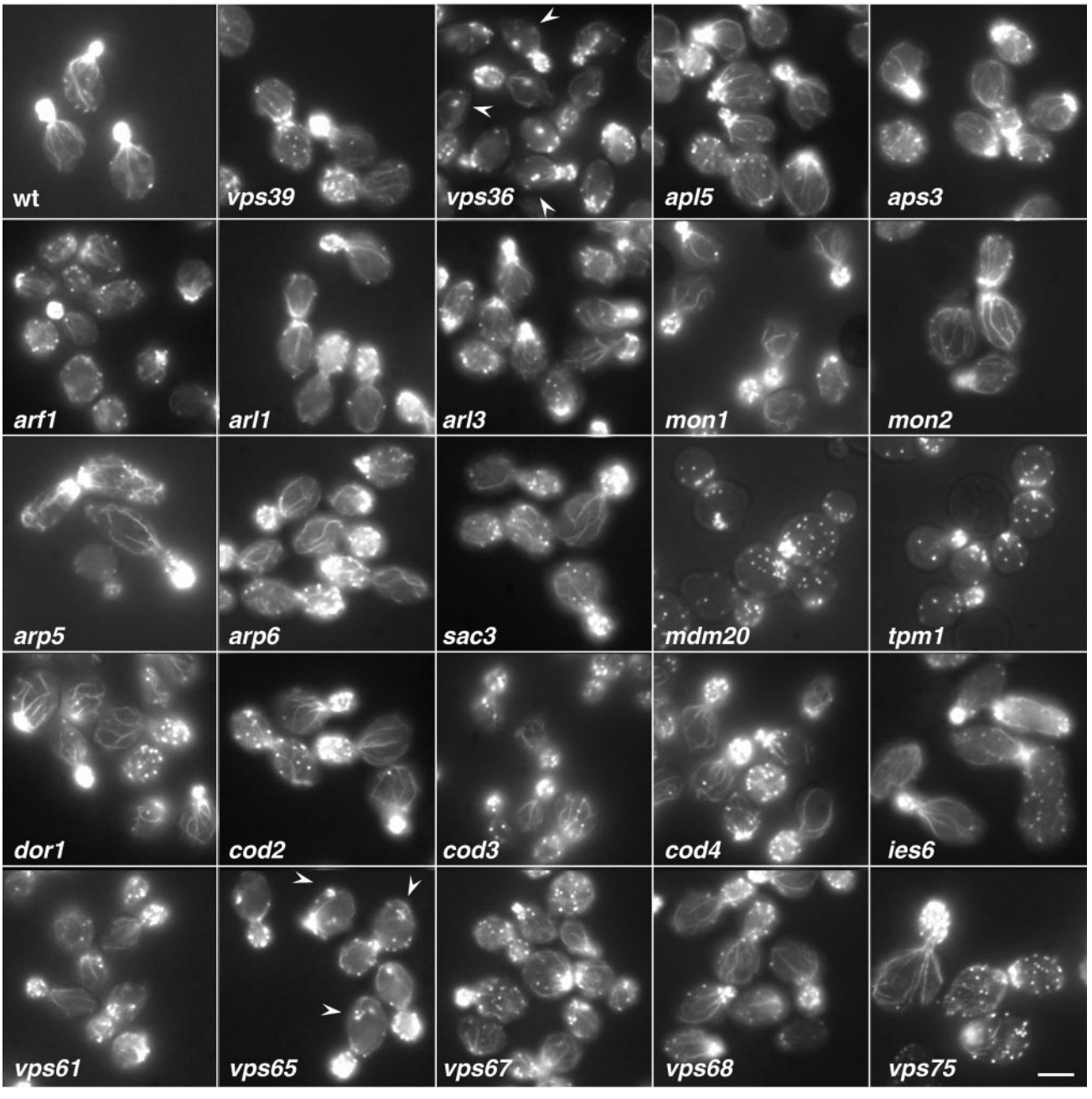

Figure 4. Visualization of the actin cytoskeleton in wild-type and mutant strains by light microscopy. The actin cytoskeleton was examined by labeling fixed log-phase cells with 1.1 μM Oregon green-phalloidin at 30°C for ~16–20 h as described in MATERIALS AND METHODS. Results are shown for wild-type (wt), *vps39*, and 23 selected vacuolar sorting mutants. The most common phenotype observed was the decrease in number or an absence of polarized actin cable in the mother cells, as is seen in *arf1*, *mdm20*, *tpm1*, and *vps61*. Other phenotypes included reduced polarized actin cables with the appearance of a large actin aggregate in the mother cell, seen in *vps36* and *vps65* (arrowheads), and slightly disorganized actin filaments, observed in *arp5* and *arp6*. Bar, 9 μm.

iting fragmented vacuoles (Figure 3), whereas the vacuoles of *cod3* were smaller and often exhibited unusual tubular structures (Figure 3). All of the mutants with the exception of *cod2* displayed defects in α-factor secretion with *cod3* having the most severe defect (Figure 5). The observations that these mutants share only some of the phenotypes and have some distinct phenotypes is consistent with previous reports, and may suggest that some of the proteins have additional roles besides their function in the sec34/35 complex.

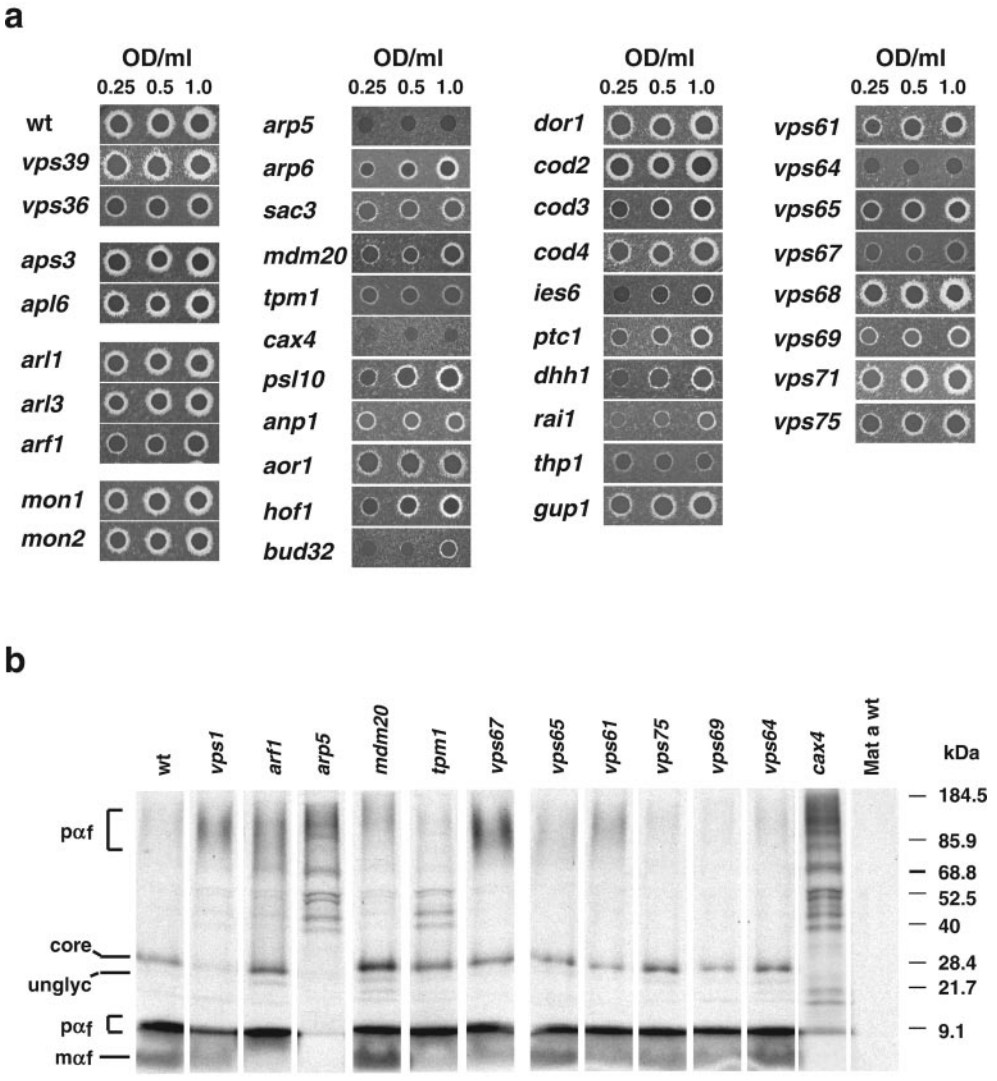

Figure 5. Analysis of α-factor secretion and processing. (a) To test for α-factor secretion, wild-type and mutant strains were grown to log phase and serially diluted to the concentrations indicated, and equal amounts were spotted on YEPD plates containing a freshly spread lawn of the α-factor–sensitive strain RC634 (MAT a, *sst1-3*), as described in MATERIALS AND METHODS. The plates were then incubated at 30°C for 48 h to assess α-factor secretion as indicated by the relative growth inhibition of the *sst1-3* mutant strain (halo). (b) To test for processing of α-factor, strains were metabolically labeled with [^{35}S]methionine at 25°C for 7.5 min as described in MATERIALS AND METHODS. Mature α-factor (mαf), α-factor–processing intermediates and pro-α-factor were immunoprecipitated with anti-α-factor antibodies, and resolved by SDS-PAGE (4–20% acrylamide gradient gels). Various forms of α-factor were detected, including pro-α-factor (pαf) that includes a range of high molecular weight intermediates containing the proregion and various degrees Golgi-derived glycosylation (~ 90–150 kDa), unglycosylated core-α-factor (unglyc) (~20 kDa), ER core-glycosylated α-factor (core) (~26 kDa), low molecular weight intermediates in α-factor processing (interαf) that most likely represent the tetrapeptide in various states of processing with the proregion removed (~6–8 kDa), and the mature α-factor (mαf), the 13-amino acid terminally processed secreted form (~2 kDa).

Hypothetical ORFs

Fifteen hypothetical ORFs were among the strong-moderate genes (Table 1 and Supplemental Table 1). For convenience, we designated these ORFs *VPS61-VPS75*. Many of these are small open reading frames (under 300 amino acids) and may have been missed in previous screens that depended on random mutagenesis, as the smaller target size of the genes lowered the probability of them being hit. Of these new putative ORFs, the deletion of two, *VPS61* (YDR136C) and *VPS67* (YKR020W), resulted in secretion of high levels of CPY (Figure 1) and caused a significant block in the processing of CPY, PrA, and ALP (Figure 2). Interestingly, *vps67* has similar vacuolar morphology to *cod3*, exhibiting small fragmented vacuoles as well as some tubular structures (Figure 3). *VPS61* is a small ORF opposite of 5′-untranslated region of *RGP1*, which has previously been implicated in vacuole protein sorting. Thus, it is still unclear whether deletion of *VPS61* is responsible for all or some of the phenotypes observed. However *rgp1*Δ did not display a defect in ALP processing (our unpublished data), suggesting that the lack of Vps61p may be responsible for this defect. Deletion of the other ORFs resulted in secretion of moderate levels of CPY (Figure 1; and Table 3) as well as defects in CPY and PrA processing that were less severe (Figure 2 and Table 3). In addition, many of them exhibited defects in α-factor secretion and/or processing (Figure 5). *vps67* and *vps64* (deletion of YDR200C) displayed the most severe phenotype (Figure 5), with little to no mature α-factor being secreted. Intriguingly, although *vps67* accumulates α-factor–processing intermediates (Figure 5B), *vps64*, which has a more severe secretion defect, exhibits almost normal levels of mature α-factor and α-factor–processing intermediates compared with wild type. Although in many of the strains analyzed, aberrant Kex2p localization and recycling to the Golgi compartment may result in delayed processing of α-factor and thus indirectly cause an α-factor secretion defect, this is not always the case. Some of the proteins involved in vacuole protein sorting, such as Vps64p, may be directly involved in the secretion of mature α-factor as well.

Table 4. Summary of phenotypes

Aberrant Intracellular Trafficking (73 Analyzed):
- Affect CPY and PrA Sorting (53): *adh1, aor1, arl1, arl3, arp5, arp6, bro1, bud14, ckb1, cod2, cod4, cod5, dhh1, did4, dor1, gyp1, hex3, hof1, ies6, irs4, kre25, mak3, mak10, mrl1, mvp1, pkr1, psl10, ptc1, rai1, rgp1, rpla27, sac3, sna2, snf3, sys1, thp1, tpm1, vma4, vps22, vps36, vps37, vps44, vps55, vps62, vps65, vps66, vps68, vps70, vps71, vps72, vps73, vps74, yaf9*
- Affect CPY, PrA and ALP Sorting (20): *apl5, apl6, apm3, aps3, arf1, bud32, cax4, cod3, gos1, gup1, mdm20, mon1, mon2, ric1, vps39, vps61, vps64, vps67, vps69, vps75*
- Affect α-factor secretion (41): *arf1, arp5, arp6, bro1, bud14, bud32, cax4, cod2, cod3, cod4, cod5, dhh1, dor1, gup1, hof1, ies6, irs4, mdm20, mvp1, pkr1, psl10, ptc1, rai1, rgp1, sac3, thp1, tpm1, vma4, vps22, vps36, vps37, vps44, vps55, vps61, vps64, vps66, vps67, vps68, vps69, vps71, vps73*

Aberrant vacuole morphology (71 Analyzed):
- Class B (35): *aor1, arf1, arl1, arl3, bud14, ckb1, cod2, cod4, cod5, dhh1, dor1, gos1, hof1, ies6, irs4, mdm20, mon1, mon2, pkr1, ptc1, ric1, rgp1, snf3, sys1, thp1, tpm1, vps61, vps64, vps66, vps67, vps69, vps71, vps72, vps73, vps75*
- Class C (2): *cax4, gup1*
- Class D (1): *bud32*
- Class E (4): *vps22, vps36, vps37, vps44*
- Class F (16): *arp5, arp6, bro1, hex3, mak10, mvp1, rai1, sac3, thp1, vma4, vps55, vps62, vps65, vps68, vps71, yaf9*

Aberrant Actin Cytoskeleton at 30°C (71 Analyzed):
- Disorganized actin cables (2): *arp5, arp6*
- Reduced number of polarized actin cables (9): *anp1, aor1, arf1, cax4, hof1, mdm20, psl10, tpm1,* and *vps52*
- Reduced actin cables and aberrant actin patches (4): *vps36, vps61, vps65, vps67*

This table groups the 73 deletion strains analyzed (listed in Table 3) according their shared phenotypes. For vacuole morphology, the strains were classified according to the designations established by Raymond *et al*. (1990). Class A strains, or those exhibiting normal vacuolar morphologies are not listed. Similarly only the strains exhibiting an aberrant cytoskeleton at 30°C are listed. Due the slow growth rates of *rpl27a* and *adh1,* neither the vacuole morphology nor the actin cytoskeleton was analyzed for these strains.

Of the 15 new ORFs, seven displayed some homology to mammalian gene products. However, only Vps74p (product of YDR372C) exhibited homology to a known protein, having ~40% amino acid sequence identity to a mammalian protein termed GMx33 (Wu *et al*., 2000) or GPP34/Golgi phosphoprotein 3 (Bell *et al*., 2001).

Because several actin-related proteins were found to be important for CPY sorting in our screen, it seemed possible that some of the new ORFs could encode proteins involved in the organization or stability of the actin cytoskeleton. Thus, we examined the actin cytoskeleton through staining F-actin with Oregon green-phalloidin in all of the new open reading frame deletions, as well as some additional control strains (Figure 4; our unpublished data). Of the new ORFs examined, only *vps61*, *vps65* (deletion of YLR322W), and *vps67* displayed defects in the actin cytoskeleton at 30°C. Interestingly, both *vps36* and *vps65* had a similar staining pattern, displaying a greatly reduced number of polarized actin cables as well as a large aggregate (indicated by arrowheads) in the mother cells (Figure 4). Although it is still unclear whether this aberrant structure is associated with any cellular organelles or whether it is simply a cytosolic actin aggregate, these observations further confirm that the need to establish a normal actin cytoskeleton may be important in intracellular Golgi-to-vacuole trafficking. Further characterization of these proteins and their interaction with the actin cytoskeleton will help to clarify the role of actin in trafficking to the vacuole.

Concluding Remarks

Many more genes had been previously implicated in the CPY pathway than in the ALP pathway (reviewed by Burd *et al*., 1998; Conibear and Stevens, 1998; Lemmon and Traub, 2000; Mullins and Bonifacino, 2001a). In fact, only the genes encoding the four subunits of AP-3 were known to be specifically involved in the ALP pathway (Cowles *et al*., 1997a; Stepp *et al*., 1997). Other gene products that controlled the ALP pathway were shared with the CPY pathway. These included Vps39p and Vps41p, which play dual roles in the budding of AP-3–coated intermediates from the late-Golgi complex (Rehling *et al*., 1999; Darsow *et al*., 2001) and tethering/fusion of both CPY and ALP carriers to the vacuole (Price *et al*., 2000; Wurmser *et al*., 2000). They also included other proteins involved in vesicle formation in the late-Golgi complex and tethering to the vacuole (reviewed by Burd *et al*., 1998; Conibear and Stevens, 1998; Mullins and Bonifacino, 2001a). More than two-thirds of all known *VPS* genes, however, were specific to the CPY pathway.

The work reported herein resulted in the identification of many new genes involved in vacuolar protein sorting. The fact remains, however, that the majority of them controls trafficking along the CPY pathway (summarized in Table 4). This could reflect a bias due to the use of CPY secretion as the assay for identification of the mutants. More likely, however, is that the overabundance of CPY pathway genes reflects the complex roles of the PVC in this pathway. The interposition of the PVC between the Golgi complex and the vacuole enables the recycling of cargo receptors such as the CPY and PrA receptor, Vps10p. In addition, the PVC allows for certain membrane proteins to be transported into the vacuole lumen through invagination of its limiting membrane (Odorizzi *et al*., 1998; Katzmann *et al*., 2001). This process is important for the delivery of some vacuole resident proteins (e.g., carboxypeptidase S) into the lumen and for the regulated turnover of proteins whose primary function is in other compartments (e.g., Kex2p).

Our studies did nonetheless identify several novel proteins involved in the ALP pathway. Mon2p was particularly interesting because its absence affected the ALP pathway more than the CPY pathway. The absence of Mon1p, Cod3p, Vps61p, and Vps67p also impaired both pathways, with the CPY pathway being more affected than the ALP pathway. These proteins could thus mediate either formation of both CPY and ALP carriers at the late-Golgi complex or tethering/fusion of Golgi-derived ALP carriers or PVC-derived CPY carriers to the vacuole. It is likely that screens specifically designed for analysis of ALP, Vam3p, or Nyv1p missorting could result in the identification of novel ALP pathway genes. Given that even AP-3 subunit mutants secrete some CPY, however, it is plausible that some ALP pathway genes may be represented among the "weak" genes identified in our screen (Table 2 and Supplemental Table 2).

We do not expect the list of genes identified in our study to represent the complete repertoire of vacuolar protein sorting genes, for the following reasons: 1) Some *VPS* genes might be essential and therefore would not be represented in the collection. For example, Sec18p is the product of an essential gene that plays a role in vesicle fusion in various pathways, including transport to the vacuole (Graham and Emr, 1991). 2) Other *VPS* genes might be redundant, as is the case for those encoding Gga1p and Gga2p (Dell'Angelica *et al.*, 2000; Hirst *et al.*, 2000; Costaguta *et al.*, 2001; Zhdankina *et al.*, 2001). 3) Some knockout strains might cease secretion of CPY due to an adaptive or compensatory response, as is the case for clathrin mutants (Seeger and Payne, 1992). 4) Genes <100 codons were not targeted for deletion. 5) Manifestation of the CPY secretion phenotype might be dependent on the genetic background of the parental strains. Most of the deletion mutants used herein were made in the BY4743 background, whereas most previously characterized *vps* mutants were derived from other strains (e.g., SEY6210, SEY6211, and SF839–1D). 6) Some genes may not have been deleted due to technical problems. For example, *vps11* and *vps15* mutants are not present in the collection. 7) Some genes could have been erroneously identified, or the mutants mishandled. Given the high accuracy with which *VPS* genes were identified in our screen, however, we suspect that the cases of mistaken identity are few.

Our studies support the conclusion that the vacuolar protein sorting machinery is highly conserved between yeast and higher eukaryotes. BLAST searches by using the strong and moderate genes as queries revealed that >50% of them have mammalian homologs. This percentage is considerably higher than the 31% of all yeast genes that have definite homologs among mammals (Botstein *et al.*, 1997). This confirms the presumption that studies of vacuolar protein sorting in yeast are particularly relevant to the understanding of lysosome biogenesis in mammals. The biochemical and functional characterization of the novel gene products identified in this study should considerably further our understanding of the molecular mechanisms that underlie the biogenesis of the yeast vacuole and mammalian lysosomes.

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REFERENCES

Amerik, A.Y., Nowak, J., Swaminathan, S., and Hochstrasser, M. (2000). The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. Mol. Biol. Cell *11*, 3365–3380.

Bell, A.W., *et al*. (2001). Proteomics characterization of abundant Golgi membrane proteins. J. Biol. Chem. *276*, 5152–65.

Bensen, E.S., Yeung, B.G., and Payne, G.S. (2001). Ric1p and the Ypt6p GTPase function in a common pathway required for localization of trans-Golgi network membrane proteins. Mol. Biol. Cell *12*, 13–26.

Bonangelino, C.J., Catlett, N.L., and Weisman, L.S. (1997). Vac7p, a novel vacuolar protein, is required for normal vacuole inheritance and morphology. Mol. Cell. Biol. *17*, 6847–6858.

Bonifacino, J.S., and Dell'Angelica, E.C. (1998). Immunoprecipitation. In: Current Protocols in Cell Biology, ed. J.S. Bonifacino, M. Dasso, J.B. Harford, J. Lippincott-Schwartz, and K. Yamada, New York: John Wiley & Sons.

Botstein, D., Chervitz, S.A., and Cherry, J.M. (1997). Yeast as a model organism. Science *277*, 1259–1260.

Burd, C.G., Babst, M., and Emr, S.D. (1998). Novel pathways, membrane coats and PI kinase regulation in yeast lysosomal trafficking. Semin. Cell Dev. Biol. *9*, 527–533.

Catlett, N.L., and Weisman, L.S. (2000). Divide and multiply: organelle partitioning in yeast. Curr. Opin. Cell Biol. *12*, 509–516.

Chardin, P., Paris, S., Antonny, B., Robineau, S., Beraud-Dufour, S., Jackson, C.L., and Chabre, M. (1996). A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. Nature *384*, 481–484.

Chen, C.Y., and Graham, T.R. (1998). An arf1Delta synthetic lethal screen identifies a new clathrin heavy chain conditional allele that perturbs vacuolar protein transport in *Saccharomyces cerevisiae*. Genetics *150*, 577–589.

Conibear, E., and Stevens, T.H. (1998). Multiple sorting pathways between the late Golgi and the vacuole in yeast. Biochim. Biophys. Acta *1404*, 211–230.

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.

Costaguta, G., Stefan, C.J., Bensen, E.S., Emr, S.D., and Payne, G.S. (2001). Yeast Gga coat proteins function with clathrin in Golgi to endosome transport. Mol. Biol. Cell *12*, 1885–1896.

Cowles, C.R., Odorizzi, G., Payne, G.S., and Emr, S.D. (1997a). The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. Cell *91*, 109–118.

Cowles, C.R., Snyder, W.B., Burd, C.G., and Emr, S.D. (1997b). Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. EMBO J. *16*, 2769–2782.

Darsow, T., Burd, C.G., and Emr, S.D. (1998). Acidic di-leucine motif essential for AP-3-dependent sorting and restriction of the functional specificity of the Vam3p vacuolar t-SNARE. J. Cell Biol. *142*, 913–922.

Darsow, T., Katzmann, D., Cowles, C.R., and Emr, S.D. (2001). Vps41p function in the alkaline phosphatase pathway requires homo-oligomerization and interaction with AP-3 through two distinct domains. Mol. Biol. Cell *12*, 37–51.

Darsow, T., Rieder, S.E., and Emr, S.D. (1997). A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. J. Cell Biol. *138*, 517–529.

De Duve, C., and Wattiaux, R. (1966). Functions of lysosomes. Annu. Rev. Physiol *28*, 435–492.

Dell'Angelica, E.C., Puertollano, R., Mullins, C., Aguilar, R.C., Vargas, J.D., Hartnell, L.M., and Bonifacino, J.S. (2000). GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. J. Cell Biol. *149*, 81–94.

Donaldson, J.G., and Jackson, C.L. (2000). Regulators and effectors of the ARF GTPases. Curr. Opin. Cell Biol. *12*, 475–482.

Drake, M.T., Zhu, Y., and Kornfeld, S. (2000). The assembly of AP-3 adaptor complex-containing clathrin-coated vesicles on synthetic liposomes [In Process Citation]. Mol. Biol. Cell *11*, 3723–3736.

Gall, W.E., Higginbotham, M.A., Chen, C., Ingram, M.F., Cyr, D.M., and Graham, T.R. (2000). The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. Curr. Biol. *10*, 1349–1358.

Graham, L.A., Powell, B., and Stevens, T.H. (2000). Composition and assembly of the yeast vacuolar H(+)-ATPase complex. J. Exp. Biol. *203*, 61–70.

Graham, T.R., and Emr, S.D. (1991). Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast sec18 (NSF) mutant. J. Cell Biol. *114*, 207–218.

Hermann, G.J., King, E.J., and Shaw, J.M. (1997). The yeast gene, MDM20, is necessary for mitochondrial inheritance and organization of the actin cytoskeleton. J. Cell Biol. *137*, 141–53.

Hirst, J., Lui, W.W., Bright, N.A., Totty, N., Seaman, M.N., and Robinson, M.S. (2000). A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. J. Cell Biol. *149*, 67–80.

Huang, C.F., Buu, L.M., Yu, W.L., and Lee, F.J. (1999). Characterization of a novel ADP-ribosylation factor-like protein (yARL3) in *Saccharomyces cerevisiae*. J. Biol. Chem. *274*, 3819–3827.

Jones, E.W. (1977). Proteinase mutants of *Saccharomyces cerevisiae*. Genetics *85*, 23–33.

Jungmann, J., and Munro, S. (1998). Multi-protein complexes in the cis Golgi of *Saccharomyces cerevisiae* with alpha-1,6-mannosyltransferase activity. EMBO J. *17*, 423–434.

Kamei, T., Tanaka, K., Hihara, T., Umikawa, M., Imamura, H., Kikyo, M., Ozaki, K., and Takai, Y. (1998). Interaction of Bnr1p with a novel Src homology 3 domain-containing Hof1p. Implication in cytokinesis in *Saccharomyces cerevisiae*. J. Biol. Chem. *273*, 28341–28345.

Katzmann, D.J., Babst, M., and Emr, S.D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell *106*, 145–155.

Kim, D.W., Sacher, M., Scarpa, A., Quinn, A.M., and Ferro-Novick, S. (1999). High-copy suppressor analysis reveals a physical interaction between Sec34p and Sec35p, a protein implicated in vesicle docking. Mol. Biol. Cell *10*, 3317–3329.

Klionsky, D.J., and Emr, S.D. (1989). Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. *8*, 2241–2250.

Kojima, H., Hashimoto, H., and Yoda, K. (1999). Interaction among the subunits of Golgi membrane mannosyltransferase complexes of the yeast *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. *63*, 1970–1976.

Kornfeld, S., and Mellman, I. (1989). The biogenesis of lysosomes. Annu. Rev. Cell Biol *5*, 483–525.

Kranz, A., Kinner, A., and Kolling, R. (2001). A family of small coiled-coil-forming proteins functioning at the late endosome in yeast. Mol. Biol. Cell *12*, 711–723.

Kucharczyk, R., Dupre, S., Avaro, S., Haguenauer-Tsapis, R., Slonimski, P.P., and Rytka, J. (2000). The novel protein Ccz1p required for vacuolar assembly in *Saccharomyces cerevisiae* functions in the same transport pathway as Ypt7p. J. Cell Sci. *113*, 4301–4311.

Lee, F.J., Huang, C.F., Yu, W.L., Buu, L.M., Lin, C.Y., Huang, M.C., Moss, J., and Vaughan, M. (1997). Characterization of an ADP-ribosylation factor-like 1 protein in *Saccharomyces cerevisiae*. J. Biol. Chem. 272, 30998–31005.

Lemmon, S.K., and Traub, L.M. (2000). Sorting in the endosomal system in yeast and animal cells. Curr. Opin. Cell Biol. *12*, 457–466.

Liu, H.P., and Bretscher, A. (1989). Disruption of the single tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. Cell *57*, 233–242.

Mizuta, K., Park, J.S., Sugiyama, M., Nishiyama, M., and Warner, J.R. (1997). RIC1, a novel gene required for ribosome synthesis in *Saccharomyces cerevisiae*. Gene *187*, 171–178.

Moreau, V., Galan, J.M., Devilliers, G., Haguenauer-Tsapis, R., and Winsor, B. (1997). The yeast actin-related protein Arp2p is required for the internalization step of endocytosis. Mol. Biol. Cell *8*, 1361–1375.

Mullins, C., and Bonifacino, J.S. (2001a). The molecular machinery for lysosome biogenesis. Bioessays *23*, 333–343.

Mullins, C., and Bonifacino, J.S. (2001b). Structural requirements for function of yeast GGAs in vacuolar protein sorting, alpha-factor maturation, and interactions with clathrin. Mol. Cell. Biol. *21*, 7981–7994.

Munn, A.L. (2001). Molecular requirements for the internalization step of endocytosis: insights from yeast. Biochim. Biophys. Acta *1535*, 236–257.

Muren, E., Oyen, M., Barmark, G., and Ronne, H. (2001). Identification of yeast deletion strains that are hypersensitive to brefeldin A or monensin, two drugs that affect intracellular transport. Yeast *18*, 163–172.

Novick, P., Osmond, B.C., and Botstein, D. (1989). Suppressors of yeast actin mutations. Genetics *121*, 659–674.

Odorizzi, G., Babst, M., and Emr, S.D. (1998). Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell *95*, 847–858.

Ooi, C.E., Dell'Angelica, E.C., and Bonifacino, J.S. (1998). ADP-ribosylation factor 1 (ARF1) regulates recruitment of the AP-3 adaptor complex to membranes. J. Cell Biol. *142*, 391–402.

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.

Peters, C., Bayer, M.J., Buhler, S., Andersen, J.S., Mann, M., and Mayer, A. (2001). Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature *409*, 581–588.

Peyroche, A., Paris, S., and Jackson, C.L. (1996). Nucleotide exchange on ARF mediated by yeast Gea1 protein. Nature *384*, 479–481.

Piper, R.C., Bryant, N.J., and Stevens, T.H. (1997). The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. J. Cell Biol. *138*, 531–545.

Poon, P.P., Nothwehr, S.F., Singer, R.A., and Johnston, G.C. (2001). The Gcs1 and Age2 ArfGAP proteins provide overlapping essential function for transport from the yeast trans-Golgi network. J. Cell Biol. *17*, 17.

Price, A., Wickner, W., and Ungermann, C. (2000). Proteins needed for vesicle budding from the Golgi complex are also required for the docking step of homotypic vacuole fusion. J. Cell Biol. *148*, 1223–1229.

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.

Reggiori, F., Black, M.W., and Pelham, H.R. (2000). Polar transmembrane domains target proteins to the interior of the yeast vacuole. Mol. Biol. Cell *11*, 3737–3749.

Rehling, P., Darsow, T., Katzmann, D.J., and Emr, S.D. (1999). Formation of AP-3 transport intermediates requires Vps41 function. Nat. Cell Biol *1*, 346–353.

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

Robinson, J.S., Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol. Cell. Biol. *8*, 4936–4948.

Rothman, J.H., Howald, I., and Stevens, T.H. (1989). Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*. EMBO J. *8*, 2057–2065.

Schafer, D.A., and Schroer, T.A. (1999). Actin-related proteins. Annu. Rev. Cell Dev. Biol. *15*, 341–363.

Seeger, M., and Payne, G.S. (1992). A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. EMBO J. *11*, 2811–2818.

Sekiya-Kawasaki, M., Botstein, D., and Ohya, Y. (1998). Identification of functional connections between calmodulin and the yeast actin cytoskeleton. Genetics *150*, 43–58.

Seol, J.H., Shevchenko, A., and Deshaies, R.J. (2001). Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. Nat. Cell Biol. *3*, 384–391.

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

Siniossoglou, S., and Pelham, H.R. (2001). An effector of Ypt6p binds the SNARE Tlg1p and mediates selective fusion of vesicles with late Golgi membranes. EMBO J. *20*, 5991–5998.

Spelbrink, R.G., and Nothwehr, S.F. (1999). The yeast GRD20 gene is required for protein sorting in the trans-Golgi network/endosomal system and for polarization of the actin cytoskeleton. Mol. Biol. Cell *10*, 4263–4281.

Stearns, T., Kahn, R.A., Botstein, D., and Hoyt, M.A. (1990). ADP ribosylation factor is an essential protein in *Saccharomyces cerevisiae* and is encoded by two genes. Mol. Cell. Biol. *10*, 6690–6699.

Stepp, J.D., Huang, K., and Lemmon, S.K. (1997). The yeast AP-3 complex is essential for the efficient delivery of alkaline phosphatase by the alternate pathway to the vacuole. J. Cell Biol. *139*, 1761–1774.

Taunton, J., Rowning, B.A., Coughlin, M.L., Wu, M., Moon, R.T., Mitchison, T.J., and Larabell, C.A. (2000). Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J. Cell Biol. *148*, 519–530.

TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. EMBO J. *15*, 6483–6494.

Van Valkenburgh, H., Shern, J.F., Sharer, J.D., Zhu, X., and Kahn, R.A. (2001). ADP-ribosylation factors (ARFs) and ARF-like 1 (ARL1) have both specific and shared effectors: characterizing ARL1-binding proteins. J. Biol. Chem. *276*, 22826–22837.

VanRheenen, S.M., Cao, X., Sapperstein, S.K., Chiang, E.C., Lupashin, V.V., Barlowe, C., and Waters, M.G. (1999). Sec34p, a protein required for vesicle tethering to the yeast Golgi apparatus, is in a complex with Sec35p. J. Cell Biol. *147*, 729–742.

Vida, T.A., and Emr, S.D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J. Cell Biol. *128*, 779–792.

Whyte, J.R., and Munro, S. (2001a). The Sec34/35 Golgi transport complex is related to the exocyst, defining a family of complexes involved in multiple steps of membrane traffic. Dev. Cell *1*, 527–537.

Whyte, J.R., and Munro, S. (2001b). A yeast homolog of the mammalian mannose 6-phosphate receptors contributes to the sorting of vacuolar hydrolases. Curr. Biol. *11*, 1074–1078.

Winther, J.R., Stevens, T.H., and Kielland-Brandt, M.C. (1991). Yeast carboxypeptidase Y requires glycosylation for efficient intracellular transport, but not for vacuolar sorting, in vivo stability, or activity. Eur. J. Biochem. *197*, 681–689.

Winzeler, *et al*. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science *285*, 901–906.

Wu, C.C., Taylor, R.S., Lane, D.R., Ladinsky, M.S., Weisz, J.A., and Howell, K.E. (2000). GMx33: a novel family of trans-Golgi proteins identified by proteomics. Traffic *1*, 963–975.

Wurmser, A.E., Sato, T.K., and Emr, S.D. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the ypt7 GTPase to SNARE-dependent docking and fusion. J. Cell Biol. *151*, 551–562.

Yahara, N., Ueda, T., Sato, K., and Nakano, A. (2001). Multiple roles of Arf1 GTPase in the yeast exocytic and endocytic pathways. Mol. Biol. Cell *12*, 221–238.

Yamashiro, C.T., Kane, P.M., Wolczyk, D.F., Preston, R.A., and Stevens, T.H. (1990). Role of vacuolar acidification in protein sorting and zymogen activation: a genetic analysis of the yeast vacuolar proton-translocating ATPase. Mol. Cell. Biol. *10*, 3737–3749.

Zhdankina, O., Strand, N.L., Redmond, J.M., and Boman, A.L. (2001). Yeast GGA proteins interact with GTP-bound Arf and facilitate transport through the Golgi. Yeast *18*, 1–18.