Genome-wide Analysis of AP-3–dependent Protein Transport in Yeast

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The evolutionarily conserved adaptor protein-3 (AP-3) complex mediates cargo-selective transport to lysosomes and lysosome-related organelles. To identify proteins that function in AP-3–mediated transport, we performed a genome-wide screen in *Saccharomyces cerevisiae* for defects in the vacuolar maturation of alkaline phosphatase (ALP), a cargo of the AP-3 pathway. Forty-nine gene deletion strains were identified that accumulated precursor ALP, many with established defects in vacuolar protein transport. Maturation of a vacuolar membrane protein delivered via a separate, clathrin-dependent pathway, was affected in all strains except those with deletions of *YCK3*, encoding a vacuolar type I casein kinase; *SVP26*, encoding an endoplasmic reticulum (ER) export receptor for ALP; and AP-3 subunit genes. Subcellular fractionation and fluorescence microscopy revealed ALP transport defects in *yck3Δ* cells. Characterization of *svp26Δ* cells revealed a role for Svp26p in ER export of only a subset of type II membrane proteins. Finally, ALP maturation kinetics in *vac8Δ* and *vac17Δ* cells suggests that vacuole inheritance is important for rapid generation of proteolytically active vacuolar compartments in daughter cells. We propose that the cargo-selective nature of the AP-3 pathway in yeast is achieved by AP-3 and Yck3p functioning in concert with machinery shared by other vacuolar transport pathways.

INTRODUCTION

Subcellular compartmentalization by membrane-bounded organelles is a fundamental feature of eukaryotic cells. This organization allows for physical and functional segregation of subcellular processes. An important example of this compartmentalization is the lysosome, which is an acidic organelle that serves as a major site for protein degradation within eukaryotic cells. Degradative enzymes are sequestered within lysosomes ensuring that only material delivered to the organelle is subject to destruction.

Compartmentalization of lysosome functions necessitates transport pathways for lysosomal biogenesis, maintenance of function, and transfer of molecules targeted for turnover. Genetic analysis of proteins that are targeted to the lysosome-like vacuole of *Saccharomyces cerevisiae* has defined six such trafficking routes, each involving vesicle-mediated transport (Bryant and Boyd, 1993). Two of these pathways involve protein transport from the cytoplasm to the vacuole: the cytoplasm to vacuole transport pathway (CVT) and starvation-induced autophagy. A third pathway, the endocytic pathway, delivers cell surface and extracellular molecules to the vacuole. Yet another route provides for vacuole inheritance during cell division. The inheritance pathway directs vesicles derived from the maternal vacuole into the new bud where vesicle fusion seeds formation of a daughter cell vacuole. Finally, there are two vacuolar biosynthetic pathways that originate from the secretory pathway, one clathrin dependent and the other clathrin-independent. Newly synthesized vacuolar components destined for both pathways

are transported from the endoplasmic reticulum (ER) to the Golgi complex. At the *trans*-Golgi network (TGN), the proteins enter into either the clathrin-dependent pathway that passes through endosomes to the vacuole or into a clathrin-independent pathway that proceeds directly to vacuole.

Clathrin-dependent transport between the TGN and endosomes relies on several evolutionarily conserved clathrin adaptors: monomeric GGA proteins and the heterotetrameric adaptor protein-1 (AP-1) complex (Bonifacino, 2004; Traub, 2005). These adaptors function as protein interaction platforms by binding to the coat protein clathrin and other coat-associated proteins, as well as recognizing sorting signals in the cytoplasmic domains of transmembrane cargo proteins. In this way, adaptors play central roles in vesicle formation by coupling cargo selection to assembly of a protein coat.

Most vacuolar proteins are transported through the clathrin-dependent pathway, including the soluble protease carboxypeptidase Y (CPY), the membrane protease carboxypeptidase S (CPS), and membrane components of the V-type H⁺-ATPase responsible for acidifying the vacuole (Bowers and Stevens, 2005). This pathway flows through multivesicular endosomes, where membrane proteins destined for the lumen of the vacuole are sorted into luminal vesicles that are subsequently delivered to the vacuole. There is limited information on sorting signals for the clathrin-dependent pathway in yeast, in part because this pathway seems to act as a default route for membrane proteins lacking cytoplasmic sorting signals (Roberts *et al.*, 1992; Redding *et al.*, 1996).

The clathrin-independent pathway to the vacuole is distinguished by a requirement for the AP-3 adaptor complex, a four-subunit complex with homology to AP-1. AP-3 is evolutionarily conserved and consists of two large subunits, $\beta 3$ and δ ; a medium subunit $\mu 3$; and a small subunit $\sigma 3$ (Odorizzi *et al.*, 1998). Only a few proteins that transit via the AP-3 pathway have been identified in yeast; among these

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are alkaline phosphatase (ALP) (Cowles *et al.*, 1997a; Stepp *et al.*, 1997), the target membrane-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (t-SNARE) Vam3p (Cowles *et al.*, 1997a), yeast casein kinase 3 (Yck3p) (Sun *et al.*, 2004), and the Niemann-Pick Type C homologue Ncr1p (Berger *et al.*, 2007). Each of these proteins is localized to the limiting vacuolar membrane rather than intravacuolar vesicles, suggesting that the AP-3 pathway bypasses multivesicular endosomes and functions as a selective and direct route from the TGN to the vacuolar membrane. Cargoes of the AP-3 pathway contain either acidic dileucine-like or YXX Φ (where Φ is a bulky hydrophobic amino acid and X is any amino acid) signals that are necessary for sorting into the pathway (Darsow *et al.*, 1998; Vowels and Payne, 1998a; Sun *et al.*, 2004).

Formation of AP-3 vesicles in yeast, unlike vesicles with AP-1 and GGA proteins, seems to be independent of clathrin. AP-3 does not strongly associate with clathrin, nor do mutations in clathrin alter the rate of ALP transport to the vacuole (Seeger and Payne, 1992; Vowels and Payne, 1998a; Yeung *et al.*, 1999). Only one other protein in yeast has been characterized with selective function in AP-3 vesicle formation in yeast, Vps41p. Vps41p is a member of the homotypic vacuole fusion and vacuole protein sorting (HOPS) complex (Nakamura *et al.*, 1997; Seals *et al.*, 2000; Wurmser *et al.*, 2000). The HOPS complex functions in tethering vesicles to the vacuole, and, in this capacity, it is necessary for delivery of both clathrin-dependent and AP-3-dependent cargo (Bowers and Stevens, 2005; Ostrowicz *et al.*, 2008). However, Vps41p also seems to play a selective role in AP-3 vesicle formation through an interaction with the AP-3 δ subunit (Rehling *et al.*, 1999; Darsow *et al.*, 2001). Vps41p has features similar to clathrin, but whether this protein is a structural component of the AP-3 coat remains unresolved (Conibear and Stevens, 1998; Ybe *et al.*, 1999).

Mammalian AP-3 also participates in protein transport to lysosomes (Robinson and Bonifacio, 2001; Di Pietro and Dell'Angelica, 2005). Much like the yeast adaptor, mammalian AP-3 seems to act in a pathway to the lysosomal limiting membrane that bypasses multivesicular late endosomes. However, in mammalian cells, AP-3 localizes predominantly at early endosomes instead of the Golgi (Peden *et al.*, 2004; Theos *et al.*, 2005), suggesting that AP-3 pathways in yeast and mammalian cells originate at distinct organelles. Another difference between yeast and mammalian AP-3 is that the mammalian adaptor binds clathrin (Dell'Angelica *et al.*, 1998), although the functional significance of this interaction has not been fully established (Newell-Litwa *et al.*, 2007).

In addition to a ubiquitous role in lysosomal protein traffic, mammalian AP-3 acts in cell type-specific pathways to specialized lysosome-related organelles such as melanosomes and platelet dense granules (Di Pietro and Dell'Angelica, 2005; Huizing *et al.*, 2008). Defects in transport to lysosomes and lysosome-related organelles caused by mutations in the AP-3 β subunit lead to the inherited human disease Hermansky-Pudlak syndrome (HPS) (Dell'Angelica *et al.*, 1999; Huizing *et al.*, 2002; Jung *et al.*, 2006), a disease characterized by oculocutaneous albinism and prolonged bleeding times (Di Pietro and Dell'Angelica, 2005; Huizing *et al.*, 2008). A disease similar to HPS is manifested in mice bearing mutations in AP-3 subunits (Di Pietro and Dell'Angelica, 2005; Newell-Litwa *et al.*, 2007; Huizing *et al.*, 2008).

Mutations in other genes also cause HPS and HPS-like diseases in both humans and mice, and the products of these genes have been associated with trafficking pathways to lysosomes and lysosome-related organelles (Di Pietro and

Dell'Angelica, 2005; Huizing *et al.*, 2008). One such protein, Vps33a, is a component of the mammalian HOPS complex that also contains Vps41 (Suzuki *et al.*, 2003). Most of the other proteins do not seem to have homologues in yeast, although a recent report describes limited sequence homologies between HPS4 and the yeast Ccz1p protein involved in vacuolar protein transport (Hoffman-Sommer *et al.*, 2005). Thus, although not readily apparent by sequence conservation, there is the possibility that other HPS proteins will have functional analogues in yeast.

To more fully understand the mechanisms of transport through the AP-3 pathway and identify additional candidate disease genes, having a complete catalogue of genes/proteins involved in AP-3 pathway function would be advantageous. For this reason, we have carried out a systematic, genome-wide screen in yeast to identify genes involved in AP-3-dependent ALP transport to the vacuole. We identified a complement of genes that serve a variety of functions from processing enzymes, to vesicle budding, targeting, and vesicle fusion. Many of these genes have well established roles in protein transport through both the clathrin and AP-3 pathways. Only deletions in AP-3 subunit genes and YCK3 selectively affected the AP-3 pathway. We also observed that the ER export receptor for ALP, Svp26p, is required for transport of a subset of type II membrane proteins. Finally, our results indicate that the vacuolar inheritance pathway is important for timely production of a proteolytically mature vacuole in newly budded cells.

MATERIALS AND METHODS

Yeast Media, Plasmids, and Strains

Yeast strains were grown in YPD [1% Bacto-yeast extract (Difco, Detroit, MI), 2% Bacto-peptone (Difco), 2% dextrose], YPD + 200 μ g/ml G418, or SD (0.67% yeast nitrogen base [Difco], 2% dextrose) supplemented with 20 μ g/ml L-histidine, uracil, and L-tryptophan, and 30 μ g/ml L-leucine, adenine, and L-lysine. Solid media contained 2% agar. Yeast transformations were performed using the lithium acetate method (Ito *et al.*, 1983). Cell densities were measured by spectrophotometry. One OD₆₀₀ corresponds to 10⁷ cells per milliliter. Sequences of oligonucleotides used in this study are available on request.

Plasmids pYCK3, *pyck3-1*, and *pyck3-2* are described in Sun *et al.* (2004). *pyck3-1* bears an in frame deletion of a portion of YCK3 encoding Yck3p Δ 409-462. *pyck3-2* bears a substitution mutation changing amino acid 444 from tyrosine to histidine. pGFP-ALP is a multicopy plasmid encoding a fusion of GFP to the N terminus of full-length ALP (Cowles *et al.*, 1997a). pBS207, a low copy centromeric plasmid contains a fragment of chromosome XIV (coordinates 67948–78118) encoding full-length MON2 (Jochum *et al.*, 2002).

Strains used in this study were obtained from the single gene deletion libraries constructed by the international deletion consortium (Winzeler *et al.*, 1999) constructed in BY4741 (*MAT α ura3 Δ 0 leu2 Δ 0 his3 Δ 1 met15 Δ 0*) and BY4742 (*MAT α ura3 Δ 0 leu2 Δ 0 his3 Δ 1 lys2 Δ 0*) (Brachmann *et al.*, 1998). Deletion of *SVP26* was performed by standard polymerase chain reaction (PCR)-based methods (Longtine *et al.*, 1998) to generate GY4865 (*MAT α ura3-52 his3 Δ 200 leu2-3 lys2-801 trp1 Δ 901 suc2 Δ 9 mel svp26 Δ ::HIS3*) in SEY6210 (Robinson *et al.*, 1988). *MAT α cps1 Δ pho8 Δ* was generated by crossing *MAT α pho8 Δ* with *MAT α cps1 Δ* from the single gene deletion collections, sporulating, and dissecting the sporulated diploid to generate GY4866.

Preparation of Cell Extracts from Single-Gene Deletion Libraries

The 96-well format *S. cerevisiae* *MAT α* and *MAT α* single gene deletion libraries were obtained from Open Biosystems (Huntsville, AL) and ResGen (now Invitrogen, Carlsbad, CA), respectively. Each plate of the 96-well format gene deletion libraries was replicated into a 96-well plate (Cole-Parmer Instrument, Vernon Hills, IL) containing 200 μ l/well of YPD + 200 μ g/ml G418. Cells were grown for 2 d at 30°C. Fifty microliters from each well was transferred to 2 ml of YPD in a 96-deep well plate (Matrix, Hudson, NH). Individual magnetic stir-bars (VP Scientific, San Diego, CA) were added and stirred continuously using a magnetic stir device (VP Scientific) for 5 h at 30°C. Cells were then sedimented by centrifugation at 1500 $\times$ g for 5 min. The supernatant was discarded, and 100 μ l of cracking buffer (10% glycerol, 5% SDS, 2% β -mercaptoethanol, 50 mM Tris-Cl, pH 6.8, and 0.02% bromophenol blue) was added to each well then transferred to 96-well PCR plates (Thermo Fisher

Scientific, Waltham, MA). Plates were incubated at 99°C for 3 min, and samples were frozen at -80°C until further processing.

Immunoblotting ALP for Single-Gene Deletion Library Extracts

Five microliters of each lysed strain in cracking buffer was subjected to electrophoresis through a 10% SDS-polyacrylamide gel. Gels were transferred onto polyvinylidene difluoride (PVDF) membranes at 100 V for 60 min in 24 mM Tris base, 192 mM glycine, and 20% methanol buffer. Membranes were treated with blocking buffer (5% milk, 0.1% Tween 20 in phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 2 mM KH₂PO₄]) for 1 h and then incubated overnight at 4°C in a rabbit anti-Pho8p antibody (Seeger and Payne, 1992b) diluted in blocking buffer. Next, 25 µl/ml concentrated *cps1Δ* *pho8Δ* cell extract was added to antibody dilutions, and the mixture was preincubated for 30 min before incubation with PVDF membranes to reduce nonspecific signals. Bound rabbit antibodies were detected with goat anti-rabbit horseradish peroxidase conjugated antibody (Bio-Rad, Hercules, CA). 10 µL/ml *pho8Δ* extract was added to the secondary antibody dilution in blocking buffer and preincubated for 30 min. Membranes were washed in PBS and 0.1% Tween 20 and developed using ECL-Plus (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Digital images were acquired with a fluorescence imager (GE Healthcare).

To prepare concentrated *cps1Δ* *pho8Δ* extract, *MATa* *cps1Δ* *pho8Δ* cells were grown in YPD to 10 OD₆₀₀/ml, 2 l were harvested by centrifugation, and frozen in liquid nitrogen. The frozen cells were mechanically lysed in a blender, and the lysate was resuspended in 75 ml of 1% SDS and heated at 100°C for 5 min. The lysate was then clarified by centrifugation at 100,000 × g for 45 min at 4°C.

Cell Extract Preparation, Immunoblotting, and Immunoprecipitation for Analysis of Individual Strains

Three stationary overnight cultures in YPD from each mating type of a gene deletion strain were diluted to 0.125 OD₆₀₀ in YPD and grown to 0.5–1 OD₆₀₀/ml, yielding six samples per deletion strain. Approximately 5 OD₆₀₀ cells were harvested by centrifugation at 1500 × g for 3 min in 13 × 100-mm glass test tubes (Thermo Fisher Scientific). Lysates were prepared by mechanical disruption of the pellet by adding 50 µl of LSB (10% glycerol, 2% SDS, 2% β-mercaptoethanol, 50 mM Tris-Cl, pH 6.8, and 0.02% bromophenol blue) and 250 µl of 0.5-mm glass beads (Biospec, Bartlesville, OK) to the pellet and agitating with a Vortex mixer (Thermo Fisher Scientific). Lysates were resuspended in 400 µl of LSB and incubated at 100°C for 5 min. Immunoblots were performed as described above using rabbit anti-Pho8p, rabbit anti-Cps1p (generated using glutathione transferase fused to Cps1p amino acids [aa]46–576 as antigen), or rabbit anti-Gda1p (generated using 6 histidine-tagged Gda1p aa25–518). Pho8p and Cps1p antibodies and secondary antibodies were treated with concentrated *pho8Δ* *cps1Δ* extract as described above. Gda1p antibodies and secondary antibodies were treated with a concentrated *gda1Δ* cell extract prepared as described above for *pho8Δ* *cps1Δ* extract.

Radiolabeling and immunoprecipitations for ALP and CPS were performed as described in Seeger and Payne (1992b) and Costaguta *et al.* (2006).

Quantitation of Immunoblots and Immunoprecipitations

Digital images of immunoblots and immunoprecipitations were analyzed using ImageJ (<http://rsb.info.nih.gov/ij/>). Bands were quantified by measuring the total integrated density of a band after background subtraction. The precursor ALP (pALP) to total ALP (tALP) ratio represents the pALP band density divided by the sum of the pALP, mature ALP (mALP), and soluble ALP (sALP) band densities. A ratio of pALP/tALP was determined for each of the six samples per gene deletion strain. These six calculated ratios per strain were averaged together, and a standard deviation was calculated. Wild-type strains were also grown in sextuplicate and a pALP/tALP ratio, and standard deviation was determined. Two tailed *t* tests were performed for each deletion strain pALP/tALP ratio compared with the wild-type ratio. A strain was determined to display a processing defect if the deletion strain pALP/tALP was greater than wild-type, with *p* < 0.05. The pALP/tALP ratio for the deletion strain was then normalized to the wild-type ratio by dividing the deletion strain ratio by the wild-type ratio. The normalized pALP/tALP ratio is reported in Table 1.

Defects in CPS maturation were scored in a qualitative manner due to overlap between precursor and mature forms of the protein. Both pCPS and mCPS migrate as two differentially glycosylated forms. The lower molecular weight form of pCPS comigrates with the higher molecular weight form of mCPS—we term this the intermediate form. Thus, only the higher molecular weight precursor form can be used as a reliable diagnostic for maturation defects. Strains were scored as having no CPS maturation defect if a two band pattern corresponding to the intermediate and mature forms was detected. Strains were scored as having a severe defect if a two-band pattern was detected consisting of the higher-molecular-weight precursor and the intermediate-molecular-weight form, with little or no lower-molecular-weight mCPS. Strains were scored as having a moderate defect if three bands were

detected—the higher-molecular-weight precursor form, the intermediate-molecular-weight form, and the lower-molecular-weight mature form.

Cell Lysis and Differential Centrifugation

Cell lysis and differential centrifugation was carried out as described in Yeung and Payne (2001), with the following modifications: 50 OD₆₀₀ cells were harvested; cells were converted to spheroplasts with 0.05 U of Zymolyase (Seikagaku, Tokyo, Japan) per OD₆₀₀ cells; and the lysis buffer was 200 mM sorbitol, 50 mM Tris, pH 6.8, 50 mM NaCl, 2 mM EDTA, and protease inhibitors.

N-[3-Triethylammoniumpropyl]-4-[*p*-diethylaminophenyl]hexatrienyl] Pyridinium Dibromide (FM4-64) Staining and Fluorescence Microscopy

The *MATa* wild-type strain BY4741 and *MATa* deletion strains *yck3Δ*, *apl6Δ* (*β3Δ*), and *vac17Δ*, all harboring pGFP-ALP were grown in liquid SD medium with appropriate supplements overnight to 0.2–0.4 OD₆₀₀/ml. Cells were harvested by centrifugation at 1500 × g for 3 min, the supernatant was decanted, and the cells resuspended in the residual liquid (200 µl). Cells were stained using 200 µM FM4-64 for 20 min, washed, and incubated for 60 min at room temperature as described in Vida and Emr (1995) and imaged using an Axioobserver Z1 1.0 spinning disk confocal microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

Genome-wide Screens for ALP Maturation Defects

To screen for AP-3 pathway defects, we monitored the proteolytic maturation of ALP. ALP, a type II membrane protein, is synthesized as a 74-kDa glycosylated zymogen (pALP) that is proteolytically processed upon delivery to the vacuole to yield a 72-kDa integral membrane mature form (mALP) and a 66-kDa soluble mature form (sALP) (Klionsky and Emr, 1989). In wild-type cells, virtually all ALP is present in the mature forms as a consequence of the rapid rate of pALP transport to the vacuole and proteolytic processing. In contrast, cells with defects in AP-3-mediated transport accumulate pALP, which is easily distinguished from the mature forms by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting (Lorenz *et al.*, 2007). Thus, as a systematic approach to identify genes involved in ALP trafficking, we used immunoblotting to assess pALP accumulation in the sets of haploid *MATa* and *MATα* strains bearing single deletions of all nonessential genes.

Deletion strains were grown in 96-well plates to mid-logarithmic phase, and then lysates were prepared and analyzed by SDS-PAGE and immunoblotting with an ALP antibody. A strain with a partial ALP maturation defect (*yck3Δ*) was included in each 96-well plate for quality control to ensure reproducibility of the assay. Strains displaying pALP to ALP ratios significantly greater than wild-type strains, using *yck3Δ* as a general guide, were initially scored as positive. Examples of the data are presented in Figure 1, revealing pALP accumulation in *vac8Δ*, *yck3Δ*, and *apl6Δ* cells (Figure 1A, top, lane 4; and bottom, lanes 2 and 6).

Strains scored as positive in the initial screen were grown individually and retested for pALP accumulation by immunoblotting. In addition, we tested a few strains with deletions of vacuolar H⁺-ATPase subunits that did not display ALP maturation defects in the genome-wide screens but that were expected to score positive because deletions of other subunits resulted in maturation defects. In total, 4848 single gene deletion strains in the *MATa* library and 4871 strains in the *MATα* library were screened; 49 strains exhibited ALP maturation defects when retested (Table 1).

The specificity of the ALP maturation defect was assessed by analyzing in parallel the processing of ALP and another type II vacuolar membrane protein, CPS. CPS, like ALP, is synthesized in a precursor form that is proteolytically matured in the vacuole (Spormann *et al.*, 1992). However, CPS

Table 1. ALP maturation defects in strains from gene deletion libraries

Single gene deletion strain	Gene product function	ALP defect	CPS defect	Significance of ALP defect, p value
AP-3				
<i>apl5Δ</i>	δ Subunit of AP-3	3.1	—	<0.001
<i>apl6Δ</i>	β Subunit of AP-3	3.1	—	<0.001
<i>apm3Δ</i>	μ Subunit of AP-3	2.3	—	<0.001
<i>aps3Δ</i>	σ Subunit of AP-3	3.1	—	<0.001
Vesicle formation				
<i>vps1Δ</i>	Dynamin homologue	2.7	M	<0.001
<i>arf1Δ</i>	ARF GTPase, acts in vesicle coat assembly	2.3	M	<0.001
<i>drs2Δ</i>	Lipid flippase	3.2	M	<0.001
Vacuolar proteases				
<i>pep4Δ</i>	Vacuolar serine protease, activates other vacuolar proteases	7.2	S	<0.001
<i>prb1Δ</i>	Vacuolar serine protease	5.3	M	<0.001
HOPS/CORVET				
<i>vps41Δ</i>	HOPS subunit/AP-3 vesicle formation	6.3	S	<0.001
<i>vam6Δ (vps39Δ)</i>	HOPS subunit/Ypt7 guanine nucleotide exchange factor	6.5	S	<0.001
<i>pep5Δ (vps11Δ)</i>	HOPS/CORVET core subunit	7.2	S	<0.001
<i>pep3Δ (vps18Δ)</i>	HOPS/CORVET core subunit	7.4	S	<0.001
<i>vps16Δ</i>	HOPS/CORVET core subunit	7.5	S	<0.001
<i>vps8Δ</i>	CORVET subunit, interacts with Vps21p	2.3	M	<0.001
<i>vps3Δ</i>	CORVET subunit	3.8	S	<0.001
Rabs and vesicle targeting factors				
<i>ypt7Δ</i>	Rab GTPase for tethering/fusion to vacuole	5.6	S	<0.001
<i>vps45Δ</i>	Sec1/Munc-18 family, interacts with Pep12p	3.8	S	<0.01
<i>vps21Δ</i>	Rab GTPase for tethering/fusion to endosome	1.8	M	<0.001
<i>vps9Δ</i>	Vps21 guanine nucleotide exchange factor	3.6	S	<0.001
SNAREs and fusion				
<i>vam3Δ</i>	Vacuole membrane t-SNARE	6.2	S	<0.001
<i>vam7Δ</i>	Vacuole membrane t-SNARE	6.2	S	<0.001
<i>pep12Δ</i>	Endosome t-SNARE	4.6	S	<0.001
Vacuolar inheritance				
<i>vac8Δ</i>	Vacuolar membrane protein that binds Vac17p	3.4	M	<0.001
<i>vac17Δ</i>	Links Vac8p to the myosin, Myo2p	4.8	S	<0.001
Phosphatidylinositol synthesis				
<i>fab1Δ</i>	Phosphatidylinositol-3-phosphate 5-kinase	4.0	M	<0.01
<i>vac14Δ</i>	Activator of Fab1p	2.9	M	<0.001
<i>vps15Δ</i>	Protein kinase subunit of phosphatidylinositol 3 (PI3)-kinase complexes I and II	5.0	S	<0.001
<i>vps34Δ</i>	PI3-kinase	6.2	S	<0.001
<i>vps30Δ</i>	Subunit of PI3-kinase complexes I and II	2.9	M	<0.001
<i>vps38Δ</i>	Subunit of PI3-kinase complex I	3.2	M	<0.001
Retromer				
<i>pep8Δ (vps26Δ)</i>	Retromer subunit	1.7	M	<0.01
<i>vps5Δ</i>	Retromer subunit	1.6	M	=0.001
<i>vps35Δ</i>	Retromer subunit, cargo binding	2.3	M	<0.001
<i>vps29Δ</i>	Retromer subunit	2.4	M	<0.01
<i>vps17Δ</i>	Retromer subunit	1.4	M	=0.02
v-type H ⁺ -ATPase				
<i>tfp1Δ</i>	Subunit A of V ₁ domain	4.0	S	=0.02
<i>vma2Δ^a</i>	Subunit B of V ₁ domain	1.7	M	<0.01
<i>vma5Δ^a</i>	Subunit C of V ₁ domain	1.4	M	<0.001
<i>vma8Δ^a</i>	Subunit D of V ₁ domain	1.6	M	<0.001
<i>vma7Δ</i>	Subunit F of V ₁ domain	3.2	S	<0.001
<i>vma10Δ</i>	Subunit G of V ₁ domain	1.7	M	<0.05
<i>vph1Δ</i>	Subunit a of V ₀ domain	3.3	M	<0.001
<i>cup5Δ</i>	Subunit c of V ₀ domain	3.7	S	<0.001
<i>tfp3Δ</i>	Subunit c' of V ₀ domain	2.9	S	=0.1
<i>ppa1Δ</i>	Subunit c'' of V ₀ domain	2.0	S	<0.05
<i>vma6Δ</i>	Subunit c''' of V ₀ domain	5.4	S	<0.05
<i>vph2Δ</i>	v-ATPase assembly	5.3	S	<0.01
<i>vma22Δ</i>	v-ATPase assembly	3.8	S	=0.01
Miscellaneous				
<i>yck3Δ</i>	Type I casein kinase involved in homotypic vacuole fusion	3.2	—	<0.001
<i>svp26Δ</i>	Adaptor for sorting ALP into COP-II vesicles at ER	4.2	—	<0.001
<i>ccz1Δ</i>	Acts in vesicle fusion to the vacuole in a complex with Mon1p	4.4	M	<0.001
<i>mon1Δ</i>	Acts in vesicle fusion to the vacuole in a complex with Ccz1p	4.4	M	<0.001

Three colonies from each mating type of a single-gene deletion strain were individually analyzed for ALP and CPS maturation defects by immunoblotting. Band densities for ALP were quantified and an average ratio of pALP to total ALP was determined for the six samples (see *Materials and Methods*). The ALP defect for a gene deletion strain is expressed as the pALP/tALP ratio of the deletion strain divided by the pALP/tALP ratio of wild-type cells. Statistical significance of the ALP defect was calculated from the values and standard deviations. CPS maturation is reported as having no defect (—), moderate defect (M), or severe defect (S) as determined by the presence or absence of precursor and mature forms of CPS (see *Materials and Methods*). Neither *MATa* or *MATα* library contained valid deletion strains for *vps33Δ* (HOPS subunit), *pep7Δ* (vesicle targeting to endosomes), *vma4Δ* (subunit E of V₁ domain of ATPase), or *vma21Δ* (v-ATPase assembly) as determined by sequencing the bar codes of the relevant strains.

^a ALP maturation defects in these strains were not detected in the genome-wide screens.

Figure 1. Example of results from analysis of ALP maturation in strains from the *MATa* deletion library. (A) Indicated strains were grown in YPD, extracts were prepared, and ALP maturation was assessed by SDS-PAGE and immunoblotting. Strains scored as positive for pALP accumulation are designated with an asterisk. The positions of precursor (p), mature (m), and soluble (s) forms of ALP are indicated. The abundance of the sALP form can vary between experiments depending on growth state and other undetermined factors. (B) Example of results from analysis of ALP and CPS maturation in multiple samples of each strain scored as positive in the genome-wide screen. Three individual colonies from each of the indicated gene deletion strains and WT cells were independently cultured, cell extracts were prepared, and ALP and CPS were detected by immunoblotting. Forms of ALP are indicated as in A. The position of precursor (p) and mature (m) forms of CPS are indicated. The forked line indicates the intermediate band position where pCPS and mCPS forms comigrate. Examples of strains scored in Table 1 as no defect (*yck3Δ*), moderate defect (*pep8Δ*), and severe defect (*vam3Δ*) are shown.

is transported via the clathrin-dependent pathway (Cowles *et al.*, 1997b). Consequently, defects specific for the AP-3 pathway should affect ALP but not CPS maturation. Three independent samples of each mating type of each positive strain were monitored for ALP and CPS maturation by immunoblotting (Figure 1B). Quantitation of the fraction of pALP present in each sample demonstrated that all the strains exhibited statistically significant accumulation of pALP compared with wild-type cells (Table 1). For CPS, the presence of partially overlapping glycosylated precursor and mature forms necessitated a more qualitative measure of maturation (Figure 1B and Table 1). Nevertheless, each strain exhibited reproducible patterns of CPS that could be reliably assessed for maturation defects (Table 1).

Most strains with ALP maturation defects could be grouped according to established functional categories or protein complexes (Table 1). In all cases except for AP-3 subunit gene deletions, *svp26Δ*, and *yck3Δ*, deletions of genes that affected ALP maturation also affected CPS maturation. Thus, the analysis yielded only six gene deletions

with selective effects on ALP maturation, four of which were AP-3 subunits. Additionally, *vac8Δ* and *vac17Δ* strains displayed defects in both ALP and CPS maturation that have not been reported previously. Vac8p and Vac17p function in vacuole inheritance (Weisman, 2006).

Among the deletions affecting both ALP and CPS maturation are genes expected to influence ALP maturation through direct roles in either maturation or transport. These include the master vacuolar maturation proteases Pep4p and Prb1p that are required for ALP and CPS processing in the vacuole (Jones, 1984), proteins that act in vesicle formation such as Vps1p, and Arf1p (Odorizzi *et al.*, 1998; Bowers and Stevens, 2005), and components of the machinery responsible for AP-3 and endosomal pathway vesicle docking and fusion with the vacuole such as the HOPS complex, the rab GTPase Ypt7p, and SNAREs (Bowers and Stevens, 2005; Ostrowicz *et al.*, 2008).

Other gene deletions identified in the screen are likely to inhibit ALP transport/maturation indirectly. For example, proteins involved in the clathrin-dependent pathway such as Rab Vps21p, the Vps21p guanine nucleotide exchange factor Vps9p, the endosomal t-SNARE Pep12p, and the Vps34p phosphatidylinositol 3-kinase complex I, are necessary for transport of the maturation proteases Pep4p and Prb1p to endosomes (Bowers and Stevens, 2005). In these mutants, reduced levels of Pep4p and Prb1p in the vacuole can account for the ALP maturation defects. A similar explanation may apply to the weak ALP maturation defects observed in strains with deletions of genes encoding subunits of the retromer complex, which mediates endosome to Golgi retrieval of vacuolar protein sorting receptors (Seaman *et al.*, 1998). Retromer mutations could also affect ALP maturation by blocking a retrieval pathway from the vacuole membrane that passes through endosomes to the TGN (Bryant *et al.*, 1998). This pathway is involved in retrieval of the v-SNARE Vti1p that participates in ALP delivery to the vacuole (Vti1p acts in several transport steps and is essential for viability, thus *vti1Δ* is not represented in the haploid deletion mutant collection).

Deletions of *FAB1* and *VAC14* inhibit production of phosphatidylinositol 3,5 bisphosphate, resulting in perturbations in several vacuole pathways that could affect ALP and CPS maturation, including the vacuole to endosome retrieval pathway described above and the vacuolar inheritance pathway (Cooke, 2002; Shaw *et al.*, 2003; Weisman, 2006). However, we did not observe defects in ALP or CPS maturation in either *MATa* or *MATa* cells lacking Vac7p, an activator of the Fab1p lipid kinase (Bonangelino *et al.*, 1997), and we also noted variable maturation defects in different *fab1Δ* strains, suggesting further analysis is needed to determine whether Fab1p and its activators are necessary for ALP and CPS maturation.

Finally, deletions of many of the genes encoding subunits or assembly factors for the V-type H⁺ ATPase were identified. Acidification by the V-ATPase is required for optimal activity of the vacuolar maturation enzymes and for efficient protein transport to the vacuole, particularly through the endosomal pathway (Kane, 2006). Additionally the V₀ membrane component has been proposed to play a more direct role in vacuole membrane fusion (Peters *et al.*, 2001). Whether the variability observed between different subunit deletions reflects distinct functions will require additional investigation.

ALP Transport Is Specifically Affected in *yck3Δ* Cells

Our immunoblotting analysis of ALP and CPS in *yck3Δ* suggested a specific defect in ALP traffic in this strain. Yck3p

Figure 2. Specific defect in ALP transport in *yck3* Δ cells. (A) Pulse-chase immunoprecipitation of ALP. Cells from WT, *apl6* Δ -AP-3 β subunit gene deletion strain ($\beta 3\Delta$), and *yck3* Δ strains were metabolically labeled with [³⁵S]methionine for 7 min, and labeling was quenched with nonradioactive amino acids. Samples of cells were removed at the indicated times after initiation of the chase. Cells were lysed, ALP was immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE. Gels were exposed to a phosphor screen and digitally imaged. (B) Pulse-chase immunoprecipitation of CPS. Pulse-chase analysis of CPS was performed as described in A, except that samples were removed at 0, 10, 20, and 40 min after addition of the chase. (C) Subcellular fractionation of *yck3* Δ cells. Cell lysates from wild-type (WT), *apl6* Δ ($\beta 3\Delta$), and *yck3* Δ strains were fractionated by differential centrifugation to generate low-speed ($300 \times g$ for 5 min) supernatant (S₁), medium-speed ($10,000 \times g$ for 15 min) supernatant and pellet (S₂, P₂), and high-speed ($200,000 \times g$ for 17 min) supernatant and pellet (S₃, P₃) fractions. Fractions were analyzed by SDS-PAGE and immunoblotted for ALP, the Golgi protein Kex2p, and the vacuole membrane (Vac) protein Vph1p. (D) ALP maturation in sorting defective Yck3p mutants. Cells from the *MATa yck3* Δ strain harboring empty vector, or plasmids containing wild-type YCK3 or two mutant *yck3* genes (*yck3-1*, *yck3-2*) that lack a YDSI tyrosine-based AP-3 pathway sorting sequence (Sun *et al.*, 2004). Cells were cultured in selective media, cell extracts were prepared and subjected to immunoblotting for ALP.

is a type I casein kinase that is localized to the vacuole membrane and plays a role in regulating vacuole morphology and inheritance (Sun *et al.*, 2004; LaGrassa and Ungermann, 2005). A role for this kinase in protein traffic to the vacuole has not been described. To more carefully assess the specificity of the trafficking defect we characterized ALP and CPS maturation by pulse-chase immunoprecipitation. Wild-type, *yck3* Δ , and *apl6* Δ cells were pulse labeled for 10 min, and then they were subjected to chase regimens before lysis and immunoprecipitation of ALP and CPS. In wild-type cells, maturation of ALP was >50% complete after the labeling period and complete by 10 min of chase (Figure 2A, lanes 1–4). In contrast, in the *yck3* Δ cells, >50% of ALP was

in the precursor form after the 5-min chase and significant levels of precursor persisted at the 15-min chase point (Figure 2A, lanes 9–12). This corresponds to roughly a threefold delay in ALP maturation in *yck3* Δ cells. By comparison, cells lacking the AP-3 β subunit ($\beta 3\Delta$) displayed a severe ALP maturation defect, with the precursor form predominant throughout the chase period (Figure 2A, lanes 5–8). These results suggest an important but not required function of Yck3p in ALP transport to the vacuole. CPS is matured more slowly than ALP in wild-type cells, presumably because of the intermediate endosome stage in the clathrin-dependent pathway (Figure 2, A and B, lanes 1–4). Maturation of CPS was not significantly delayed in $\beta 3\Delta$ or *yck3* Δ cells (Figure 2B, lanes 5–12). Together, the results from immunoblotting and pulse-chase immunoprecipitation provide evidence that maturation of ALP is selectively affected in *yck3* Δ cells. This phenotype is similar to, but less severe than, those of AP-3-deficient cells, suggesting that Yck3p acts in the AP-3 pathway.

To determine whether the ALP maturation delay in *yck3* Δ cells is due to a defect in ALP transport to the vacuole, we analyzed the subcellular distribution of pALP by differential centrifugation. Extracts from wild-type, *yck3* Δ and $\beta 3\Delta$ cells were first sedimented at $300 \times g$ to remove unbroken cells, and then the resulting supernatant (S₁) was sedimented at $13,000 \times g$ to pellet larger organelles including the ER and vacuoles (P₂). The $10,000 \times g$ supernatant (S₂) was then sedimented at $200,000 \times g$ to pellet smaller organelles such as the Golgi and transport vesicles (P₃). Fractions were then analyzed by immunoblotting for ALP, Kex2p (TGN/endosome marker), and Vph1p (vacuole membrane marker). In all three strains, mALP pelleted together with the Vph1p in P₂ (Figure 2C, lanes 2, 6, and 10), whereas sALP was mostly present in S₃ due to release from vacuoles during the lysis procedure (Figure 2C, lanes 4, 8, and 12). In $\beta 3\Delta$ and *yck3* Δ cells, pALP, like Kex2p, sedimented predominantly in P₃, indicating a defect in ALP transport to the vacuole in the two strains.

As an independent test of ALP traffic, localization of GFP-tagged ALP was assessed by fluorescence microscopy. To visualize vacuoles, cells were also stained with FM4-64, which is delivered to the vacuole via the endocytic pathway (Vida and Emr, 1995). In wild-type cells, GFP-ALP colocalized with FM4-64 at vacuole membranes (Figure 3, WT). In contrast, in $\beta 3\Delta$ cells GFP-ALP was present both at the vacuole together with FM4-64 and also at cytoplasmic puncta that did not label with FM4-64 (Figure 3, $\beta 3\Delta$). A similar but less severe localization defect was apparent in *yck3* Δ cells (Figure 3, *yck3* Δ). The punctate distribution of GFP-ALP in $\beta 3\Delta$ and *yck3* Δ cells is consistent with the pALP transport defect observed by differential centrifugation and likely reflects accumulation of pALP in Golgi and/or endosomes. Together, results from analysis of *yck3* Δ cells identify Yck3p as a factor specifically required for ALP transport between the Golgi and the vacuole.

The normal localization of Yck3p to the vacuole limiting membrane suggests a role in regulating transport vesicle targeting/fusion to the vacuole. The established role of Yck3p in controlling vacuole morphology supports this idea because many proteins involved in vacuole morphology also participate in transport vesicle targeting and fusion to the vacuole membrane (Ostrowicz *et al.*, 2008). Through such functions, these proteins are required for both the AP-3 and clathrin-dependent pathways (Bowers and Stevens, 2005). Indeed, our screen identified severe pALP and pCPS accumulation in strains lacking components of the HOPS complex and vacuole fusion machinery (Table 1). However, the

Figure 3. Mislocalization of GFP-ALP in *yck3Δ* but not *vac17Δ* cells. WT, $\beta 3\Delta$, *yck3Δ*, and *vac17Δ* cells harboring a plasmid encoding GFP-ALP were grown to early-logarithmic phase in minimal media, collected by centrifugation, and stained with FM4-64 for 20 min. The cells were then washed and allowed to internalize the dye for 60 min. Cells were imaged by confocal microscopy to visualize GFP-ALP and FM4-64 localization. Arrowhead indicates a vacuole probably generated *de novo*.

selective effects of *yck3Δ* on pALP transport imply a more specific role for Yck3p in the AP-3 pathway. Yck3p is a palmitoylated, membrane-associated protein that is delivered to the vacuole through the AP-3 pathway (Sun *et al.*, 2004), raising the possibility that sorting into the pathway confers functional specificity. An example of this mechanism was recently reported for the mammalian AP-3 pathway cargo, phosphatidylinositol-4-kinase type II α (Craigie *et al.*, 2008). To test whether Yck3p function in ALP transport requires sorting into the AP-3 pathway, we analyzed ALP maturation in cells expressing mutant forms of Yck3p that carry alterations of the YDSI signal that directs sorting into the AP-3 pathway. Although these mutants are not efficiently sorted into the AP-3 pathway, they still localize to the vacuole membrane, at least in part because of default transport through the endosomal route (Sun *et al.*, 2004). As assessed by immunoblotting, cells expressing these mutants as the sole source of Yck3p displayed no accumulation of pALP (Figure 2D). These data suggest that sorting into the AP-3 pathway is not required for Yck3p function in ALP transport.

ER Export of a Subset of Type II Integral Membrane Proteins Is Delayed in *svp26Δ* Mutants

In addition to *yck3Δ* and AP-3 subunit deletions, *svp26Δ* affected maturation of ALP but not CPS (Table 1). A specific effect of *svp26Δ* on ALP transport was reported previously in a study that characterized Svp26p as a transmembrane adaptor required for incorporation of ALP into COP-II vesicles at the ER (Bue *et al.*, 2006). In this study, *svp26Δ* did not affect transport of CPY, a soluble luminal protein, or Gas1p, a GPI-linked membrane protein. A separate study described mislocalization of the Golgi mannosyltransferase Ktr3p to the ER in *svp26Δ* cells (Inadome *et al.*, 2005), suggesting that

Figure 4. Selective defects in type II membrane protein transport in *svp26Δ* cells. (A) Pulse-chase immunoprecipitation of ALP. Wild-type (WT), *apl6Δ* ($\beta 3\Delta$), and *svp26Δ* cells were analyzed by pulse-chase immunoprecipitation of ALP as described in the legend to Figure 2A, except that chase samples were removed at 0, 30, 60, and 90 min. (B) Pulse-chase immunoprecipitation of CPS. Pulse-chase immunoprecipitation of CPS was performed as described in the legend to Figure 2B, except that samples were removed at 0, 20, 40, and 60 min after addition of the chase. (C) Pulse-chase immunoprecipitation of Gda1p. Pulse-chase immunoprecipitation of Gda1p from the indicated strains was performed as described in the legend to Figure 2A, except that samples were removed at 0, 20, 40, and 60 min after addition of the chase. UG, unglycosylated form of Gda1p (does not diminish in size after treatment with endoglycosidase H [data not shown]); ER, endoplasmic core glycosylated form of Gda1p; G, Golgi glycosylated forms of Gda1p (Vowels and Payne, 1998). (D) Subcellular fractionation of *svp26Δ* cells. Subcellular fractionation was performed as described in the legend to Figure 3. White arrowheads indicate the accumulated, glycosylated form of Gda1p not present in WT extract. Endoglycosidase H treatment reduces all forms of Gda1p (G, Golgi form; ER, endoplasmic reticulum form; UG, unglycosylated form) in both WT and *svp26Δ* strains to the UG form (data not shown). Fractions were immunoblotted for Gda1p, the ER protein Sec63p, and Kex2p.

Svp26p may serve as an adaptor for multiple proteins. Both ALP and Ktr3p are type II membrane proteins, a feature that could be important for recognition by Svp26p. To further define the specificity of Svp26p, we assessed maturation of ALP and CPS, another type II vacuole membrane protein, in *svp26Δ* cells by pulse-chase immunoprecipitation. ALP maturation was severely hindered in *svp26Δ* cells, as expected, with a delay commensurate with that displayed in AP-3-deficient cells (Figure 4A). CPS maturation was unaffected in *svp26Δ* cells, indicating that not all type II membrane proteins require Svp26p for transport from the ER (Figure 4B).

We also examined trafficking of another type II membrane protein, the Golgi guanosine diphosphatase Gda1p. In wild-

type cells, residence of Gda1p in the Golgi complex allows continued exposure to glycosyltransferases that extend oligosaccharide chains on Gda1p, causing a progressive size increase over time (Vowels and Payne, 1998b; Figure 4C, lanes 1–4). Mutations that prevent ER-to-Golgi transport result in accumulation of a core-glycosylated form of GD-Pase that does not change size (Vowels and Payne, 1998b). Accordingly, the size of Gda1p over time was monitored in wild-type and *svp26Δ* cells by pulse-chase immunoprecipitation. Unlike Gda1p in wild-type cells, which gradually increased in size during the chase period (Figure 4C, lanes 1–4), Gda1p in *svp26Δ* cells did not display a size shift until 40 min after the chase and even at the 60-min chase time exhibited substantial amounts of the ER form (Figure 4C, lanes 5–8). These data are consistent with an impediment in export of Gda1p from the ER in *svp26Δ* cells.

To more directly assess Gda1p localization, the subcellular distribution of Gda1p was examined in wild-type and *svp26Δ* strains by differential centrifugation. In wild-type cell extracts, several Gda1p species were detected by immunoblotting, including a major 70-kDa form and minor 67- and 57-kDa forms (Figure 4D, Gda1p, lane 1). Treatment with endoglycosidase H reduced all forms to 57 kDa, indicating that the higher-molecular-weight forms are glycosylated (unpublished data). All Gda1p forms in wild-type cells cofractionated with the Golgi marker Kex2p in P3 (Figure 4D, Gda1p and Kex2p, lane 5). In *svp26Δ* cell extracts, a new species of ~60 kDa was detected which corresponded in size to that expected for ER form (Figure 4D, Gda1p, lane 2, arrowhead). Additionally, there was a shift in the ratio of the higher-molecular-weight forms with the 67-kDa form most prominent. The 60-kDa form sedimented with the ER marker Sec63p (Figure 4D, Gda1p and Sec63p, lane 8), whereas the higher-molecular-weight species fractionated mostly in P3. Mislocalization of the 60-kDa Gda1p in *svp26Δ* cells is consistent with accumulation in the ER, and together with the results of the pulse-chase immunoprecipitation, provides evidence that Gda1p requires Svp26p for export from the ER. This result, combined with the earlier studies, indicates that Svp26p plays a role in transport of only a subset of type II membrane proteins from the ER to the Golgi, including Gda1p, Ktr3p, and ALP but not CPS.

ALP Maturation Is Defective in Vacuole Inheritance Mutants

Our original screen detected both ALP and CPS maturation defects in *vac8Δ* and *vac17Δ* strains. VAC8 and VAC17 encode proteins involved in vacuole inheritance during cell division (Weisman, 2006). Vac8p is associated with the vacuole membrane and also participates in cytoplasmic to vacuole transport and homotypic vacuole fusion (Wang *et al.*, 1998, 2001). Vac17p, which acts preferentially in vacuole inheritance, links Vac8p to the type-V myosin Myo2p (Ishikawa *et al.*, 2003). Myo2p directs movement of tubulovesicular vacuole inheritance structures along the actin cytoskeleton from the mother cell vacuole into the newly forming bud in which they seed formation of a new vacuole (Hill *et al.*, 1996). Roles for Vac8p or Vac17p in vacuolar protein maturation and/or vesicle-mediated protein transport have not been reported.

To further evaluate the ALP and CPS maturation defects, *vac8Δ* and *vac17Δ* cells were analyzed by pulse-chase immunoprecipitation. The results of this analysis revealed an unusual maturation pattern. In the case of ALP, ~50% was mature by the 10-min chase point but the level of precursor did not decline substantially at later chase points (Figure

Figure 5. ALP and CPS maturation is delayed in *vac8Δ* and *vac17Δ* cells. (A) Pulse-chase immunoprecipitation of ALP. Pulse-chase immunoprecipitation of ALP from the indicated strains was performed as described in the legend to Figure 2A except that chase samples were removed at 0, 5, 10, 15, and 20 min. (B) Pulse-chase immunoprecipitation of CPS. Pulse-chase immunoprecipitation of CPS was performed as described in the legend to Figure 4B. (C) Subcellular fractionation of *vac8Δ* and *vac17Δ* cells. Subcellular fractionation *vac8Δ* and *vac17Δ* cells and immunoblotting analysis was performed as described in the legend to Figure 2C.

5A). Similar biphasic maturation kinetics were observed for CPS (Figure 5B).

To determine the subcellular localization of the accumulated pALP in the inheritance mutants, we performed subcellular fractionation of *vac8Δ* and *vac17Δ* cells. Unlike pALP in trafficking mutants such as *β3Δ* and *yck3Δ*, which fractionated in P₃, pALP in *vac8Δ* and *vac17Δ* strains sedimented in P₂ with mALP and the vacuolar membrane marker Vph1p (Figure 5C, ALP and Vph1p, lanes 6 and 10). Consistent with these results, GFP-ALP in *vac17Δ* cells was localized to the vacuolar limiting membrane with FM4-64 (Figure 3). These results provide evidence that pALP in *vac8Δ* and *vac17Δ* cells is transported to vacuole membranes but a portion is inefficiently matured.

The inheritance defect in *vac8Δ* and *vac17Δ* cells provides a possible explanation for the unusual maturation kinetics of ALP and CPS. Vacuole inheritance mutants can generate vacuoles de novo through an unknown mechanism (Weisman *et al.*, 1987; Gomes De Mesquita *et al.*, 1997). However, such vacuoles do not receive contents from the maternal vacuole that contains the active form of protease A responsible for maturation of ALP and CPS. Consequently, in contrast to inherited vacuoles, de novo-generated vacuoles are not expected to be immediately proteolytically active. Thus, the relatively stable population of pALP and pCPS in *vac8Δ* and *vac17Δ* cells may represent those precursors delivered to de novo-formed vacuoles. In contrast, newly synthesized pALP and pCPS that is delivered to the maternal vacuoles is expected to be matured with essentially normal kinetics.

Over time, vacuoles formed de novo can become proteolytically active through autocatalytic activation of proteases A and B that are delivered from the biosynthetic pathway (Woolford *et al.*, 1986; Nebes and Jones, 1991). If the stable population of pALP is indeed present in de novo-generated vacuoles, then pALP should be matured once the vacuole

Figure 6. Accumulated pALP in *vac17Δ* cells matures after 120 min delay. (A) Pulse-chase immunoprecipitation of ALP from WT and *vac17Δ* cells was performed as described in the legend to Figure 2A except that chase samples were removed at 0, 30, 60, 90, 120, 150, 180, and 210 min. (B) Bands in A were quantified as described in *Materials and Methods*, and the ratio of pALP to total ALP was calculated.

acquires sufficient levels of active protease A. To test this prediction, we monitored maturation of ALP in *vac17Δ* cells by using a pulse-chase regimen that extended the chase period over 3.5 h (Figure 6). The level of pALP maturation after 30 min reached a plateau that persisted for 90 min (Figure 6, A and B). However, after 2 h ALP processing resumed, and virtually all of the pALP was mature by the 3.5-h time point. A similar result was obtained with *vac8Δ* cells (unpublished data). These data conform to the model that a percentage of pALP in *vac8Δ* and *vac17Δ* cells is transported to proteolytically inactive vacuoles formed de novo in the absence of the inheritance pathway. Our results suggest that the vacuole inheritance pathway is important for the timely biogenesis of functional vacuoles in newly forming daughter cells during cell division.

DISCUSSION

As an approach to identify the full complement of proteins required for transport through the AP-3 pathway in yeast, we have systematically surveyed collections of viable gene knockout strains by using maturation of vacuolar ALP as an indicator of AP-3 pathway function. Among the genes identified through this strategy are those that encode proteins involved in vesicle budding, targeting, and fusion, as well as other processes such as proteolytic processing in the vacuole, organelle acidification, vacuole inheritance, and protein export from the ER. Our study uncovered a selective role for the vacuolar casein kinase Yck3p in AP-3 pathway function, the only protein other than AP-3 that was required specifically for AP-3-dependent traffic.

Yck3p is one of four type I casein kinases in yeast and is unique in localizing to the vacuole (Wang *et al.*, 1996; Sun *et al.*, 2004). Recently, LaGrassa and Ungermann (2005) pro-

Figure 7. Model for Yck3p function. Vps41p phosphorylation by Yck3p targets Vps41p for function at the Golgi in AP-3-dependent vesicle formation. Unphosphorylated Vps41p functions as a component of HOPS in vesicle docking/fusion at the vacuole membrane. See text for details. Phosphorylated Vps41p may function at the Golgi as part of the HOPS complex although it is pictured acting independently. The site of Vps41p dephosphorylation and release from AP-3 vesicles is unknown.

vided initial insight into Yck3p function, reporting that *yck3Δ* cells display a defect in maintenance of vacuole fragmentation triggered by hyperosmotic stress. The study presented evidence that the fragmentation maintenance defect results from an up-regulation of vacuole fusion driven by increased tethering activity of the HOPS complex in the absence of Yck3p. Phosphorylation of Vps41p, a HOPS subunit, was demonstrated to depend on Yck3p. Based on these findings, it was proposed that Yck3p-mediated Vps41 phosphorylation inhibits HOPS tethering activity, thereby decreasing vacuole fusion and maintaining the fragmented state in hypertonic conditions. By analogy to the role of Vps41p in AP-3 vesicle formation, the authors also speculated that Vps41p might act in vacuole fragmentation and suggested that phosphorylation could control the distribution of Vps41p functioning in vacuole fragmentation versus fusion. Our finding that *yck3Δ* cells are defective in transport of ALP to the vacuole is consistent with a role for Vps41p phosphorylation in regulating vesicle formation and, importantly, indicates that Yck3p plays a role under normal growth conditions as well as in response to hypertonic stress.

We envision a model for Yck3p function in the AP-3 pathway that integrates the mechanism proposed to control stress-induced vacuole fusion (LaGrassa and Ungermann, 2005). In this model, Yck3p serves to regulate transition of the bifunctional Vps41p between two functional states—one state involved in AP-3 vesicle formation (phosphorylated) and the other state involved in vesicle targeting/fusion (dephosphorylated) at the vacuole (Figure 7). According to this model, deletion of *YCK3* would shift the balance of Vps41p toward targeting/fusion at the vacuole. Consistent with this prediction, *yck3Δ* cells display increased levels of vacuole-associated Vps41p in vivo under normal growth conditions and more avid association of Vps41p with vacuole membrane in vitro (LaGrassa and Ungermann, 2005). Redistrib-

tion of unphosphorylated Vps41p to the vacuole would allow fusion of vesicles from both the AP-3 pathway and the clathrin-dependent pathway but would deplete the pool of Vps41p available to participate in AP-3 vesicle formation, thereby accounting for the specific defect in ALP transport in *yck3Δ* cells. To determine whether the *yck3Δ* defect can be overcome simply by increasing levels of Vps41p, ALP maturation was monitored in *yck3Δ* cells expressing *VPS41* from a multicopy plasmid (Supplemental Figure S1). However, the ALP defect persisted in these cells, suggesting that phosphorylation by Yck3p may be required for Vps41p function in AP-3 vesicle formation. Alternatively, Vps41p may function in AP-3 dependent traffic at the Golgi as part of a complex, conceivably HOPS. In this scenario, overexpression of Vps41p without other members of the complex would not increase AP-3-specific Vps41p activity.

The apparently stable membrane association of Yck3p imposes a spatial constraint on Yck3p activity that may be important for models of AP-3 pathway-specific function. As an AP-3-dependent cargo, it is possible that the pathway-specific activity of Yck3p occurs during transport through the pathway. However, our results indicate that sorting-defective Yck3p mutants supported wild-type levels of ALP maturation, making it unlikely that Yck3p transport through the AP-3 pathway confers specificity. The Yck3p sorting mutants are reported to localize primarily at the vacuole membrane, probably due to transport through the clathrin-dependent endosomal pathway (Sun *et al.*, 2004). Thus, function of the sorting mutants in ALP transport suggests that the vacuole membrane is the site from which Yck3p provides AP-3 pathway-specific activity. This possibility is compatible with the Vps41p phosphorylation model described in Figure 7—the pool of unphosphorylated Vps41p involved in tethering could serve as substrate for vacuole-localized Yck3p, thereby generating the phosphorylated form of Vps41p proposed to function in AP-3 vesicle formation.

As an approach to address the importance of membrane association in Yck3p function, we assessed ALP maturation in cells expressing a palmitoylation-defective mutant of Yck3p (Sun *et al.*, 2004). This mutant supported normal ALP maturation (Supplemental Figure S1). However, differential fractionation revealed significant but not complete mislocalization to the soluble fraction. Similarly, Vancura *et al.* (1994) reported that expression of a palmitoylation-defective mutant of the plasma membrane casein kinase I, Yck2p, rescued defects in Yck2p-deficient cells, although the mutant kinase was substantially mislocalized. It seems that, in these cases, a small fraction of properly localized kinase may be sufficient to retain function, or these kinases can provide function from the cytoplasm.

Casein kinase I activity has also been associated with AP-3 function in mammalian cells (Faundez and Kelly, 2000). In this case, however, the $\beta 3$ subunit of AP-3 was identified as a kinase target. Inhibition of the kinase reduced AP-3 recruitment to synaptic vesicles in vitro and synaptic vesicle budding in vivo. In these studies, the role of Vps41 was not examined. Conversely, the possibility that AP-3 is a target of Yck3p has not been addressed in yeast. A more complete identification of kinase targets and functional tests of phosphorylation site mutants in both yeast and animal cells will be needed to define the regulatory mechanisms and determine the extent of similarity across species.

Our screen also identified Svp26p as a factor required for ALP maturation. During the course of our work, Svp26p was reported to function as an ER receptor for ALP, neces-

sary for sorting ALP into COP II vesicles (Bue *et al.*, 2006). Another group observed that Svp26p interacted with the Golgi glycosyltransferase Ktr3p and *svp26Δ* caused mislocalization of Ktr3p to the ER, results consistent with a role for Svp26p as an ER export receptor for Ktr3p (Inadome *et al.*, 2005). Our results provide evidence that another Golgi membrane protein, Gda1p, also accumulates in the ER in *svp26Δ* cells, supporting the view that Svp26p is a general ER cargo receptor, directing multiple cargoes into COP II vesicles. It is currently unknown how Svp26p recognizes cargo. ALP, Ktr3p, and Gda1p do not share significant sequence homology, yet all are type II integral membrane proteins, raising the possibility that membrane topology could constitute part of the binding determinant for Svp26p. However, our observation that CPS, another type II membrane protein, is not affected by *svp26Δ* indicates that membrane topology is not the sole factor for Svp26p recognition. Importantly, the common topologies of CPS and the three Svp26-dependent proteins should now allow a straightforward chimeric protein approach to map the elements that target cargo to Svp26p. Single homologues of Svp26p are present in a number of eukaryotic species, including humans (Bue *et al.*, 2006). Considering the evolutionary conservation of both Svp26p and COP II proteins, analysis of Svp26p cargo recognition has the potential to provide insight into a fundamental mechanism of protein export from the ER in eukaryotic cells.

During the polarized growth of yeast cells, the vacuole inheritance pathway directs transfer of tubular/vesicular elements of the mother cell vacuole into the emerging daughter bud through an actin and myosin-based mechanism (Hill *et al.*, 1996). This pathway promotes distribution of vacuoles to both mother and daughter cells during cell division. The role of vacuole inheritance is not completely clear, because daughter cells can synthesize vacuoles de novo in vacuole inheritance mutants. Our analysis of the ALP and CPS maturation defects discovered in *vac8Δ* and *vac17Δ* cells provides evidence that the vacuole inheritance pathway is important for the timely generation of a proteolytically active vacuole. In these vacuole inheritance mutants we observed that precursor ALP is delivered to vacuole membranes but only a fraction undergoes rapid maturation. Based on these findings, we suggest that precursor vacuolar enzymes are efficiently transported to two populations of vacuoles, one population that is proteolytically active and the other population that becomes active only after several hours. Slowly activating vacuoles would arise in daughter cells through de novo biogenesis of organelles that, because of the inheritance pathway defect, would initially lack active proteases normally transferred from the mother cell vacuole. Without acquisition of preactivated forms of the master processing proteases, generation of a fully active hydrolytic compartment would be delayed until newly synthesized protease zymogens are delivered by the biosynthetic transport pathways and undergo autocatalytic activation. Thus, it seems that the vacuolar proteolytic maturation system, which restricts activation of hydrolase precursors to the appropriate compartment, imposes a significant time delay in de novo formation of active vacuoles. Our study suggests that the inheritance pathway surmounts this problem by seeding daughter cell vacuoles with preactivated maturation proteases and other vacuolar hydrolases from the maternal vacuole.

Our use of a direct screen for ALP maturation in two collections of genome-wide yeast gene deletions identified a total of forty-nine strains with maturation defects. Notably, only five of these mutants exhibited selective defects in post-ER ALP transport, those with deletions of single AP-3 subunit genes and *yck3Δ*. Does this represent the complete

set of factors required exclusively for traffic through the AP-3 pathway in yeast? In addition to our systematic screen for ALP maturation defects, several other genetic approaches have been applied to identify AP-3 pathway-specific proteins. A systematic screen of ~15% of an independent gene deletion library by immunoblotting did not reveal new AP-3 pathway-specific mutations (Avaro *et al.*, 2002). Emr and colleagues carried out two independent screens of randomly mutagenized yeast for AP-3 pathway mutants (Darsow *et al.*, 2001). These approaches were sufficiently powerful to identify an AP-3 pathway-specific allele of *VPS41* but did not yield mutations in genes other than those encoding AP-3. Finally, a systematic screen for mutants with defects in sorting through the endosomal pathway also identified AP-3 mutants, probably due to indirect effects of AP-3 mutations on the endosomal pathway (Bonangelino *et al.*, 2002). In contrast to our work, this study reported *mon2Δ* as a mutation that caused stronger defects in ALP maturation than in sorting of carboxypeptidase Y through the clathrin-dependent pathway. We specifically tested *MATa* and *MATα* haploid *mon2Δ* strains but did not detect ALP maturation defects by immunoblotting and pulse-chase immunoprecipitation. However, the screen by Bonangelino *et al.* (2002) was carried out with the library of diploid strains homozygous for each gene deletion. We confirmed that the *mon2Δ* strain from the homozygous diploid collection accumulates ALP precursor. However, a plasmid expressing wild-type Mon2p that complemented the monensin sensitivity of the *mon2Δ* diploid strain did not restore ALP maturation, indicating that the ALP maturation defect in this strain is not due to the absence of Mon2p (unpublished data). These results suggest that Mon2p is not an AP-3 pathway-specific factor, a conclusion strongly supported by the recently reported role of Mon2p in Gga protein localization (Jochum *et al.*, 2002).

Considering the combined results of our direct systematic screen together with previous systematic and random screens, it is reasonable to suppose that AP-3 and Yck3p are the only proteins that function preferentially in the AP-3 pathway in yeast. Full AP-3 pathway function in yeast is therefore achieved by a very small set of pathway-specific components acting in concert with factors common to multiple trafficking pathways, some of which, like Vps41p, may be multifunctional with AP-3 pathway-specific activities. The strong evolutionary conservation of both specific and shared elements of the AP-3 pathway in yeast suggests that this set of proteins serves as a core foundation for elaboration of the machinery necessary to accommodate more complicated trafficking patterns in specialized metazoan cells.

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