Plate-based assay of AP-3 traffic in budding yeast

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SUMMARY

AP-3 (adaptor complex 3) mediates traffic between late Golgi or endolysosomal organelles, and late endolysosomal organelles. In mammals, mutations in AP-3 cause Hermansky-Pudlak Syndrome type 2, cyclic neutropenias, and a form of epileptic encephalopathy. In budding yeast, AP-3 carries cargo directly from the *trans* Golgi to the lysosomal vacuole. Despite the pathway's importance, and its discovery two decades ago, rapid screens and selections for AP-3 mutants have not been available. We now report GNSI, a synthetic, genetically-encoded reporter that allows rapid plate-based assessment of AP-3 functional deficiency, using either chromogenic or growth phonotype readouts. This system identifies defects in both the formation and consumption of AP-3 carrier vesicles and will be adaptable to high-throughput screening in both plate array and liquid batch culture formats. A plasmid encoding GNSI has been deposited in the Addgene repository.

INTRODUCTION

Transport vesicle budding is mediated by coat protein complexes that shape the nascent carrier. Generally, coat complexes associate with adaptors that populate the transport vesicles with specific integral membrane cargo molecules. The heterotetrameric adaptor protein (AP) complexes 1 and 2 belong to a family of coat protein adaptors originally identified on the basis of their association with clathrin. AP-1 and AP-2 function with clathrin coat proteins to mediate vesicular transport from the trans-Golgi network and the plasma membrane, respectively. Several additional AP complexes have subsequently been identified (Dacks and Robinson, 2017). AP-3 functions at trans-Golgi and/or early endosomal compartments, sorting its cargo molecules into transport vesicles that deliver their content to lysosomes and lysosome-related organelles. This functional assignment emerged from the discovery that mutations in AP-3 subunits correspond to many of the earliest known genetic mutations that affect pigmentation in laboratory strains of fruit flies and mice (Feng et al., 1999; Kantheti et al., 1998; Ooi et al., 1997; Simpson et al., 1997). In humans, mutations that disrupt AP-3 function cause an autosomal recessive disorder known as Hermansky-Pudlak Syndrome type 2, characterized by albinism, clotting deficiency, and a host of other abnormalities due to the malfunction of cell types that rely on lysosome-related organelles (Dell'Angelica, 2009; Horwitz et al., 2004).

The identification of an AP-3-dependent protein transport pathway from trans-Golgi compartments to the lysosome-like vacuole in *Saccharomyces cerevisiae* highlighted the early evolution of AP-3 in eukaryotes (Cowles et al., 1997; Stepp et al., 1997). The discovery of the yeast AP-3 pathway also gave way to expectations of the development of genetic screens that could be used to characterize this transport pathway. Meeting these expectations, however, has proven difficult because yeast can use partially redundant pathways to deliver cargoes to the vacuole. Consequently, screens for genes that influence AP-3 function have relied on laborious assays of vacuolar ALP processing: either preparation of cell extracts followed by SDS-PAGE and immunoblotting for ALP, or $^{[35]}$S pulse-chase, followed by cell extract preparation, anti-ALP immunoprecipitation, SDS-PAGE, and autoradiography (Anand et al., 2009;

Bonangelino et al., 2002). To date, only the immunoblot approach has been applied at genome scale, and this required a multi-year effort (Anand et al., 2009). We now report a rapid and scalable phenotypic assay of AP-3 deficiency in yeast, based on a synthetic, genetically-encoded reporter. This system reports AP-3 function using colorimetric or growth phenotype readouts, as well as by fluorescence microscopy, and will facilitate high-throughput mutational analyses of the AP-3 complex and its cofactors.

RESULTS & DISCUSSION

Translational fusions to Suc2, the major invertase (INV) enzyme of budding yeast, have been a mainstay in studies of yeast endocytic trafficking. For example, carboxypeptidase Y (CPY) traffics from the late Golgi to the endosome, then to the vacuole, through a mechanism that requires endosome-Golgi recycling of Vps10, a transport receptor for CPY. The CPY itinerary defines the vacuolar protease sorting (*VPS*) pathway (Bryant et al., 1998). Loss of Vps10, or *vps* mutations that disrupt Vps10 cycling *in trans*, result in exocytosis of CPY into the periplasmic space between the plasma membrane and cell wall. CPY-INV fusions, as well as fusions of vacuolar proteases A and C to INV, were used in pioneering screens that led to identification of dozens of *VPS* genes (Bankaitis et al., 1986; Johnson et al., 1987; Robinson et al.,

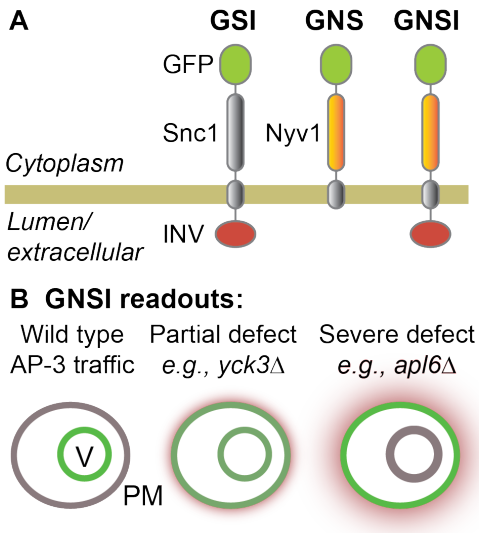

Fig. 1. Optical and enzymatic reporters of endocytic recycling (GSI) and AP-3 (GNS, GNSI) traffic. **A.** Schematic diagram of the reporters. **B.** Known behavior of GNS, and predicted behavior of GNSI. In wild type cells, the GFP signal of GNS is localized almost exclusively to the vacuole, and the invertase (INV) reporter enzyme is degraded in the vacuole lumen. In AP-3 deficient cells, GNS is targeted to the plasma membrane and the invertase (INV) reporter enzyme is exposed to the extracellular space, where it can catalyze the conversion of a colorless, cell-impermeant substrate into a quantifiable colored product, depicted here as a halo.

1988). More recently, the v/R-SNARE Snc2 was fused to both green fluorescent protein (GFP) and invertase. The resulting reporter, here called GSI (Fig. 1A) was used to assay the integrated levels of endocytosis and exocytosis in high-throughput screens of arrayed yeast gene deletion mutants (Burston et al., 2009; Dalton et al., 2015; Dalton et al., 2017).

The v/R-SNARE protein Nyv1 is normally targeted to the limiting membrane of the yeast vacuole through the AP-3 pathway (Reggiori et al., 2000; Wen et al., 2006). Nyv1 sorting requires interaction a YXXΦ-like motif in Nyv1's N-terminal longin domain (YGTI) with Apm3, the AP-3 μ-chain (Wen et al., 2006). If AP-3 function is deficient, Nyv1 is mis-routed into the endosomal system. Nyv1 then enters the multivesicular body (MVB), and is destroyed in the lumen of the lysosomal vacuole (Wen et al., 2006).

Previously, Pelham's group (Reggiori et al., 2000) studied a chimera called GNS (Fig. 1A). In GNS, GFP-marked Nyv1 cytoplasmic domain, is fused to the transmembrane anchor of the v/R-SNARE, Snc1. In wild type cells GNS, like GFP-Nyv1, is targeted to the vacuole limiting membrane. In cells defective for AP-3 function, however, GNS is diverted not to the MVB but rather to the plasma membrane. GNS has since been used as an optical reporter of AP-3–mediated traffic (Cabrera et al., 2009; Cabrera et al., 2010; Wen et al., 2006). Based on these results, we reasoned that fusion of invertase to the lumenal/ extracellular C-terminus of GNS would yield a reporter (GNSI; Fig. 1A) that could be used to monitor AP-3 traffic using colony-based or liquid culture systems (Fig. 1B). We constructed the GNSI chimera, and expressed it from a single-copy plasmid under the constitutive *CYC1* promoter (Fig. 2). We then assessed GNSI as an AP-3 reporter using three readouts.

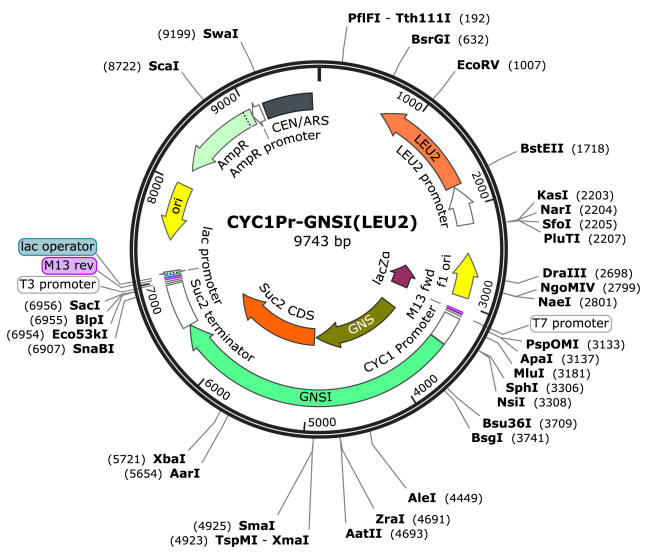

Fig. 2. Map of the GNSI reporter plasmid.

First, we asked whether GNSI faithfully recapitulates the subcellular localization of the original GNS reporter (Reggiori et al., 2000). As shown in Fig. 3, it does so. In wild type cells, GNSI is targeted efficiently to the vacuole limiting membrane. In cells lacking AP-3 (*apl6*Δ) or in cells defective for terminal docking and fusion of AP-3 vesicles at the vacuolar target membrane (*vam3*^{tsf}) GNSI accumulated largely on the plasma membrane. In cells with less penetrant AP-3 deficiency (*ypt7*Δ) , plasma membrane localization was weaker and observed only in a subset of the cells. In experiments using high-copy (rather than single-copy) vectors, or promoters stronger than *CYC1pr*, we noted that GNSI had a tendency to accumulate in the endoplasmic reticulum (unpublished data). Thus, extreme overexpression should be avoided with GNSI. With this caveat, the subcellular localization of GNSI closely mirrors the localization of the original GNS reporter.

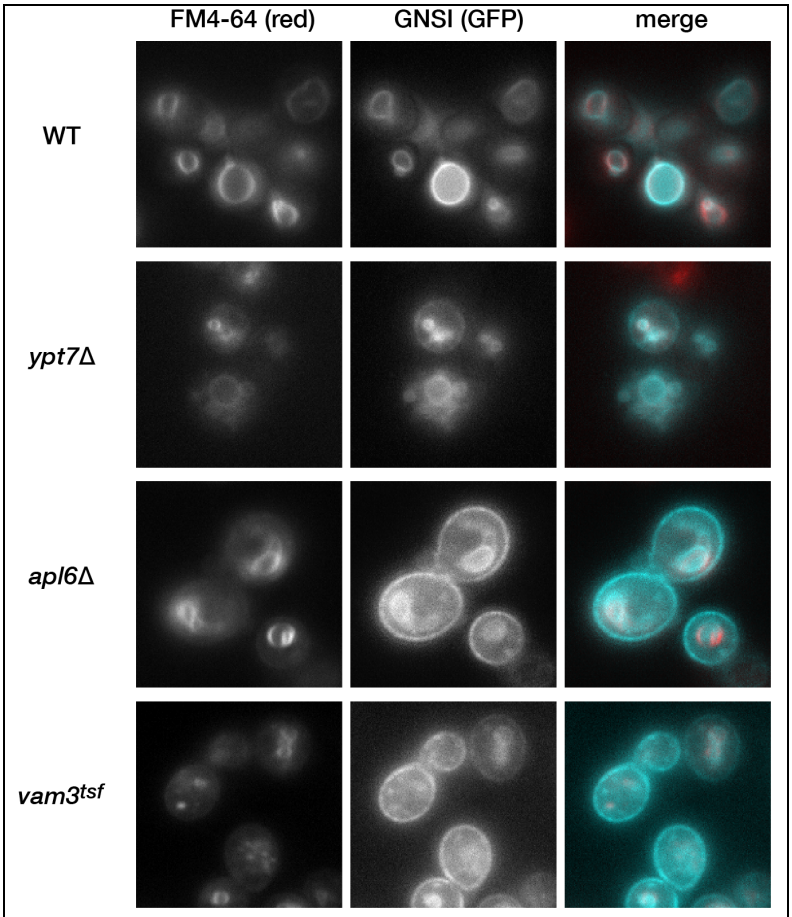

Fig. 3. Subcellular localization of the GNSI probe. Cells were grown to mid-log phase at 30° C, and vacuoles were pulse-chase labeled with the red styryl dye FM4-64. Wide-field fluorescence micrographs are shown. In the color merge panels, the GFP signal is false-colored cyan. All strains here and in the following figures are in the SEY6210 genetic background.

Next, we tested whether a chromogenic assay that measures glucose liberation by invertase could be used for plate-based assay of AP-3 activity (Darsow et al., 2000). As shown in Fig. 4, colored invertase reaction product strongly accumulated in strains defective for AP-3 traffic. Again, strains previously reported to exhibit more severe

trafficking defects using proteolytic processing of ALP as a readout also yielded stronger colorimetric signals with the GNSI reporter.

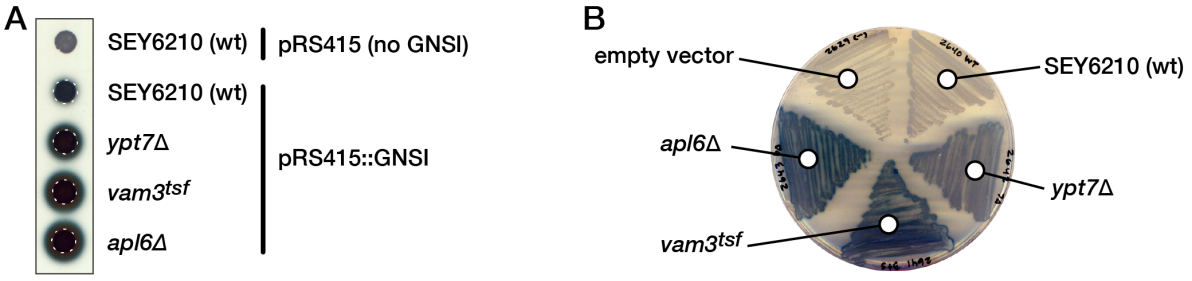

Fig. 4. Agar overlay assay of defects in AP-3 trafficking. Liberation of glucose by invertase exposed to the extracellular (periplasmic) space was detected as described in Methods. Dark precipitate indicates the presence of glucose. **B.** Colonies grown following inoculation with a pin manifold (96-well spacing) are assayed. Dashed circles denote the colony diameters. **B.** Streaked plate sectors containing many colonies each are assayed. All strains are in the SEY6210 genetic background.

Finally, we asked if GNSI could be used in growth selections. Mutants lacking extracellular invertase exhibit growth defects when sucrose rather than dextrose is the primary carbon source. Thus, we tested whether *suc2*Δ cells with AP-3 deficiency would exhibit enhanced growth on sucrose plates containing the pH indicator dye bromocresol purple. As shown in Fig. 5A, cells expressing GNSI grew into larger colonies, and more strongly acidified the medium, when mutations compromising AP-3 traffic were present. As expected, the stringency of this growth selection was enhanced when oxidative respiration was blocked with the uncoupling toxin antimycin A (Fig. 5B). Thus, the GNSI reporter will allow positive selection, with controllable stringency, of mutants defective in AP-3 traffic.

In summary, GNSI is a convenient reporter of AP-3 traffic, which does not require laborious preparation and analysis of cell extracts, metabolic labeling, or fluorescence microscopy. This system will allow rapid and high-throughput analyses of AP-3 structure-function, genome-scale screening, and secondary screening of potential

AP-3 co-factors identified through, for example, proteomics-based approaches (our unpublished results).

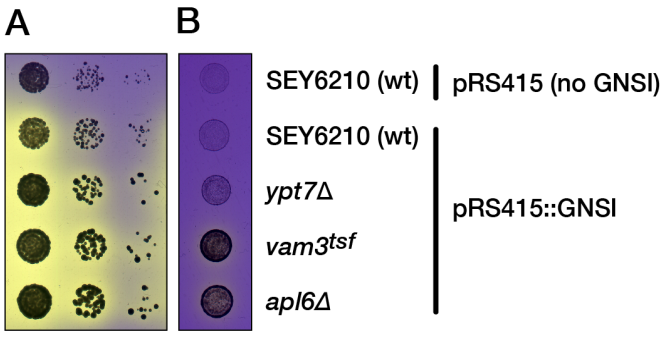

Fig. 5. Growth phenotypes on sucrose indicator media. **A**. Ten-fold serial dilutions were inoculated with a pin manifold onto 2% sucrose media containing bromocresol purple. **B**. Cells were inoculated as on the first column of A, but using media containing 10 µg/mL antimycin A.

A limitation of the plasmid-borne GNSI reporter demonstrated here is that it can be used only in strains with the *suc2* genotype (*e.g.,* SEY6210; Robinson et al., 1988). However, by replacing the chromosomal *SUC2* locus with *GNSI*, in cells also bearing the "magic marker" haploid selection locus (Tong et al., 2001), it will be possible to rapidly introduce *GNSI*::*suc2*Δ0 into any compatible mutant background using automated mating and sporulation procedures (Tong and Boone, 2005). Construction of strains for this purpose is underway in our laboratories. These strains will facilitate both genome-scale screens for mutants defective in AP-3 transport, and high-density deep-scan mutagenesis (DMS) structure-function studies (Fowler et al., 2014) of specific AP-3 transport factors, including the AP-3 complex itself.

METHODS

Cloning. pGNS416, encoding GFP-Nyv1-Snc1TMD (Reggiori et al., 2000) was a gift from F. Reggiori. pHB4, encoding GSI (in earlier work called GSS) (Burston et al., 2009) was a gift from E. Conibear. To construct *pCYC1pr-GNSI*(*LEU2*), the yeast vector pRS415 was linearized using XhoI and SacI. Using a 3-piece Gibson assembly procedure, three purified PCR products were cloned into the linearized vector. The *CYC1* promoter (290bp) was cloned from yeast genomic DNA using primers *CYC1* Promoter Forward and *GFP-CYC1* Reverse. The *GNS* cassette was amplified from

pGNS416 using primers *CYC1-GFP* Forward and *TMD* Reverse. The invertase cassette (including the Snc1 TMD, SUC2 ORF, and SUC2 terminator) was amplified from pHB4 using primers TMD Forward and SUC2term Reverse. The resulting plasmid was amplified in *E. coli*, mapped using PCR, and fully sequenced using dideoxy chain termination chemistry. DNA constructs were designed, and the map in Fig. 2 was produced, using the SnapGene package (GSL Biotech). *pCYC1pr-GNSI*(*LEU2*) and a full DNA sequence file are available from the Addgene repository (plasmid #111450).

Yeast strains and media.

Microscopy. All strains shared the SEY 6210 genetic background (Robinson et al., 1988). FM4-64 pulse chase was done as described (Vida and Emr, 1995) with a 20 min exposure to the dye followed by a 30-45 min chase in media without the dye. Epifluorescence micrographs were acquired using an Olympus IX71 microscope equipped with an Andor 885 camera, an intermediate 1.5X magnification lens, and a 60× 1.4NA objective.

Invertase agar overlay assay. The invertase agar overlay assay was done as described (Darsow et al., 2000). The cells were grown on synthetic complete (SC) –Leu, +2% raffinose plates. Reactions were developed for 15 minutes before being photographed.

Sucrose indicatior plate assay. Cells were pinned onto 2% sucrose indicator media (Darsow et al., 2000) containing bromocresol purple (Thermo-Fisher Scientific), in the absence or presence of 10 µg/mL antimycin A (Fisher Scientific).

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REFERENCES

Anand, V. C., L. Daboussi, T. C. Lorenz, and G. S. Payne. 2009. Genome-wide analysis of AP-3-dependent protein transport in yeast. *Mol Biol Cell.* 20:1592-1604.

Bankaitis, V. A., L. M. Johnson, and S. D. Emr. 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc Natl Acad Sci U S A.* 83:9075-9079.

Bonangelino, C. J., E. M. Chavez, and J. S. Bonifacino. 2002. Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae. *Mol Biol Cell.* 13:2486-2501.

Bryant, N. J., R. C. Piper, S. R. Gerrard, and T. H. Stevens. 1998. Traffic into the prevacuolar/endosomal compartment of Saccharomyces cerevisiae: a VPS45-dependent intracellular route and a VPS45-independent, endocytic route. *Eur J Cell Biol.* 76:43-52.

Burston, H. E., L. Maldonado-Báez, M. Davey, B. Montpetit, C. Schluter, B. Wendland, and E. Conibear. 2009. Regulators of yeast endocytosis identified by systematic quantitative analysis. *J Cell Biol.* 185:1097-1110.

Cabrera, M., L. Langemeyer, M. Mari, R. Rethmeier, I. Orban, A. Perz, C. Brocker, J. Griffith, D. Klose, H. J. Steinhoff, F. Reggiori, S. Engelbrecht-Vandre, and C. Ungermann. 2010. Phosphorylation of a membrane curvature-sensing motif switches function of the HOPS subunit Vps41 in membrane tethering. *J Cell Biol.* 191:845-859.

Cabrera, M., C. W. Ostrowicz, M. Mari, T. J. LaGrassa, F. Reggiori, and C. Ungermann. 2009. Vps41 phosphorylation and the Rab Ypt7 control the targeting of the HOPS complex to endosome-vacuole fusion sites. *Mol Biol Cell.* 20:1937-1948.

Dacks, J. B., and M. S. Robinson. 2017. Outerwear through the ages: evolutionary cell biology of vesicle coats. *Curr Opin Cell Biol.* 47:108-116.

Dalton, L., M. Davey, and E. Conibear. 2015. Large-scale analysis of membrane transport in yeast using invertase reporters. *Methods Mol Biol.* 1270:395-409.

Dalton, L. E., B. D. Bean, M. Davey, and E. Conibear. 2017. Quantitative high-content imaging identifies novel regulators of Neo1 trafficking at endosomes. *Mol Biol Cell.*

Darsow, T., G. Odorizzi, and S. D. Emr. 2000. Invertase fusion proteins for analysis of protein trafficking in yeast. *Methods Enzymol.* 327:95-106.

Dell'Angelica, E. C. 2009. AP-3-dependent trafficking and disease: the first decade. *Curr Opin Cell Biol.* 21:552-559.

Feng, L., A. B. Seymour, S. Jiang, A. To, A. A. Peden, E. K. Novak, L. Zhen, M. E. Rusiniak, E. M. Eicher, M. S. Robinson, M. B. Gorin, and R. T. Swank. 1999. The beta3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness. *Hum Mol Genet.* 8:323-330.

Fowler, D. M., J. J. Stephany, and S. Fields. 2014. Measuring the activity of protein variants on a large scale using deep mutational scanning. *Nat Protoc.* 9:2267-2284.

Horwitz, M., K. F. Benson, Z. Duan, F. Q. Li, and R. E. Person. 2004. Hereditary neutropenia: dogs explain human neutrophil elastase mutations. *Trends Mol Med.* 10:163-170.

Johnson, L. M., V. A. Bankaitis, and S. D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell.* 48:875-885.

Kantheti, P., X. Qiao, M. E. Diaz, A. A. Peden, G. E. Meyer, S. L. Carskadon, D. Kapfhamer, D. Sufalko, M. S. Robinson, J. L. Noebels, and M. Burmeister. 1998. Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. *Neuron.* 21:111-122.

Ooi, C. E., J. E. Moreira, E. C. Dell'Angelica, G. Poy, D. A. Wassarman, and J. S. Bonifacino. 1997. Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the Drosophila eye color mutant garnet. *EMBO J.* 16:4508-4518.

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

Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr. 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.

Simpson, F., A. A. Peden, L. Christopoulou, and M. S. Robinson. 1997. Characterization of the adaptor-related protein complex, AP-3. *J Cell Biol.* 137:835-845.

Tong, A. H., and C. Boone. 2005. Synthetic Genetic Array Analysis in Saccharomyces cerevisiae. *Methods Mol Biol.* 313:171-192.

Tong, A. H., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader, N. Page, M. Robinson, S. Raghibizadeh, C. W. Hogue, H. Bussey, B. Andrews, M. Tyers, and C. Boone. 2001. Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science.* 294:2364-2368.

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

Wen, W., L. Chen, H. Wu, X. Sun, M. Zhang, and D. K. Banfield. 2006. Identification of the yeast R-SNARE Nyv1p as a novel longin domain-containing protein. *Mol Biol Cell.* 17:4282-4299.