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Genomic Analysis of Homotypic Vacuole Fusion

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Yeast vacuoles undergo fission and homotypic fusion, yielding one to three vacuoles per cell at steady state. Defects in vacuole fusion result in vacuole fragmentation. We have screened 4828 yeast strains, each with a deletion of a nonessential gene, for vacuole morphology defects. Fragmented vacuoles were found in strains deleted for genes encoding known fusion catalysts as well as 19 enzymes of lipid metabolism, 4 SNAREs, 12 GTPases and GTPase effectors, 9 additional known vacuole protein-sorting genes, 16 protein kinases, 2 phosphatases, 11 cytoskeletal proteins, and 28 genes of unknown function. Vacuole fusion and vacuole protein sorting are catalyzed by distinct, but overlapping, sets of proteins. Novel pathways of vacuole priming and docking emerged from this deletion screen. These include ergosterol biosynthesis, phosphatidylinositol (4,5)-bisphosphate turnover, and signaling from Rho GTPases to actin remodeling. These pathways are supported by the sensitivity of the late stages of vacuole fusion to inhibitors of phospholipase C, calcium channels, and actin remodeling. Using databases of yeast protein interactions, we found that many nonessential genes identified in our deletion screen interact with essential genes that are directly involved in vacuole fusion. Our screen reveals regulatory pathways of vacuole docking and provides a genomic basis for studies of this reaction.

INTRODUCTION

Membrane fusion is required for selective delivery of proteins from one organelle to another and for the maintenance of low organelle copy number. Fusion is catalyzed by a cascade of interacting proteins, including integral membrane SNAREs, chaperones such as Sec18p/NSF, Sec17p/α-SNAP and LMA1, GTPases of the Rab and Rho families, GTPase effectors, calcium channels, and calcium-responsive proteins. Certain lipids, such as phosphoinositol phosphatides, are also needed, both to recruit proteins to organelles and to generate signaling molecules. The complexity of membrane fusion has so far made it difficult to enumerate all the responsible factors and to connect them in a coherent scheme of catalysis.

Yeast vacuoles offer several advantages for studying membrane fusion (Wickner and Haas, 2000). Vacuoles are readily visualized in intact cells and undergo constant fission and fusion. Consequently, defects in fusion are readily seen as vacuole fragmentation. Large vacuoles are not required for cell growth under laboratory conditions, and thus strains with deletions of genes encoding vacuole fusion catalysts are viable. Vacuoles can be purified in large amounts and stored frozen. Vacuoles fuse during incubation in vitro, and this fusion can be assayed colorimetrically. This reaction occurs in ordered stages of priming, docking, and fusion.

Priming occurs on separate vacuoles and is needed for productive vacuole associations (Mayer *et al.*, 1996). Priming is initiated by ATP hydrolysis by Sec18p. Priming drives the release of Sec17p, disassembly of *cis*-SNARE complexes (Ungermann *et al.*, 1998), and transfer of the LMA1 cochaperone from Sec18p to the activated t-SNARE Vam3p (Xu *et al.*, 1997, 1998). Priming requires phosphatidylinositol 4,5-bisphosphate (PI[4,5]P2; Mayer *et al.*, 2000) and ergosterol (Kato and Wickner, 2001). Priming allows HOPS (homotypic fusion and vacuole protein sorting)/VPS class C complex, a complex of at least 6 proteins (Vps 11, 16, 18, 33, 39, and 41), to be transferred to Ypt7p (Price *et al.*, 2000; Seals *et al.*, 2000), catalyzing the conversion of Ypt7p to its active GTP form (Wurmser *et al.*, 2000) and thereby initiating docking. Docking, which also requires the vacuole membrane potential $\Delta\mu_H+$ (Ungermann *et al.*, 1999), Rho GTPases (Eitzen *et al.*, 2001; Muller *et al.*, 2001), and phosphoinositides (Mayer *et al.*, 2000), concludes with *trans*-pairing of SNAREs (Ungermann *et al.*, 1998) and calcium release from the vacuole (Peters and Mayer, 1998). Calcium-bound calmodulin binds at V0 (the integral membrane domain of the vacuolar H^+-ATPase) and triggers V0-V0 association in trans (Peters *et al.*, 2001). On protein phosphatase 1 (PP1) action (Peters *et al.*, 1999), LMA1 is released (Xu *et al.*, 1998) and fusion occurs. Despite this progress, few subreactions of vacuole fusion have been reconstituted with pure components, and the

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connections between these steps in the pathway remain obscure.

Nine of the known proteins that catalyze vacuole fusion are encoded by *VAM* genes. The *vam* mutants were identified (Wada *et al.*, 1992) by nonselective screening for abnormal *va*cuole *m*orphology. Six of the vacuole morphology (*VAM*) genes encode subunits of the HOPS complex (Vam1p = Vps11p, Vam9p = Vps16p, Vam8p = Vps18p, Vam5p = Vps33p, Vam6p = Vps39p, and Vam2p = Vps41p), Vam3p is the vacuolar t-SNARE, Vam7p is the vacuolar SNAP25 SNARE homologue, and Vam4p is the GTPase Ypt7p. Although each of the nine original *VAM* genes are allelic with a known *VPS* gene, the initial screen for *vam* mutants was not saturated. We have therefore taken a genomic approach to identify additional catalysts of vacuole fusion, exploiting a collection of 4828 yeast strains with deletions in each nonessential gene and visualizing the vacuole with the fluorescent vital dye FM4-64. The new *VAM* genes identified in this manner define novel pathways whose roles can be confirmed through the use of selective inhibitors of in vitro fusion of wild-type vacuoles. They reveal a striking and unexpected complexity of the priming and docking stages of homotypic vacuole fusion.

MATERIALS AND METHODS

FM4-64 and antibody to carboxypeptidase Y (CPY) were from Molecular Probes (Eugene, OR). Anti-mouse immunoglobulin G-POD was from Boehringer-Mannheim (Indianapolis, IN). Libraries of strains with deletion of each of the nonessential genes, in homozygous diploid (BY4743) and haploid (BY4739, BY4741, BY4742) backgrounds, were purchased from Research Genetics (Huntsville, AL).

Deletion Screen

Microtiter plates containing 96 yeast deletion strains were thawed, and 5–25 μl of each culture was used to inoculate 1 ml of YPD with 3 μM FM4-64 and 20 μg/ml G418. Cultures were grown for 12–36 h at 30°C with constant shaking before microscopic examination. Strains with vacuole morphology defects were streaked to single colonies and examined by at least two individuals. Microscopic examination and phenotype scoring was performed without reference to strain identity.

CPY Secretion

The CPY secretion assay was performed according to the method of Roberts *et al.* (1991) with minor modifications; single colonies were picked from YPD-agar plates and suspended in 200 μl of YPD, and 5 μl of each suspension was spotted onto YPD-agar plates and allowed to dry before filter overlay and incubation.

Vacuole Isolation

Vacuoles were isolated (Hass, 1995) and stored frozen (Seals *et al.*, 2000).

Fusion Reaction

Fusion reactions (Hass, 1995) contained 3 μg each of vacuoles from BJ3505 (MATa, pep4::HIS3. prb1-Δ1.6 R, lys2–208, trp1-Δ101, ura3-52, gal2, can) and DKY6281 (MATa, leu2-3112, ura 3-52, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, pho8::TRP1) in reaction buffer (200 mM sorbitol, 20 mM 1,4-piperazinediethanesulfonic acid-KOH, pH 6.8, 119 mM NH_4Cl, 3.9 mM $MgCl_2$, 0.8 mM ATP [Amersham Pharmacia Biotech, Piscataway, NJ], 18.8 mM creatinine kinase [Roche Molecular Biochemicals, Summerville, NJ], 23 mM creatine phosphate [Roche Molecular Biochemicals], and 0.8 mM CoA). All reactions contained 7.8 μg of HMA, a high molecular weight fusion-enhancing fraction purified from yeast cytosol by gel filtration on Sephacryl 200HR (Amersham Pharmacia Biotech). Inhibitors (Calbiochem, San Diego, CA) were prepared as 35× concentrated stock solutions: 420 mM 2-aminoethoxydiphenyl borate (2-APB) in dimethyl sulfoxide (DMSO), 6.12 mM Ruthidium Red in PS (200 mM sorbitol, 10 mM 1,4-piperazinediethanesulfonic acid-KOH, pH 6.8), 15 mM cyclopiazonic acid in DMSO, 10.5 mM thapsigargin in DMSO, and 2 mM ET-18-OCH_3 in ethanol. Antibodies to yeast vacuole proteins (α-YPT7, 33 μg/ml; α-Sec17, 150 μg/ml; α-Vam3, 75 μg/ml) were purified from the sera of rabbits immunized with the specific recombinant protein by adsorption to protein A-Sepharose. KLH-conjugated YPT7 peptide (TEAFEDDYNDAINIR) was synthesized by Biosynthesis and was added at 1 μg per reaction from a stock solution of 1 mg/ml in PS buffer.

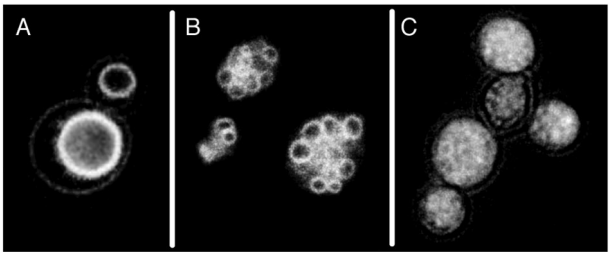

Figure 1. Vacuole morphologies. Single yeast colonies were picked from YPD-agar plates, inoculated into 1.0 ml of YPD with 3 μM FM4-64, and grown overnight at 30°C with constant shaking. Samples of 3 μl were examined by fluorescence microscopy with a rhodamine filter set on a standard microscope (Carl Zeiss, Thornwood, NY) with backlighting and photographed with T-MAX 400 Pro film (Kodak, Rochester, NY). A–C show yeast with wild-type, class B, and class C vacuoles, respectively.

RESULTS

Using the fluorescent vacuolar vital stain FM4-64, we scored 4828 nonessential gene deletions in yeast for vacuole morphology. Cultures of each strain were grown at 30°C overnight in rich media, stained with FM 4-64, and screened by fluorescence light microscopy for vacuole morphology without reference to the strain identities. Wild-type yeast typically have one to three large vacuoles (Figure 1A). Abnormal vacuole morphologies were categorized as in earlier *VPS* screens (Rothman and Stevens, 1986; Banta *et al.*, 1988). Class B mutants have multiple small vacuoles (Figure 1B), class C mutants have highly fragmented vacuoles (Figure 1C), class D mutants have single, grossly enlarged vacuoles, and class E mutants have an enlarged "prevacuolar" compartment surrounded by numerous membrane vesicles. Of 4828 nonessential genes, 714 deletions caused an altered vacuole morphology (see table on journal website). A subset of these were of class B and C, consistent with defects in vacuole fusion. Notably, our results match closely those previously reported for *vps/vam* mutants bearing distinct vacuolar fragmentation phenotypes. Of 11 previously reported *vps/vam* mutants (*VPS*5, 11, 16, 17, 18, 33, 39, 41, 43, 52, and 54) described by others as bearing either class B or C vacuoles (Bankaitis *et al.*, 1986; Rothman *et al.*, 1986; Weisman *et al.*, 1990; Wada *et al.*, 1992; Conibear and Stevens, 2000), we

Table 1. Known catalysts

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
SNAREs				
YOR106w	VAM3/PTH1	100%C	+++	Syntaxin (t-SNARE) homologue
YGL212w	VAM7/VPS43	100%C	++	SNAP-25 homologue
HOPS complex				
YLR148w	VPS18/VAM8	100%C	+++	HOPS subunit
YMR231w	VPS11/VAM1	100%C	+++	HOPS subunit
YDL077c	VPS39/VAM6	100%C	+++	HOPS subunit, Ypt7p GEF
YPL045w	VPS16/VAM9	100%C	+++	HOPS subunit
YLR396c	VPS33/VAM5	100%C	+++	HOPS subunit, Sec1p homologue
YDR080w	VPS41/VAM2	100%C	+++	HOPS subunit, AP-3 coat protein
Vacuolar V-ATPase				
YKL080w	VMA5/VATC	10%C 60%D	++	V1 sector V-ATPase subunit
YLR447c	VMA6	25%C 50%D 25%E	++	V0 sector V-ATPase subunit
YPL234c	VMA11/TFP3	40%C 60%D	++	V-ATPase proteolipid
YHR026w	VMA16/PPA1	40%C 60%D	++	V-ATPase proteolipid
YHR060w	VMA22/VPH6	20%C 70%D	++	V-ATPase assembly protein
YOR270c	VPH1	75%B 10%C	++	V0 sector V-ATPase subunit
Other known catalysts				
YML001w	YPT7/VAM4	100%C	++	Ypt/Rab GTPase
YNL015w	PB12	50%B	+	LMA1 subunit
YEL013w	VAC8	60%B 15%E	+	Docking factor required for vacuole inheritance

detected all 11 in this screen and all had either a class B or C phenotype.

Many deletion strains with a class B or C phenotype showed only a small percentage of cells with vacuole fragmentation, and some of these genes encode proteins that are unlikely to directly regulate fusion. For example, many of these genes encode nonessential transcription factors, ribosomal subunits, or nuclear pore proteins. These proteins may not be directly involved in fusion but, rather, might affect the expression of proteins that directly catalyze the fusion reaction. These considerations allowed the selection of 137 candidate *VAM* genes (Tables 1–9). Screening these for secretion of CPY revealed 26 new gene deletions with a moderate to strong *VPS* phenotype. In total, only 50 gene deletions of the 137 *VAM* genes, including 24 already characterized *VPS* genes, gave rise to a moderate or strong *VPS* phenotype. This suggests that there is distinction as well as overlap between pathways of traffic to the vacuole and homotypic vacuole fusion.

Seventeen of the genes identified in the deletion screen encode proteins that were previously known from biochemical studies to be involved in vacuole fusion (Table 1). These include the Vam3p and Vam7p SNAREs, the GTPase Ypt7p, its six HOPS effector subunits, multiple subunits of the vacuolar H^+-ATPase (needed for the vacuolar membrane potential and for *trans*–V0-V0–pairing during fusion), the chaperone IB2, and Vac8p, a protein with armadillo repeats that is required for vacuole fusion (Veit *et al.*, 2001; Wang *et al.*, 2001). Many proteins that are required for vacuole fusion are also essential for cell growth and thus are not represented in the screen. These include Sec17p, Sec18p, Vti1p, Ykt6p, calmodulin, and protein phosphatase 1. Strains with a deletion of the gene for Nyv1p have normal vacuole morphology, even though this SNARE is localized to the vacuole and is found in *cis*- and *trans*-SNARE complexes (Ungermann *et al.*, 1998). Vacuoles from *nyv*1Δ strains fuse poorly, and antibody to Nyv1p blocks the in vitro fusion of normal vacuoles (Nichols *et al.*, 1997). Other SNAREs may compensate for the absence of Nyv1p function in vivo, as suggested by genetic studies of other yeast-trafficking reactions (Fischer von Mollard *et al.*, 1997; Grote and Novick, 1999).

Membrane traffic is regulated by protein phosphorylation and dephosphorylation (Cabanoils *et al.*, 1999; Marash and Gerst, 2001). We found that normal vacuole copy number requires 16 kinases and two protein phosphatases, in addition to two regulatory subunits of PP1 (Table 2). Several of the kinases are members of known families, such as casein kinases or mitogen-activated protein (MAP) kinase cascades, but most are categorized only as kinases by sequence homology. Other kinases and phosphatases are members of the *STE* pathway of mating hormone response and delivery to the vacuole. Vacuole morphology depends on two putative regulatory subunits of protein phosphatase 1 (Glc7p), an enzyme that regulates the last step of vacuole fusion (Peters *et al.*, 1999).

Lipid metabolism is important for vacuole fusion (Table 3). Five steps of the ergosterol biosynthetic pathway are essential for normal vacuole copy number, although the deletion of *ERG*4, which encodes the final enzyme of the pathway, gave only a partial vacuole morphology defect. These observations suggest that ergosterol or its immediate precursor zymosterol is needed for vacuole fusion. Our further studies, reported elsewhere (Kato and Wickner, 2001), showed that vacuolar ergosterol is required for normal Sec18p-mediated priming and thus regulates the initial commitment to vacuole fusion.

PI(4,5)P2 is needed for vacuole docking (Mayer *et al.*, 2000). The phosphatidylinositol 4-kinases Stt4p and Pik1p, as well as the phosphatase *Sac*Ip, are needed for normal vacuole morphology (Audhya *et al.*, 2000). Arf1p, which activates the PI4P 5-kinase activity of Mss4p (Donaldson and Jackson, 2000), and Glo3p, which is an Arf-GTPase–activat-

Table 2. Protein modification

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
Protein kinase related				
YAL040c	CLN3/DAF1	30%B 30%E	+	G1/S-cyclin, interacts with Cdc28p
YAR018c	KIN3/NPK1	15%B	+	Ser/Thr protein kinase
YBR059c	AKL1	40%B	+	Ser/Thr protein kinase
YBR097w	VPS15/VPT15	30%C 50%D	+++	Protein kinase, interacts with Vps34p
YDL025c		15%B 10%E	+	Ser/Thr protein kinase
YDR507c	GIN4/ERC47	50%B 10%C	+	Ser/Thr protein kinase required for septin organization
YER123w	YCK3/CK13	30%B 20%C 10%D	++	Casein kinase I isoform
YFL033c	RIM15	30%B	+	Ser/Thr protein kinase, regulates IME2
YGL215w	CLG1	25%B	+	Cyclin-like protein, associates with Pho85p
YGR188c	BUB1	10%B 60%D	+	Ser/Thr protein kinase, affects microtubules
YJL095w	BCK1	20%B 10%C	+	MEKK family protein kinase
YKL048c	ELM1/ECM41	80%B	+	Protein kinase-regulating pseudohyphal growth
YKL139w	CTK1	50%B 10%C	++	C-terminal domain kinase, α subunit
YLR362w	STE11	50%B	+	MAP kinase kinase kinase (MAPKKK)
YPL150w		40%B	+	Ser/Thr protein kinase
YOR061w	CKA2	20%B 25%C 20%D	+	Casein kinase II catalytic subunit
YBR028c		15%B	+	Protein kinase with similarity to Ypk2p and Ypk1p
YDR477w	SNF1/CAT1	50%B	+	Protein kinase, derepression of glucose-repressed genes
Protein phosphatase related				
YAL016w	TPD3/FUN32	30%B 30%C	+	PP2Ap regulatory subunit, ceramide activated
YDL006w	PTC1/TPD1	60%B	+	PP2C Ser/Thr phosphatase for MAP kinases
YBR050c	REG2	20%B 10%C	+	Possible regulatory subunit for Glc7p
YBL058w	SHP1	25%B 10%D	+	Possible regulatory subunit for Glc7p
Protein acetyltransferase related				
YPR131c	NAT3	80%B	−	Protein *N*-acetyltransferase

ing protein (GAP) (Dogic *et al.*, 1999), are also required for low vacuole copy number (Table 5). Surprisingly, we found that the further metabolism of PI(4,5)P2 is required for normal vacuole morphology. Deletion of INP54, which encodes a PI(4,5)P2 5-phosphatase, causes striking vacuole fragmentation (Table 3), as do multiple deletions of the redundant

Table 3. Lipids

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
Phosphoinositol related				
YDR173c	IPK2/ARG82	50%B 10%C	++	Inositol polyphosphate multikinase
YOL065c	INP54	50%B	++	Phosphatidylinositol polyphosphate 5-phosphatase
YDR017c	KCS1	80%B	+	Inositol hexaphosphate kinase
YPL268w	PLC1/GSL2	50%B 10%C	+	Phosphoinositide-specific PLC
YER019w	ISC1	30%B 20%C	++	PLP C-like activity
YLR240w	VPS34/VPT29	20%C 80%D	+++	Phosphoinositide 3-kinase, associates with Vps15p
YDR323c	VPS19/PEP7	50%C	+++	Coordination of PI3K and Rab signaling
Ergosterol related				
YLR056w	ERG3/SYR1	60%B	+	C-5 sterol desaturase
YMR015c	ERG5/CYP61	80%B	−	Cytochrome P450
YML008c	ERG6/ISE1	60%B	+	Zymosterol methylation
YNL280c	ERG24	30%B 20%C	−	C-14 sterol reductase
YER044c	ERG28	30%B 30%C	+	C-4 sterol demethylation
Sphingolipid/phospholipid related				
YLR260w	LCB5	40%B 40%C	−	Phosphorylation of sphingosines
YCR034w	FEN1/GNS1	75%B 10%C	−	Fatty acid elongation
YLR372w	SUR4/VBM1	100%C	−	24–26-carbon fatty-acid conversion
YOR196c	LIP5	30%B	++	Lipoic acid synthetase
YOR221c	MCT1	30%B	+	Malonyl CoA:acyl carrier protein transferase
YKL055c	OAR1	30%B	+	3-Oxoacyl-[acyl-carrier-protein] reductase
YBR177c	EHT1	20%B 10%C 10%E	+	Alcohol acyl transferase

Table 4. Cations

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
Calcium related				
YAL026c	DRS2/ATC4	50%B	−	P-type Ca-ATPase
YBR131w	CCZ1	20%B 80%C	++	Required for growth in high calcium, caffeine, or zinc
YEL010w	SPF1/COD1	60%D	++	Putative Ca-ATPase
YGL167c	PMR1/DER5	40%B	+	P-type Ca-ATPase
YGR262c	–	50%B 10%C	+	Similar to calcium/calmodulin-binding protein kinase
YAL058w	CNE1/FUN48	70%B	+	Similar to mammalian calnexin and calreticulin
Metal related				
YBR037c	SCO1	50%B	+	Putative copper transporter
YOL152w	FRE7	50%B	+	Copper regulated protein
YMR038c	LYS7	40%B	+	Copper chaperone for Sod1p
YDR456w	VPS44/VPL27	30%B 30%C 20%E	+++	Na^+/H^+ antiporter

PI4P phosphatases INP51, 52, and 53 (Stolz *et al.*, 1998). The need for PI(4,5)P2 hydrolysis as well as synthesis underscores the central role of PI(4,5)P2 in docking, both as a regulatory ligand and second messenger. PI(4,5)P2 is known to regulate the mammalian Arp2/3 complex (Rohatgi *et al.*, 2000), in accord with the observation (Table 6) that deletion of the only nonessential component of this complex, ARC18, causes vacuole fragmentation. PI(4,5)P2 can be hydrolyzed to diacylglycerol and inositol trisphosphate (IP3) by two phospholipase C (PLC) enzymes. IP3 is converted to IP(4–8) by inositol polyphosphate multikinase and inositol hexaphosphate kinase. Deletion of any of these four genes causes a strong vacuole fragmentation phenotype (Table 3).

Proteins that mediate divalent cation transport and homeostasis also affect vacuole morphology (Table 4). Calcium is required for vacuole fusion. It is released from the vacuole lumen late in docking (Peters and Mayer, 1998), complexes with calmodulin, and regulates the formation of *trans*-V0:V0:Vam3p complexes (Peters *et al.*, 2001). Additionally, three proteins related to copper transport and one sodium/proton antiporter are required for the maintenance of low vacuole copy number.

In addition to Ypt7p (Table 1), several other GTPases and GTPase effectors were identified in our screen (Table 5). These include Arf1p and its GAP, Glo3p, which regulate PI(4,5)P2 biosynthesis (Jones *et al.*, 2000), and exchange factors and GAPs for Rho1p, an essential GTPase that is required for normal cell polarity (Hall, 1998) and for the docking stage of vacuole fusion (Eitzen *et al.*, 2001). Other GTPases and effectors do not have a clear link to vacuole fusion, and their effects may be direct or indirect.

Cytoskeletal proteins also govern vacuole copy number (Table 6). Intact microtubules are known to be needed for vacuole integrity in vivo (Guthrie and Wickner, 1988), although tubulin has not been shown to be needed for in vitro vacuole fusion. Actin is found on isolated vacuoles (P. Slusarewicz, A. Merz, G.E., and W.W., unpublished results) and we have recently reported that the Rho GTPases Cdc42p and Rho1p, which regulate actin polymerization, are required for vacuole docking (Eitzen *et al.*, 2001). Cla4p, Vrp1p, which interacts directly with Bee1p, the yeast WASp, and Arc18p, a subunit of the yeast Arp2/3 complex, are part of a well-characterized regulatory cascade in yeast and mammals (Higgs and Pollard, 1999; Winter *et al.*, 1999; Evangelista *et al.*, 2000; Rohatgi *et al.*, 2000). The finding that these proteins regulate vacuole copy number provides an important link in studies of the role of vacuole-bound actin.

Table 5. G-Protein related

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
YDL192w	ARF1	70%B	+	GTPase involved in coat formation, activates Mss4p
YER122c	GLO3	30%B 20%C	+	Arf1p/Arf2p GAP
YKR001c	VPS1	60%B	+++	Dynamin-like GTPase
YLR262c	YPT6	40%B 40%C	++	Endosomal Rab-like GTPase
YLR371w	ROM2	20%B 40%D	+	Rho1p GEF
YBR200w	BEM1/SRO1	50%B	−	Interacts with Cdc42p
YER155c	BEM2/SUP4	60%D	−	Rho1p GAP
YPL161c	BEM4/ROM7	50%B 30%E	+	Bud emergence protein, interacts with Rho GTPases
YOR070c	GYP1	50%B 25%E	+	Ypt4p/Sec4p GAP
YDR389w	SAC7	30%B	+	Rho1p GAP
YBR025c		60%B 25%e	+	Uncharacterized GTPase
YCR002c	CDC10	40%B	−	Septin, GTPase

Table 6. Actin/tubulin

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
Actin/polarity related				
YLR337c	VRP1/END5	20%B 20%C 10%D	+	Verprolin homologue, WASP-interacting protein
YLR370c	ARC18	30%B 20%D 30%E	+	ARP2/3 complex subunit
YDR484w	VPS52/SAC2	50%B 45%C	+++	Vps52p-Vps53p-Vps54p complex subunit
YDR027c	VPS54/LUV1	45%B 50%C	+++	Vps52p-Vps53p-Vps54p complex subunit
YDR129c	SAC6/ABP67	30%B 30%C 30%E	−	Fimbrin homologue, F-actin bundling protein
YLR144c	ACF2	30%B 30%E	−	Cortical actin assembly
YMR238w	DFG5	30%B 30%E	−	Cell polarity
YNL298w	CLA4/ERC10	45%B 30%E	+	Protein kinase required for cytokinesis
Microtubule related				
YCL029c	BIK1/ARM5	60%B 20%E	+	Microtubule-associated protein
YGL216c	KIP3	25%B 25%C	+	Kinesin-related protein
YOR349w	CIN1	25%B 25%E	+	Microtubule stability

Many VPS proteins have well-established roles in vacuole fusion (Table 1), and others with known catalytic activities are categorized accordingly. However, many *VPS* genes that are needed for normal vacuole morphology have no known catalytic function or have functions without obvious relationship to vacuole fusion per se (Table 7). Of the 137 genes considered in detail here, 24 were already designated *VPS* genes, and each of these deletion strains showed a *vps* phenotype of CPY secretion. Of the remaining 113 deletion strains, only 26 had a moderate or severe *vps* phenotype, suggesting substantial differences between homotypic vacuole fusion and heterotypic trafficking pathways to the vacuole.

Other proteins, with known or imputed roles in trafficking, are also needed for normal vacuole morphology (Table 8). Some, such as clathrin heavy and light chains or SNAREs, probably act indirectly by preventing trafficking of needed fusion catalysts to the vacuole. SNAREs may also act promiscuously in vacuole fusion, as suggested by the ability of certain SNAREs, when overexpressed, to compensate for the loss of others (Gotte and Gallwitz, 1997; Darsow *et al.*, 1998; Tsui *et al.*, 2001). Further studies are needed to resolve this question.

Signaling Pathways of Vacuole Docking

Normal vacuole morphology requires Isc1p and Plc1p, the PLC enzymes that hydrolyze PI(4,5)P2 to diacylglycerol plus IP3, and Ipk2p and Kcs1p, which further phosphorylate IP3 to IP6 and IP4-(PP)2 (Table 3; Saiardi *et al.*, 2000). This suggests that IP3 or its derivatives may regulate later steps of vacuole docking, including the docking-dependent release of calcium from the vacuole (Belde *et al.*, 1993). To provide an independent test of these genetic inferences, we have tested pharmacological inhibitors of PLC and of calcium channels (Figure 2). In vitro vacuole fusion is blocked by the PLC inhibitor ET-18-OCH3 (Arthur and Bittman, 1998) as well as by calcium channel inhibitors such as Ruthenium Red, cyclopiazonic acid, 2-APB, and thapsigargin (Belde *et al.*, 1993; Takahashi *et al.*, 1994; Thomas and Hanley, 1994; Buratti *et al.*, 1995; Calvert and Sanders, 1995; Herrmann-Frank *et al.*, 1996; Maruyama *et al.*, 1997). To order their inhibitory action in the vacuole fusion pathway, we exploited the antibody to a Ypt7p peptide. This antibody blocks Ypt7p action at the start of docking but can be reversed by addition of the cognate peptide (Eitzen *et al.*, 2001). Fusion (Figure 2A, lane 2) is blocked by anti-Ypt7p (Figure 2A, lane 3) but not by the antibody that was premixed with peptide antigen (Figure 2A, lane 4). Vacuoles that were incubated at 27°C with this antibody could be deblocked by peptide addition after 25 min (Figure 2A, lane 5) and had then acquired resistance to the antibody to Sec17p, indicative of the completion of priming (Figure 2A, lane 6, anti-Sec17p from t = 0; lane 7, anti-Ypt7p from t = 0, addition of Ypt7-peptide + anti-Sec17p at t = 25). Because

Table 7. Other VPS

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
YDR495c	VPS3/PEP6	30%C 60%D	+++	Involved in vacuolar segregation
YOR069w	VPS5/PEP10	100%B	+++	Nexin-like, involved in protein sorting
YAL002w	VPS8/VPT8	30%C 30%D 30%E	+++	Required for Vps10p localization, Golgi retrieval
YOR132w	VPS17/VPT3	95%B	+++	Retromer complex subunit
YCL008c	VPS23/STP22	50%D 25%B	+++	Unknown
YKL041w	VPS24/VPT24	50%B 50%E	+++	Protein sorting to prevacuolar compartment
YPL065w	VPS28/VPT28	60%E	+++	May control endosomal export
YLR025w	VPS32/SNF7	30%B 30%D 30%E	+++	Protein sorting to prevacuolar compartment
YJL154c	VPS35/VPT7	50%B	+++	Retromer complex subunit

Table 8. Other trafficking-related genes

Locus	Common name(s)	Vacuolar phenotype(s)	CPY secretion	Function
YDR099w	BMH2/SCD3	20%B 10%C 20%E	+	14-3-3 protein
YLR373c	VID22	30%B	+	Targeting to prevacuolar compartment
YKL054c	VID31	30%B 10%C 10%E	+	Vacuolar import and degradation
YGR167w	CLC1	50%B 40%C 10%E	++	Clathrin light chain, coat protein
YGL206c	CHC1	25%B 25%C	++	Clathrin heavy chain, coat protein
YHL031c	GOS1	70%B	+	Synaptobrevin (v-SNARE) homologue
YOL018c	TLG2	50%B	++	Syntaxin (t-SNARE) homologue
YOR036w	VPS6/PEP12	75%C 25%E	+++	Syntaxin (t-SNARE) homologue

Ypt7p action is essential for *trans*-pairing of SNAREs (Ungermann *et al.*, 1998), the reaction did not acquire resistance to anti-Vam3p during the period of blockade by anti-Ypt7p (Figure 2A, lanes 8 and 9). Using this assay, we found that primed vacuoles remain fully sensitive to each of the PLC and calcium channel blockers (Figure 2A, lanes 10–19, filled bars), showing that these act after priming is complete. In a complementary approach, these same inhibitors were added at various times to aliquots of an ongoing fusion reaction (Figure 2B). Although each ligand inhibits completely when added from the start, the reaction acquires resistance to antibody to Vam3p as docking is completed (Ungermann *et al.*, 1998), whereas the acquisition of resistance to microcystin LR is kinetically indistinguishable from fusion (Mayer *et al.*, 1996). Resistance to each putative inhibitor of calcium channels or PLC is acquired only at, or shortly after, docking but well before fusion. Thus, PLC action and calcium flux occur at, or shortly after, docking.

DISCUSSION

Of the 4828 nonessential genes of yeast, ~700 showed at least some abnormal vacuole morphology. However, most of these encode proteins of a known function or that reside in a subcellular locale, which strongly indicates that their effects are indirect. We found 137 genes that may be directly needed for normal vacuole size and copy number in growing cells and are, in this regard, *VAM* genes. It is clear that there is only modest overlap between the *VPS* and *VAM* genes. In one study (Banta *et al.*, 1988), mutants in 25 of the 30 *VPS* genes had normal vacuole structure and thus were not *VAM* genes. In a later study (Raymond *et al.*, 1992), several class A (normal vacuole appearance) *vps* mutants (*vps*13, 44, and 46) had only 21–40% secretion of CPY, whereas others (*vps*8, 10, 29, 30, 35, and 38) had 62–84% CPY secretion, similar to the ranges for class B or class C *vps* mutants. Thus, a strong *vam* phenotype of fragmented vacuoles is not required for a strong *vps* phenotype. Conversely, of the 137 *VAM* genes listed here, only 50 are moderate or strong *VPS* genes. Many gene deletions that cause striking vacuole fragmentation phenotypes do not secrete significant amounts of CPY and thus are not *VPS* genes. For example, none of the *erg* deletions have a moderate or strong *vps* phenotype, even though several show striking *vam* fragmentation phenotypes (Table 3; Kato and Wickner, 2001). Thus, there are many genes involved in regulating or catalyzing vacuole fusion that do not affect trafficking to the vacuole, and others that affect this heterotypic traffic, at vesicle budding, movement, and fusion, that do not affect homotypic vacuole fusion.

Many *VAM* genes, such as those needed for ergosterol biosynthesis, function at a nonvacuolar site to make a product that itself traffics to the vacuole and is more directly involved, and some of these genes will only affect fusion indirectly. Nevertheless, several criteria assure us that this set of 137 *VAM* genes is highly enriched in direct catalysts of the in vitro fusion reaction: 1) The screen selected each of the nonessential genes that encode proteins that are biochemically established as catalyzing vacuole fusion. 2) Functional clusters of genes were evident from the screen, for ergosterol biosynthesis (Kato and Wickner, 2001), phosphoinositide metabolism (Mayer *et al.*, 2000), and actin regulation (Eitzen and Wickner, unpublished data), and these have been confirmed biochemically by adding specific inhibitors to the in vitro fusion reaction. 3) Genetic and proteomic relationships (Uetz *et al.*, 2000; Ito *et al.*, 2001; www.proteome.com) link many of these *VAM* genes to essential genes that directly participate in vacuole fusion. Of the 137 genes identified in our screen, 28 are in uncharacterized open reading frames and 17 encode known catalysts of vacuole fusion (Table 9). Of the remaining 92 genes, 21 can be directly tied to the reaction: two regulate protein phosphatase 1 (Table 2), six are directly involved in inositol phosphatide metabolism, five others are directly involved in ergosterol biosynthesis (Table 3), three mediate the Rho-GTPase regulation of actin (Table 6), four are effectors of Rho1p, and one activates Mss4p (Table 5).

The first novel functional gene cluster to emerge from the genetic screen was for ergosterol biosynthesis (Kato and Wickner, 2001). Striking vacuole fragmentation was seen in several ergΔ strains, and this was confirmed by direct inhibition of the in vitro fusion of wild-type vacuoles by filipin, nystatin, and amphotericin B, each a specific ligand of ergosterol. This was shown to be specific, in that addition of ergosterol or cholesterol to vacuoles overcame ergosterol deficiency or drug inhibition. Kinetic analysis showed that inhibition was seen only at the priming stage of the reaction and indeed represents a direct requirement of ergosterol for Sec18p-mediated Sec17p release (Kato and Wickner, 2001).

Roles of PI(4,5)P2

Our current *VAM* deletion screen has revealed that vacuole fusion is governed by highly interrelated pathways

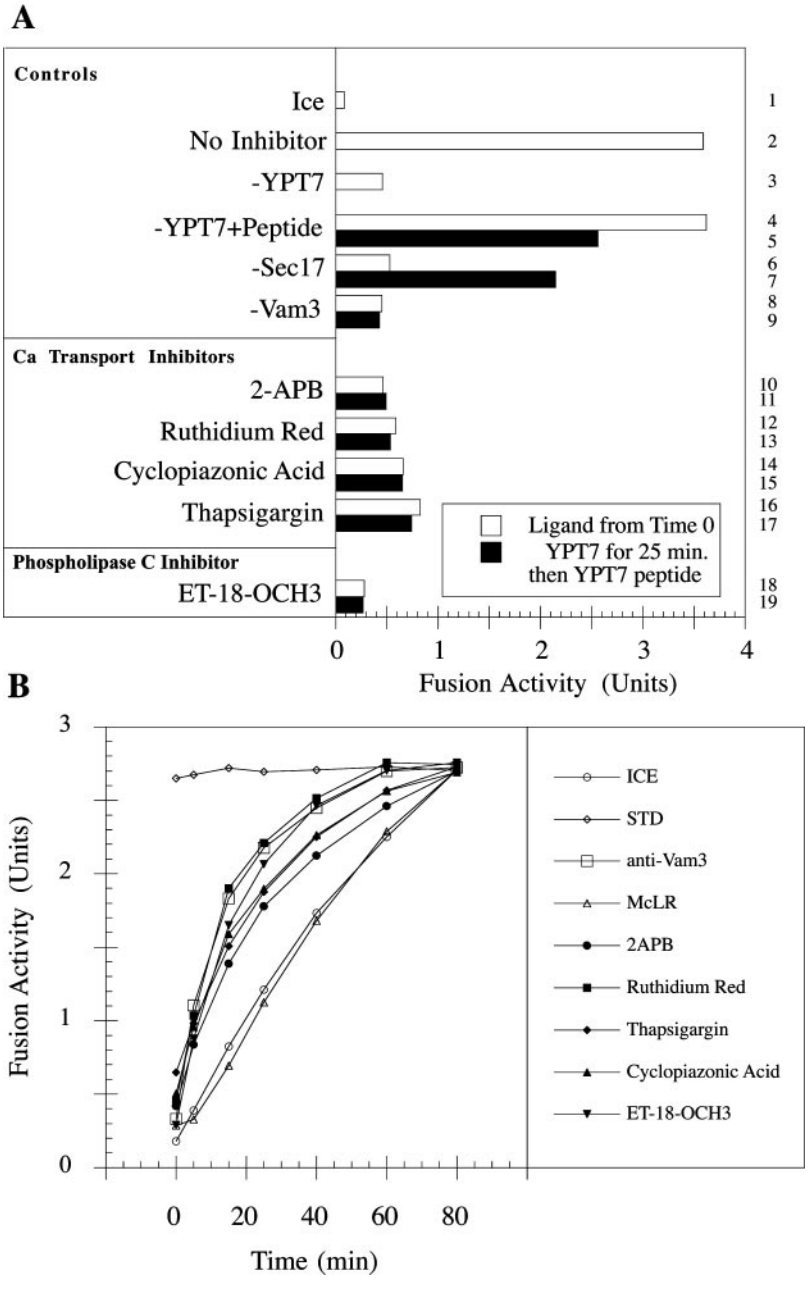

Figure 2. Inhibitors of docking. (A) A 44× scale (1.32 ml) fusion reaction was prepared as described in MATERIALS AND METHODS. Portions (30 μl) were dispensed into individual microfuge tubes, and each reaction was brought to a final volume of 38 μl with ligand or PS buffer as indicated. After incubation on ice for 5 min, the tubes were transferred to a 27°C water bath for 90 min and assayed for alkaline phosphatase activity. The remaining master reaction was treated with anti-YPT7 antibody for 5 min on ice and then for 15 min at 27°C. YPT7 peptide was added, and 30-μl portions were dispensed into microfuge tubes. Ligand or PS was added to a final volume of 38 μl, and the reaction was allowed to proceed for 80 min at 27°C before assay for alkaline phosphatase activity. (B) Aliquots (30 μl) from a 1.98-ml fusion reaction were transferred to ice or mixed with 1 μl of PS buffer or the indicated inhibitor at the indicated time. After a total incubation of 80 min at 27°C, all tubes were placed on ice for 5 min and then assayed for alkaline phosphatase activity.

of PI(4,5)P2 synthesis, PI(4,5)P2 hydrolysis by phosphatases and phospholipases, IP3 phosphorylation, and PI(4,5)P2-dependent regulation of actin polymerization. These interrelated pathways are summarized in Figure 3. They reveal an unexpected complexity of the docking phase of vacuole fusion, and yet there is precedent for each component having a role in other fusion reactions and biochemical evidence ties these pathways together.

PIP2 turnover, as well as synthesis, is essential for docking. The clustering of *VAM* genes in the pathways of PI(4,5)P2 biosynthesis and hydrolysis (Figure 3) is in accord with the finding (Mayer *et al.*, 2000) that this lipid has a central role in docking. Each step of synthesis and degradation of PI(4,5)P2 is required for normal vacuole morphology. Thermosensitive PI 4-kinase has been shown to have fragmented vacuoles at nonpermissive temperature (Audhya *et al.*, 2000). PI(4P) 5-kinases are activated by Arf1p (Jones *et al.*, 2000); deletion of *ARF*1 or *GLO*3, the GAP for Arf1p, yields fragmented vacuoles (Table 5). INP54, which removes the 5-phosphate from PI(4,5)P2, is needed for normal vacuole morphology, as are the redundant phosphatases *INP*51, 52, or 53. These genetic findings are strengthened by the sensitivity of docking to added bacterial PLC (Mayer *et al.*, 2000), which degrades PI.

Table 9. Orphan open reading frames

Locus	Vacuolar phenotype(s)	CPY secretion
YCL016c	50%B	+
YDR200c	70%B	+
YDR223w	10%B 40%E	+
YDR433w	80%B	+
YEL044w	40%B 40%D	+++
YER083c	30%B 30%C 30%E	+
YGL024w	75%B	−
YGL223c	90%B	+++
YJL075c	40%B	−
YJL184w	40%C 20%D	+
YLL002w	60%B	+
YLR091w	90%C	−
YLR204w	70%D	+
YLR261c	60%B 10%C	++
YLR320w	50%B	+
YLR322w	25%B 25%C	+
YML013c-A	35%B 35%C	++
YMR269w	85%B	−
YNL080c	70%B	+
YNL281w	70%B	+
YNL297c	60%B	+
YOL008w	50%B	−
YOL035c	60%B	+
YOL050c	70%B	+
YOL063c	50%B	+
YOR068c	90%B	+++
YOR359w	25%B 25%C	+
YPL055c	50%B	++

Three genes (YLR320w, YNL281w, and YOR068c) are adjacent to genes that are also required for normal vacuole morphology. Further analysis will be required to determine whether these deletions act by altering the expression level of an adjacent gene.

Although yeast has no obvious homologue of the mammalian IP3 receptor calcium channel proteins, the sensitivity of the reaction to inhibitors of such channels suggests a functional homologue may be responsible for docking-dependent calcium release from the vacuole (Peters and Mayer, 1998), especially because this release does not use the well-characterized vacuolar calcium transport proteins (Ungermann *et al.*, 1999). IP3 can induce calcium release from yeast vacuoles (Belde *et al.*, 1993).

IP3 is a biologically significant product of PI(4,5)P2 hydrolysis by PLC. Yeast have two PLC isoforms, *PLC*1 and *ISC*1, and deletion of either alters vacuole structure (Audhya *et al.*, 2000), suggesting a role for PLC isoforms in the catalysis of vacuole fusion. PLC activity and the generation of IP3 are central for the induction of regulated calcium flux across the endoplasmic reticulum (Parker *et al.*, 1996) and vacuolar membranes (Belde *et al.*, 1993; Calvert *et al.*, 1995). Because 1) calcium signaling is required for vacuole fusion, 2) the precursor of IP3, PI(4,5)P2, is required for docking (Mayer *et al.*, 2000), and 3) deletion of either of the two PLC isoforms, *PLC*1 or *ISC*1, results in vacuole fragmentation, we used several inhibitors of PLC and of IP3-gated calcium channels to verify the participation of PLC and IP3, respectively, in vacuole fusion. Inhibitors of both PLC and of IP3 receptors inhibit vacuole fusion (Figure 2), thus supporting their involvement in the reaction. Higher-order, hyperphosphorylated forms of inositol have been implicated in several trafficking events in the cell as well as in vacuole biogenesis. This screen showed that deletion of either of two phosphoinositol polyphosphate kinases, *IPK*2 and *KCS*1, results in fragmented vacuoles, as noted before (Saiardi *et al.*, 2000).

Other categories of phosphatidylinositol-modifying enzymes were also revealed through this screen. These include the synaptojanin-like PI(4,5)P2 5-phosphatase-INP gene family and two high-order phosphoinositol kinases, *IPK*2 and *KCS*1. Single deletion of *INP*54 or double deletion of *INP*51 and *INP*52 (Soltz *et al.*, 1998) result in fragmented vacuoles. The precise role of these phosphatases and their products are currently unknown. Other studies (Malecz et al., 2000) have suggested potential roles for inositol polyphosphate 5-phosphatases, such as those of the INP family and synaptojanin, in the regulation of the actin cytoskeleton by virtue of their association with the GTP-bound form of Rac and through genetic interactions with *SAC*6, a yeast fimbrin homologue essential for the assembly of normal actin structures (Adams *et al.*, 1991). Because actin may be involved in the fusion of vacuolar membranes (Eitzen and Wickner, unpublished data), these data may represent a regulatory role for INP family-generated inositol metabolites in the fusion of vacuolar membranes.

A third functional gene cluster to emerge from this screen regulates vacuole-bound actin. Cla4p, Vrp1p, and Arc18p are elements in a well-studied cascade by which Rho GTPases regulate F-actin assembly (Higgs and Pollard, 2000; Rohatgi *et al.*, 2000). Each of these three genes shows a two-hybrid relationship to the essential Myo3p motor protein, itself a member of this regulatory pathway (Evangelista *et al.*, 2000; Lechler *et al.*, 2000), and Cla4p binds to Cdc42p (Mitchell and Sprague, 2001; Mosch *et al.*, 2001). We have recently shown that two Rho GTPases, Rho1p and Cdc42p, act after Ypt7p in the docking phase of vacuole fusion (Eitzen *et al.*, 2001) and that actin mutations or actin-specific drugs (latrunculin B and jasplakinoloid) affect the reaction. Although the functional role of actin in vacuole docking is not known, the deletion screen provides critical insight that can be combined with biochemical approaches to establish novel pathways of the fusion reaction.

We speculate that there is an intimate relationship among actin regulation through Rho family GTPases, the need for ergosterol, phosphoinositide signaling, and calcium flux. PI(4,5)P2 (Rozelle *et al.*, 2000) and Cdc42p directly regulate the activity of Bee1p/WASp and thus actin dynamics, whereas PLC has been shown to regulate actin polymerization and calcium release. Rho-GTPases, Arf1p, and their effectors have been shown, in conjunction with PLC, to directly modulate the activities of PI4P 5-kinases and the levels of PI(4,5)P2 in vivo (Weernink *et al.*, 2000a,b). PI(4,5)P2 has been shown in other systems to directly activate the nucleotide exchange activities of Rho proteins (Zheng *et al.*, 1996). Further studies have shown that PI4P 5-kinase activities, PI(4,5)P2 generation, and F-actin generation occur preferentially on membrane rafts and that these activities are sensitive to methyl-β-cyclo-

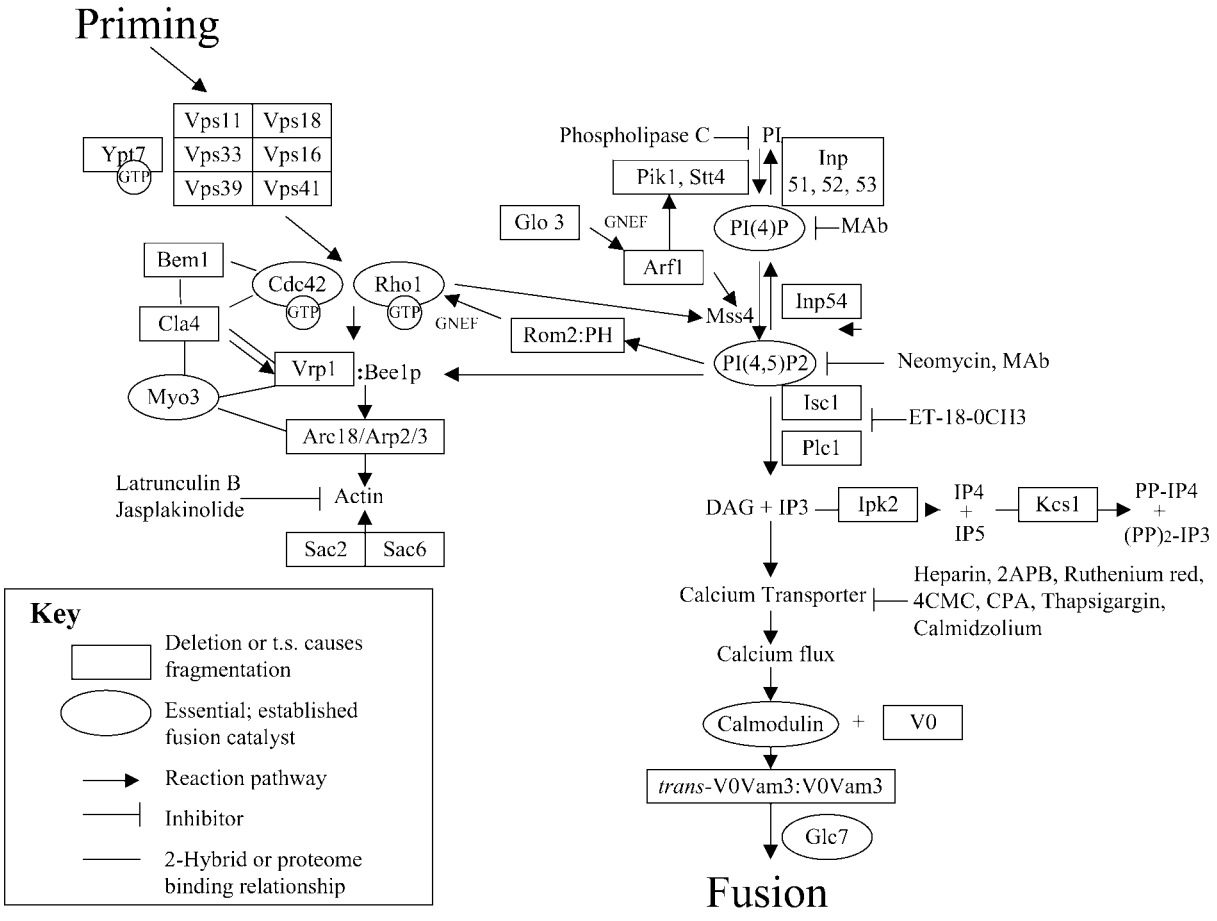

Figure 3. Signaling cascades of homotypic vacuole fusion. Genes that are needed for normal vacuole morphology in vivo are in rectangles. Arrows indicate reaction pathways, in yeast and in other eukaryotes.

dextrin (Rozelle *et al.*, 2000), thus implicating sterol as a central structural catalyst for the integration of these fusion-associated events. Several important aspects of the fusion reaction can be linked to membrane microdomains known to be enriched in ergosterol in yeast. If these pathways are indeed interrelated in the context of homotypic vacuole fusion, they might signal from Ypt7p at the start of docking through activation of Rho family GTPases to the calcium flux seen at the end of docking (Figure 3).

The deletion screen is limited to nonessential genes, and yet many essential genes that participate in other pathways are required for vacuole fusion. Their roles may be discovered through biochemical analysis of the in vitro fusion reaction or by their relationships to other catalysts of vacuole fusion. One example of this is shown in Figure 4. Interactions among genes may be inferred from biochemical data that is compiled at www.proteome.com (indicated by dark connector lines), typically reflecting protein copurification, directly related function, or coimmunoprecipitation from extracts (www.proteome.com), or from comprehensive two-hybrid analysis of yeast (Uetz *et al.*, 2000; Ito *et al.*, 2001), as indicated by thin dotted connector lines. Eleven genes identified in our deletion screen (Figure 4, ovals) have no binary connections with each other, either through two-hybrid or proteome relationships. However, these 11 genes can be connected in this manner by the addition of three essential genes, known from biochemical studies to be required for the vacuole fusion reaction (Figure 4, indicated by stars), and eight other "connector" genes (Figure 4, rectangles). Two of these eight connector genes, *TOR*2 and *CDC*24, are essential and thus were not in the deletion screen. Cdc24p is the guanine nucleotide exchange factor for Cdc42p (Zheng *et al.*, 1994). Thus, this modest network has suggested the involvement of additional gene products. Nonessential connector genes may encode proteins that have redundant functions and thus not show a phenotype on single deletion. Gene clusters such as that shown (Figure 4) provide attractive targets for further investigation.

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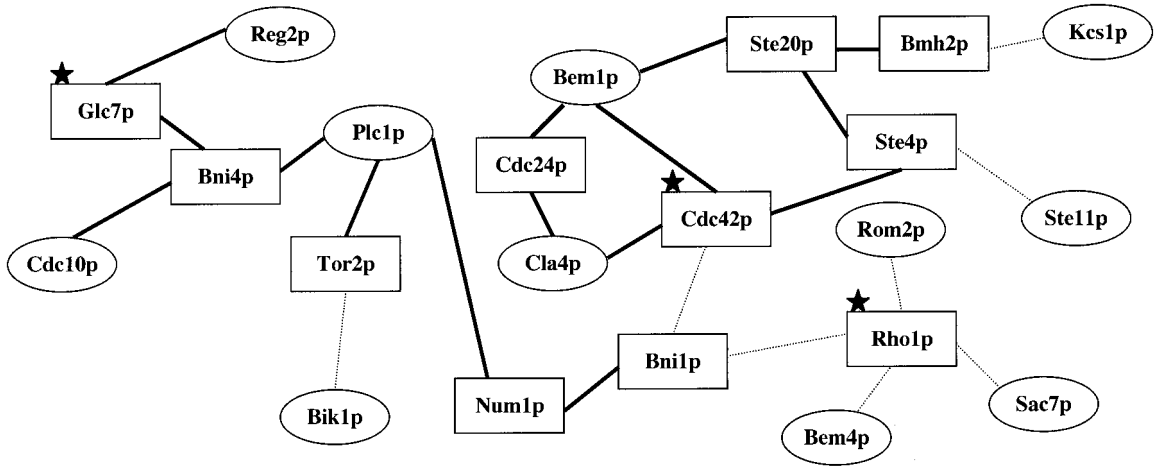

Figure 4. Clustered genetic interactions. Ovals indicate genes that were detected in our screen as necessary for normal vacuole morphology. Two-hybrid and proteome relationships can connect these genes when the genes in rectangles are added. Thick lines represent experimentally determined protein-protein interactions other than two-hyrid interactions, compiled from www.proteome.com. Thin lines represent protein-protein interactions from two-hybrid analyses. Stars represent genes encoding proteins that are known from biochemical studies to be involved in the vacuole fusion reaction.

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